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Citation	Shiwakoti, S., Sintim, H. Y., Poudyal, S., Bufalo, J., Cantrell, C. L., Astatkie, T., ... & Zheljazkov, V. D. (2015). Diurnal Effects on Mentha canadensis Oil Concentration and Composition at Two Different Harvests. HortScience, 50(1), 85-89.
DOI	
Publisher	American Society for Horticultural Science
Version	Version of Record
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsofuse

HortScience 50(1):85–89. 2015.

Diurnal Effects on *Mentha canadensis* Oil Concentration and Composition at Two Different Harvests

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Additional index words. Mentha arvensis, essential oil concentration, essential oil composition, menthol, menthone

***Abstract*. Japanese cornmint, also known as menthol mint (*Mentha canadensis* L. syn *M. arvensis* L.), is an essential oil crop cultivated in several countries in Asia and South America. The plant is currently the only commercially viable source for natural menthol as a result of the high concentration of menthol in the oil compared with other crops. The hypothesis of this study was that harvesting at regular intervals within a 24-hour period would have an effect on essential oil concentration and composition of Japanese cornmint grown at high altitude in northern Wyoming. Flowering plants were harvested every 2 hours on 7 to 8 Aug. and on 14 to 15 Aug. and the essential oil was extracted by steam distillation and analyzed by gas chromatography–mass spectroscopy (GC-MS). The effects of harvest date (Harvest 1 and Harvest 2) and harvest time (12 times within a 24-hour period) were significant on oil concentration and yield of menthol, but only harvest date was significant on the concentration of menthol in the oil. The interaction effect of harvest date and harvest time was significant on water content and on the concentrations of menthol and menthofuran in the oil and on the yield of limonene, menthol, and menthofuran. Overall, the oil concentration in grams per 100 g dried material for the two harvests (1.26 and 1.45, respectively), the concentration of menthol in the oil (67.2% and 72.9%, respectively), and menthol yield (1066 to 849 mg/100 g dried biomass) were higher in plants at Harvest 2 as compared with plants at Harvest 1. The oil concentration was higher in plants harvested at 1100 HR or at 1300 HR and lowest in the plants harvested at 1500 HR. Menthol yield was the highest in plants harvested at 1300 HR and lowest in the plants harvested at 0700 HR, 1900 HR, or at 0300 HR. This study demonstrated that harvesting time within a 24-hour period and harvest date (maturity of the crop) may affect essential oil concentration and composition of Japanese cornmint grown at high altitude in northern Wyoming.**

Japanese cornmint (*Mentha canadensis* L. syn *M. arvensis* L.) is an industrial crop cultivated for its essential oil, which has wide personal uses and industrial applications. Japanese cornmint is the only commercially viable source for production of natural menthol because its essential oil contains high concentrations of menthol (Galeotti et al., 2002; Lawrence, 2007; Zheljazkov et al., 2010a). Japanese cornmint is grown in India, China, Japan, Paraguay, and Brazil (Lawrence, 2007; Singh and Saini, 2008), and to a limited extent in eastern Europe (Topalov and Zheljazkov, 1991; Zheljazkov et al., 1996).

Menthol is an important monoterpene used in the pharmaceutical, therapeutic, food, and cosmetic industries (Lawrence, 2007). Menthol and the dementholized oil have a stable market (Lawrence, 2007). Menthol and Japanese cornmint essential oil has been used for treatment of insomnia, nasal congestion, inflammation, irritable bowel syndrome, bad breath, headache, and other infections (Farco and Grundmann, 2013; Patel et al., 2007). Besides its medical uses, menthol is used widely in personal care products such as moisturizers, lip balms, chewing gums, and bathing products (Lawrence, 2007; Ram et al., 2006).

The United States is a major importer and user of menthol and dementholized oil; however, there is no production of natural menthol in this country. Introducing Japanese cornmint to the United States will provide a new high-value crop for American growers and domestic production of natural menthol. Previous studies indicated feasibility of growing Japanese cornmint in Mississippi (Zheljazkov et al., 2010a, 2010b) and in Wyoming (Zheljazkov et al., 2013). Although there has been research on the timing of harvest representing the stage of development of the crop on Japanese cornmint in the south of the United States (Zheljazkov et al., 2010b), there is no information on how harvest time within a 24-h period would affect oil concentration and composition of Japanese cornmint grown in a more northern climate.

