Comparison of volatile leaf terpenoids from *Lippia dulcis* (Verbenaceae) obtained by steam distillation and pentane liquid extraction

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ABSTRACT

Comparison of pentane extraction and steam distillation of intact leaves of *Lippia dulcis* (hierbia dulce, ex Mexico) yielded 2.13% (DW basis) vs. 0.13% for pentane extraction (18 h, shaking). Analyses of the oils showed (pentane; distilled): camphor (33.9, 21.2%), camphene (1.8, 12.7%), 6-methyl-5-hepten-2-one (7.9, 3.4%), (+)-hernandulcin, the sweet sesquiterpene (9.2, 5.9%), 3-methyl-2-cyclohexene-1-one (2.7, 0.4%). The differences in hernandulcin by extraction method seems to be due to the different effects of pentane solvent on intact leaves (vs. steam volatilization by distillation). The pentane extract was also high in free, long chain fatty acids such as linoleic, hexadecanoic, and octadecanoic acids. Quantative data are presented for 72 components. Varying GC injector temperature from 100°C to 220°C revealed degradation of hernandulcin between 200°C and 220°C. Published on-line **www.phytologia.org** *Phytologia 96(3): 252-259 (July 1, 2014).* ISSN 030319430

KEY WORDS: *Lippia dulcis,* leaf terpenoids, hernandulcin, pentane extraction, steam distillation, degradation.

Lippia dulcis Trevir. (Verbenaceae) is a sweet-tasting, woody herb sold as hierbia dulce, hierbia buena, yerba dulce, Orozuz and 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). The reader is referred to Compadre, Robbins and Kinghorn (1986) who give an excellent historical review of the folk-lore and traditional medicinal uses of the species.

The nomenclature has been subject to controversy. Moldenke (1934) considered *Lippia dulcis* as part of *Phyla* and renamed it *Phyla dulcis* (Trevir.) Moldenke, Torreya 34: 9, 1934. Floras in Ecuador (Jorgensen and Leon-Yanez, 1999) and Nicaragua (Stevens, et al., 2001) continue to place it in *Phyla*. The Germplasm Resources Information Network (GRIN), USDA (http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl? 429352, search 20 Aug. 2014) currently recognizes *Phyla dulcis*. However, IPNI (International Plant Names Index, Kew) recognizes *Lippia dulcis*, with *Phyla dulcis* treated as a synonym (search 20 Aug. 2014). Marx et al. (2010), in a study of the phylogeny and classification of the Verbenaceae using seven cp DNA markers, found *Phyla* species to occur in a well-supported clade, four *Lippia* species in a clade with *Lantana*; *Lippia dulcis* occurred outside both the *Phyla* and *Lantana* clades. *Lippia* is a large genus of over 200 species, and their study only included six *Lippia* species. Nevertheless, because the traditional species of *Phyla* formed a well-supported clade, with the exclusion of *Lippia dulcis*, their study supports the concept that *Lippia dulcis* is not a part of *Phyla*.

More recently, 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 (Table 1). 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 (Table 1). 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 (1986) 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 because 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.

Oliveira et al. (2010) reported no camphor and 19.2% hernandulcin (Table 1) in plants grown in Brazil extracted by supercritical CO_2. 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 was to run 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 about 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 in nature. See Adams (1991) for a diagram of this type of steam distillation apparatus.

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
Present study	Mexico (seeds, Chiltern Seeds, UK)[5]	21.2	9.2	pentane overnight
Present study	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.

