Comparison of intensely sweet volatile leaf oils of *Lippia dulcis* (Verbenaceae) with low and high camphor from Brazil and Mexico

Robert P. Adams
Biology Department, Baylor University, Box 97388, Waco, TX 76798, USA Robert_Adams@baylor.edu

and

Patricia Francisco de Oliveira
Lab de Controle de Processos, Dept. de Engenharia Quimica e Engenharia de Alimentos,
Univ. Federal de Santa Catarina (UFSC), P. O. Box 476, 88010-970, Florianopolis, SC, Brazil

ABSTRACT

Lippia dulcis is a sweet-tasting, woody herb but the sweetness is often mixed with a camphorous taste. *Lippia dulcis* oil from Brazil was found to be low in camphor (trace), whereas the oil from Mexico had considerable camphor (33.9%). The *Lippia* oil from Brazil was high in 6-methyl-5-hepten-2-one (10.5%), α-copaene (8.6%), (E)-caryophyllene (10.6%), bicyclogermacrene (6.6%), δ-cadinene (7.2%), epi-α-bisabolol (6.5%) and hernandulcin (8.8%). In addition, it contained β-cedrene and α-calacorene, compounds found in cedarwood oils. The *Lippia* oil from Mexico was high in camphene (12.7%), limonene (4.6%), camphor (33.9%), α-copaene (4.0%), (E)-caryophyllene (6.0%) and hernandulcin (5.9%). In addition, it contained alkanes and acids (docosane, tricosane, tetracosane, pentacosane, linoleic acid and octadecanoic acid) that were not found in the *Lippia* oil from Brazil. The Brazil germplasm with low-camphor and high hernandulcin is worthy of conservation, as it could be an important alternative source of sweeteners.
Published on-line www.phytologia.org *Phytologia 98(3): 207-214 (July 6, 2016).* ISSN 030319430.

KEY WORDS: *Lippia dulcis,* leaf terpenoids, hernandulcin, Brazil, Mexico, steam distillation, supercritical CO_2, degradation.

Lippia dulcis Trevir. (Verbenaceae) is a sweet-tasting, woody herb sold as hierbia dulce, hierbia buena, yerba dulce, Orozuz and yet other names in Mexico and central America (Compadre, Robbins and Kinghorn, 1986). The sweet taste is due to the presence of hernandulcin, a bisabolane-type sesquiterpene. It has been rated as 1000 times sweeter than sucrose (Compadre, Robbins and Kinghorn, 1986). Unfortunately, most of the oils also contain copious amounts of camphor, so the sweetness is mixed with a camphorous taste.

The nomenclature has been subject to controversy and has been reviewed by Adams et al. (2014). But it might be mentioned that O'Leary and Mulgura (2012), in their revision of the genus *Phyla*, explicitly excluded *Phyla dulcis* and *P. stochaedifolia* from the genus, stating "these are considered here to be better placed under *Lippia*, given that both species lack malpighiaceous hairs, which are characteristic of the genus *Phyla* and are woody shrubs rather than the herbaceous habit noted for all *Phyla* species considered herein." In the present report, it is treated as *Lippia dulcis*.

Reports on the composition of the leaf terpenoids of *L. dulcis* have been variable. Nayal et al. (2009) and Gornmann et al. (2008) reported 32.6% camphor and 10% hernandulcin from *L. dulcis* grown from seeds from M. P. Gupta, Panama. However, the same laboratory (Nayal et al. 2009) reported 0.02% camphor and 14.5% hernandulcin from plants grown from Panama seeds (ex M. P. Gupta seed lot). It may be that the report by Gommann et al. (2008), from that same laboratory, erroneously reported the composition of Mexico *L. dulcis* for their 'Panama' plants.

Kaneda et al. (1992) found no camphor but 0.154% hernandulcin in market plants from Valle de Anton, Cocle, Puerto Rico, sold for the treatment of respiratory ailments. This plant (identified as *L. dulcis*) was listed in the Flora of Panama as *Phyla scaberrima* (A. L. Juss.) Moldenke. Kaneda et al. (1992) also identified a new sweet sesquiterpene, (+)-4β-hydroxyhernandulcin as well as (-) epi-hernandulcin from their Panama plants. Mori and Kato (1986a,b) synthesized all four isomers of hernandulcin and noted that only the 6S, 1'S isomer (i.e., (+) hernandulcin) was sweet.

