Headspace Analysis of the Volatile Oils of *Agastache*†

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Equilibrium headspace analysis in combination with gas chromatography/mass spectroscopy was used to identify volatile compounds released by the inflorescences and leaves from individual plants of *Agastache foeniculum*, *Agastache rugosa*, and putative hybrids. Methylchavicol was the major constituent in most populations tested. The inflorescences produced from 2 to 6 times more volatiles per gram than did the leaves. *A. rugosa* produced more volatiles than did *A. foeniculum* and had less diversity in its volatile composition. The putative hybrid was intermediate between the two proposed parents. The headspace analysis technique gave values comparable to those of traditional volatile oil extraction methods.

INTRODUCTION

Agastache foeniculum (Pursh) O. Kuntze has been commercially cultivated as a source of nectar for honey bees (*Apis mellifera* L.) (Mayer et al., 1982). Populations of *A. foeniculum* and related species of *Agastache* have recently been evaluated as a part of a project to select superior nectar sources at the North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA (Widrlechner, 1992).

The foraging choices of honey bees are influenced by many factors, such as floral aroma. Beker et al. (1989) showed that foraging bees could distinguish between two volatile oil chemotypes of *Majorana syriaca* L. Similar observations have been noted for *Ocimum* (Darrah, 1974) and *Medicago sativa* L. (Kauffeld and Sorensen, 1971; Loper et al., 1974). Pham-Delegue et al. (1989) recently used headspace analysis to identify components of the floral aroma of *Helianthus annuus* L. that allow bees to distinguish among different varieties.

An evaluation of the essential oils of *Agastache* populations grown at the NCRPIS has recently been conducted using gas chromatography of hydrodistillates (Charles et al., 1991). Evaluation determined that there are important interpopulational and interspecific differences in essential oil composition. The studies cited above suggest that such variation could have significant consequences for honey bee foraging preferences.

Another factor that could influence bee preference is the Nasonov pheromone (Sladen, 1901). The Nasonov pheromone is a specific mixture of essential oils that honey bees spray onto nectar sources to mark and direct other bees to them (Sladen, 1901). The Nasonov pheromone contains seven compounds: (*E*)-citral (geranial), (*Z*)-citral (neral), nerol, geraniol, nerolic acid, geranic acid, and (*E,E*)-farnesol, each of which attracts honey bees (Williams et al., 1981). Nykänen et al. (1989) analyzed the essential oil of a Canadian population of *A. foeniculum* and found it to contain 0.1% geraniol. The presence of Nasonov pheromone components in the floral aroma of particular populations of *Agastache* could bias bee preference and might be useful as a tool for researchers to select attractive types.

The present study uses headspace analysis in combination with gas chromatography/mass spectroscopy to identify volatile compounds given off by the leaves and inflorescences of individual plants of *Agastache* to assess the degree of inter- and intrapopulation aroma variability and also to determine whether components of the Nasonov pheromone are present in the aromas of these plants.

The results of this study are also compared with the findings of Charles et al. (1991) to determine if headspace analysis may be as effective as hydrodistillation in capturing essential oil components of *Agastache*.

MATERIALS AND METHODS

Headspace Sample Collection. Samples of *Agastache foeniculum*, *Agastache rugosa* (Fisch. and C. A. Mey.) O. Kuntze, and suspected *rugosa* × *foeniculum* hybrids were collected from a plot at the NCRPIS, Ames, IA. Five populations of *A. foeniculum* (A3064, A3481, A4546, A4550, and A7569), two populations of *A. rugosa* (A4721 and A4992), and three putative hybrids were used in this study (Table I). Single-plant samples were collected between 8:00 and 9:00 a.m., placed into plastic bags (containing a small amount of water to prevent wilting), and transported to the laboratory. Six to eight grams of inflorescences or 7–9 g of leaves was accurately weighed and placed, without damaging the tissue, into glass sampling bottles that were 11 cm long by 4 cm in diameter. The bottles were immediately sealed with headspace sampling caps containing Teflon-coated septa and aluminum seals (Ong, 1988) and equilibrated at 20 °C for 4 h before sampling.

