HortScience 47(6):785–789. 2012.

Effects of Distillation Time on the *Pinus ponderosa* Essential Oil Yield, Composition, and Antioxidant Activity

Valtcho D. Zheljazkov[1]
University of Wyoming, Sheridan Research and Extension Center, 663 Wyarno Road, Sheridan, WY 82801

Tess Astatkie
Nova Scotia Agricultural College, Department of Engineering, 50 Pictou Road, P.O. Box 550, Truro, Nova Scotia B2N 5E3, Canada

Vicki Schlegel
University of Nebraska–Lincoln, Department of Food Science and Technology, 326 Food Technology Complex, Lincoln, NE 68583

Additional index words. Pinus ponderosa, essential oil content, essential oil composition, alpha-pinene, beta pinene, limonene, germacrene

Abstract. **This study evaluated the effect of distillation time (DT; 1.25, 2.5, 5, 10, 20, 40, 80, 160, 240, and 360 min) on essential oil yield, composition, and the antioxidant activity of ponderosa pine essential oil. Pine essential oil yield increased with length of the DT and reached maximum at 160 min DT. The major oil constituents were alpha-pinene and beta-pinene, ranging from 17% to 40% and from 21% to 29%, respectively, of the total oil. Overall, the concentration of alpha-pinene and beta-pinene was high at the initial DT (5–20 min) and decreased with increasing DT. The concentration of myrcene (range, 0.9% to 1.5%) was lowest at 5 min DT, then increased at 10 min DT, and did not change with longer DT. Overall, the concentrations of most other constituents (delta-3-carene, limonene, cis-ocimene, alpha-terpinyl acetate, germacrene-D, alpha-muurolene, gamma-cadinene, delta-cadinene, and germacrene-D-4-ol) were low at the initial DT and increased with increasing DT. Total yields (a function of oil yield and the concentration of individual constituents) of all constituents were generally the lowest at 5 min DT, increased with increasing DT, and reached maximum at 160 min DT. The antioxidant capacity of the pine oil in this study varied between 7.0 and 14.5 μmole Trolox/g and was unaffected by DT. This study demonstrated that DT can significantly modify the essential oil yield and composition of ponderosa pine needles. Furthermore, DT could be used to obtain pine oil with targeted chemical profiles. This report can also be used as a reference point for comparing literature reports, in which different DTs are used to extract essential oil of ponderosa pine.**

Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws), sometimes referred to as western yellow pine, is one of the most widely spread and important pines in the western United States, including Wyoming (Barbour et al., 2008). It belongs to genus Pinus (pinaceae family), which includes more than 100 species, distributed mostly in the Northern Hemisphere. Ponderosa pine is an important source for various products (Barbour et al., 2008), whereas the pine forests provide an important wildlife habitat (Kalies et al., 2010). The essential oil of ponderosa pine also has antimicrobial properties (Himejima et al., 1992; Krauze-Baranowska et al., 2002). Ponderosa pine essential oil has a wide internal and international market. For example, Krauze-Baranowska et al. (2002) reported that ponderosa pine essential oil had stronger antifungal activity against *Fusarium culmorum*, *F. solani,* and *F. poae* than oils of *Pinus resinosa* or *Pinus strobes*.

Large quantities of residual leaf biomass are produced from logging ponderosa pine or after heavy storms when trees are damaged. To reduce forest density and probability for wildfires, trees in the pine forests are often cut, generating additional biomass (Farnsworth et al., 2003; Kelkar et al., 2006). This offers an opportunity for the use of this residual biomass [mostly leaves (needles) and small branches] to be used for essential oil production. In some areas of the western United States, ponderosa pine has been severely affected by the Western pine beetle (*Dendroctonus brevicomis* LeConte) (Costello and Schaupp, 2011; Negrón et al., 2008) rendering additional biomass for potential use as pine essential oil.

The essential oil synthesis and accumulation in plants is affected by a number of factors, as reviewed recently by Figueiredo et al. (2008). It has been demonstrated with some other essential oil species that the length of the DT may affect essential oil yield and composition, but not oil bioactivity (Cannon et al., 2012; Zheljazkov et al., 2012). Some researchers used 180 min DT for extraction of ponderosa pine essential oil (Kurose et al., 2007). However, there is no comprehensive study in the literature on the effect of DT on ponderosa pine essential oil yield, composition, and bioactivity. We hypothesized that DT may affect essential oil composition, antioxidant activity, and, hence, may be used as a tool for obtaining pine oil with desirable characteristics to meet specific market requirements. The objective of this study was to evaluate the effect of DT (5, 10, 20, 40, 80, 160, 240, and 360 min) on yield, composition, and the antioxidant activity of ponderosa pine essential oil.