The presence of a large amount of camphor in the Mexican plants is of considerable interest since there are conflicting reports of none (or trace amounts of camphor in plants) from Panama, Puerto Rico and Columbia (Table 1). Because camphor is very heat-stable, it seems unlikely that the trace or absence of camphor in the Panama, Puerto Rico, Columbia and Brazil samples is due to decomposition; more likely, it is due to the lack of camphor in these plants. Although all the studies cite "plants identified by taxonomist," it is very possible that some of the samples may have been mis-identified or there may be chemical races or chemotypes present in *L. dulcis,* as suggested by Souto-Bachiller et al. (1997). Souto-Bachiller et al. (1997) concluded that 'tzonpelic xihuitl' ascribed to Francisco Hernandez by Aztec physicians more than 400 years ago (Anderson, 1977) is, in fact, 'yerba dulce' of Puerto Rico. Research on geographic variation in the leaf oils of *L. dulcis* is needed to clarify the problem.

Souto-Bachiller et al. (1996) collected seeds in 1990 from plants in Orocovis, Puerto Rico. They obtained high hernandulcin yields (18-26 mg/g), with no camphor from germinated shoots (6-8 weeks old). After repeated sub-culturing for five years, there were little effects on the oil composition, implying that the oils are genetically stable.

Table 1. Reports on the amounts of camphor and hernandulcin in *Lippia dulcis*.

publication	plant source	camphor %	hernandulcin %	extraction
Compadre et al. 1986	Mexico (local markets)[1]	53.2	0.004[#]	steam distilled, 2h
Nayal et al. 2009	Mexico (Helenion Nursery, Germany)[2]	32.6	10.1	distilled, 4h
Gornmann et al. 2008	Panama (seeds, M. P. Gupta, Panama)[3]	32.6	10.0	steam distilled, 4h
Nayal et al. 2009	Panama (seeds, M. P. Gupta, Panama)[2]	0.02	14.5	distilled, 4h
Kaneda et al. 1992	Puerto Rico (market,Valle de Anton)[1]	none	0.154	petroleum ether
Souto-Bachiller et al. 1997	Puerto Rico (plants, ex Orocovis)[1]	<0.01	22.0	pentane & CH_2Cl_2
Moreno-Murillo etal. 2010	Colombia (plants, ex Tenza Valley)[4]	none	1.1*	hydro-distillation, 3h
Oliveira, et al. 2010.	Brazil(local plants?)[1]	none	19.2	supercritical CO_2
Adams et al. (2014)	Mexico (seeds, Chiltern Seeds, UK)[5]	21.2	9.2	pentane overnight
Adams et al. (2014)	Mexico (seeds, Chiltern Seeds, UK)[5]	33.9	4.5	steam distilled, 4h

[1]dried and milled; [2]air dried, 30°C and cut; [3]dried and cut; [4]fresh or dried?; [5]fresh leaves.
*(ca. 4-5%, hernandulcin was mostly decomposed during GC analysis)
[#]severely decomposed during GC analysis.

Recently, Attia, Kim and Ro (2012) reported on molecular cloning of (+)-epi-β-bisabolol synthase as a precursor to the biosynthesis of hernandulcin.

Compardre et al. (1986) discovered that hernandulcin decomposes upon heating to 140°C. They tried to compensate for this problem by running their GC injector at 70°C, but this is too low to quantitatively transfer a board mixture of volatile components to the GC column. Souto-Bachiller et al. (1997) found a solution to the problme: they ran a narrow bore injector liner (0.75 mm bore) so that the dead volume is small and the sample quickly transferred from their injector (220°C) to the cool (60°C) column. Even using this method, they appeared to have decomposition of hernandulcin, as indicated by the presence of 6-methyl-5-hepten-2-one and 3-methyl-2-cyclohexen-1-one (putative decomposition

products of hernandulcin). Souto-Bachiller et al. (1997) extracted with pentane and dichloromethane (sequentially with combined extracts) because they thought that distillation would lead to decomposition. It may be that they were considering water-distillation where the plants are placed in water and boiled to co-produce steam and volatile oil. Water-distillation (or hydro-distillation) is well known to produce artifacts due action of acids from the leaching of organic leaf acids into the water (Adams, 1991). A safer steam distillation can be performed in all glass units, with the plant materials suspended above boiling water, so that the oil is not exposed to leached-out organic acids. As the maximum temperature reached is 100°C, and contact is only with glass, this type of steam distillation eliminates decomposition for all but the most labile components found in nature. See Adams (1991) for a diagram of this type of steam distillation apparatus.

