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Quantification of carvone, cineole, perillaldehyde, perillyl alcohol and sobrerol by isocratic high-performance liquid chromatography

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Abstract

A simple and rapid isocratic HPLC assay is presented for the analysis and quantification of monoterpenes, i.e., carvone, cineole, perillaldehyde, perillyl alcohol and sobrerol in the diet of laboratory animals. The monoterpenes were extracted from the diet using 90% methanol in water. The analysis for the monoterpenes was performed by HPLC using a Whatman PartiSphere C_{18} column with UV detection. The mobile phase was isocratic methanol–water (72:28, v/v) for carvone, cineole, perillaldehyde and perillyl alcohol and methanol–water (65:35, v/v) for sobrerol. The method was simple with good repeatability (R.S.D.=4.2–16.1%), reproducibility (R.S.D.=3.7–19%), and accuracy (R.S.D.=5.5–23.3%). The detection limit of the monoterpenes extracted from the diet was 2 μg/g for carvone, perillaldehyde and sobrerol, 20 μg/g for perillyl alcohol and 100 μg/g for cineole. Excellent resolution was achieved between the monoterpenes and the constituents of the diet. The method demonstrated applicability to monitoring the formulation of monoterpenes in the diet of laboratory animals.

Keywords: Food analysis; Carvone; Cineole; Perillaldehyde; Perillyl alcohol; Sobrerol; Monoterpenes

1. Introduction

Recently, much attention has been directed toward the chemopreventive and anticarcinogenic activity of naturally occurring non-nutrient dietary constitutes [1–3]. The monoterpenes including carvone, cineole, perillaldehyde, perillyl alcohol and sobrerol are present in a wide variety of food. In laboratory animals, monoterpenes administered in the diet have demonstrated the ability to prevent the formation and/or to cause the regression of chemically induced colon, hepatic, mammary, and pancreatic cancers [4–9]. The USA National Cancer Institute and others are currently evaluating monoterpenes for prevention of cancer in bioassays using laboratory animals. In these bioassays the monoterpenes are administered in the diet which requires the quality control monitoring of (1) their formulation in the diet to ensure accuracy and uniform distribution and (2) their stability — including loss due to evaporation and degradation during storage and presentation of the feed to the animals. Routine quality control requires a procedure that is simple, rapid and cost-effective.

A gas-chromatographic method has been reported for determining menthol and perillyl alcohol [4,10] and an HPLC method for menthol [11]. However, there is no report of a procedure suitable for monitoring and quantification of carvone, cineole, perillal-

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dehyde, perillyl alcohol and sobrerol in diet. In this study, we describe a simple and rapid isocratic HPLC assay for their determination and quantification. The assay is applicable to quality control testing of their formulation and stability in the diet.

2. Experimental

2.1. Chemicals and reagents

Cineole, (*S*)-(−)-perillyl alcohol and (*S*)-(−)-perillaldehyde were obtained from Aldrich (Milwaukee, WI, USA); D-(+)-carvone from Acros Organics (New Jersey, USA); and DL-sobrerol from Sigma (St. Louis. MO, USA). HPLC grade methanol was obtained from Fisher (Fair Lawn, NJ, USA). Other chemicals were of the highest purity commercially available. Water was distilled and then purified with a Model D4754 nanopure ultrapure water system (Barnstead Thermolyne, Dubuque, IA, USA).

Stock solutions of monoterpenes (5 mg/ml) were prepared in 90% methanol and kept at 4°C for up to 2 weeks. The stock solutions were diluted with 90% methanol to prepare working standard solution of 800 μg/ml for cineole and perillyl alcohol and 50 μg/ml for carvone, perillaldehyde and sobrerol. A 50-μl aliquot of these solutions was used daily as a control to check the conditions of the HPLC procedure. Calibration graphs for each compound were constructed by plotting the peak area against concentration. Triplicate injections were made for each concentration.

2.2. Chromatography

A Waters HPLC system (Milford, MA, USA) was used that consisted of a Model 510 pump, a Model U6K universal injector, a Model 481 Lambda Max UV–Vis LC spectrophotometer and a Model 730 Data Module. Separation was carried out with a Whatman PartiSphere C_{18} column (250 mm×4.6 mm I.D., 5 μm particle) (Clifton, NJ, USA) and an isocratic mobile phase consisting of methanol–water (72:28, v/v) for carvone, cineole, perillaldehyde, perillyl alcohol and methanol–water (65:35, v/v) for sobrerol with a flow-rate of 1.3 ml/min and at room temperature. The detection wavelengths were 220 nm for detecting carvone, perillyl alcohol and perillaldehyde and 200 nm for sobrerol and cineole. These wavelengths were chosen since they gave the maximum absorbance. Prior to injection of a sample, the column was equilibrated with the mobile phase at a flow-rate of 1.3 ml/min for at least 20 min or until a steady baseline was obtained. Quantification was based on integration of peak areas.

