Distillation Time Changes Oregano Essential Oil Yields and Composition but Not the Antioxidant or Antimicrobial Activities

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Abstract. Oregano (Origanum vulgare L.) is an important medicinal, culinary, and essential oil plant. Oregano essential oil is extracted from either leaves or shoots through steam distillation. Researchers and industry in various countries reported different distillation times (DTs) for oregano; however, there are no reports on optimum DT. This study evaluated the effect of DT (1.25, 2.5, 5, 10, 20, 40, 80, 160, 240, 360 min) on essential oil yield, composition, and antioxidant activity of the oregano essential oil. In general, the concentration of the low boiling essential oil constituents (alpha-thujene, alpha-pinene, camphene, 1-octen-3-ol, myrcene, alpha-terpinene, paracycmen, beta-phellandrene/limonene, gamma-terpinene, cis-sabinene hydrate, terpinolene) were highest at the shortest DT (1.25 or 2.5 min), reduced with increasing DT up to 40 min, and then stayed the same. However, the concentration of the major oil constituent, carvacrol, was lowest at the shortest DT of 1.25 min (18%) and increased steadily with increasing DT up to 40 min, where it leveled at 80% to 82%. The concentration of other higher boiling constituents (borneol, 4-terpineol, beta-bisabolene, beta-caryophyllene) was maximum at 5 to 20 min DT. Maximum yield of the low boiling constituents was achieved at relatively short DT, at ~20 min DT, and peaked again at 240 min DT. Maximum yields of alpha-terpinene, beta-phellandrene/limonene, and gamma-terpinene were reached at 240 min DT. Maximum yields of paracycmen cis-sabinene hydrate, terpinolene, and transsabinene hydrate were also achieved at 240 min DT, but yields at 20 min DT were not different. Yields of borneol, 4-terpineol, carvacrol, beta-caryophyllene, and beta-bisabolene also were highest at 240 min DT. Distillation time at 20, 80, or 360 min did not alter antioxidant or antimicrobial activity of oregano oil. The relationship between the concentration and yield of the constituents with DT was adequately modeled by the asymptotic and Michaelis-Menten nonlinear regression models, respectively. Results demonstrated that 1) DT can be used to obtain oregano essential oil with differential composition; 2) maximum essential oil yield of steam-distilled oregano leaves could be obtained at 240 min DT; and 3) reports on oregano essential oil yield and composition using different DTs may not be comparable. Results from this study will aid in comparing published reports on oregano essential oil that used different lengths of DT.

Materials and Methods

Steam distillation and distillation times. Bulk certified dried leaves of Origanum vulgare L. were purchased from Starwest Botanicals (Rancho Cordova, CA). The experiment was carried out at the University of Wyoming Sheridan Research and Extension Center in 2011. Sample amount for all distillations was 250 g of dried oregano leaves. Oregano essential oil was extracted through the traditional steam distillation method using 2-L steam distillation units, as described previously for peppermint and spearmint (Zheljazkov and Astatkie, 2011; Zheljazkov et al., 2010).

The following DTs were studied: 1.25 min, 2.5 min, 5 min, 10 min, 20 min, 40 min, 80 min, 160 min, 240 min, and 360 min, all in three replicates. These DTs were measured from the beginning of the distillation, when the first drop of essential oil was deposited until the end of the distillation, when the heating was turned off, the vapor pressure reduced, and the Florentine vessel (a separator) removed from the apparatus. The oil was measured on an analytical balance and kept in a freezer at ~5 °C until analyses. The oil yield (content) was calculated as grams of oil per 100 g of dried oregano leaves.

Gas chromatography analysis of essential oil. Oregano essential oil samples (all in three replicates per DT) were analyzed on a Hewlett-Packard 6890 gas Chromatograph equipped with a flame ionization detector.
Packard gas chromatograph 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 60:1, 0.5 μL, inlet 220 °C; oven temperature programmed 60 °C for 1 min, 10 °C/minute to 250 °C. The column was HP- INNOWAX (crosslinked polyethylene glycol; 30 m × 0.32 mm × 0.5 μm) and the flame ionization detector temperature was 275 °C.