Adams et al. (2014) examined the effects of inlet injector temperature on the degradation of hernandulcin using a series of analyses, by increasing the injector temperature (Fig. 1). Hernandulcin content was lowest at 100°C, then increased to 160°C, then declined from 200°C to 220°C (Fig. 1). It seems likely that the high variance at 100°C and lower amount of the less volatile sesquiterpene, hernandulcin, is due to incomplete volatilization in the injector and selective loss of hernandulcin in the split line. The decline at 220°C is due to decomposition of hernandulcin (Fig. 1). The rather constant nature of 6-methyl-5-hepten-2-one (Fig. 1), followed by the sudden increase at 220°C, seems to indicate that most 6-methyl-5-hepten-2-one is a natural product in the oil and only a small portion was derived from the decomposition of hernandulcin (220°C, Fig. 1). Alternatively, there could have been some decomposition of hernandulcin during harvest, storage and/ or extraction.

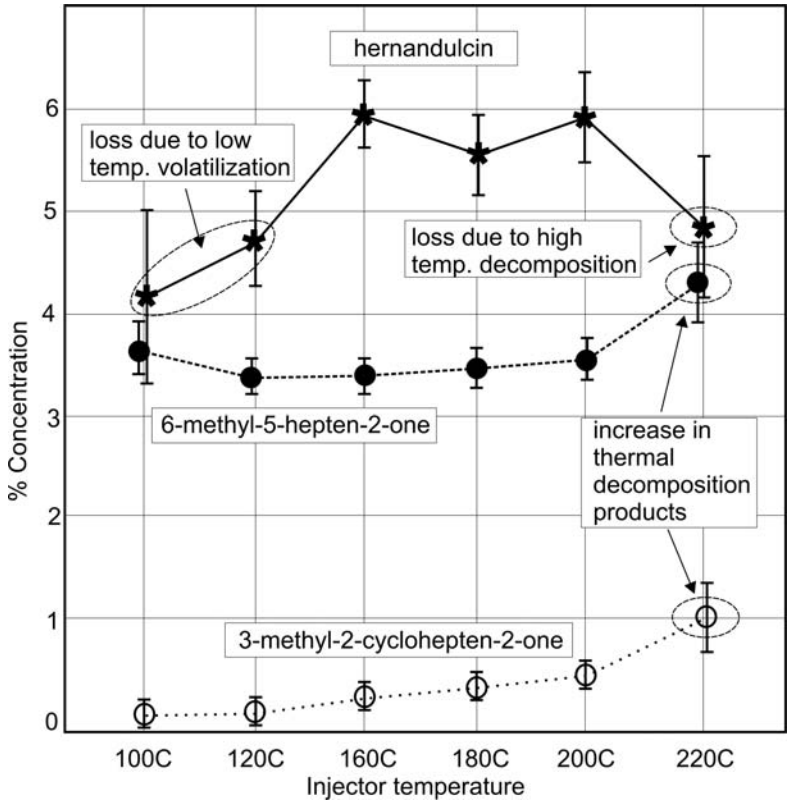

Figure 1. Plots of hernandulcin versus putative decomposition products: 6-methyl-5-hepten-2-one and 3-methyl-2-cyclohepten-2-one with changes in the injector temperature for GC analyses. (from Adams et al., 2014).

The concentration of 3-methyl-2-cyclohepten-2-one was very stable from 100°C to 200°C, then increased at 220°C (Fig. 1). This suggests that the increase at 220°C is due to the decomposition of hernandulcin. Small amounts of 3-methyl-2-cyclohepten-2-one may be naturally present in the oil.

Oliveira et al. (2010) reported 19.2% hernandulcin but no camphor in plants grown in Brazil extracted by supercritical CO_2. These plants appear unusual in having little or no camphor (Table 1). Low camphor *Lippia dulcis* plants have been reported from Panama (0.02%, Nayal et al., 2009); Puerto Rico (none, Kaneda et al, 1992, or <0.01%, Souto-Bachiller et al. 1997); Columbia (none, Moreno-Murillo et al., 2010) and Brazil (none, Oliveira et al. 2010).