Gas Chromatographic Analysis. Volatile analyses were performed on a Varian 3700 gas chromatograph equipped with an FID detector and a Hewlett-Packard 3390A integrator. A fused-silica (1.0-μm film thickness) DB-5 (0.25 mm i.d. × 30 m) capillary column (J&W Scientific, Folsom, CA) was used throughout this study. The column (nitrogen), makeup gas (nitrogen), oxygen, and hydrogen flow rates were 1.5, 28.5, 300, and 30 mL/min, respectively. The injector and detector temperatures were set at 200 and 250 °C, respectively. The oven temperature was programmed from 40 to 230 °C at 10 °C/min with an 8-min hold at 230 °C. The detector sensitivity was 1×10^{-12} amp/s.

Sample Analysis. The method of Wilson et al. (1992) was used for all headspace analyses. A 5-mL Hamilton syringe was

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Table I. Origins of *Agastache* Populations

species	population	origin
A. foeniculum	A3064	Manitoba: Morden, cultivated plants at the Agriculture Canada Research Station
A. foeniculum	A3481	Michigan: Washtenaw Co., cultivated plants at the Matthaei Botanical Gardens Ann Arbor: originally received from the University of Washington School of Pharmacy, Seattle
A. foeniculum	A4546	Iowa: Story Co., cultivated plants grown by W. John Johnson, Rte. 4, Ames
A. foeniculum	A4550	North Dakota: Barnes Co., Kathryn Quad, Little Yellowstone Co. Pk., T137N R58W, SW 1/4 of SW 1/4 of Sec. 36, elevation 1330 ft; growing on edge of woods on east-facing slope with *Solidago* and *Aster*
A. foeniculum	A7569	Minnesota: Cass Co., Powell Twp., 1.5 mi east of Backus on west side of Lindsey Lake
A. rugosa	A4721	commercial seed from Seeds Blum, Boise, ID
A. rugosa	A4992	Iowa: Polk Co., cultivated plants grown by Clifford Jantz, 4115 E. Garden Ave., Des Moines
putative hybrid	OH	appeared in a row of A4992 in a test plot at the North Central Plant Introduction Station, Ames, IA
putative hybrid	J1, J2	observed in a population of open-pollinated seedlings grown from seed harvested from a row of A4721 growing in a private garden, Ames, IA

Table II. Results of Headspace Injections from Single-Plant Samples of *A. foeniculum* Inflorescences (Data Are Expressed as a Percentage of Total Peak Area)

		retention	A4546 inflor			A7569 inflor	A3064 inflor				A3481 inflor			A4550 inflor		
compd	peak	time, min	1[a]	2	3	1	1	2	3	4	1	2	3	1	2	4
α-pinene	1	6.69	6.0	–[b]	11.2	3.5	–	–	–	–	7.8	12.0	13.8	–	11.8	25.4
α-camphene	2	6.97	6.8	–	11.1	2.9	–	–	–	–	6.1	11.0	14.6	–	–	–
β-myrcene	3	7.60	39.3	11.2	12.8	37.3	–	–	–	–	4.9	4.6	–	17.6	8.8	–
α-limonene	5	8.32	8.7	50.4	24.5	–	5.1	9.5	6.8	3.0	–	–	–	–	18.6	46.6
*[c]	6	8.38	12.3	–	–	22.8	–	–	–	–	–	–	–	–	–	–
*	7	8.59	11.9	–	–	10.5	–	–	26.3	–	27.9	29.6	47.3	35.4	23.3	18.7
linalool	8	9.47	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*	10	10.28	–	–	–	2.6	–	–	–	–	–	–	–	–	–	–
methylchavicol	12	11.16	–	23.2	14.7	7.4	94.9	90.5	66.9	97.0	43.2	31.9	–	47.0	25.4	9.4
*	13	11.24	5.3	–	–	–	–	–	–	–	–	–	–	–	–	–
citral	14	11.71	4.8	–	–	4.7	–	–	–	–	–	–	–	–	–	–
*	16	12.30	–	–	–	–	–	–	–	–	–	–	–	–	–	–
bornyl acetate	17	12.60	–	–	–	–	–	–	–	–	–	–	5.3	–	–	–
*	18	12.95	–	–	14.0	–	–	–	–	–	–	–	–	–	–	–
*	19	13.06	–	–	–	4.0	–	–	–	–	–	–	–	–	–	–
β-caryophyllene	22	14.71	–	–	–	–	–	–	–	–	3.0	5.7	12.6	–	–	–
*	23	14.89	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*	24	15.42	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*	25	15.55	–	–	11.7	3.5	–	–	–	–	7.2	5.9	6.6	–	–	–
δ-cadinene	26	15.66	5.1	–	–	3.9	–	–	–	–	–	–	–	–	11.9	–
*	30	17.64	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*	32	20.41	–	15.3	–	–	–	–	–	–	–	–	–	–	–	–