Assays for in vitro antiileishmanial, antimicrobial, and antimalarial activity; assay for cytotoxicity; and antioxidant activity. Oregano essential oil from 20, 80, and 360 min DT was submitted for antioxidant activity by the ORAC·eq method as described previously (Huang et al., 2002a, 2002b). Briefly, samples of extracted oil were prepared for antioxidant capacity tests by mixing 10 ± 1 μg oil with 1 mL of water and acetonitrile (1:1) with 7% methyl-β-cyclodextrins (w/v). The fluorescent probe, fluorescein (8.16 × 10⁻³ μmol·mL⁻¹), 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 μs 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 fluorescence values plateaued. From these data, the area under the decay curve was calculated using a one-way analysis of variance. For each response, the validity of model assumptions was verified by examining the residuals as described in Montgomery (2009). Most of the concentration responses required cubic root transformation to meet the normality assumption. However, the means reported in the tables and figures are backtransformed values to their original scale. Because the effect of DT was significant (P < 0.05) on all responses, multiple means comparison was completed using Duncan’s multiple range test 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 model to describe the relationship between DT and all concentration response variables except beta-bisabolene (where there was no relationship) was the asymptotic regression model (Eq. 1). However, the relationship between DT and all yield response variables except 1-octen-3-ol (where the relationship was very weak) was the Michaelis-Menten regression model (Eq. 2). Both models are nonlinear, and their parameters were estimated iteratively using the NLIN Procedure of SAS (SAS Institute Inc., 2008).

\[
Y = \Theta_1 - \Theta_2(\exp(-\Theta_3 x)) + \epsilon \quad (1)
\]

\[
Y = \Theta_{1x} \times x - \Theta_{2x} + \epsilon \quad (2)
\]

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

**Results**

Effect of distillation time on oregano essential oil yield. Oregano essential oil yield (often referred to as content) ranged from 0.114% at the shortest DT to 2.3% at 240 min DT (Table 1). Increasing DT up to 240 min increased essential oil yields (Table 1; Fig. 1). Longest DT (360 min) reduced oil yield relative to the 240 min but increased yield relative to the shorter DT (shorter than 240 min). Range of oil constituents and response to DT. With the higher boiling point constituents, the maximum concentrations shifted from 1.25 or 2.5 min DT to 5, 10, or 20 min DT (Table 3; Fig. 2). Transsabinene hydrate was highest at 0.9% to 1.0% in 1.25 to 5 min DT and reduced with increasing DT to 20 min; further increase in DT did not change the concentrations of these constituents. Cis-sabinene hydrate was 19.4% at 1.25 min DT and reduced to 2.5% to 3% at 40–360 min DT. The concentration of cis-sabinene hydrate was 0.15% to 1%, whereas concentration of terpineol was 0.12% to 0.85%; the concentration of both constituents was highest at 1.25 to 2.5 min DT and reduced with increasing DT to 80 or 40 min, respectively (Table 2).

With the higher boiling point constituents, the maximum concentrations shifted from 1.25 or 2.5 min DT to 5, 10, or 20 min DT (Table 3; Fig. 2). Transsabinene hydrate was highest at 2% to 2.2% at 2.5 to 10 min DT, and then decreased again at 20 min and at 40 min DT. Further increase in DT did not change the concentrations relative to the 40 min DT. Beta-bisabolene was 0.69% at 1.25 min DT, increased to 1.4% at 10 to 20 min DT, and decreased to reach 0.96% at 160 min DT. Further increase in DT actually increased it up to the longest DT. A 10-fold concentration difference for most essential oil constituents resulted between the shortest and longest DT. However, the concentration of the major oil constituent, carvacrol, was lowest at the shortest DT (1.25 min) and increased steadily with increase in DT up to 40 min, where it leveled. Alpha-thujene decreased from 2.2% at 1.25 min DT to 0.17% to 0.22% at 40 to 360 min DT (Table 1; Fig. 1). Alpha-pinene decreased from 3.4% at 1.25 min DT to 2.7% at 40 min to 0.35% at 40 to 360 min DT with decreases in 

### Table 1. Mean essential oil (EO) content (%), and the concentrations (%) of alpha-thujene, alpha-pinene, camphene, 1-octen-3-ol, and myrcene obtained from the 10 distillation times (DTs).²

| DT (min) | EO yield | Alpha-thujene | Alpha-pinene | Camphene | 1-Octen-3-ol | Myrcene |
|----------|----------|---------------|--------------|----------|-------------|---------|
| 1.25     | 0.114 b  | 2.2 a         | 3.36 a       | 2.18 a   | 0.37 a      | 6.06 a  |
| 2.5      | 0.166 gh | 1.9 b         | 2.83 b       | 1.80 a   | 0.75 b      | 4.92 b  |
| 5        | 0.263 g  | 1.3 c         | 1.99 c       | 1.30 b   | 0.63 c      | 3.75 c  |
| 10       | 0.404 f  | 1.0 d         | 1.47 d       | 0.96 c   | 0.49 d      | 2.87 d  |
| 20       | 1.028 e  | 0.5 e         | 0.69 e       | 0.46 d   | 0.24 e      | 1.37 e  |
| 40       | 1.477 d  | 0.2 f         | 0.35 f       | 0.24 e   | 0.13 f      | 0.73 f  |
| 80       | 1.975 c  | 0.2 f         | 0.11 f       | 0.21 ef  | 0.11 fg     | 0.65 f  |
| 160      | 2.022 c  | 0.2 f         | 0.29 f       | 0.20 ef  | 0.10 g      | 0.61 f  |
| 240      | 2.312 a  | 0.2 f         | 0.32 f       | 0.22 ef  | 0.09 g      | 0.64 f  |