Souto-Bachiller et al. (1997) described their collection location of *L. dulcis* as "in Sector Negro, Orocovis, we found abundant samples of this species at the country estate of Mrs. Maria Ortolaza, Road No. 143, Km 30.7. A specimen is deposited at the herbarium, Biology Dept., UPRM." After correspondence with James Ackerman, Dir. of UPRRP Natural History Collections, Univ. of Puerto Rico, in 2014, a graduate student, Fabiola Areces, visited the estate of Mrs. Ortolaza in Nov., 2014 and found that Mrs. Ortolaza had died and that her estate was now in disrepair. A search for plants of *Lippia dulcis* at the estate revealed that the garden plot had been abandoned and was grown over with wild vegetation. No *Lippia dulcis* plants could be found. Thus, the garden plants of *Lippia dulcia,* may remain the only known source of this rare low-camphor genotype.

We recently analyzed leaves from the plants that Oliveira et al. (2010) used for supercritical CO_2 extraction. The purpose of this study was to compare the steam distilled oils of the Brazilian *Lippia dulcis* with the volatile leaf oil of Mexican materials.

MATERIALS AND METHODS

Plant material: Lippia dulcis seeds were obtained from Chiltern Seeds, UK, via M. Attia, University of Calgary, Canada and grown in the greenhouse. Vegetatively propagated plants were grown under partial shade in pots or in the field of the experimental farm at Prairie View A&M University. Fresh leaves were collected from young plants. In addition, we were able to obtain leaves of *Lippia dulcis* from plants grown in a garden in Florianopolis, Brazil.

Essential oils analysis - 30.1 g FW (4.79 g DW) of fresh, greenhouse, mature leaves with 2 mg of methyl decanoate added (as an internal standard) steam distilled for 4 h using a modified circulatory Clevenger-type apparatus (Adams 1991). Extracts were concentrated (diethyl ether trap-removed) with nitrogen and stored at -20ºC until analyzed. Extracted leaves were oven dried to a constant weight (48 hr, 100ºC) for the determination of oil yield as [oil wt./(oil wt. + oven dried extracted foliage wt.)]. The extracted oils were analyzed on a HP5971 MSD mass spectrometer: 0.2 ul of a 10% solution (in diethyl ether) oil injected, split, 1:10, temperature programmed, linear, 60º - 246ºC at 3ºC/min. (62 mins.), carrier gas He, flow 34.96 cm/sec or 1.02 ml/min, injector 160ºC, detector 240ºC, scan time 1/sec, directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25-micron coating thickness, fused silica capillary column (see Adams 2007, p. 4, for detailed operating conditions). Identifications were made by searches of our volatile oil library (Adams 2007) using HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantification was by flame ionization detector on an HP 5890 gas chromatograph operated under the same conditions as the GCMS (above) using the HP Chemstation software.

RESULTS AND DISCUSSION

The volatile oils yields were very different, with Brazil *Lippa* yielding 0.37% and Mexico, 2.13% (Table 2) The oils were quite different with most monoterpenes being a trace or absent in the Brazilian *Lippia* oil (Table 2). The *Lippia dulcis* oil from Brazil was the low in camphor (trace), whereas the oil from Mexico had considerable camphor (33.9%).

The *Lippia* oil from Brazil was high in 6-methyl-5-hepten-2-one (10.5%), α-copaene (8.6%), (E)-caryophyllene (10.6%), bicyclogermacrene (6.6%), δ-cadinene (7.2%), epi-α-bisabolol (6.5%) and

hernandulcin (8.8%). In addition, it contained β-cedrene and α-calacorene, compounds found in cedar wood oils. It is note-worthy that not only is camphor very low, but all the monoterpenes are also low or missing in the Brazil oil. It appears the entire monoterpene pathway has been blocked in the Brazil plants.

The *Lippia* oil from Mexico was high in camphene (12.7%), limonene (4.6%), camphor (33.9%), α-copaene (4.0%), (E)-caryophyllene (6.0%) and hernandulcin (5.9%). In addition, it contained alkanes and acids (docosane, tricosane, tetracosane, pentacosane, linoleic acid and octadecanoic acid,) not found in the *Lippia* oil from Brazil.

The exact origin of the plants from Brazil is not known. They were obtained from a nursery garden dealer, so they are likely items of trade commerce. Because *Lippia dulcis* is thought to be non-native to Brazil, it may be this germplasm came from low-camphor plants in Panama, Columbia or even from Puerto Rico. In any case, this germplasm with low-camphor and high hernandulcin is worthy of conservation as it could be an important alternative source of sweeteners.

