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Predicting terpene content in dried conifer shoots using near infrared spectroscopy

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Abstract

Terpenes are phytochemicals found in multiple plant genera, especially aromatic herbs and conifers. Terpene content quantification is costly and complex, requiring the extraction of oil content and gas chromatography analyses. Near infrared (NIR) spectroscopy could provide an alternative quantitative method, especially if calibration can be developed with the spectra of dried plant material, which are easier and faster to acquire than oil-based spectra. Here, multispecies NIR spectroscopy calibrations were developed for total terpene content (mono- and sesquiterpenes) and for specific terpenes (α-pinene, β-pinene and myrcene) with five conifers species (*Picea glauca*, *Picea rubens*, *Pinus resinosa*, *Pinus strobus* and *Thuja occidentalis*). The terpene content of fresh shoot samples was quantified with gas chromatography. The NIR spectra were measured on freeze-dried samples ($n = 137$). Using a subset of the samples, modified partial least squares regressions of total terpene and the three individual terpenes content were generated as a functions of the NIR spectra. The standard errors of the internal cross-validations (values between 0.25 and 2.28) and the ratio of prediction to deviation ratios (RPD values between 2.20 and 2.38) indicate that all calibrations have similar accuracy. The independent validations, however, suggest that the calibrations for total terpene and α-pinene content are more accurate (respective coefficient of determination: $r^2 = 0.85$ and 0.82). In contrast, calibrations for β-pinene and myrcene had a low accuracy (respectively: $r^2 = 0.62$ and 0.08), potentially because of the low concentration of these terpenes in the species studied. The calibration model fits (i.e., r^2) are comparable to previously published calibration using the spectra of dried shoot samples and demonstrate the potential of this method for terpenes in conifer samples. The calibration method used could be useful in several other domains (e.g. seedling breeding program, industrial), because of the wide distribution of terpenes and especially of pinenes.

Keywords

Terpenoids, evergreen, calibration, cedar, spruce, pines, plant resistance

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Introduction

Terpenes and terpenoids are organic compounds found in multiple plant genera, but are especially abundant in aromatic plants and in conifers.[1] In addition to serving basic physiological functions in plant growth and development,[2] terpenes are a key component mediating interactions among plants, animals and microorganisms.[2] These compounds can have antibacterial and antifungal activity[3–5] and can also be toxic, irritant or carcinogenic to vertebrates.[1,6–8] Several small terpenes (monoterpenes and sesquiterpenes) are volatile and can thus attract or repulse herbivores and pollinators.[1,2] Aside from their functions in ecological interactions, volatile terpenes are the principal component of resins and essential oils[9] and thereby commonly used in cosmetics, cleaning products, food and medicine.[2,9–12]

Measuring terpene concentration and composition is a costly and lengthy procedure, usually requiring the extraction of the oil content of the plants, followed by gas chromatography analyses.[13,14] Near infrared (NIR) spectroscopy could address these limitations if the NIR spectra of plant tissues can be correlated with their terpene content. Successful calibration of total terpene content or specific terpenes content with NIR spectra has been reported with oil-based samples.[13,15,16] Oil extraction, however, can

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require a relatively large amount of biomass (e.g. 100 g of dried, ground plant sample),[13] a luxury not always available in experimental studies. A few studies correlated the spectra of dried leaves to oil-based laboratory analyses with success, notably with aromatic herbs[13,17] and Eucalyptus trees,[18] and this procedure has yet to be tested for conifer trees (but see Ercioglu et al.[13] for coniferous shrubs).

As part of a project on plant chemical defence against mammalian herbivores, this study aimed to develop multispecies NIR spectroscopy calibrations for total terpene content (mono- and sesquiterpenes) and for specific terpenes with five conifers species (*Picea glauca* (Moench) Voss, *Picea rubens* Sarg., *Pinus resinosa* Aiton, *Pinus strobus* L. and *Thuja occidentalis* L.). This project was limited by the amount of biomass available, as several chemical analyses (i.e. terpene, nitrogen, phenolics, fibre content) were planned on the samples. The objective was thus to develop a calibration between the spectra of dried plant samples, which required less biomass and preparation, and laboratory analyses of terpenes realized with small amounts of fresh samples.