²Within each column, means followed by the same letter are not significantly different at 5%.
Yield of essential oil constituents. The yield of essential oil constituents indicates how much of the individual constituent was actually extracted at different DT and was calculated from the essential oil yield and the concentration of individual oil constituents at any given DT. Overall, the yields of the oil constituents were lowest at the shortest DT and increased with increasing DT (Tables 4, 5, and 6; Figs. 3 and 4). Maximum yield of the low boiling constituents alpha-thujene, alpha-pinene, camphene, and myrcene was achieved at relatively
short DT, at \( \approx 20 \) min DT, and then peaked again at 240 min DT (Table 4; Fig. 3). The yield of 1-octen-3-ol was also maximized at 20 min DT; further increase in DT reduced the yield relative to the one at 20 min DT (Table 4). Yields of all of these constituents at 360 min DT were lower than the ones at 240 min DT.

Table 3. Mean concentrations (%) of transsabinene hydrate, borneol, 4-terpinenol, carvacrol, beta-caryophyllene, and beta-bisabolene obtained from the 10 distillation times (DTs).

| DT (min) | Transsabinene hydrate (%) | Borneol (%) | 4-Terpinenol (%) | Carvacrol (%) | Beta-caryophyllene (%) | Beta-bisabolene (%) |
|---------|---------------------------|-------------|------------------|---------------|------------------------|---------------------|
| 1.25    | 0.86 bc                   | 2.34 c      | 1.46 d           | 17.9 f        | 1.49 b                 | 0.69 e              |
| 2.5     | 1.06 a                    | 3.24 b      | 2.10 b           | 27.7 e        | 2.07 a                 | 0.99 cd             |
| 5       | 0.97 ab                   | 3.77 a      | 2.39 a           | 37.4 d        | 2.20 a                 | 1.28 b              |
| 10      | 0.78 c                    | 4.02 a      | 2.57 a           | 44.8 c        | 2.20 a                 | 1.54 a              |
| 20      | 0.75 c                    | 2.58 c      | 1.68 c           | 68.2 b        | 1.33 b                 | 1.49 a              |
| 40      | 0.40 d                    | 1.64 d      | 1.17 e           | 79.5 a        | 0.90 c                 | 1.10 bcd            |
| 80      | 0.32 d                    | 1.29 de     | 0.95 f           | 81.8 a        | 0.77 c                 | 1.00 cd             |
| 160     | 0.29 d                    | 1.22 c      | 0.94 f           | 80.6 a        | 0.76 c                 | 0.96 d              |
| 240     | 0.35 d                    | 1.30 de     | 0.97 f           | 79.9 a        | 0.83 c                 | 1.16 bc             |
| 360     | 0.31 d                    | 1.30 de     | 1.01 f           | 81.8 a        | 0.85 c                 | 1.18 bc             |

Within each column, means followed by the same letter are not significantly different at 5%.

The yields of alpha-terpinene, beta- phellandrene/limonene, and gamma-terpinene increased with increasing DT and reached maximum at 240 min DT; yields at 360 min DT, although lower than at 240, were not significantly different. The fitted asymptotic regression models for the concentration of each constituent are shown in Fig. 2.

Fig. 2. Plot of the concentration of nine constituents vs. distillation time along with the fitted asymptotic regression model. Equations of the fitted models are shown within each plot. Because there was no significant relationship for beta-bisabolene, only the scatterplot is shown.
Table 4. Mean yields (mg) of alpha-thujene, alpha-pinene, camphene, 1-octen-3-ol, and myrcene obtained from the 10 distillation times (DTs).z