Diurnal variations in essential oil content and composition of aromatic plants from the same family have been reported previously for clary sage (*Salvia sclarea* L.) (Shevchenko, 1973; Tsvetkov and Balinova-Tsvetkova, 1976), for lavender (*Lavandula angustifolia* Mill.) (Hassiotis et al., 2010), and for basil (*Ocimum gratissimum* L.) (De Vasconcelos Silva et al., 1999). Additionally, a recent study on 'Native' spearmint in northern Wyoming reported a significant effect of diurnal changes on spearmint essential oil content and composition (Bufalo et al., 2015). The effect of diurnal changes on Japanese cornmint in North America has not been investigated. Such information would be important for domestic producers of Japanese cornmint. Therefore, the objective of this study was to evaluate the effect of harvest time within a 24-h period (0700 HR, 0900 HR, 1100 HR, 1300 HR, 1500 HR, 1700 HR, 1900 HR, 2100 HR, 2300 HR, 0100 HR, 0300 HR, and 0500 HR) and harvest date (Harvest 1 and Harvest 2) on essential oil concentration and composition of Japanese cornmint.

Materials and Methods

Field and laboratory experiments. The study was conducted in 2013 at the Sheridan Research and Extension Center Experimental Fields (long. 44°45.686′ N, long. –106°55.479′

W, elevation 1170 m above sea level). In this study, we used Japanese cornmint cultivar Arvensis 3 of *Mentha canadensis* L. (synonym *M. arvensis* L.). The Japanese cornmint plantation was established in 2011 with land preparation, weed control, fertilization, and irrigation as described previously (Zheljazkov et al., 2013).

Harvesting and drying. Japanese cornmint was harvested twice with a 2-week period between two harvests in Aug. 2013. Plants were harvested at ≈10 to 12 cm above the soil surface; samples included all parts (stems, leaves, and inflorescences). The samples were obtained at each harvest time within the 24-h period on 7 Aug. and 8 Aug. for the first harvest date and on 14 Aug. and 15 Aug. for the second harvest date. The Japanese cornmint was flowering at the time of both harvests. During the first harvest, the mints had ≈25% of open flowers on the inflorescence of the main stem, whereas during the second harvest, the mint plants had almost 100% open flowers on the main stem. Japanese cornmint usually is harvested at full flowering to ensure the highest essential oil concentration and the highest concentration of menthol in the oil (Topalov, 1989). Harvests within the 24-h period were conducted every 2 h from 0700 HR to 0500 HR on the next day. Each harvest was replicated three times; hence, the total number of biomass samples, 500 g fresh weight each, was 72. The 72 samples for essential oil extraction were dried at a shady place in a well-ventilated barn (20 to 25 °C) for 4 weeks before the oil extraction. To determine the water content of each sample at harvest, a second set of fresh samples, 1 kg each, was generated from each plot and dried in a dryer at 65 °C until constant weight was achieved.

Distillation. The 72 dried Japanese cornmint biomass samples were steam-distilled for 60 min in 2-L steam distillation units to extract the essential oil as described previously (Gawde et al., 2009; Zheljazkov et al., 2010a, 2010b).

The beginning of each distillation was measured when the first drop of essential oil was deposited from the condenser and into the separator. At the end of the 60 min, the power was turned off, and the oil and the water were decanted from the separator into glass vials. The oil was separated from the water; oil weight was measured on an analytical scale, and the oil was stored in a freezer at –14 °C until all samples were extracted and could be analyzed. The essential oil concentration (yield) was calculated as grams of oil per 100 g of dry herbage.

Gas chromatography–flame ionization detection quantification of essential oil components. Japanese cornmint oil samples from all treatments and replications were analyzed by GC–flame ionization detection (FID) on an Agilent 7890 A GC System (Agilent Technologies, Santa Clara, CA) using GC equipped with a DB-5 column (30 m × 0.25-mm fused silica cap. column, film thickness of 0.25 µm) operated using the following conditions: injector temperature, 240 °C; column temperature, 60 to 240 °C at 3 °C/min, held at 240 °C for 5 min; carrier gas, helium; injection volume, 5 µL (split on FID, split ratio 25:1); detector temperature for FID was 300 °C. Commercial standards (R)-(+)-limonene, (-)-menthol, (+)-menthofuran, (-)-menthone were obtained from Fluka, Analytical (Sigma-Aldrich, St. Louis, MO). Linearity was imposed by using response factors (RF) and regression coefficients independently. Response factors were calculated using the equation RF = DR/C, where DR was the detector response in peak area (PA) and C is the concentration of the analyzed substance. The chromatograms of each of the essential oil samples from all harvests and replicates were compared with the chromatograms from standards. Target peaks were confirmed by retention time. Confirmed integrated peaks were used to determine the percentage of each chemical constituent in the essential oil. The RF of the target chemical constituent was used to determine the percentage of that constituent in each essential oil sample using the equation (PA/RF/C) × 100 = % (peak area/response factor/concentration).