[a] Individual plant number. [b] No peak observed. [c] Unknown compound.

flushed three times with the equilibrium headspace of inflorescences or leaves before a 2-mL sample was injected at a rate of 1 mL/min. Liquid nitrogen was used to cryofocus the samples on column before the temperature program was begun. The splitter (1:20) was turned on 30 s after the injection of the headspace sample.

A 10-μL Hamilton syringe was flushed twice with 0.5 μL of the headspace volatiles above each of the pure standards [α-pinene, α-camphene, β-pinene, α-limomene, β-myrcene, bornyl acetate, eugenol, linalool, methylchavicol, methyleugenol, pulegone, β-caryophyllene (*E*)-citral, nerol, geraniol] followed by flushing with ambient air five times before 0.5 μL was injected into the injection port. The splitter (1:20) was turned on before volatile headspace standards were injected.

Each headspace analysis of the leaves and inflorescences was replicated five times with one headspace sample taken from each of five sampling bottles. Standard compound headspace samples were replicated three times in the same manner as the plant samples.

Methane was injected regularly to ensure that the septa and syringe seals were not leaking or the syringes plugged.

Compound Identification. Identification of the headspace volatile composition of individual plants within each *Agastache* population was performed by (1) retention time comparison and co-injection with standard compounds, (2) Kovats indices comparison with literature values, and (3) gas chromatography/mass spectroscopy (Adams, 1989; Jennings and Shibamoto, 1980). Two gas chromatograph/mass spectrometers were used in this study. A Hewlett-Packard 5970 Series mass spectrometer (Hewlett-Packard Co., Palo Alto, CA) coupled with a Varian Aerograph Series 1520 gas chromatograph was used for headspace volatile analysis. Because the mass spectrometers were less sensitive than the gas chromatograph, steam volatile oils (ASTA, 1985) were determined for dried bulk samples of *A. foeniculum*, *A. rugosa*, and the putative hybrids. Triplicate analyses were made for each bulk sample, and duplicate injections of each volatile oil sample were used to characterize the composition of each oil by gas chromatography/mass spectroscopy. The analyses of the volatile oils were performed by Chemical Instrument Services, Iowa State University, using a Finnigan 4000 with a Incos data system (Finnigan MAT, San Jose, CA).

RESULTS

Methylchavicol was found in the headspace of inflorescences and leaves in all but four samples (two inflorescences and one leaf sample from *A. foeniculum* and one leaf sample from a putative hybrid), representing all populations tested (Tables II–V). α-Limonene was found in the headspace of leaves and inflorescences of all populations except the putative hybrids and *A. foeniculum* A3481 (inflorescences and leaves) and A7569 (inflorescences). Charles et al. (1991) also found that *A. foeniculum* A3481 leaves lacked α-limonene. α-Pinene was present in headspace of most populations of *A. foeniculum* but absent in *A. rugosa* and putative hybrid populations.

Headspace volatiles from *A. foeniculum* contained 0.0–97.0% methylchavicol in the inflorescences and 0.0–90.2% methylchavicol in their leaves (Tables II and III). Methyl chavicol was also the dominant volatile in the headspace of the inflorescences and leaves of *A. rugosa* with 55.7–86.2 and 69.7–97.4%, respectively (Table IV). The pu-