Material and Methods

Steam distillation and distillation times. Fresh pine needles and branches less than 2 mm in diameter were used in this study. The samples were generated from a single tree (≈60–70 years old) from the shelterbelt tree project at the University of Wyoming Sheridan Research and Extension Center in the spring of 2011. To avoid variables associated with drying of the biomass, the pine needles were harvested before each set of distillations, fresh samples were generated within 5 min of the harvest, and the samples were distilled as soon as the material was loaded into the bioflasks. Distillation was performed on 500 g of fresh pine needles plus branches less than 2 mm thick. Distillations were performed in three replicates for each one of the DTs. The pine essential oil was extracted in 2-L steam distillation units as described previously for other essential oil crops, peppermint and spearmint (Zheljazkov and Astatkie, 2011; Zheljazkov et al., 2010).

Ten DTs were evaluated in this study: 1.25, 2.5, 5, 10, 20, 40, 80, 160, 240, and 360 min, all in three replicates. However, the 1.25 and 2.5 min DT did not yield sufficient essential oil for analyses and were excluded from this study. All DTs were measured from the beginning of the actual distillation. At the end of each DT, the heating was turned off and the Florentine vessel removed from the apparatus to collect the oil. The weight of the resulting essential oil was measured on an analytical balance, and the essential oil yield (content) was calculated as the amount (g) of oil per weight (kg) of fresh plant tissue. The oils were kept in a freezer at –5 °C until analyses.

Gas chromatography analysis of essential oil. The ponderosa pine essential oil samples from each treatment and replicate were analyzed on a Hewlett-Packard gas chromatograph (GC) 6890 GC with an autosampler [carrier gas helium, 40 cm·sec^{-1}, 11.7 psi (60 °C), 2.5 mL·min^{-1} constant flow rate; injection: split

Received for publication 5 Mar. 2012. Accepted for publication 23 Apr. 2012.
This research was supported by the University of Wyoming funds awarded to Dr. Valtcho D. Zheljazkov.
We thank Mrs. Adrienne Olson for her help with the extraction of the ponderosa pine oil.
[1]To whom reprint requests should be addressed; e-mail valtcho.pubs@gmail.com or vjeliazk@uwyo.edu.

60:1, 0.5 µL, inlet 220 °C; oven temperature program: 60 °C for 1 min, 10 °C·min^{-1} to 250 °C]. The GC column was HP-INNOWAX (crosslinked polyethylene glycol; 30 m × 0.32 mm × 0.5 µm), and the flame ionization detector (FID) temperature was 275 °C. For almost all samples, 99% of the compounds seen on the GC-FID was identified; the lowest identification was 98.3%. The identification of the individual compounds were done by GC–mass spectroscopy.

Antioxidant activity of selected pine essential oils. The antioxidative capacity of the essential oils was determined by the oxygen radical absorbance capacity method as described by Huang et al. (2002a, 2002b). Briefly, samples of extracted oils were prepared for antioxidant capacity tests by mixing 10 ± 1 mg oil with 1 mL of water and acetone (1:1) with 7% methyl-β-cyclodextrins (w:v). The fluorescent probe, fluorescein (8.16 × 10^{-5} mM), was incubated with different concentrations of Trolox (which served as the standard) and the oil samples for 10 min, 3 min of which was with shaking. After incubation, the reaction was activated by adding 153 mM 2, 2'-azobis (2-amidinopropane) hydrochloride, i.e., the radical initiator. All samples/standards were prepared in 96 well plates and monitored with a BMG Labtech FLUOstar Optima microplate reader (Durham, NC). Fluorescence was measured every 1.5 min at an excitation and emission wavelength of 485 nm and 520 nm, respectively, until the decreasing fluorescence values plateaued. From these data, the area under the decay curve was calculated and the results are shown as µmol Trolox equivalents/g of oil extract. Each sample was tested in triplicate.