ACKNOWLEDGEMENTS

This research was supported in part with funds from Baylor University. Thanks to Aruna Weerasooriya, Prairie View A & M University for providing plant material Mexican *L. dulcis* material. Thanks to James Ackerman, Dir. of UPRRP Natural History Collections, Univ. of Puerto Rico and Fabiola Areces for investigating the status of low-camphor plants in Puerto Rico.

LITERATURE CITED

Adams, R. P. 1991. Cedarwood oil - Analysis and properties. In: Linskens, H.-F., Jackson, J. F. (eds.). Modern Methods of Plant Analysis, New Series: Oil and Waxes. Springler-Verlag, Berlin. pp. 159–173.

Adams, R. P. 2007. Identification of essential oil components by gas chromatography/ mass spectrometry. fourth ed. Allured Publishing, Carol Stream, IL.

Adams, R. P., A. Weerasooriya and M. Gao. 2014. Comparison of volatile leaf terpenoids from *Lippia dulcis* (Verbenaceae) obtained by steam distillation and pentane liquid extraction. Phytologia 96: 252-259.

Anderson, F. J. 1977. An illustrated history of the herbals. Columbia University Press, Columbia, NY. Chp. 30, p. 235.

Attia, M., S-U. Kim and D-K. Ro. 2012. Molecular cloning and characterization of (+)-epi-α-bisabolol synthase, catalyzing the first step in the biosynthesis of the natural sweetener, hernandulcin, in *Lippia dulcis*. Arch. Biochem. Biophysics 527: 37-44.

Compadre, C. M., E. F. Robbins and A. D. Kinghorn. 1986. The intensely sweet herb, *Lippia dulcis* Trev.: Historical uses, field inquiries, and constituents. J. Ethnopharmacology 15: 89-106.

Gornemann, T., R. Nayal, H. H. Pertz. 2008. Antispasmodic activity of essential oil from *Lippia dulcis* Trev. J. Ethnopharmacology 117: 166-169.

Kaneda, N., I-S. Lee, M. P. Gupta, D. D. Soejarto and A. D. Kinghorn. 1992. (+)-4β-hydroxyhernandulcin, a new sweet sesquiterpene from the leaves and flowers of *Lippia dulcis*. J. Natural Products 55: 1136-1141.

Mori, K. and M. Kato. 1986a. Synthesis of (6s,1's)-(+)-hernandulcin, a sweetener, and its stereo isomers. Tetrahedron 42: 5895-5900.

Mori, K. and M. Kato. 1986b. Synthesis and absolute configuration of (+)-hernandulcin, a new sesquiterpene with intensely sweet taste. Tetrahedron Letters 27: 981-982.

Moreno-Murillo, B., C. Quijano-Celis, A. Romero R. and J. A. Pino. 2010. Essential oil of *Lippia dulcis* grown in Columbia. Natural Product Communications 5: 613-614.

Nayal, R., M. Y. Abajy and M. F. Melzig. 2009. Comparison of essential composition and cytotoxicity of *Lippia dulcis* Trev. from Mexico and Panama. Intl. J. Essential Oil Therapeutics 3: 91-100.

O'Leary, N. and M. E. Mulgura. 2012. A taxonomic revision of the Genus *Phyla* (Verbenaceae). Ann. Missouri Bot. Gard. 98: 578-596.

Oliveira, P. F., R. A. F. Machado, A. Bolzan and D. Barth. Seasonal variation on the yield of *Lippia dulcis* Trev. extract obtained by supercritical CO_2. http://www.isasf.net/fileadmin/files/Docs/Graz/HtmlDir/Papers/P09.pdf

Souto-Bachiller, F. A., M. DeJesus-Echevarria and O. Cardenas-Gonzalez. 1996. Hernandulcin is the major consituent of *Lippia dulcis* Trev. (Verbenaceae). Natural Product Letters 8: 151-158.

Souto-Bachiller, F. A., M. DeJesus-Echevarria, O. Cardenas-Gonzalez, M. F. Acuna-Rodriguez, P. A. Melendez and L. Romero-Ramsey. 1997. Terpenoid composition of *Lippia dulcis*. Phytochemistry 44: 1077-1086.

Table 2. Comparison oil compositions of Brazil *Lippia dulcis* (cultivated) vs. Mexico *Lippia* or Orozuz steam distilled (4h). t < 0.1%, NI = not integrated. GC injector run at 160°C.