Table III. Results of Headspace Injections from Single-Plant Samples of *A. foeniculum* Leaves (Data Are Expressed as a Percentage of Total Peak Area)

compd	peak	retention time, min	A4546 leaves	A3064 leaves		A3481 leaves		A4550 leaves	
			2[a]	1	2	1	3	1	4
α-pinene	1	6.69	–[b]	–	–	13.3	12.4	6.1	15.4
α-camphene	2	6.97	–	–	–	7.7	–	5.8	–
β-myrcene	3	7.60	–	–	–	5.4	5.5	14.0	10.9
α-limonene	5	8.32	1.5	–	17.7	–	–	7.2	–
*[c]	6	8.38	–	–	–	–	–	–	–
*	7	8.59	–	–	16.8	13.7	15.9	50.9	26.4
linalool	8	9.47	–	–	8.6	–	–	–	–
*	10	10.28	–	17.5	9.5	–	–	–	–
methylchavicol	12	11.16	90.2	63.9	27.9	16.4	42.0	–	36.3
*	13	11.24	–	–	–	–	–	–	–
(*E*)-citral	14	11.71	–	18.6	–	–	–	–	–
*	16	12.30	1.5	–	–	–	–	–	–
bornyl acetate	17	12.60	–	–	–	–	–	–	–
*	18	12.95	1.5	–	11.3	8.2	10.2	–	–
*	19	13.06	–	–	–	–	–	–	–
β-caryophyllene	22	14.71	2.0	–	–	13.3	–	8.5	–
*	23	14.89	1.5	–	–	–	–	–	–
*	24	15.42	1.6	–	8.2	–	–	–	–
*	25	15.55	–	–	–	21.9	5.0	6.7	–
δ-cadinene	26	15.66	–	–	–	–	–	–	11.0
*	30	17.64	–	–	–	–	9.0	–	–
*	32	20.41	–	–	–	–	–	–	–

[a] Individual plant number. [b] No peak detected. [c] Unknown compounds.

Table IV. Composition of the Headspace from the Leaves and Inflorescences of *A. rugosa*

compd	peak	retention time, min	% of total peak area						
			A4992 inflor	A4721 inflor			A4992 leaves	A4721 leaves	
			1[a]	1	2	3	4	1	3	4
β-myrcene	3	7.60	0.5	2.2	–[b]	0.2	0.4	–	0.4	1.8
α-limonene	5	8.32	11.9	33.2	25.0	20.1	19.8	2.6	17.7	24.4
*[c]	9	9.63	0.6	–	–	–	–	–	–	–
*	10	10.28	–	2.9	5.2	0.1	0.8	–	0.4	–
*	11	10.58	–	–	–	–	1.8	–	–	–
methylchavicol	12	11.16	86.2	55.7	57.0	75.1	73.8	97.4	79.7	69.7
*	18	12.95	–	3.7	6.2	0.5	0.7	–	0.7	1.1
β-caryophyllene	22	14.71	0.8	2.2	1.8	1.7	2.1	–	1.1	1.7
*	24	15.42	–	–	4.9	–	–	–	–	–
*	27	15.78	–	–	–	0.7	0.2	–	–	–
*	28	15.97	–	–	–	0.8	–	–	–	–
*	29	17.30	–	–	–	–	–	–	–	1.4
*	31	18.08	–	–	–	0.8	0.3	–	–	–

[a] Individual plant number. [b] No peak detected. [c] Unknown compounds.

Table V. Composition of the Headspace from the Leaves and Inflorescences of Putative *Agastache* Hybrids

compd	peak	retention time, min	% of total peak area			
			OH inflor	J3 inflor	OH leaves	J1 leaves
α-limonene	5	8.32	75.2	16.6	73.1	87.5
*[a]	7	8.59	5.1	–[b]	7.6	–
methylchavicol	12	11.16	0.8	74.0	–	8.4
(+)-pulegone	15	11.86	6.1	–	–	–
*	17	12.61	–	2.1	–	–
methyleugenol	20	14.08	–	2.4	–	–
β-caryophyllene	22	14.71	–	2.7	–	4.0
*	24	15.42	0.9	2.1	–	–
δ-cadinene	26	15.66	–	–	6.5	–
*	29	17.30	11.9	–	–	–
*	32	20.41	–	–	12.8	–

[a] Unknown compound. [b] No peak detected.

tative hybrids contained 0.8–74.0% methylchavicol in the inflorescences and 0.0–8.4% in the leaves (Table V). The populations of *A. foeniculum* that were low in methylchavicol (0.0–27.9%) contained greater amounts of α-pinene, myrcene, limonene, unknown peak 7, and unknown peak 25 (Tables II and III).