Statistical methods. The effect of DT on essential oil content and the concentration and yield of alpha-pinene, beta-pinene, myrcene, delta-3-carene, limonene, cis-ocimene, linalyl anthranilate, alpha-terpinyl acetate, germacrene-D, alpha-muurolene, gamma-cadinene, delta-cadinene, and germacrene-D-4-ol was determined using one-way analysis of variance. For each response, the validity of model assumptions was verified by examining the residuals as described in Montgomery (2009). Because the effect of DT was significant ($P < 0.05$) on all responses, multiple means comparison was completed using Duncan's multiple range test method at the 5% level of significance and letter groupings were generated. The analysis was completed using the GLM Procedure of SAS (SAS Institute Inc., 2008).

The most appropriate regression model to describe the relationship between DT and concentration response variables was either the Michaelis-Menton (Eq. 1) or the Power (Eq. 2). Both are nonlinear. However, the relationship between DT and yield response variables except 1-Octen-3-ol (where the relationship was very weak) was adequately described by either the nonlinear Michaelis-Menton (Eq. 1) or the linear third-order polynomial (Eq. 3). The parameters of the nonlinear models were estimated iteratively using the NLIN Procedure of SAS (SAS Institute Inc., 2008).

$$Y = \frac{\theta_1 x}{\theta_2 + x} + \varepsilon \quad (1)$$

$$Y = \theta_1 x^{-\theta_2} + \varepsilon \quad (2)$$

$$Y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \varepsilon \quad (3)$$

where Y is the dependent (response) variable, x is the independent (DT) variable, and the error term ε is assumed to have normal distribution with constant variance.

Results

Pine essential oil yield. Pine essential oil yield (content) increased with increasing length of the DT and reached a maximum of 1.02 g·kg^{-1} at 160 min DT (Fig. 1). Further increase in DT actually slightly reduced essential oil yield relative to the one at 160 min DT.

Pine essential oil composition as a function of distillation time. Alpha-pinene and beta-pinene were the major oil constituents in pine oil, ranging from 17% to 40% and from 21% to 29% of the total oil, respectively (Fig. 1). Overall, the concentration of these two low-boiling oil constituents (alpha-pinene and beta-pinene) was high at the initial shorter DT (5–20 min) and decreased with increasing DT. However, the concentration of other low-boiling constituent myrcene (range, 0.9% to 1.5%) was lowest at 5 min DT, then increased at 10 min DT, and did not change with further increase of DT. The concentration of delta-3-carene (range, 3.0% to 4.4% of the oil) was low at 5 min DT, increased at 10 min, and in general stayed at ≈3.5% to 4.4% in all other DTs. The concentration of limonene (range within 2.0% to 3.7% of the oil) was low at 5 min DT, increased at 10 min, and then reached a maximum at 240–360 min DT. Cis-ocimene (range within 0.08% to 1.2% of the oil) followed a similar trend as the concentration was low at 5 min DT, increased at 10 min DT, then increased again at 40 min DT, and reached a maximum at 240–360 min DT.

The concentration of linalyl anthranilate (range within 5% to 9%) was lowest at the 360 min DT, higher at the 10 min DT, and peaked at the 80 min DT; the other treatments were not different from the 10 min or 80 min DT. The concentration of alpha-terpinyl acetate (0.87% to 1.6%) was lowest at 5 to 40 min DT, increased at 80 min DT, and then plateaued with increasing DT (Fig. 1). The concentration of germacrene-D (range within 0.1% to 19%) was the lowest at 5 min DT, increased with longer DT reaching a maximum at 80 min DT, and did not change further with increasing DT (Fig. 1). The concentration of alpha-muurolene (range within 0% to 2.2%) was not detectable at 5 min, was low at 10–40 min DT, increased at 80 min DT, and did not increase further with increasing DT. However, its concentration at 160 min was lower than that at 360 min, but not different from other treatments that had measurable amounts.

The concentrations of gamma-cadinene (range within 0.4% to 1.8%) and delta-cadinene (range within 0.4% to 4.6%), respectively, were low in the initial shorter DT and increased with DT to reach maximum at 240–360 min DT. The concentration of germacrene-D-4-ol was not detectable at 5 min DT and then stayed at 0.5% to 0.9% unaffected by DT 10–360 min (Fig. 1).