Material and methods

Samples

Nursery grown conifer seedlings were used. These seedlings were produced by the ministère des Forêts, de la Faune et de Parcs (Québec government) as part of an experimental assisted migration project. All seedlings were grown from seeds in spring 2017 at the provincial nursery in Berthierville, Québec, Canada. Plants for this study were sampled at the end of the second growing season (2018). The seedlings from each species were produced from three different seed provenances, spanning a latitudinal gradient in eastern North America ranging from 65°N to 72°N. Ten seedlings were randomnly selected per provenance, for a total of 30 samples per species (150 samples in total). From these seedlings, half were harvested in late summer (August 2018), and the remaining half after winter bud formation (between 28 September and 30 October, depending on the species; hereafter October). The use of several seed provenance and of two sampling periods allowed us to cover a wider range of terpene concentrations, because terpene content can vary geographically[19] and increase during the growing season.[20] Plant samples including both foliage and fine twigs were used, as mammalian herbivores (mostly cervids and leporids), are known to consume both.

Terpene analyses

The terpene analyses were conducted by a private laboratory (PhytoChemia, Saguenay, QC, Canada) using gas chromatography of non-polar extracts. In contrast to traditional distillation methods, this analysis only required ca. 2 g of fresh shoots (foliage and twigs). August samples were conserved at room temperature in plastic centrifuge tubes (capacity of 10 mL) and analyzed less than a week after collection. October samples were kept frozen (−10°C) in the same tubes for a maximum of five weeks before analyses, to account for differences among species in the timing of winter bud formation.

The samples were weighed (approximately 1–2 g) in a ball-mill container, to which were added 5 mL of pentane and 80 μL of a stock solution of methyl octanoate (1.10 mg mL^{-1} in methanol) as well as two stainless-steel balls. The container was hermetically sealed and agitated at 30 concussions per second for 5 minutes. After a brief period of settling, the container was reopened. An aliquot of pentane was then filtered over a 45 μm filter into a 1.5 mL amber screw cap vial for gas chromatography (GC) analyses. Samples were then injected on an Agilent 6890 N GC equipped with a non-polar DB-5 column (10 m × 0.10 mm × 0.10 μm) as well as a flame ionization detector. Representative samples were also injected on an Agilent 7890 A GC coupled to an Agilent 5975 C InertXL EI/CI mass spectrometer, equipped with either an HP-5MS column (30 m × 0.25 mm × 0.25 μm), to validate compounds identification. Selected compounds were identified from their retention indexes as calculated from C7 to C40 straight-chain alkane standards, and mass spectra.[21] Quantification of target compounds was obtained using predicted response factors against the methyl octanoate internal standard.[22] Concentrations were expressed in milligrams of volatile compounds per gram of fresh needles. A Limit of Quantification of 0.1 mg g^{-1} (rounded) was used for *Picea* spp. and *Thuja*, and of 0.01 mg g^{-1} for *Pinus*, because of their lower number of detectable compounds. Compounds under these thresholds were ignored; we also ignored resin-based compounds and retained only volatile compounds (mono- and sesquiterpenes). Analyses could not be replicated because the small remaining sample mass was required for additional chemical analyses. Consequently, the standard error of the laboratory analysis could not be estimated. The reported standard deviation for this method is low (5-20%) and known to increase with decreasing compound concentration.[22]

Laboratory analyses identified a total of 50 specific terpenes in all species, and of three unidentified compounds in *Picea rubens* (see complete list of terpenes in Table A1, Appendix 1). The rest of this study will focus on total terpene concentration and on the concentration of three terpenes present in all species (Table 1): α-pinene, β-pinene (coeluted with sabinene, as a minor component) and myrcene. Four other terpenes present in all species were excluded, either because of their extremely low concentrations (α-humulene and β-caryophyllene) or because they occurred in coelutions for a subset of species (camphene and limonene). The terpenes concentration

Table 1. Mean, standard deviation, minimal and maximal values for content in total terpene, α-pinene, β-pinene and myrcene in the samples of five conifer species.

