Response Factor Considerations for the Quantitative Analysis of Western Redcedar (*Thuja plicata*) Foliar Monoterpenes*

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Abstract

A method is described for quantitative analysis of monoterpenes in western redcedar (*Thuja plicata*) foliage by gas chromatography with flame ionization detection. Response factors for monoterpenes identified in redcedar are evaluated to determine similarities among monoterpene responses. Evaluation demonstrates that redcedar monoterpenes yield detector responses that fall into two groups. One monoterpene from each group is used as a standard for quantitative analysis. Redcedar monoterpenes are quantitated by comparing analyte response with the response factor of one of the standards in single-point calibrations. Homogenized foliage samples are extracted with ethyl acetate and the extracts passed through a solid phase extraction column of graphitized carbon to remove plant pigments. Method bias and repeatability are evaluated by fortifying foliage samples with (1S)-(+)-carvone and (1S)-(+)-2-carene and subjecting the samples to the extraction and analysis procedures. Detection limits are also assessed from fortified samples. Excellent recovery (> 95.0%) and precision (< 5%) are obtained from the analysis of 2-carene from fortified samples. Carvone recovery is approximately 80% with excellent precision (< 4%). The method limits of detection obtained from 2-carene and carvone fortified samples are 4.7 and 13.5 µg/g, respectively.

Introduction

Owing to volatility, gas chromatography (GC) is the method of choice for monoterpene analysis in conifers (1). Because of the availability of enantioselective stationary phases, GC is also widely employed for the analysis of monoterpene enantiomers (2). Detection of monoterpenes can be achieved by either mass spectrometry (MS) or flame ionization detection (FID). MS offers the advantage of spectral identification of the analytes. However, its usefulness for quantitative analysis can be limiting because response factors (RF) are not constant over typical concentration ranges of interest (3). Thus, an external standard is required for each analyte and single-point calibrations would not be valid for quantitative analysis. Conversely, FID provides no spectral information but is much better suited for quantitative analysis because detector response is presumed to be directly proportional to the number of carbon atoms in the molecule (4).

Essential oils present in conifers serve many roles in plant-animal interactions. Research of these interactions in conifers has demonstrated that the abundance and distribution of monoterpenes play important roles in mammal and insect behavior (5–9). Furthermore, the enantiomeric composition of conifer monoterpenes has been shown to influence insect behavior (10,11). In contrast to plant-insect interactions, studies of conifer-mammal interactions rarely account for the enantiomeric composition of essential oil constituents.

The goal of this research was to develop an analytical method for the determination of monoterpenes in western redcedar (*Thuja plicata*) foliage that provides quantitative information for future studies of black-tailed deer (*Odocoileus hemionus columbianus*) monoterpene preferences. To achieve this goal, comparisons were made among monoterpene RFs, and a quantitative method was designed with single-point calibration versus a minimum number of external standard compounds. An additional objective was to employ a chromatographic system to yield quantitative and qualitative data regarding monoterpene enantiomers in redcedar.

Experimental

Equipment

A Hewlett-Packard Model 5890 Series II GC equipped with electronic pressure control and FID (Agilent Technologies, Wilmington, DE) was used for chromatographic analyses. The GC

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was equipped with two fused-silica capillary columns linked in series with a fused-silica fitting (Press-tight, Restek, Bellefonte, PA). The first column (attached to the injection port) was a 30-m × 0.25-mm β-cyclodextrin capillary column with a 0.25-µm film thickness (DB-CDX-B, Agilent Technologies). The second column (attached to the first) was a 30-m × 0.25-mm 5% phenyl–methylpolysiloxane capillary column with a 0.25-µm film thickness (DB-5.625, Agilent Technologies).

A freezer mill (model 6850, SPEX CertiPrep Inc., Metuchen, NJ) was employed to homogenize the foliage samples and a vacuum packaging system (Food Saver Professional II, Tilia International, San Francisco, CA) was used to seal frozen, homogenized samples in disposable bags until analysis. A horizontal mechanical shaker (Eberbach, Ann Arbor, MI) and bench-top centrifuge (Fisher Scientific, Pittsburgh, PA) were used in the preparation of sample extracts. Plant pigments were removed from the extracts with 250-mg graphitized, nonporous, carbon, solid-phase extraction (SPE) columns (3-mL reservoir) (Supelco, Bellefonte, PA). The plungers from 3-mL disposable syringes were used to force extracts through the SPE columns (Becton Dickinson and Company, Franklin Lakes, NJ).