Statistical analysis. The effect of harvest date and harvest time (12 levels: 0700 HR, 0900 HR, 1100 HR, 1300 HR, 1500 HR, 1700 HR, 1900 HR, 2100 HR, 2300 HR, 0100 HR, 0300 HR, and 0500 HR) on water (%), oil concentration (oil content g/100 g dry weight), the concentration (%), and yield (mg/100 g dried material) of limonene, menthol, menthone, and menthofuran was determined using analysis of variance of a 2 × 12 factorial design. The analysis was completed using the Mixed Procedure of SAS (SAS Institute Inc., 2010), and the validity of model assumptions (normal distribution and constant variance of the error terms assumptions; randomization of the run orders ensured the independence of the error terms assumption) was verified by examining the residuals as described in Montgomery (2013). The normality assumption was violated for limonene yield; however, it was valid after applying a square root transformation. Although the letter groupings are generated using the transformed values, the presented means are back-transformed to the original scale. For the responses with significant ($P < 0.05$) and marginally significant (P value between 0.05 and 0.1) interaction effect, the significance of the main effect(s) was ignored and multiple means comparison was completed by comparing the least squares means of the 24 combinations of harvest and time using the lsmeans statement of the Mixed Procedure of SAS (SAS Institute Inc., 2010). However, for the responses with a non-significant interaction effect, but significant main effect(s), least squares means of the corresponding two harvest dates and/or 12 harvest times were compared (SAS Institute Inc., 2010). In both significant main effect and interaction effect cases, because a large number of means is being compared, letter grouping was done at the 1% level of significance to reduce the overinflation of Type I experimentwise error rate.

Results and Discussion

The main effects of harvest date and harvest time were significant on oil concentration (content) and the yield of menthol, whereas only the main effect of harvest date was significant on the concentration of menthol in the oil (Table 1). The interaction effect of harvest date and harvest time (time of the day) was significant on water content, on the concentrations of menthone and menthofuran in the oil, and on the yield of limonene, menthone, and menthofuran (Table 1).

The concentration of menthone (6.8% to 15.4% of the total oil) was the highest in the oil of the plants harvested at 1300 HR during Harvest 1 and was the lowest in the oil of the plants harvested at 0700 HR during Harvest 2 (Fig. 1). The concentration of menthofuran (1.1% to 1.76% of the total oil) was the highest in plants harvested at 0100 HR during Harvest 2 and the lowest in the plants harvested at 0700 HR or 1700 HR to 2100 HR during Harvest 2. The concentration of menthofuran in plants during Harvest 1 was unaffected by the time of harvest.

Limonene yield (a function of oil concentration and the concentration of limonene in the oil) was higher in plants during Harvest 2 at 1300 HR and 0100 HR and lower in Harvest 1 plants harvested at the harvest times other than 1100 HR and 1300 HR (Fig. 2). On the other hand, menthone yield was the highest in Harvest 1 plants harvested at 1100 HR and 1300 HR and the lowest in plants at 0300 HR during Harvest 1 or at 0700 HR during Harvest 2 (Fig. 2). Menthofuran yield was the highest in plants at 0100 HR during Harvest 2 and the lowest in plants at 0700 HR during Harvest 2 (Fig. 2). There was no significant difference in menthofuran yield in plants harvested during Harvest 1 (Fig. 2).

Overall, the oil concentration (content) in dry tissue was the highest in plants harvested at 0100 HR, but it was not significantly higher than that harvested at 0900 HR to 1700 HR, 2100 HR to 2300 HR, and at 0500 HR (Table 2). Also, generally, menthol yield was the highest in plants harvested at 1300 HR and lower in the plants harvested at 0700 HR, 1900 HR, and 0300 HR (Table 2).