	Sample size	Total terpene				α-pinene				β-pinene				Myrcene			
		Mean	SD	Min.	Max.	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
All species	137	7.8	4.3	1.6	19.5	1.2	0.9	0.1	4.7	0.6	0.5	0.1	2.5	0.3	0.3	0.03	2.2
Per species																	
Picea glauca	30	11.4	3.8	4.6	19.5	1.2	0.4	0.4	2.1	0.3	0.1	0.1	0.6	0.5	0.4	0.2	2.2
Picea rubens	29	10.1	3.2	3.9	18.5	1.4	0.4	0.6	2.4	0.2	0.1	0.1	0.5	0.4	0.3	0.1	1.3
Pinus resinosa	29	2.8	0.9	1.6	4.7	1.1	0.4	0.6	2.8	1.3	0.5	0.5	2.5	0.05	0.01	0.03	0.08
Pinus strobus	19	4.5	1.4	1.8	7.0	2.9	1.0	1.2	4.7	0.8	0.3	0.4	1.4	0.2	0.1	0.03	0.5
Thuja occidentalis	30	8.7	2.7	5.3	16.9	0.2	0.1	0.1	0.4	0.6	0.3	0.2	1.7	0.3	0.1	0.2	0.6
Calibration set	107	7.9	4.2	1.7	19.5	1.2	0.9	0.1	4.2	0.6	0.5	0.1	2.1	0.3	0.3	0.03	2.2
Validation set	30	7.2	4.5	1.6	16.1	1.4	1.1	0.1	4.7	0.6	0.6	0.1	2.5	0.3	0.2	0.03	0.9

Note: The means presented here are combined values for seedlings of three seed provenances per species and two sampling periods. Content is reported as mg g^{-1}.

differed among species but also between sampling periods: terpene content was higher in October than in August (Table A2 and Figure A1; Appendix 1).

NIR spectral acquisition

The remaining shoots of each seedling were collected for NIR spectral acquisition and additional chemical analyses. For the smaller species, *Pinus* spp. and *Thuja*, the samples also included the main stem, with the exception of the last few centimeters at the bottom. The seedlings were cut in small parts and frozen in paper bags (−10°C); this manipulation lasted less than 15 min, to limit volatile compounds release between cutting and freezing.[23,24] Samples were kept frozen until they could be freeze-dried for 48 hours (max. conservation time: 4 months). The dried samples were milled using a 2 mm sieve (Ultra Centrifugal Mill, Type ZM200, RETSCH).

The samples were scanned immediately after milling between 400.0 and 2,498.2 nm (intervals of 0.5 nm), using a FOSS NIRS DS500 near infrared spectrophotometer (FOSS Analytical A/S, HillerØd, Denmark; room temperature of 22°C). The samples were placed in a FOSS small sample cup (diameter of 7 cm; code 60048084) with a cover to ensure a good coverage of the sample on the glass (approximate depth of sample >2 mm). Not all samples could be scanned, because of the low amount of biomass available for some of them; 137 spectra were thus collected for the NIR models out of 150 samples available (see Table 1 for sample size per species).

NIR calibration and validation

The calibrations between laboratory values of terpene content and NIR spectra were developed on a subset of samples, and the remaining samples were used for model validation. The samples were divided randomly in a calibration set (n = 107) and a validation set (n = 30). The calibration set was distributed among species, and included 24 *Picea glauca*, 24 *P. rubens*, 20 *Pinus resinosa*, 14 *P. strobus* and 25 *Thuja occidentalis*. Modified partial least squares regression were used for calibration development,[25] using full (leave-one out) cross-validation,[26] without removing outliers. All models were constructed and validated using WinISI 4.8.0 (FOSS Analytical A/S, Hillerød, Denmark). Following WinISI guidelines, eight combinations of derivatives (first or second) and scatter correction (no correction, standard normal variate and detrend, standard normal variate only)[27,28] were compared. For all models, the gap over which the derivative is calculated was set at 16, and the first and second smoothing factors at 16 and 1, respectively. The equation with the lowest standard error of the cross validation (SECV) and the highest 1-variance ratio (1-VR) was selected. The presence of outliers in the observations was verified with global Mahalanobis distances. Observations with values above 3 are generally considered to be outliers using WinISI.[29]

Calibration accuracy was evaluated using several indicators: the number of MPLS (modified partial least squares) factors,[26] the coefficient of determination of the full-cross validation (r^2), the performance-to-deviation ratio (RPD)[30] and the range error ratio (RER). The RPD was calculated as the ratio of the standard deviation of the calibration set to the standard error of the prediction. A ratio of 1 indicates inaccurate predictions, and Williams and Sobering[30] suggest that values over 2.5 are satisfactory for screening, while values between 5 and 10 are adequate for quality control. The RER was calculated as the ratio of the range of concentration in the calibration set to the calibration standard error. Williams[31] recommends RER values above or equal to 10.