| DT (min) | Alpha-thujene | Alpha-pinene | Camphene | 1-Octen-3-ol | Myrcene |
|---------|---------------|--------------|----------|--------------|---------|
| 1.25    | 2.54 e        | 3.86 d       | 2.51 f   | 1.04 ef      | 6.98 f  |
| 2.5     | 3.11 df       | 4.68 cd      | 2.99 ef  | 1.24 e       | 8.17 ef |
| 5       | 3.47 ed       | 5.23 bc      | 3.42 de  | 1.65 d       | 9.86 de |
| 10      | 3.79 ed       | 5.71 bc      | 3.79 cd  | 2.18 b       | 11.3 cd |
| 20      | 4.77 ab       | 7.09 a       | 4.77 ab  | 2.46 a       | 14.1 ab |
| 40      | 3.24 cde      | 5.10 bc      | 3.48 de  | 1.92 c       | 10.7 cd |
| 80      | 4.04 bc       | 6.01 b       | 4.24 bc  | 2.17 b       | 12.8 ab |
| 160     | 3.71 cd       | 5.87 b       | 4.11 bcd | 2.92 bc      | 12.3 bc |
| 240     | 4.86 a        | 7.48 a       | 5.09 a   | 2.15 b       | 14.9 a  |
| 360     | 3.73 cd       | 5.92 b       | 4.09 bcd | 0.87 f       | 12.6 bc |

zWithin each column, means followed by the same letter are not significantly different at 5%.

Table 5. Mean yields (mg) of alpha-terpinene, para-cymene, beta-phellandrene/limonene, gamma-terpinene, cis-sabinene hydrate, and terpinolene obtained from the 10 distillation times (DTs).z

| DT (min) | Alpha-terpinene | Para-cymene | Beta-phellandrene/limonene | Gamma-terpinene | Cis-sabinene hydrate | Terpinolene |
|---------|-----------------|-------------|---------------------------|-----------------|---------------------|-------------|
| 1.25    | 5.80 e          | 29.8 f      | 2.26 e                    | 22.3 e          | 1.15 g              | 0.97 g      |
| 2.5     | 7.21 e          | 32.3 f      | 2.75 e                    | 27.3 e          | 1.76 f              | 1.27 f      |
| 5       | 9.14 d          | 40.4 e      | 3.44 d                    | 36.2 d          | 2.48 e              | 1.74 e      |
| 10      | 10.9 cd         | 48.1 de     | 4.16 c                    | 43.0 cd         | 3.16 cd             | 2.19 d      |
| 20      | 14.3 b          | 60.9 ab     | 5.46 b                    | 56.5 b          | 3.97 ab             | 2.97 ab     |
| 40      | 11.4 c          | 49.0 cd     | 4.47 c                    | 44.7 c          | 2.95 dc             | 2.12 d      |
| 80      | 13.7 b          | 55.7 bcd    | 5.32 b                    | 53.5 b          | 3.16 cd             | 2.66 cd     |
| 160     | 13.4 b          | 56.1 bcd    | 5.12 b                    | 51.9 b          | 2.97 dc             | 2.63 c      |
| 240     | 16.4 a          | 66.9 a      | 6.16 a                    | 63.9 a          | 4.39 a              | 3.16 a      |
| 360     | 14.1 b          | 57.5 bc     | 5.32 b                    | 54.5 b          | 3.57 bc             | 2.77 bc     |

zWithin each column, means followed by the same letter are not significantly different at 5%.

Table 6. Mean yields (mg) of transsabinene hydrate, borneol, 4-terpinenol, carvacrol, beta-caryophyllene, and beta-bisabolene obtained from the 10 distillation times (DTs).z

| DT (min) | Transsabinene hydrate | Borneol | 4-Terpinenol | Carvacrol | Beta-caryophyllene | Beta-bisabolene |
|---------|-----------------------|--------|--------------|-----------|-------------------|-----------------|
| 1.25    | 0.97 f                | 2.6 g  | 1.6 f        | 20 f      | 1.68 g            | 0.77 f          |
| 2.5     | 1.77 e                | 5.4 f  | 3.5 e        | 46 f      | 3.44 f            | 1.65 ef         |
| 5       | 2.54 d                | 9.9 e  | 6.3 d        | 99 ef     | 5.78 e            | 3.40 e          |
| 10      | 3.08 d                | 18.9 d | 11.9 c       | 187 e     | 9.76 d            | 7.35 d          |
| 20      | 7.72 a                | 26.4 bc| 17.2 b       | 703 d     | 13.7 c            | 15.3 c          |
| 40      | 5.85 e                | 24.2 c | 17.2 b       | 1175 c    | 13.3 c            | 16.3 c          |
| 80      | 6.21 bc               | 25.5 c | 18.7 b       | 1615 b    | 15.1 b            | 19.7 b          |
| 160     | 5.93 c                | 24.7 c | 18.9 b       | 1631 b    | 15.3 b            | 19.4 b          |
| 240     | 8.16 c                | 30.0 a | 22.5 a       | 1848 a    | 19.1 a            | 26.5 a          |
| 360     | 6.83 b                | 28.3 ab| 22.0 a       | 1782 a    | 18.5 a            | 25.7 a          |

zWithin each column, means followed by the same letter are not significantly different at 5%.