Chemicals

High-performance liquid chromatography grade methanol (EM Science, Hawthorne, NY) and ethyl acetate (Fisher Scientific, Fair Lawn, NJ) were the solvents used in this method. (1S)-(–)-α-Pinene, (1R)-(+)-camphene, (1S)-(–)-β-pinene, myrcene, (1S)-(+)-2-carene, α-terpinene, *p*-cymene, (1S)-(–)-limonene, γ-terpinene, (1S,4R)-(–)-α-thujone, (1S)-(+)-carvone, and (1S)-(+)-terpinen-4-ol were obtained from Aldrich Chemical Company (Milwaukee, WI). α-Thujene was obtained from Indofine Chemical Company (Somerville, NJ). (1R)-(+)-Sabinene was obtained from Fluka Chemica-BioChemika (Ronkonkoma, NY) and terpinolene was from TCI America (Portland, OR).

Qualitative standard solutions

Four concentrated, qualitative standard solutions were prepared in a manner that minimized contributions to monoterpenes of interest from impurities found in the technical materials (Table I). For example, the α-thujene technical material contained significant α-pinene. Thus, these two monoterpenes were not present in the same solution. The concentration of each monoterpene was targeted at 1000 µg/mL in each solution of ethyl acetate. Dilutions of each concentrated solution were made in ethyl acetate to produce five working solutions (yielding a total of 20 standard solutions), whose monoterpene concentrations ranged from approximately 5.0 to 100 µg/mL.

Table I. Identity of Monoterpenes Employed in Four Qualitative Standard Solutions

Solution	Monoterpenes
A	*p*-cymene, myrcene, and terpinolene
B	α-thujone, carvone, γ-terpinene, and 2-carene
C	camphene, α-terpinene, α-thujene, and sabinene
D	β-pinene, terpinen-4-ol, α-pinene, and limonene

Chiral calibration standard solutions

Chiral calibration solutions were prepared by dilution of individual (1S)-(–)-α-pinene and (1S,4R)-(–)-α-thujone concentrated standard solutions prepared in ethyl acetate. Six solutions were prepared with α-pinene concentrations ranging from 0.89 to 487 µg/mL and seven α-thujone solutions with concentrations ranging from 1.06 to 4930 µg/mL (one of seven solutions contained α-thujone only).

Working standard solution

A single, mixed, quantitative chiral working standard solution was prepared in ethyl acetate from the concentrated calibration standard solutions to yield an α-pinene concentration of 121 µg/mL and an α-thujone concentration of 1060 µg/mL.

Matrix fortification solutions

Fortification solutions were prepared for use in the preparation of fortified conifer samples to be analyzed for method evaluation. A mixed fortification solution containing (1S)-(+)-2-carene and (1S)-(+)-carvone was prepared in methanol. The concentration of 2-carene was 3.28 mg/mL, and the carvone concentration was 311 mg/mL. Fortification solutions for assessing the method limit of detection (MLOD) were prepared in methanol by dilution of the mixed fortification standard. The first MLOD fortification solution had a 2-carene concentration of 328 µg/mL (and a carvone concentration of 31.1 mg/mL). The second MLOD fortification solution had a carvone concentration of 778 µg/mL (and a 2-carene concentration of 8.20 µg/mL).

Inspection of detector responses

FID responses produced by the monoterpenes were first addressed without performing an enantiomeric separation. With only the DB-5.625 capillary column in place, 1-µL splitless (1.0-min purge time) injections of the qualitative standard solutions were made into the GC. The injection port temperature was 200°C and the detector temperature was 325°C. The initial oven temperature of 40°C was held for 0.5 min, followed by a 5°C/min ramp to an intermediate temperature of 110°C, and a 20°C/min ramp to a final temperature of 300°C. The run time was 24 min. The helium carrier gas was delivered using electronic pressure programming to provide a constant linear velocity of 39 cm/s (initial pressure 18.6 psi). The split vent flow was 55 mL/min. The FID gases were nitrogen (make-up gas, 30 mL/min), hydrogen (30 mL/min), and air (400 mL/min).

A single injection of each qualitative standard solution was made. The detector responses for each terpene were subjected to linear regression analysis, and RFs (concentration in micrograms per milliliter divided by area peak response) were calculated. The RFs were subjected to an analysis of variance to determine whether they varied among monoterpenes. A Tukey's test of multiple comparisons was made to distinguish the monoterpene RFs that varied from the others (12).