The validation set was used to realize an independent validation of the selected models. The presence of outliers was verified in the validation set using the Global Mahalanobis distances and the Neighbourhood Mahalanobis distances. A sensitivity analysis was performed to evaluate the robustness of our calibration to

the completely random division of the samples in the calibration and validation sets. Four additional divisions in calibration and validation sets were generated by selecting each 5th observation until the calibration set equalled 107. Each division was created with a different starting point. New calibrations and validations were then created for each of these additional sets.

Results and discussion

Using laboratory analyses of fresh plant samples, multispecies NIR calibrations were developed to predict the terpene content of dried samples for five conifer species. There were no outliers in the calibration set, as indicated by the low mean Global Mahalanobis distance (1.00, SD: 0.4), below the recommended threshold of 3. Based on the internal full-cross validation, all calibrations had comparable accuracy (r^2 values in Table 2). The model for myrcene, however, included a higher number of MPLS factors, which indicates a higher level of uncertainty.[26] The values of performance-to-deviation ratio (RPD) were all slightly below the recommended 2.5 rule-of-thumb[30] (Table 2), and suggest that the overall accuracy of calibrations is low. The range error ratio (RER), however, was above 10.0 for all calibrations.

The independent validation demonstrated differences in accuracy among calibrations. The calibrations for total terpene content and for α-pinene content presented a higher coefficient of determination for the relation between predicted and laboratory values (r^2 values 0.85 and 0.82, respectively; Table 3). Based on the slope of the models, the calibrations for total terpene and α-pinene seems reliable to extreme values in terpene or in α-pinene concentrations; a slope close to one is considered reliable to extreme values.[26] The validation set did not present outliers (mean global Mahalanobis distance $= 1.0 \pm 0.5$), and was similar to the calibration set (mean neighbourhood Mahalanobis distance $= 0.5 \pm 0.3$), although some of the terpene content value were outside the range of the calibration dataset (Table 1). Both calibrations were robust to the selection of the calibration and validation set, as shown by the similar calibrations obtained with four different divisions of the samples in calibration and validation sets (Appendix 1, Table A3). However, the additional calibrations for α-pinene presented lower RDP and RER, suggesting a completely random division of samples in calibration and validation set was a better method than selecting nth samples. Total terpene content is a global measure that can be used as a proxy of plant resistance to herbivores.[32] For example, higher terpene content has been linked to lower consumption of conifers by several cervids such as *Cervus elaphus*,[33] *Alces alces*[34] and *Odocoileus hemionus*.[35] Specific terpenes including α-pinene can also decrease consumption by large herbivores.[36,37] Pinenes are the main constituent of pine oil and consequently the most abundant naturally occurring terpenes.[38] They are also present in numerous other species, including aromatic herbs,[13] thereby suggesting that the method used here could be applied to other species, coniferous or not.

The independent validation suggest that predictions were less accurate for β-pinene content, although the slope of the calibration indicates it is reliable to extreme values[26] (Table 3). The calibration for myrcene content had a very low accuracy, and should not be used for predictions (Table 3). Calibrations realized with different calibration sets were also of low accuracy (Appendix 1, Table A3). Lower accuracy could result from the low concentrations of β-pinene and myrcene in the samples as opposed to higher and more variable concentrations in total terpene content and α-pinene content

Table 3. Independent validation set ($n = 30$) results for each total terpene, α-pinene β-pinene and myrcene content, based on the comparison between terpene content obtained from the laboratory analyses of fresh samples and the predicted terpene content obtained with the calibrations presented in Table 2.

	SEP	r^2	Slope	Intercept	Bias
Total terpene	1.73	0.85	1.04	−0.07	0.23
α-pinene	0.51	0.82	1.14	−0.04	0.14
β-pinene	0.34	0.62	0.93	0.01	−0.04
Myrcene	0.30	0.08	0.25	0.17	−0.08

Table 2. Statistics for the the selected modified partial least squares (MPLS) regressions for total terpene content, myrcene, α-pinene and β-pinene content, between the NIR spectra of freeze-dried samples and the laboratory analyses of fresh samples.