DT were lower than the ones at 240 min DT (Table 5). Overall, maximum yields of para-cymene, cys-sabinene hydrate, terpinolene, and transsabinene hydrate were achieved at 240 min DT, but yields at 20 min DT time were not different from the ones at 240 min DT (Tables 5 and 6). Yields of these four constituents at 360 min DT were lower than the ones at 240 min DT. Yields of borneol, 4-terpinenol, carvacrol, beta-caryophyllene, and beta-bisabolene were maxed at 240 min DT; further increase of DT did not decrease the yields relative to the 240 min DT (Table 6; Fig. 4).

Antioxidant activity of oils from various distillation times and antimicrobial activity of bulk oil. Oregano oils from the 20, 80, and 360 min did not differ in antioxidant activity (with averages 60.8, 74.5, and 60.4 μmol Trolox equivalents/g, respectively). Additionally, bulk oregano oils at 50.0 μg/mL did not have significant antimicrobial, antileishmanial, or antimalarial activity at concentrations that would warrant bioassay-directed fractionation in a drug-discovery screening program as established by the National Center for Natural Products Research in Oxford, MS. Oregano essential oil showed lower than 50% growth inhibition of Leishmania donovani, Plasmodium falciparum clones D6 and W2, Candida krusei (6% inhibition), Candida glabrata (3% inhibition), Escherichia coli (6% inhibition), Pseudomonas aeruginosa, Cryptococcus neoformans (8% inhibition), Mycobacterium intracellulare (4% inhibition), or Aspergillus fumigatus (5% inhibition) at 50 μg/mL.1

Nonlinear regression modeling results. The fitted nonlinear regression models shown in Figures 3 and 4 suggest that the relationship between yield of the constituents and DT was the Michaelis-Menten regression model (Figs. 3 and 4). However, because the fits were not as perfect as those for concentration, these models should be used with caution for prediction purposes.

Discussion and Concluding Remarks

Indeed, various researchers reported different DTs such as 60 min (Tekippe et al., 2011), 120 min (Bisht et al., 2009; Farias et al., 2010), or 180 min (Azizi et al., 2009; Sotiriopoulou and Karamanos, 2010). However, it was not known how the results from
various reports using different DTs compare, and there was no comprehensive study on the effect of DT on oregano essential oil yield and composition. This study demonstrated that maximum essential oil yields from dried oregano leaves can be achieved at 240 min DT. This study also demonstrated that DT has a significant effect on the concentration and yields of the essential oil constituents. The concentration of carvacrol, the major oil constituent, continued to increase with increasing DT up to 240 min. Shorter DT resulted in much lower carvacrol concentrations. Most other oil constituents had higher concentrations at the shorter DT (1.25 to 5 min) and then decreased, but their yield actually increased with increasing DT. The increase in the yield of essential oil constituents suggests there were no conversions or other losses of any given oil constituent during the distillation process. With the exception of beta-bisabolene concentration, the changes in essential oil yield (content) and the concentration of the constituents as a function of DT can be modeled and predicted almost perfectly by the asymptotic regression model. However, this is not quite true for the yield of the constituents.

The results on antimicrobial activity of oregano essential oil contradict previous reports (Karakaya et al., 2011) that reported inhibition of *Listeria monocytogenes*, *Salmonella typhimurium*, and *E. coli*. The lack of significant antimicrobial activity of oregano oil tested in this study might be the result of dissimilar methods or higher concentrations used in the reported studies relative to our study where concentrations of 50 μg mL⁻¹ were used.

Although published reports on the lipophilic antioxidant capacity of oregano are limited, the values obtained in this study are within the range of many herbs and spices (Jimenez-Alverez et al., 2008; U.S. Department of Agriculture, 2010). Considering that essential oils are currently being linked to multiple health benefits and preservation qualities as a result of their ability to protect against oxidation, optimizing a lipophilic extraction process for both compositional and total oil levels is needed to ensure consistent antioxidant capacities (Bakkali et al., 2008; Sacchitti et al., 2005). The similar antioxidant values (on a per-gram basis) for the oregano oils collected at time points throughout the DT range (20, 80, and 360 min) further indicate that any given oil-based antioxidant was not converted or lost during the distillation process.
However, the total amount of antioxidant agents also increased with higher oil yields supporting the application of longer DT to oregano-based oil extractions.

This study suggests that comparing essential oil yield and composition between different reports must take into consideration the length of the steam distillation.

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