Yield of various oil constituents and oil antioxidant activity as a function of the distillation time. The yields (a function of oil yield and the concentration of individual constituents) of all constituents was generally the lowest at 5 min DT, increased with increasing DT, and reached maximum at 160 min DT (Fig. 2). The antioxidant capacity of pine oils extracted at 20, 80, and 360 min DT were 9.7, 7.0, and 14.5 µmole Trolox/g of oil, respectively, but they were not statistically different. The results suggest DT may not affect the antioxidant activity of ponderosa pine essential oil.

Relationships between distillation time and essential oil yield and composition. The relationship between essential oil content and DT as well as the relationships between the concentration of six constituents and DT were adequately described by the Michaelis-Menten model, of which the relationship between DT and the concentrations of myrcene and Cis-ocimen were very strong, suggesting that the fitted models shown in Figure 1 can be used to predict these concentrations at any DT. The estimated values of θ_1 (the first parameter) for myrcene and Cis-ocimen suggest that the maximum achievable concentrations for these constituents are 1.53% and 1.27%, respectively, and that it takes 2.5 min and 18.3 min (estimated values of θ_2, the second parameter) to get half of the maximum concentrations, respectively (Fig. 1). The relationship between DT and the concentrations of the other five constituents was adequately described by the Power (convex) model.

The relationship between DT and the yields of nine constituents was very well described by the Michaelis-Menten model (Fig. 2). Although the relationship for the yields of the other three constituents was not strong, it can be adequately described by a third-order polynomial (Fig. 2).

Concluding Discussion

Oil yields in this study were similar to the 0.30% yield reported by Krauze-Baranowska et al. (2002) and the 0.60% reported by Kurose et al. (2007). However, the essential oil composition in our study was dissimilar to the Kurose et al. (2007) study. For example, Kurose et al. (2007) reported 13% alpha-pinene, 38.2% beta-pinene, 10.5% estragole, 4.6% myrcene, 8.2% delta-3-carene, 7.5% terpineol, and other constituents. It is not clear if these differences are the result of environmental conditions [the sample for the Kurose et al. (2007) study was collected from Tsukuba, Japan, whereas our samples were collected from Wyoming], chemotype or subspecies differences, or drying [Kurose et al. (2007) used dried needles, whereas in this study the essential oil was extracted from fresh needles]. However, chemodiversity in ponderosa pine has been reported for the western United States (Thoss