Myrcene was present in most populations of *A. rugosa* but not in the putative hybrids or the inflorescences and leaves of *A. foeniculum* A3064 or the leaves of A4546. Pulegone and methyleugenol were only found in populations of the putative hybrid. Unknown peak 7 was absent in *A. rugosa* leaves and inflorescences, but it was present in one putative hybrid, in *A. foeniculum* A3481, A4550, and A7569, and in some plants from A4546 and A3064. Caryophyllene was found in all inflorescences of *A. rugosa* and in two of the putative hybrids; only the inflorescences from *A. foeniculum* A3481 contained caryophyllene (Tables II–V).

Only three *A. foeniculum* populations (A4546, A3064, and A7569) contained individual plants, which produced one of the Nasonov components [(*E*)-citral] on the inflorescences or leaves as identified by headspace analysis. Citral was not found in the volatile oils from bulked *Agastache* population samples.

The identified compounds in the headspace volatiles of *A. foeniculum*, *A. rugosa*, and the putative hybrids accounted for from 60 to 93% of the total volatiles for inflorescences and from 58 to 100% of the total volatiles from the leaves. The average total area of the headspace samples per gram of fresh weight of inflorescences (integration units per gram) was 942 000 for *A. rugosa*, 150 000 for *A. foeniculum*, and 185 000 for the putative hybrids. In contrast, the average total area per gram of fresh weight

of leaves was 562 000, 71 600, and 29 100 for *A. rugosa*, *A. foeniculum*, and the putative hybrids, respectively. In general, the inflorescences produced 2–6 times more volatiles per gram of fresh weight than did the unbroken leaves.

These headspace volatile data were supported by the results of the volatile oil analysis of inflorescences, which obtained 1.946% volatile oil for *A. rugosa*, 0.745% for *A. foeniculum*, and 1.548% for the putative hybrid. The putative hybrid produced amounts of volatile oil that were intermediate between those of the two proposed parents. This observation supports the hypothesis that these plants are interspecific hybrids between *A. foeniculum* and *A. rugosa*.

There were 75 peaks found in the volatile oils obtained from the *Agastache* spp. Eighteen of these compounds [α-pinene, α-camphene, sabinene, β-pinene, β-myrcene, α-limonene, methylchavicol, δ-cadinene, (+)-pulegone, bornyl acetate, eugenol, methyleugenol, β-caryophyllene, spathulenol, caryophyllene oxide, linalool, phytol] have been identified by mass spectroscopy. Charles et al. (1991) also reported the presence of these compounds in the volatile oil of *Agastache* spp.

DISCUSSION

There was a tremendous diversity in the compounds released by both inflorescences and leaves of *A. foeniculum*, but there was less diversity in *A. rugosa* and the putative hybrids. In agreement with previous studies (Charles et al., 1991), methylchavicol was the only compound to occur in the majority of the populations.

In *A. foeniculum*, the compounds found in the inflorescences differed greatly from even those found in the leaves of the same plant, whereas in *A. rugosa* and putative hybrids, the compounds in inflorescences and leaves of the same plant were usually similar. Our results for *A. rugosa* were similar to those reported by Maffei and Sacco (1987) for peppermint (*Mentha* × *piperita* L.).

With *A. foeniculum*, the compounds found in leaves often differed from those found in the inflorescences of the same plant. Variation in the chemical composition between leaves and inflorescences within the same plant, as demonstrated by A4450 plant 1 (methylchavicol; 47% inflorescences and 0% in the leaves), and between plants within the same population (9.4–47% methylchavicol; 0–46.6% α-limonene) are not unusual for Lamiaceae and other plant species. Guenther (1949) reported marked differences in essential oil quantities and composition for leaves and inflorescences of both *Salvia officinalis* L. and *Salvia sclarea* L. Cinnamon bark, leaves, and roots have well-documented major differences within the same plant (Richard, 1991). Cluster analysis failed to reveal a characteristic pattern of variation among samples of *A. foeniculum*.