The oil concentration and the concentration and yield of menthol in the oil were

Received for publication 1 Oct. 2014. Accepted for publication 19 Nov. 2014.
This research was funded in part by the 2011 Sun Grant Initiative Program project and by the Department of Plant Sciences funds awarded to Dr. Valtcho Jeliazkov (Zheljazkov).
We thank the people who helped us in harvesting, namely Andrew Burkhardt, Rebecca Moreland, and Jermiah Vardiman. We also thank Solomon Green III and Amber Reichley for helping with the analyses. We acknowledge the critical review of the manuscript and the suggestions by Mr. Osama Saleh of University of Wyoming and Dr. Alex Martynenko of Dalhousie University.
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Table 1. Analysis of variance *P* values for the main and interaction effects of harvest date (H) and harvest time (T) on water content, oil concentration, the concentration, and yield (yld) of limonene, menthol, menthone, and menthofuran.

Source of variation	Water	Oil concn	Limonene	Menthol	Menthone	Menthofuran	Limonene yld	Menthol yld	Menthone yld	Menthofuran yld
H	0.001	**0.001**[z]	0.304	**0.009**	0.001	0.767	0.003	**0.001**	0.001	0.003
T	0.109	**0.005**	0.471	0.214	0.284	0.001	0.040	**0.004**	0.049	0.001
H*T	**0.014**	0.174	0.159	0.635	**0.001**	**0.004**	**0.063**	0.105	**0.001**	**0.005**

[z]Significant effects that require multiple means comparison (whose results are shown in Figs. 1 and 2 and Tables 2 and 3) are shown in bold.

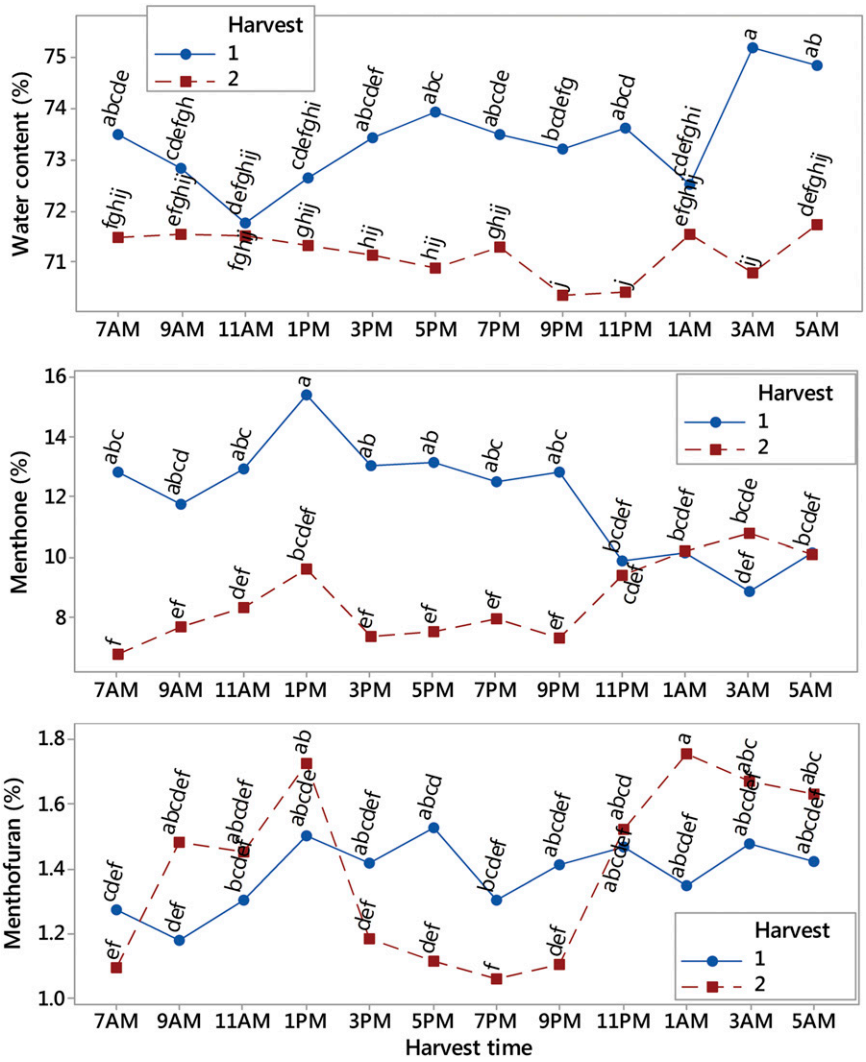

Fig. 1. Interaction plot of water content (%), and the concentrations (%) of menthone and menthofuran obtained from the two harvests and the 12 harvest times. Within each plot, means sharing the same letter are not significantly different.

higher during Harvest 2 than during Harvest 1 regardless of harvest time (Table 3). The averages at Harvest 1 and Harvest 2 were 1.26% and 1.45%, 67.2% and 72.9%, and 849 and 1066 mg/100 g dried herbage, respectively.