2.3. Rodent diet samples and extraction

American Institute of Nutrition (AIN)-76A powder diet was obtained from Dyets (Bethlehem, PA, USA). Perillyl alcohol was extensively mixed directly with the powder diet at different concentrations and stored in sealed containers at −20°C until used. Representative samples consisting of 10 g of either normal diet or diet mixed with perillyl alcohol, were added to 10 ml of 90% methanol in a 50-ml glass centrifuge tube. The tubes were capped, vortexed for 2 min and then centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant fluid was transferred to another tube and the extraction repeated once. The supernatant fluid of the two extractions were pooled and filtered through a Nalgene 0.2-μm pore size filter (Nalge, Rochester, NY, USA). A 50-μl aliquot was injected onto the HPLC column. Extraction recovery was calculated by comparing the peak area of the perillyl alcohol extracted from the diet to that of the perillyl alcohol standard injected directly onto the column. The same extraction procedure was used for the other four monoterpenes.

3. Results and discussion

Initially, the mobile phase was tried as the solvent for extraction of the monoterpenes from the diet. However, these extracts exhibited oil drops on the interphase after centrifugation, which could influence the quantitative results since the monoterpenes are oil-soluble. Production of oil drops was avoided by using 90% methanol as the solvent for the extraction. The efficiency of extraction was greater than 96.5% for all the monoterpenes tested. The retention times were 10.17 min for carvone, 13.76 min for cineole, 13.10 min for perillaldehyde, 12.55 min for perillyl alcohol and 7.56 min for sobrerol. Figs. 1 and 2

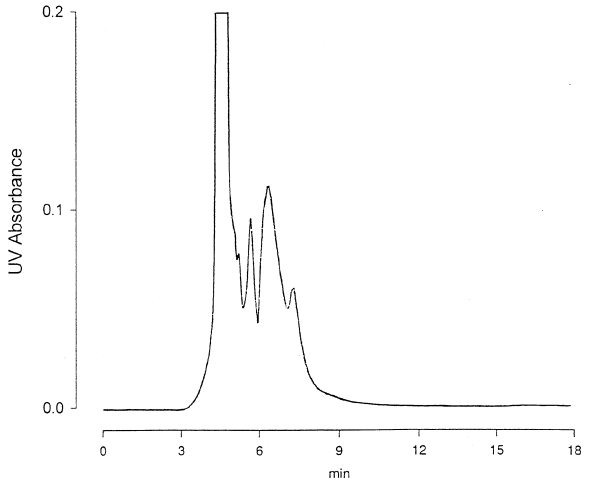

Fig. 1. Chromatogram of control AIN-76A animal diet extracted with 90% methanol. HPLC conditions: Whatman PartiSphere C_{18} column (250×4.6 mm I.D., 5 μm particle); mobile phase consisting of methanol–water (72:28, v/v); flow-rate of 1.3 ml/min; detection wavelength of 220 nm.

present chromatograms of normal diet extracted with 90% methanol using methanol–water (72:28 and 65:35, v/v, respectively) as the mobile phases. Chromatograms of extracts of normal diet contained

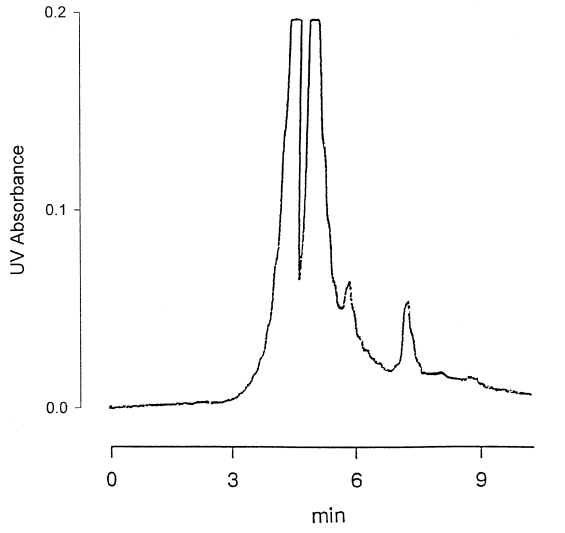

Fig. 2. Chromatogram of control AIN-76A animal diet extracted with 90% methanol. HPLC conditions: Whatman PartiSphere C_{18} column (250×4.6 mm I.D., 5 μm particle); mobile phase consisting of methanol–water (65:35, v/v); flow-rate of 1.3 ml/min, detection wavelength of 200 nm.