	Mathematical pretreatment applied	Scatter correction	Number of MPLS factors	SEP	r^2_{CV}	SECV	1-VR	RPD	RER
Total terpene	2,16,16,1[a]	SNV[b]	5	1.77	0.81	2.28	0.70	2.37	10.06
α-pinene	2,16,16,1	None	4	0.41	0.77	0.49	0.68	2.20	10.00
β-pinene	2,16,16,1	SNV and Detrend	4	0.21	0.82	0.25	0.75	2.38	11.43
Myrcene	2,16,16,1	SNV and Detrend	11	0.13	0.83	0.26	0.34	2.31	16.69

[a]The first number is the derivative used, the second is the gap over which the derivative is calculated and the last two the degrees of primary and secondary smoothing.
[b]Standard normal variate.[27]

(Table 1). Reliable calibrations for terpenes with lower concentration (including myrcene) can be obtained using NIR spectroscopy, but usually these compounds are more variable among samples.[13,15,17,28] Additionally, the laboratory methods for the identification and determination of terpenes are generally less accurate at low concentrations.[22] The increased standard deviation in laboratory measures could have undermined the development of an accurate calibration with NIR spectra. Including species with higher concentrations of β-pinene or myrcene could thus improve our calibrations, and allow for an accurate prediction of these monoterpenes. This result also suggest that a good range of concentration is required for the development of accurate calibrations, for any specific terpene.

The validation r^2 reported in this study are similar to previously published calibrations for terpenes using the spectra of freeze-dried and ground samples (*Eucalyptus melliodora*, r^2 of validation = 0.88).[28] Calibrations with higher accuracy could be obtained by using the spectra of oil samples,[15,16] but oil extraction requires a large amount of biomass. Comparatively, Schulz et al.[17] reported calibrations with a higher accuracy when using air-dried samples with a wider range of concentrations than this study (e.g. r^2 of cross-validation for 1,8-cineole = 0.93, concentration range 0.36–27.70 g/100 g). Another study using air-dried samples reported lower accuracy for calibrations with ranges of terpene concentration similar or slightly lower than in this study (e.g. r^2 of cross-validation for α-pinene = 0.24, concentration range ca. 0–15 mg g^{-1})[18]. These studies suggest it should be possible to improve the precision of the existing calibrations by adding samples with a wider range of terpene concentration. Sample manipulation could be reduced before spectra collection, or samples including only shoots (foliage and fine twigs) could be scanned. In this project, sample preparation was dictated by additional chemical analyses requirements (i.e. total phenolic compounds), and needed to include all biomass available for these analyses. Although the loss of volatile compounds was reduced by using freeze-drying, some volatile compounds were likely lost during sample preparation.[39] Scanning fresh samples could potentially reduce this loss. A calibration of *Melaleuca cajuputi* 1,8-cineole content using fresh leaves, however, reports a lower validation r^2 (0.63) and RDP (1.44) than the ones obtained here for total terpene content and α-pinene content.[14] Moreover, the amount of biomass required to scan fresh samples would be high because of the small shape of conifer foliage. Fresh leaves can also be transformed into pellets,[13] but this processing does not necessarily reduce sample manipulations.

Conclusion

Calibrations predicting the total terpene content (mono- and sesquiterpenes) and pinene content of conifer shoots from five distinct species were successfully developed. These results support the idea that freeze-dried samples can be used instead of oil samples for the spectra collection of conifer trees. The calibrations developed here, however, have a lower accuracy than recommended for quantitative analyses and could be improved by including samples with a wider range of terpene concentrations. In addition to an easier sample preparation, this methodology requires less biomass, and is thus a generally more efficient approach, compared with the processing of oil samples. Because of the wide distribution of terpenes and especially of pinenes, this method could be used to develop NIR calibration for a wide range of species and thus be used in ecology, seedling breeding programs or industrial applications.

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Data availability

The spectra data and laboratory data that support this study will be deposit in the Figshare Data Repository.

Declaration of conflicting interests

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