The DB-CDX-B column was placed in series (before the DB-5.625) for injection of the chiral calibration standard solutions into the GC. The chiral calibration standard solutions were injected in triplicate. One-microliter splitless injections were made under chromatographic conditions similar to those employed for the analyses of the qualitative standard solutions

except for the oven parameters. For enantiomeric separations, the initial oven temperature of 40°C was immediately ramped to 70°C at a rate of 1°C/min. A second temperature ramp of 5°C/min was used to elevate the temperature to 100°C, and a third ramp of 25°C brought the oven to a final temperature of 250°C, which was held for 13 min. The run time was 55 min. Additionally, a higher helium pressure (initially 33.7 psi) was required to maintain a constant linear velocity of 40 cm/s because of the effective increase in column length from using two columns in series. The detector responses for (1S,4R)-(–)-α-thujone and (1S)-(–)-α-pinene chiral calibration standard solutions were subjected to linear regression analysis and RFs (concentration and response) were evaluated for use in single-point calibration.

Sample collection

Samples were collected from trees planted at the same time as part of a larger population study and were in their eighth growing season. Green foliage was cut from two third-order branches from each compass direction of the tree. These eight within-tree foliage collections were combined to yield a unique composite sample from each tree, which maintained individual tree identities. Composite samples were retained in sample bags that were vacuum-sealed and packed in dry ice for transport and storage in a laboratory freezer at –14°C. Samples remained frozen throughout homogenization in liquid nitrogen with an automated freezer mill. Following homogenization, the foliage material was resealed in individual vacuum storage bags and returned to the freezer.

Monoterpene analysis

Between 1.00 and 1.60 g of homogenized, composite foliage (mass accurately determined and recorded) was extracted with 10.0 mL of ethyl acetate in 25-mL glass screw-top centrifuge tubes. Extractions were performed on a mechanical horizontal shaker for 10 min followed by centrifugation for 10 min. Plant pigments were removed from the extracts by loading approximately 1.5-mL aliquots onto SPE columns. The extracts were forced through the SPE columns with 3-mL syringe plungers (as if the columns were syringe bodies). The extracts were eluted directly into autosampler vials with no further clean-up. The SPE columns were not conditioned prior to the clean-up step and were used only once.

One-microliter injections were made into the GC equipped with the two capillary columns that were installed in series. The chromatographic conditions were identical to those given for the analysis of the chiral standard solutions (mentioned previously). The working standard solution (consisting of (1S,4R)-(–)-α-thujone and (1S)-(–)-α-pinene) was also injected in triplicate and the RFs were used for quantitation. The following monoterpenes were quantitated versus the α-thujone response factor: myrcene, terpinen-4-ol, α-terpinene, α-thujene, and α-thujone. Similarly, α-pinene, γ-terpinene, sabinene, 2-carene, terpinolene, β-pinene, limonene, *p*-cymene, and camphene were quantitated versus the α-pinene response factor.

For tentative identification of unknown chromatographic peaks, 1-µL injections of the extracts were also made on a GC equipped with a mass selective detector. The two capillary columns were placed on the GC in series, and identical instrumental parameters were employed (where appropriate). The detector was operated in the scan mode over the range of 33 to 300 *m/z*. Tentative identifications were made from the MS for those chromatographic peaks that did not match the retention times (t_R) of the monoterpene standards.

Method evaluation

Homogenized foliage samples from eight unique trees were fortified with 30.0 µL of the fortification solution and extracted for chiral monoterpene analysis. The fortification masses of the analytes were 98.4 µg of (1S)-(+)-2-carene and 9.33 mg of (1S)-(+)-carvone. The resulting samples represented analyte concentrations of approximately 98 and 9300 µg/g for 2-carene and carvone, respectively (assuming the sample mass to be 1.00 g). Three control samples (not fortified) were also extracted and subjected to chromatographic analysis. The mean recovery and relative standard deviation (RSD) were determined for each analyte.

Selectivity of the SPE clean-up procedure was briefly assessed by making a single injection of an ethyl acetate extract that was not subjected to clean-up and a single injection of the same extract following SPE clean-up. The detector responses of myrcene and (1S,4R)-(–)-α-thujone were compared between the two injections. The sample homogenization procedure was evaluated by performing eight replicate extractions of a single homogenized foliage sample (representing a single tree) and determining the concentration of myrcene in each replicate. Myrcene was quantitated versus (1S,4R)-(–)-α-thujone. Mean recovery and RSD were determined.