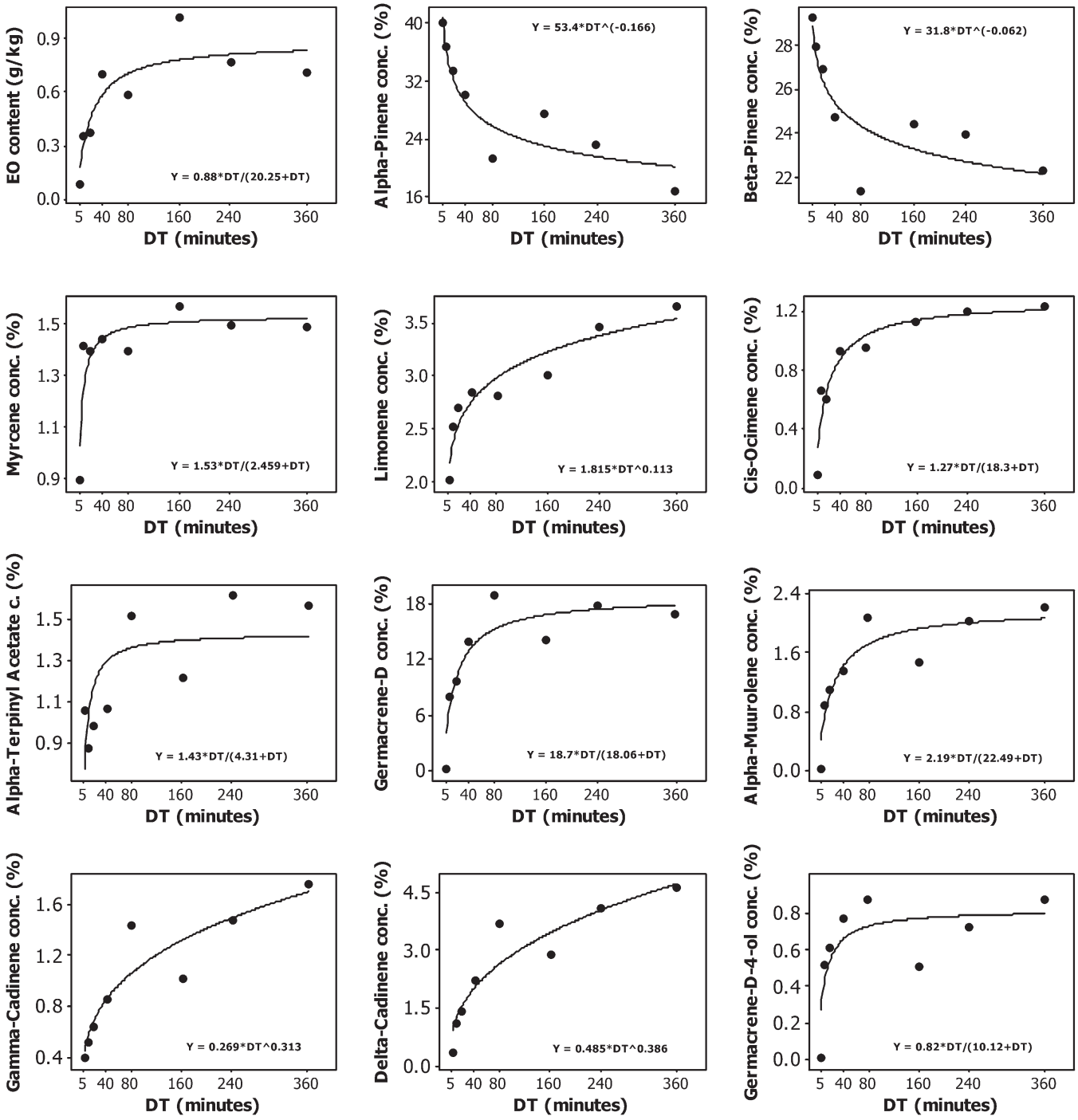

Fig. 1. Plot of essential oil (EO) content and the concentration of 11 constituents vs. distillation time (DT) along with the fitted Michaelis-Menton and Power nonlinear regression models. Equations of the fitted models are shown within each plot.

and Byers, 2006). The latter authors reported the following ranges for monoterpene concentrations in ponderosa pine oil: 18% to 69% for alpha-pinene, 2% to 57% for beta-pinene, 0% to 42% delta-carene, 1.7% to 5.2% got myrcene + alpha-phellandrene, 1.5% to 8.8% for beta-phellandrene, and 1.8% to 6.7% limonene (Thoss and Byers, 2006). Results from the latter study suggest a significant chemovariation within ponderosa pine.

An important property of essential oils is their ability to scavenge free radicals (Bakkali et al., 2008; Sacchetti et al., 2005), which, if left unchecked, can lead to the etiology and pathogenesis of various diseases (Raha and Robinson, 2000; Terranova, 2004). Such antioxidative properties have also been shown to protect against oil oxidation, the principal cause of rancidity (German, 1999). Despite the presence of essential oils in *P. ponderosa*, the antioxidative capacitiy of its essential oil has not been reported to our knowledge. The antioxidant capacity of the pine oils in this study extracted for 20, 80, and 360 min DT varied between 7.0 and 14.5 (µmol Trolox/g) and was unaffected by the DT. The results obtained from these studies (9.7, 7.0, and 14.5 µmol Trolox equivalents/g oil) fall within the range of many types of natural systems analyzed with the same assay (U.S. Department of Agriculture, 2010). The similar concentrations at the different DTs suggest that any given essential oil with antioxidative properties was not converted or lost during the distillation process. These results are analogous to findings with DT's effect on the antioxidant activity of oregano oil (Zheljazkov et al., 2012).

Results from this study confirmed the hypothesis that the length of the DT significantly modifies the essential oil yield and composition of ponderosa pine needles. However, DT had no effect on the antioxidant capacity of the pine