Time of the day is one of the main factors to be accounted for when harvesting aromatic plants for good quality and quantity of the essential oil (Filho et al., 2006; Zheljazkov et al., 2010b, 2013). The effects of harvest date and time of the day had a significant effect on the concentration of essential oil, limonene, menthol, menthone, and menthofuran and hence confirms the hypothesis of the study that harvesting at regular intervals within a 24-h period would have an effect on essential oil concentration and composition. The results also support to a certain extent the findings in a previous study with the same cultivar of Japanese cornmint in the same region (Zheljazkov et al., 2013). The highest menthone concentration of cornmint in our study was obtained at daytime harvesting (1300 HR during Harvest 1) and the lowest was obtained at morning harvesting (0700 HR during Harvest 2), average of the highest and lowest being 11.06%. This amount of menthone concentration came close to the concentration reported in most of the previous research works on Japanese cornmint. Rajeswara Rao et al. (2000), Singh et al. (2002), and Topalov and Zheljazkov (1991) reported 13.9%, 11.7%, and 12.8%, respectively, on their studies. Menthone concentrations were higher in oil obtained during Harvest 1 (25% flowering) than in oil obtained during Harvest 2 (100% flowering), which is supported by the Topalov and Zheljazkov (1991) study. The latter authors reported higher menthone concentration in oil obtained from plants harvested at 50% flowering than in oil obtained from plants harvested at 100% flowering. Similarly, Peiris et al. (1982) also reported a falling of menthone concentration as plant development progresses toward flowering. According to Peiris et al. (1982), there is a dramatic change in menthone concentration in oil obtained from plants harvested at the vegetative stage (81.4%) or at the full bloom stage (4.8%). Menthofuran is considered a toxic chemical, which is in very low concentration in plants. The highest concentration of menthofuran in our study was 1.53%, which is lower than that reported in the study by Topalov and Zheljazkov (1991) and higher than that in the studies by Lawrence (1983) and Voronina et al. (1990).

As expected, menthol was the major constituent of the Japanese cornmint oil in our study. Although menthol yield is affected significantly by timing of harvest, no distinct pattern was observed. Menthol was highest when the plants were harvested at the full-bloom stage; however, Topalov and Zheljazkov (1991) reported no difference or marginal difference between the compositions in oil obtained from 50% flowering plants and full-bloomed plants. In contrast, Peiris et al. (1982) mentioned that the concentration of menthol in the oil increases dramatically when the plants reach the full-bloom stage. The average menthol concentration in the oil of our study was 70.02% and is in agreement with other research findings on Japanese cornmint (Gasic et al., 1992; Lawrence, 1983; Peiris et al., 1982; Rajeswara Rao et al., 2000; Singh et al., 2002; Zheljazkov et al., 2013). In a study with two cultivars of Japanese cornmint in the same region as this study, menthol concentration in the oil varied from 67% to 85% depending on harvest date (Zheljazkov et al., 2013). In a study with the same cultivar of Japanese cornmint grown in Mississippi in 2008, menthol concentration of the oil varied from 78% during the first cut to 73% in the second cut (Zheljazkov et al., 2010a). Harvesting date also modified the concentration of menthol in Japanese cornmint cv. Arvensis 3 grown in Mississippi (Zheljazkov et al., 2010b).