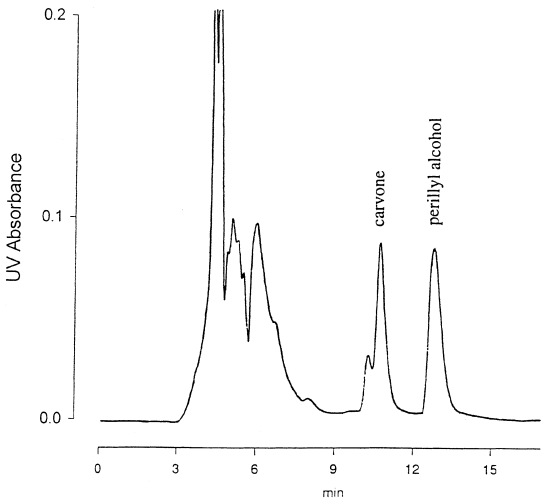

Fig. 3. Chromatogram of AIN-76A diet containing perillyl alcohol (0.1%, w/w) and extracted with 90% methanol. Carvone (4 mg/kg) was added to the diet as an internal standard. HPLC conditions: Whatman PartiSphere C_{18} column (250×4.6 mm I.D., 5 μm particle); mobile phase consisting of methanol–water (72:28, v/v); flow-rate of 1.3 ml/min, detection wavelength of 220 nm.

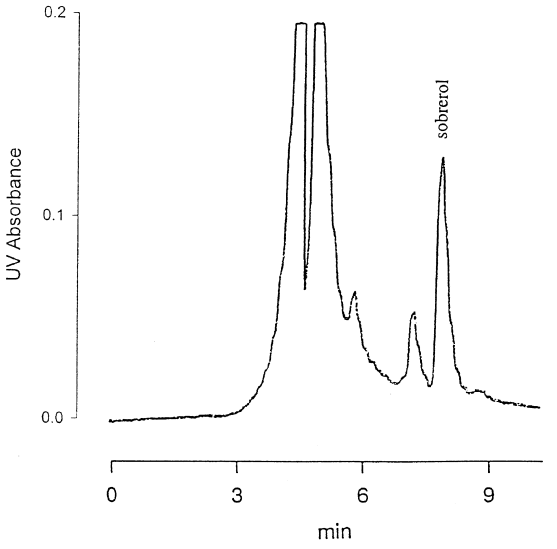

Fig. 4. Chromatogram of AIN-76A diet containing sobrerol (150 mg/kg) and extracted with 90% methanol. HPLC conditions: Whatman PartiSphere C_{18} column (250×4.6 mm I.D., 5 μm particle); mobile phase consisting of methanol–water (65:35, v/v); flow-rate of 1.3 ml/min, detection wavelength of 200 nm.

Table 1
Calibration graphs and standard addition method

Monoterpenes	Linear regression equation[a]	r^2	n[b]	Concentration range (μg/ml)[c]
Calibration				
Carvone	$y=409.6x-65.8$	0.9994	7	1–150
Cineole	$y=7.0x-82.7$	0.9989	7	50–4000
Perillaldehyde	$y=919.8x-306.0$	0.9999	7	1–250
Perillyl alcohol	$y=27.1x+134.9$	0.9987	7	10–1600
Sobrerol	$y=211.6x+574.7$	0.9938	7	1–150
Standard addition				
Carvone	$y=412.6x-60.2$	0.9948	5	1–150
Cineole	$y=6.9x-76.5$	0.9968	5	50–2000
Perillaldehyde	$y=908.5x-302.0$	0.9982	5	1–200
Perillyl alcohol	$y=28.8x+136.8$	0.9986	5	10–1500
Sobrerol	$y=212.4x+584.4$	0.9980	5	1–150

[a] y=Peak area; x=concentration (μg/ml); r^2=coefficient of determination.
[b] n=Number of different concentrations.
[c] Calibration, concentration range present; standard additions, concentration range added.

minimal absorption after 8 min (Figs. 1 and 2) when the monoterpenes were eluted. Fig. 3 presents a chromatogram obtained with an extract of diet containing perillyl alcohol (0.1%, w/w). Carvone was added as an internal standard for the extraction because it possess the same detection wavelength as perillyl alcohol and their retention time are sufficiently different to avoid overlap. Excellent resolution was also achieved between the other monoterpenes except for sobrerol, and the constituents of the diet using methanol–water (72:28, v/v) as the mobile phase. For sobrerol, a mobile phase consisting of methanol–water (65:35, v/v) gave better resolution from the constituents of the diet. Fig. 4 presents a chromatogram obtained with an extract of diet containing sobrerol (150 mg/kg).