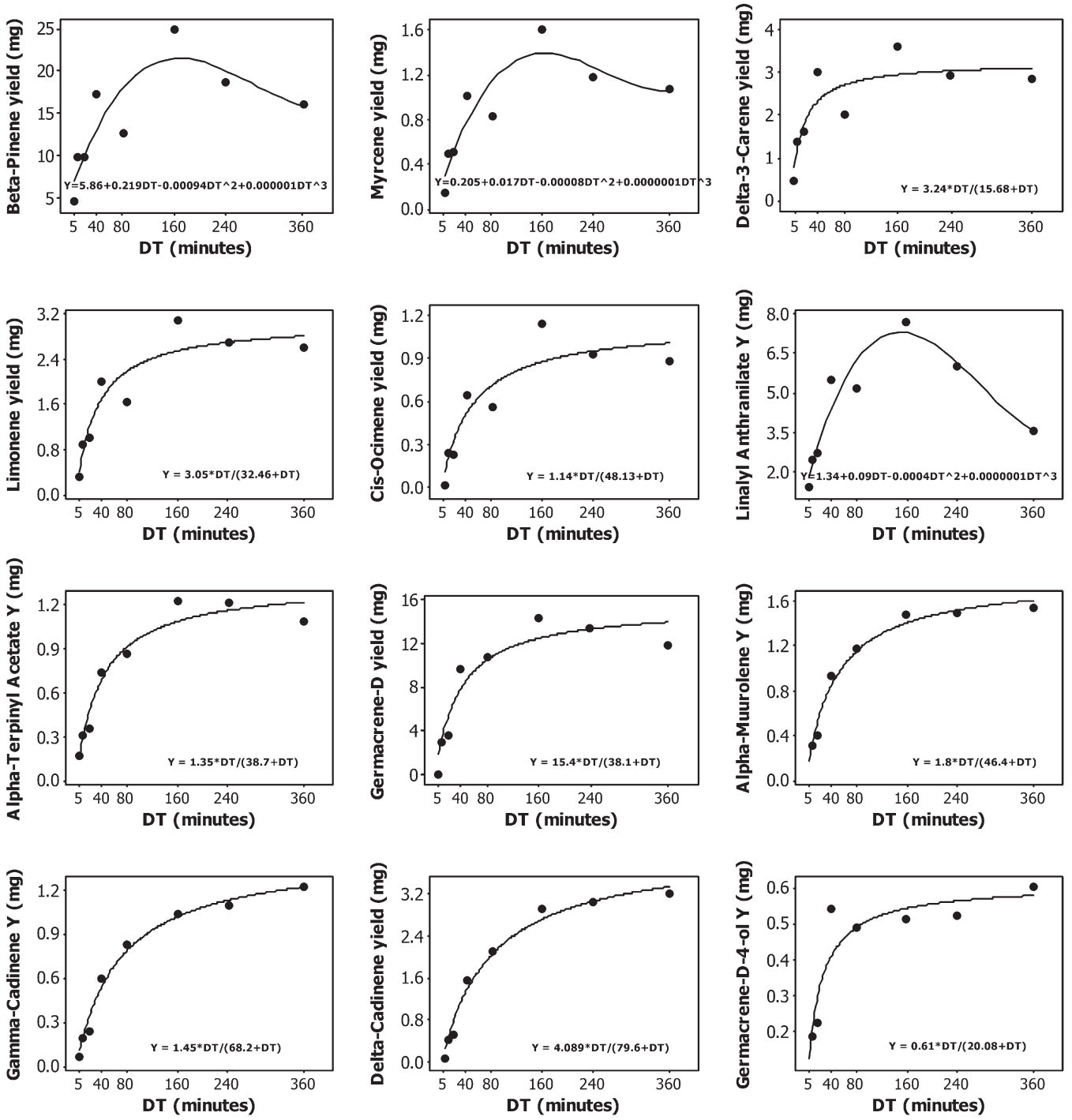

Fig. 2. Plot of vs. the yield of 12 constituents vs. distillation time (DT) along with the fitted Michaelis-Menton (nonlinear) and third-order polynomial (linear) regression models. Equations of the fitted models are shown within each plot.

essential oil. Furthermore, DT could be used as a tool for obtaining ponderosa pine oil with specific chemical profile(s). This report could act as a reference point for comparing literature reports, in which different DTs were used to extract the essential oil of ponderosa pine.

Literature Cited

Bakkali, F., S. Averbeck, D. Averbeck, and M. Idaomar. 2008. Biological effects of essential oils—A review. Food Chem. Toxicol. 46:446–475.

Barbour, J.R., X. Zhoa, and J.P. Prestemon. 2008. Timber product output implications of a program of mechanical fuel treatments applied on public timberland in the Western United States. For. Policy Econ. 10:373–385.

Cannon, J.B., C.L. Cantrell, T. Astatkie, and V.D. Zheljazkov. 2012. Modification of yield and composition of essential oils by distillation time. Ind. Crop Prod. 41:214–220.