MLOD and method limits of quantitation (MLOQ) were determined by fortifying three replicate samples with (1S)-(+)-2-carene and (1S)-(+)-carvone. The first MLOD fortification solution (30 µL) was used to deliver 9.84 µg of 2-carene to three samples. For carvone, 15 µL of the second MLOD fortification solution was used to deliver 11.7 µg to each of three different samples. The MLOD was defined as the concentration of analyte required to produce a chromatographic signal equal to three times the peak-to-peak noise. Similarly, the MLOQ was defined as the concentration of analyte required to produce a signal equal to 10 times the noise (peak to peak). Values were determined for both (1S)-(+)-2-carene and (1S)-(+)-carvone.

Results and Discussion

RFs were first evaluated without an enantiomeric separation because RFs produced by enantiomers of the same monoterpene were assumed identical. Furthermore, it was not possible to obtain many of the specific enantiomers in highly pure forms. Linear regression analyses of the data obtained from injection of the qualitative standards indicated that all 15 monoterpenes yielded linear responses over the ranges investigated ($R^2 > 0.9998$). Furthermore, the FID RFs were similar among the monoterpenes evaluated (RF range = 2.89×10^{-4} to 3.86×10^{-4}; Table II). Although it is common practice to assume equivalent RF among compounds of identical carbon number and similar structure, statistically significant differences were noted among monoterpene RFs. Multiple comparisons of the RF means indi-

cated that they could be assigned to one of two groups (Table II). One monoterpene was chosen from each group for use as a quantitative standard based on having an RF near the group median and mean. Availability of the enantiomer in high purity was also a consideration.

On the basis of the selection criteria, (1S,4R)-(–)-α-thujone and (1S)-(–)-α-pinene were selected as quantitative standards. Linear regression analyses of the detector responses obtained from the injection of the chiral calibration standard solutions indicated that α-thujone and α-pinene responses were linear and proportional over the range of concentrations investigated, indicating that single-point calibrations could be employed for quantitative analysis (Table III).

The dual-column approach provided good separation of the chiral and achiral monoterpenes of interest in western redcedar foliage (Figure 1). Conversely, enantiomeric separations using only the β-cyclodextrin column failed to resolve myrcene and (1R)-(+)-sabinene, two common hydrocarbon monoterpenes in western redcedar foliage. Employing a 5% phenyl–methylpolysiloxane column in series with the β-cyclodextrin column was necessary to achieve adequate chromatographic separation for this analysis without resulting in extremely long run times. The dual columns in series provided an excellent screening tool for all the enantiomers present in conifer foliage. This is in contrast to two-dimensional GC that relies on a heart-cutting technique to focus attention on only one region of the chromatogram. Research in the past decade has demonstrated the utility of two-dimensional GC for conifer analyses (13,14). It is an extremely powerful technique for the determination of enantiomeric excess of specific compounds (2). However, the two-dimensional technique requires a modulator device for refocusing chromatographic regions of interest eluted from the first column. Such devices are often unavailable to the typical chromatographer.

(1S)-(+)-Carvone and (1S)-(+)-2-carene were used to evaluate method recovery, bias, and limits of detection because these structurally similar monoterpenes were not present in western redcedar extracts and had chromatographic t_R near prominent monoterpenes of interest. The fortification concentration of these analytes differed by approximately two orders of magnitude because (1S,4R)-(–)-α-thujone (the predominant monoterpene in western redcedar extracts) is present in concentrations approximately one to two orders of magnitude higher than the other monoterpenes (Figure 2).

The data indicate that a very homogenous sample resulted from the automated freezer mill technique. The concentration of myrcene found in the eight replicate analyses of a single composite sample was 907.1 µg/g and very little variation was observed (RSD = 4.8%). Myrcene was arbitrarily chosen for this evaluation because it was present in each extract. These data indicated that homogenized western redcedar samples need not be subjected to replicate analyses.

Bias and recovery data indicated that neither (1S)-(+)-2-carene

Table II. Statistical Grouping of Monoterpene RFs by Tukey's Multiple Comparison Test*

Grouping	Monoterpene	Mean RF (x 10^{-4})
Group 1	Carvone	3.86
	Myrcene	3.69
	Terpinen-4-ol	3.68
	α-Thujone	3.63
	α-Terpinene	3.62
	α-Thujene	3.49
	Group 1 mean	**3.66**
Group 2	γ-Terpinene	3.22
	Sabinene	3.21
	2-Carene	3.19
	α-Pinene	3.18
	Terpinolene	3.05
	β-Pinene	3.00
	Limonene	3.00
	p-Cymene	2.95
	Camphene	2.89
	Group 2 mean	**3.08**

* The unit for RF was µg/(peak area x mL).