Linearity was checked by measuring different concentrations in the ranges 10–1600 μg/ml for perillyl alcohol, 1–250 μg/ml for perillaldehyde, 50–4000 μg/ml for cineole, 1–160 μg/ml for carvone and 1–250 μg/ml for sobrerol. The relationship between peak area and concentration of the monoterpene was linear. The slope and y-intercept for the linear regression of the calibration graph for the different monoterpenes are presented in Table 1. The standard addition method was used to check during the determination of monoterpenes for chemical interference by ingredients in the diet. For each monoterpene, the slope of the linear regression equation for the calibration and the standard addition graph were similar (Table 1).

The analytical recovery was evaluated by assaying

Table 2
Repeatability studies (n=5)

Values	Carvone	Cineole	Perillaldehyde	Perillyl alcohol	Sobrerol
High					
Mean (μg/g)	150	152	150	150	152
R.S.D.	12.4	6.7	6.3	6.0	4.6
Medium					
Mean (μg/g)	50.9	49.5	50.6	50.6	49.7
R.S.D.	7.3	7.3	5.5	11	7.2
Low					
Mean (μg/gl)	10.6		10.5	10.2	11.7
R.S.D.	13.2		16.1	4.2	9.4

Table 3
Inter-daily reproducibility studies (n=5)

Values	Carvone	Cineole	Perillaldehyde	Perillyl alcohol	Sobrerol
High					
Mean (μg/g)[a]	149	150	152	152	150
R.S.D.	15.1	4.2	6.4	4.4	6.7
Medium					
Mean (μg/g)	52.6	52.2	56.4	50.3	48.6
R.S.D.	14.4	14.2	14	13	9.4
Low					
Mean (μg/g)	10.7		11.5	10.4	10.7
R.S.D.	6.5		18.2	3.7	19

[a] Concentration of the monoterpene added to the AIN-76A diet.

diet samples spiked with different amounts of each monoterpene ranging from 100 to 1000 μg/g diet for perillyl alcohol and cineole and 10 to 100 μg/g diet for perillaldehyde, carvone and sobrerol. Replicate analyses (n=5) at each concentration were performed. The mean recoveries from the diet were 97.5±1.6% [relative standard deviation (R.S.D.)= 1.6%] for perillyl alcohol, 101.2±2.8% (R.S.D.= 2.9%) for perillaldehyde, 97.9±2.6% (R.S.D.= 2.7%) for carvone, 98.1±2.4% (R.S.D.=2.5%) for sobrerol and 102.6±2.3% (R.S.D.=2.5%) for cineole. The inter-daily repeatability, reproducibility, precision and accuracy for each monoterpene were determined on diet samples containing different (high, medium and low) concentrations. The results reported in Tables 2–4 demonstrate for the assay very satisfactory repeatability (R.S.D.=4.2–16.1%), reproducibility (R.S.D.=3.7–19%), precision (R.S.D.=5.5–23.3%) and accuracy. The detection limit (0.01 absorbance units above baseline) for the monoterpenes extracted from the diet was 2 μg/g for carvone, perillaldehyde and sobrerol, 20 μg/g for perillyl alcohol and 100 μg/g for cineole. After injecting about 200 samples onto the column, there were no appreciable changes in the pressure and quality of the absorption peaks.

Table 4
Precision and accuracy data (n=5)

Monoterpenes	Concentration (μg/g)[a]	Concentration calculated (mean±S.D.; μg/g)
Carvone	150	152±19
	100	101±6.5
	50	54±3.7
	10	11±1.1
Cineole	1000	1030±131
	500	517±28
	100	99.4±8.2
	50	50.7±3.6
Perillaldehyde	120	121±14
	60	58.6±8.4
	30	29.7±6.9
	15	14.9±2.6
Perillyl alcohol	1200	1209±168
	600	599±36
	300	301±24
	100	105±17
Sobrerol	240	245±22
	120	122±8.5
	60	60.5±4.1
	30	28.2±3.4

[a] Concentration of the monoterpene added to the AIN-76A diet.

4. Conclusions

A simple, rapid and inexpensive isocratic HPLC assay was developed for the analysis and quantification monoterpenes, i.e., carvone, cineole, perillaldehyde, perillyl alcohol and sobrerol in laboratory animal diet. The extraction of the diet and the assay are easy to perform using a standard HPLC system with a simple isocratic mobile phase. The repeatability, reproducibility, precision and accuracy of the assay are appropriate for the quality control monitoring in bioassays of the formulation and stability of monoterpenes added to the diet used to administer them to laboratory animals.

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