Costello, S.L. and W.C. Schaupp, Jr. 2011. First Nebraska State collection record of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae: Scolytinae). Coleopt. Bull. 65:21–23.

Farnsworth, A., P. Summerfelt, D.G. Neary, and T. Smith. 2003. Flagstaff's wildfire fuels treatments: Prescriptions for community involvement and a source of bioenergy. Biomass Bioenergy 24:269–276.

Figueiredo, A.C., J.G. Barroso, L.G. Pedro, and J.J.C. Scheffer. 2008. Factors affecting secondary metabolite production in plants: Volatile components and essential oils. Flavour Fragran. J. 23:213–226.

German, J.B. 1999. Food processing and lipid peroxidation. Adv. Exp. Med. Biol. 459:23–50.

Himejima, M., K.R. Hobson, T. Otsuka, D.L. Kubo, and I. Kubo. 1992. Antimicrobial terpenes from oleoresin of ponderosa pine tree *Pinus ponderosa*: A defense mechanism against microbial invasion. J. Chem. Ecol. 18:1809–1818.

Huang, D., B. Ou, M. Hampsch-Woodill, J. Flanagan, and R. Prior. 2002a. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agr. Food Chem. 50:4437–4444.

Huang, D., B. Ou, M. Hampsch-Woodill, J. Flanagan, and E.K. Demmer. 2002b. Development and validation of oxygen radical absorbance capacity assy for lipophilic antioxidants using randomly methylate B-cylodextrin as the solubility enhancer. J. Agr. Food Chem. 50:1815–1821.

Kalies, E.L., C.L. Chambers, and W.W. Covington. 2010. Wildlife responses to thinning and burning treatments in southwestern conifer forests: A meta-analysis. For. Ecol. Mgt. 259:333–342.

Kelkar, V.M., B.W. Geils, D.R. Becker, S.T. Overby, and D.G. Neary. 2006. How to recover more value from small pine trees: Essential oils and resins. Biomass Bioenergy 30:316–320.

Krauze-Baranowska, M., M. Mardarowicz, M. Wiwart, L. Pobocka, and M. Dynowska. 2002. Antifungal activity of the essential oils from some species of the genus Pinus. Z. Naturforsch. C 57:478–482.

Kurose, K., D. Okamura, and M. Yatagai. 2007. Composition of the essential oils from the leaves of nine Pinus species and the cones of three of Pinus species. Flavour Fragran. J. 22:10–20.

Montgomery, D.C. 2009. Design and analysis of experiments. 7th ed. Wiley, New York, NY.

Negrón, J.F., K. Allen, B. Cook, and J.R. Jr. Withrow. 2008. Susceptibility of ponderosa pine, *Pinus ponderosa* (Dougl. ex Laws.), to mountain pine beetle, *Dendroctonus ponderosae* Hopkins, attack in uneven-aged stands in the Black Hills of South Dakota and Wyoming USA. For. Ecol. Mgt. 254:327–334.

Raha, S., and B.H. Robinson. 2000. Mitochondria, oxygen free radicals, disease and ageing. Trends Biochem. Sci. 25:502–508.

Sacchetti, G., S. Maietti, M. Muzzoil, S. Manfedini, M. Radice, and R. Bruni. 2005. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals, and antimicrobials in foods. Food Chem. 91:621–632.

SAS Institute Inc. 2008. SAS/STAT® 9.2 user's guide. SAS Institute Inc., Cary, NC.

Terranova, F. 2004. Free radicals. Part III. Clinical aspects. Cosmetic Technology 7:21–31.

Thoss, V. and J.A. Byers. 2006. Monoterpene chemodiversity of ponderosa pine in relation to herbivory and bark beetle colonization. Chemoecology 16:51–58.

Zheljazkov, V.D. and T. Astatkie. 2011. Effect of residual distillation water of 15 plants and three plant hormones on Scotch spearmint (*Mentha* × *gracilis* Sole). Ind. Crops Prod. 33:704–709.

Zheljazkov, V.D., T. Astatkie, and V. Schlegel. 2012. Distillation time changes oregano essential oil yields and composition, but not the antioxidant or antimicrobial activities. HortScience 47:777–784.

Zheljazkov, V.D., C.L. Cantrell, T. Astatkie, and A. Hristov. 2010. Yield, content, and composition of peppermint and spearmints as a function of harvesting time and drying. J. Agr. Food Chem. 58:11400–11407.