Table III. Detector Response Data for Chiral Calibration Standards

Monoterpene	Range (µg/mL)	R^2*	Slope	*p*-Value†
(1S,4R)-(–)-α-Thujone	1.06 to 4930	0.9999	1892	0.1062
(1S)-(–)-α-Pinene	0.886 to 487	0.9994	2097	0.0857

* R^2 is the coefficient of determination.
† *p*-value is the probability associated with testing the hypothesis that the *y*-intercept of the line is zero.

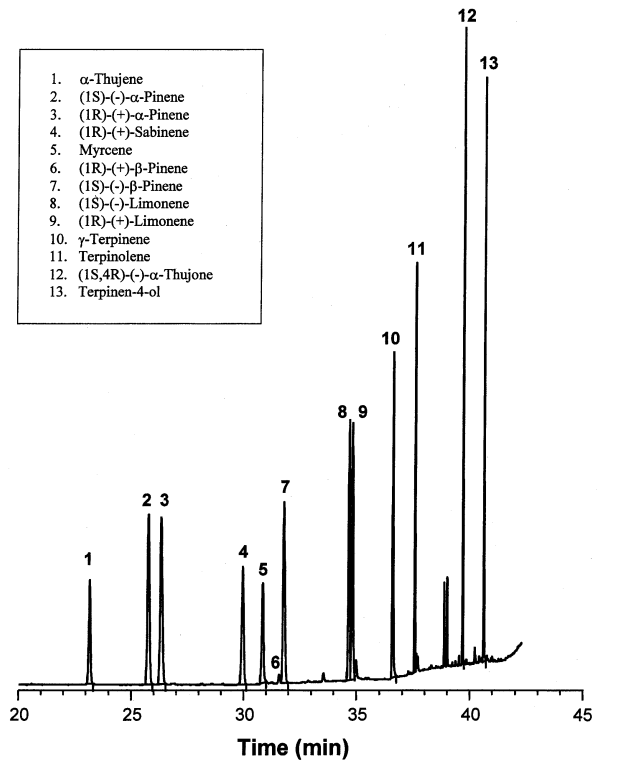

Figure 1. Chromatogram obtained from the analysis of a mixture of monoterpenes relevant to western redcedar.

nor (1S)-(+)-carvone were found in western redcedar extracts. Recovery of 2-carene from fortified samples was excellent (95.0%) and repeatability was good (RSD = 10.4%). Much of the variability observed in 2-carene recovery from the eight samples was because of a single outlier. When the recovery data from this sample were removed, the precision of 2-carene recovery was excellent (RSD = 4.2%). Recovery of carvone from fortified samples was lower than 2-carene (79.4%) but demonstrated similar precision. When evaluating the data from all eight samples, the RSD was 10.6%. However, elimination of the outlier yielded excellent precision data (RSD = 3.1%).

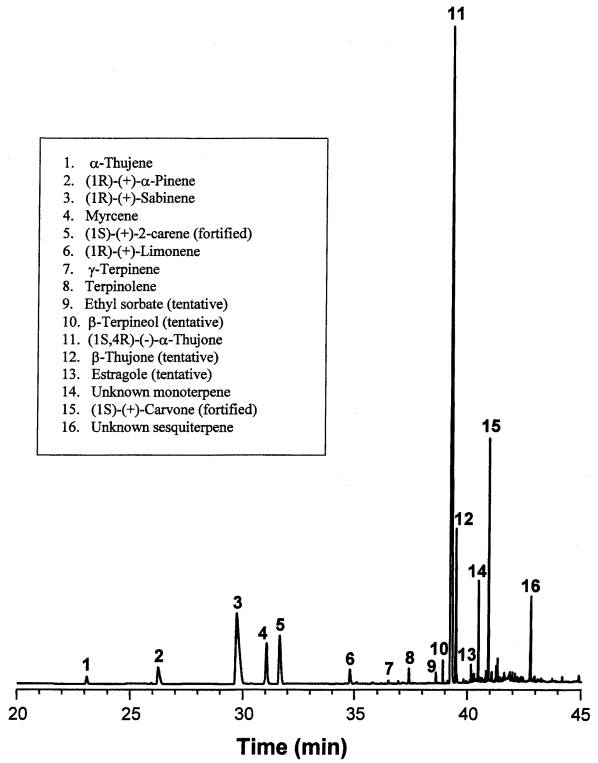

Figure 2. Chromatogram obtained from the analysis of western red cedar foliage. The 1.56-g sample was fortified with 964 µg 2-carene and 1060 µg carvone.