KI	Compound	Brazil	Mexico
	percent oil yield (DM basis)	0.37%	2.13%
846	(E)-2-hexenal	0.2	t
850	(Z)-3-hexenol	t	t
921	**tricyclene**	**-**	**0.1**
932	**α-pinene**	**-**	**2.1**
946	**camphene**	**-**	**12.7**
974	1-octen-3-ol	0.2	t
974	**β-pinene**	**-**	**0.6**
981	6-methyl-5-hepten-2-one	10.5	3.4
988	myrcene	0.1	1.4
1024	**limonene**	**t**	**4.6**
1036	benzene acetaldehyde	t	-
1046	3-me-2-cyclohexene-1-one	0.5	0.4
1086	**terpinolene**	**-**	**1.7**
1095	linalool	1.3	0.4
1100	2-methyl-butyl-2-methyl-cyclohexen-1-one	0.2	-
1103	2-methyl-butyl isovalerate	t	-
1126	4-acetyl-1-methyl-1-cyclohexene	t	-
1141	**camphor**	**t**	**33.9**
1148	citronellal	t	-
1165	**borneol**	**-**	**0.5**
1174	terpinen-4-ol	t	
1179	p-cymen-8-ol	-	0.1
1186	**α-terpineol**	**t**	**0.1**
1199	3-methyl-3-buten-1-ol, tiglate	t	-
1223	**citronellol**	**t**	**-**
1235	**neral**	**0.1**	**-**
1249	**geraniol**	**t**	**-**
1264	**geranial**	**0.2**	**-**
1284	bornyl acetate	t	-
1309	p-vinyl-guaiacol	t	-
1345	α-cubebene	0.3	0.1
1374	α-copaene	8.6	4.0
1387	β-bourbonene	1.0	0.5
1387	β-cubebene	0.1	0.1
1409	α-gurjunene	0.2	0.1
1417	(E)-caryophyllene	10.5	6.0
1419	**β-cedrene**	**0.2**	**-**
1432	α-trans-bergamotene	0.9	0.3
1440	(Z)-β-farnesene	0.5	0.2
1452	α-humulene	0.5	0.3
1454	(E)-β-farnesene	5.3	2.3
1464	9-epi-(E)-caryophyllene	0.7	0.3
1469	7-epi-1,2-dehydro-sesquicineole	1.3	0.3
1478	γ-muurolene	0.8	0.3
1480	germacrene D	3.6	1.1
1500	**bicyclogermacrene**	**6.6**	**-**
1500	α-muurolene	1.1	0.6
1505	β-bisabolene	2.6	1.0
1514	epi-cubebol	0.3	0.1
1522	δ-cadinene	7.2	3.8
1544	**α-calacorene**	**0.8**	**-**
1561	(E)-nerolidol	1.1	0.3
1577	**spathulenol**	**1.9**	**-**

KI	Compound	Brazil	Mexico
1582	caryophyllene oxide	1.4	0.6
1622	sesquiterp.,85,94,136,218	1.6	0.7
1630	muurola-4,10(14)-dien-1-β-ol	0.4	0.2
1639	caryophylla-4(12),8(13)-dien-5-β-ol	-	0.1
1644	α-muurolol	-	t
1662	**hernandulcin isomer 1,43,109,218,236**	**2.1**	**1.7**
1667	**isomer of 1662; hernandulcin isomer**	**1.2**	**1.0**
1668	β-atlantone	0.7	-
1683	α-bisabolol	-	0.1
1685	epi-α-bisabolol	6.5	2.6
1753	**sesquiterp.,41,82, 109, 232**	**0.7**	**-**
1766	**sesquiterp.,41, 109, 150, 228**	**0.6**	**-**
1794	**sesquiterp.,41, 175, 218, 248**	**0.6**	**-**
1851	**(+) hernandulcin**	**8.8**	**5.9**
1865	(-) epi-hernandulcin	1.4	0.7
1959	hexadecanoic acid	0.3	1.2
2113	hydrocarbon,71,123,55,296	0.2	0.8
2132	**linoleic acid**	**-**	**2.5**
2158	**octadecanoic acid**	**-**	**1.0**
2200	**docosane**	**-**	**t**
2300	**tricosane**	**-**	**t**
2400	**tetracosane**	**-**	**0.9**
2500	**pentacosane**	**-**	**0.1**

KI = linear Kovats Index on DB-5, 30m column.