In this study, the only identified compounds found in *A. foeniculum* that were not found by Polak and Hixon (1945) and Nykänen et al. (1989) in their studies with dried samples were camphene and myrcene. Charles et al. (1991) also found camphene is *A. foeniculum* and myrcene in *A. foeniculum* and *A. rugosa*. Nykänen et al. (1989) found myrcene but not camphene in *A. foeniculum*. The present study and Nykänen et al. (1989) also found sabinene in *A. foeniculum*, whereas Charles et al. (1991) did not.

The relative lack of diversity in the volatiles of *A. rugosa* agrees with the findings of Vogelmann (1983) and Charles et al. (1991), who found that different populations of *A. rugosa* are genetically very similar. Fujita and Fujita (1973) found a single population of *A. rugosa* that differed sharply in volatile composition from most other populations.

Table VI. Comparison of Headspace and Volatile Oil Compositions of Inflorescences of *Agastache*

compd	volatile constituent,[a] %		
	headspace	volatile oil	Charles et al. (1991)
methylchavicol			
1[b]	39.4	50.4	57.60
2	69.6	72.6	80.53
3	74.0	75.2	73.82
myrcene			
1	9.75	2.51	0.39
2	0.66		0.30
3		0.34	0.68
limonene			
1	12.37	2.16	1.78
2	22.0	4.27	5.97
3	45.9	6.49	9.72

[a] Averaged across all accessions evaluated. [b] 1, *A. foeniculum*; 2, *A. rugosa*; 3, putative hybrids.

An examination of the volatile compounds present in the headspace of the putative hybrids may provide evidence in support of their hybridity. Of the 11 compounds listed for the putative hybrids in Table V, four are found in both *A. foeniculum* and *A. rugosa*, four in *A. foeniculum* but not in *A. rugosa*, one in *A. rugosa* but not in *A. foeniculum*, and two in neither *A. foeniculum* nor *A. rugosa*. However, an analysis of extracted volatile compounds found these two compounds in the bulk samples of *A. foeniculum* and *A. rugosa* at or below 1%.

Using the same hybrids from this study, Senechal (1990) used isozyme analysis to establish the parentage of the putative hybrids. His study strongly suggests that the putative hybrids are *A. rugosa* × *foeniculum*.

The headspace analysis technique yielded a total of 32 compounds with 75 compounds by extraction techniques. This finding is not unusual (Jennings and Filsoof, 1977; Leahy and Reineccius, 1984; Takeoka et al., 1985; Wilson et al., 1992). The headspace technique yielded a higher relative percentage of lower-boiling compounds such as myrcene and α-limonene (Table VI) than the high-boiling compounds such as caryophyllene and spathulenol. Headspace, however, yielded comparable values to extraction for compounds such as methylchavicol (Table VI).

An advantage of the headspace technique is that air concentrations of volatiles can be related to physiologically active levels of compounds observed to affect bee preference. The headspace technique uses smaller samples, is faster than standard methods, and can be used to screen individual plants. Headspace techniques require no heating or extraction solvents and may produce fewer artifacts. However, the volatiles collected by headspace must be concentrated about 2~fold to obtain a positive identification by GC/MS.

Work needs to be done to determine which, if any, of these compounds other than citral attract honey bees. Another component of the headspace of one plant of *A. foeniculum*, bornyl acetate, has recently been shown to stimulate the antennae of honey bees (Thiery et al., 1990), but this olfactory stimulation has not been correlated with attractiveness.

The diversity of essential oils within *A. foeniculum* suggests that selecting plants on the basis of their olfactory attractiveness to honey bees may be feasible. Such selection, however, is desirable only if the plants selected also excel at nectar production. The great variation within populations of *A. foeniculum* as well as among them could

indicate that heritability for production of these compounds is low or that perhaps there is genetic variation both within and among these populations for the production of specific volatile compounds. These hypotheses remain to be tested.

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Registry No. α-Pinene, 80-56-8; α-camphene, 79-92-5; β-myrcene, 123-35-3; α-limonene, 138-86-3; linalool, 78-70-6; methylchavicol, 140-67-0; (*E*)-citral, 141-27-5; citral, 5392-40-5; bornyl acetate, 76-49-3; β-caryophyllene, 87-44-5; δ-cadinene, 483-76-1; (+)-pulegone, 89-82-7; methyleugenol, 93-15-2.