Conclusion

This study found diurnal changes in Japanese cornmint oil concentration and composition

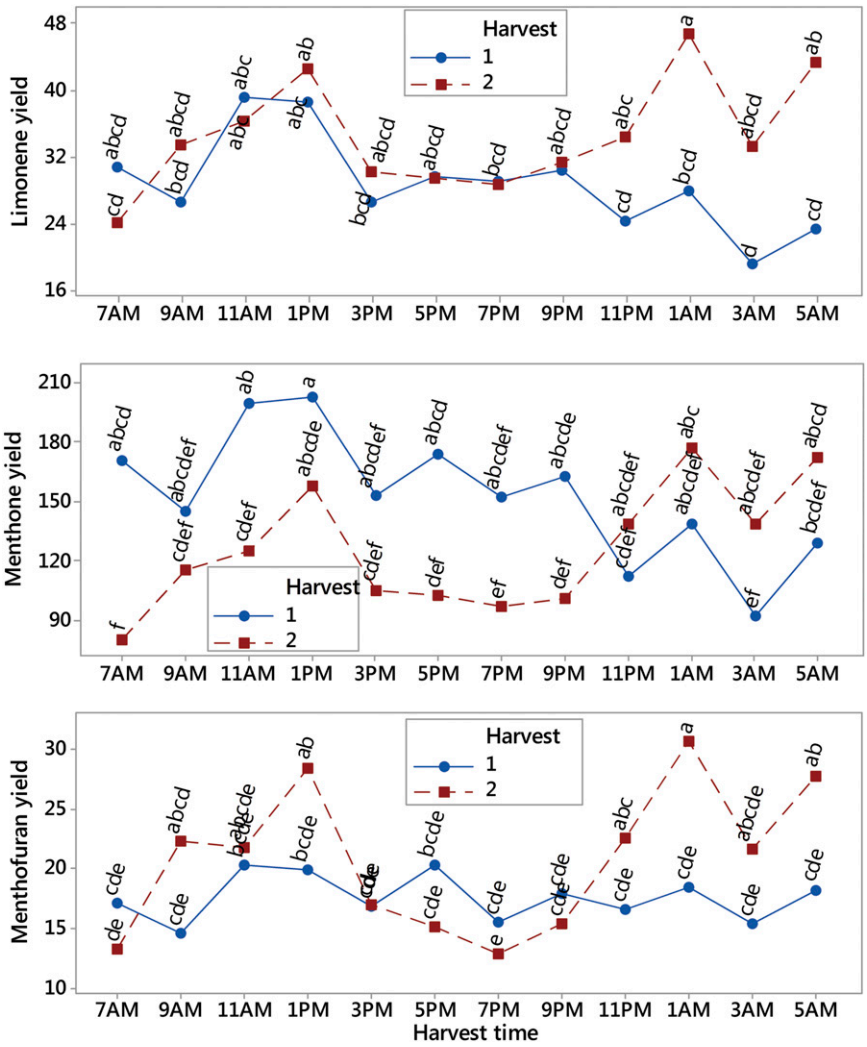

Fig. 2. Interaction plot of yields (mg/100 g dried herbage) of limonene, menthone, and menthofuran obtained from the two harvests and the 12 harvest times. Within each plot, means sharing the same letter are not significantly different.

Table 2. Mean oil concentration and menthol yield obtained from the 12 harvest times.

Time	Oil concn (g/100 g dry wt)	Menthol yield (mg/100 g dried material)
0700 HR	1.26 bc[z]	805 c
0900 HR	1.37 abc	925 abc
1100 HR	1.52 ab	1,075 abc
1300 HR	1.48 ab	1,206 a
1500 HR	1.31 abc	939 abc
1700 HR	1.33 abc	948 abc
1900 HR	1.20 c	798 c
2100 HR	1.31 abc	878 bc
2300 HR	1.30 abc	948 abc
0100 HR	1.54 a	1,124 ab
0300 HR	1.17 c	815 c
0500 HR	1.48 ab	1,030 abc

[z]Within each response variable, means followed by the same letter are not significantly different.

Table 3. Mean oil concentration, and the concentration and yield of menthol obtained from the two harvests.

Harvest	Oil concn (g/100 g dry material)	Menthol (%)	Menthol yield (mg/100 g dried material)
1	1.26 b[z]	67.2 b	849 b
2	1.45 a	72.9 a	1,066 a

[z]Within each response variable, means followed by different letters are significantly different.

grown in a northern climate. Harvest date also significantly affected oil concentration (content) and composition. However, the observed changes, although significant, were not dramatic. Therefore, from practical perspectives, Japanese cornmint grown in northern Wyoming can be harvested any time during a 24-h period. For best oil concentration and high menthol concentration in the oil, Japanese cornmint 'Arvensis 3' should be harvested during full flowering. Japanese cornmint grown under the northern Wyoming climate accumulated a significant amount of menthol.

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