Table IV. Abundance of Monoterpenes Quantitated in Foliage from Eight Western Redcedar Trees

Monoterpene	Mean (µg/g)	Concentration range
α-Thujene	74.7 (85.4)*	(25.0)† 0–170
(1R)-(+)-α-Pinene	320 (366)	(154) 0–684
(1R)-(+)-Sabinene	1300 (1490)	(414) 0–3080
Myrcene	542 (620)	(187) 0–1230
(1R)-(+)-β-Pinene	11.2 (12.8)	(0.0) 0–41.0
(1R)-(+)-Limonene	138 (158)	(59.2) 0–305
γ-Terpinene	14.0 (16.0)	(0.0) 0–33.9
Terpinolene	50.7 (57.9)	(0.0) 0–121
(1S,4R)-(–)-α-Thujone	9400 (10700)	(5880) 0–20110
(1S)-(+)-Terpinen-4-ol	74.1 (84.7)	(63.7) 0–109

* Mean concentration when outlier sample is removed (n = 7).
† Low concentration when outlier sample is removed (n = 7).

As evidenced by the pigment removal of the ethyl acetate extracts and the recovery data, the SPE clean-up procedure effectively removed pigments from the ethyl acetate extracts without affecting recovery of monoterpenes. Comparison of the chromatograms obtained from injections of an ethyl acetate extract before and after the clean-up procedure demonstrated the monoterpenes were not adsorbed to the carbon stationary phase. The myrcene response in the cleaned extract was 0.7% lower than the response obtained from injection of the raw extract. Similarly, the (1S,4R)-(–)-α-thujone response was 0.6% higher in the cleaned extract. These minor sources of bias demonstrate that the clean-up procedure had no significant impact on recovery of monoterpenes.

The MLODs determined for (1S)-(+)-2-carene and (1S)-(+)-carvone were 4.7 and 13.5 µg/g, respectively. The MLOQs were 15.7 µg/g for 2-carene and 45 µg/g for carvone. The higher detection limits determined for carvone versus 2-carene were the result of lower carvone recovery and increased chromatographic noise present near the t_R of carvone as compared with 2-carene. Measured peak to peak, the chromatographic noise was 12 times higher at the t_R of carvone versus the t_R of 2-carene.

In addition to monoterpenes identified in extracts by their t_R, a number of compounds were tentatively identified by their MS. Among these were ethyl sorbate, β-terpineol, β-thujone, estragole, and several possible sesquiterpenes. The abundance of monoterpenes varied considerably among the eight unique trees analyzed for method evaluation (Table IV). Surprisingly, one sample was devoid of all monoterpenes, including α-thujone, which was very abundant in the other samples. This observation was confirmed by subsequent reanalysis of the sample by this method. The mean concentration of α-thujone in foliage of the other seven trees was 10,700 µg/g. Only one enantiomer was identified for each chiral monoterpene observed in foliage extracts (Figure 2). Only the R(+) conformers of α-pinene, sabinene, β-pinene, and limonene were found in detectable quantities in redcedar foliage. Though it is common for one enantiomer of a monoterpene to be present in large excess versus another, it was surprising to find no detectable pairs of enantiomers. For example, the S(–) conformation was identified in great excess versus the R(+) for α-pinene, β-pinene, and limonene in essential oils from several pine and fir species, though the R(+) conformers were present (15). Similarly, the S(–) conformation of β-pinene and sabinene were found in excess in several tissues of Scots pine (16) and Douglas-fir (7), though the R(+) conformations were also observed.

Conclusion

The method described here is simple to perform and yields valuable quantitative data regarding the distribution of monoterpenes in redcedar foliage. Furthermore, quantitative analysis of a number of analytes was achieved by employing single-point calibrations with only two external standard compounds. The predominant monoterpene in western redcedar foliage is (1S,4R)-(–)-α-thujone and accounts for nearly 80% of the monoterpenes

present in the extracts. Of the minor chiral monoterpenes, only the R(+) conformers of α-pinene, sabinene, β-pinene, and limonene were present. This observation suggests that chromatographic separation of enantiomers is not necessary for quantitative analysis of *Thuja plicata* monoterpenes. Significant between-tree variability in individual monoterpene abundance was observed for all monoterpenes. In fact, the complete absence of monoterpenes was observed in the foliage from one of the trees analyzed.

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