Table 2. Comparison of column conditions vs. hernandulcin yields.

source	publication	hernandulcin %	column	injector	init. col. temp	split	liner bore
Mex.	Compadre et al. 1986	0.004[#]	DB-1	70°C	35°C	1/17	unknown
Mex.	Nayal et al. 2009	10.1	HP-5	220°C	60°C, 30 sec.	none	0.75 mm bore
Pan.	Gornmann et al. 2008	10.0	HP-5	220°C	60°C, 30 sec.	none	0.75 mm bore
Pan.	Nayal et al. 2009	14.5	HP-5	220°C	60°C, 30 sec.	none	0.75 mm bore
PR	Kaneda et al. 1992	0.154	silica gel chromatography, id by NMR (no GC/MS)				
PR	Souto-Bachiller et al. 1997	22.0	SPB-5	220°C	60°C, 30 sec.	none	0.75 mm bore
Col.	Moreno-Murillo etal. 2010	1.1*	HP-5	200°C	70°C, 2 min.	1/10	0.75 mm bore
Bra.	Oliveira, et al. 2010	19.2	OV-5	250°C	50°C	?	?
Mex.	Present study	9.2	DB-5	220°C	60°C	1/10	4.0 mm bore[$]

[$]packed with deactivated, silane treated glass wool

The purpose of this study was to compare the effects of pentane extraction and steam distillation on the composition of the volatile leaf oil of Mexican *Lippia dulcis* and to examine the effects of variation in injection temperatures on the degradation of hernandulcin.

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.

Essential oils analysis - Set: A: 10 g FW (2.2 g DW) of fresh leaves, plus 20 ng of methyl decanoate (Internal Standard) was extracted with pentane on a rotary shaker for 18 h. Set B: 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 (pentane or diethyl ether trap-removed) with nitrogen and stored at -20°C until

analyzed. Extracted leaves were oven dried to a constant weight (48 hr, 100°C) 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, 60° - 246°C at 3°C/min. (62 mins.), carrier gas He, flow 34.96 cm/sec or 1.02 ml/min, injector 220°C, detector 240°C, 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 pentane extraction (18 h) yielded 0.13% (W/W) oil, compared to 2.13% for steam distillation of 4h (Table 3). Examination of the extracted leaves after drying, revealed that the pentane-extracted leaves still had the typical sweet, camphor aroma, whereas the steam-distilled leaves were without odor. It is clear that the pentane extraction of intact leaves was not very complete. The leaves were not ground in this study to minimize the possibility of enzymatic and/ or free radical degradation of components. It is very probable that grinding the leaves would have increased the pentane extractables yields.

In addition to yields, the oils were very different in composition. The pentane extract contained 4.7 and 3.4% of the very volatile (E)-e-hexenal and (Z)-3-hexenol, versus trace in the steam distilled oil. It is common in steam distillation to lose some of the very volatile compounds. The yields of hernandulcin and epi-hernandulcin in the pentane extract was higher (9.2, 2.7%) than in the steam distilled oil (4.5, 0.7%). However, this may be due lower efficiency of pentane solvent than volatility (steam distillation) for many compounds (cf. pentane vs steam): camphene - 1.8, 12.7%; limonene - 0.7, 4.6%; camphor - 21.2, 33.9%; α-copaene - 1.5, 4.0%; (E)-caryophyllene - 2.2, 6.0%; (E)-β-farnesene - 0.8, 2.3%.

In contrast, some components were larger in the pentane extract (pentane, steam): 1-octen-3-ol - 4.1, trace; 6-methyl-5-hepten-2-one - 7.9, 3.9; benzene aldehyde - 1.8, 0.0; 3-methyl-2-cyclohexene-1-one - 4.7, 0.7; phenyl ethyl alcohol - 4.0, 0.0. The much larger amount of 1-octen-3-ol may be due to the co-solvent effects of alkanes. Phenyl ethyl alcohol may be so labile that it is degraded in the steam distilled oil.

In addition to the volatile terpenoids, large amounts of hexadecanoic, octadecanoic, and linoleic acids were removed by the pentane extractions, along with minor amounts of long chain alkanes (associated with waxy cuticles). These compounds were absent or minor in the steam distilled oil (Table 3).

The work by Kaneda et al. (1992) is relevant in that they used traditional silica gel chromatography to separate and identify (by NMR) hernandulcin, epi-hernandulcin, and 6-methyl-5-heptene-2one from *Lippia dulcis* from Panama. Although silica gel chromatography/ NMR is a slower procedure than GC/MS, no high temperatures are encountered, so degradation hernandulcin should not occur. They reported 6-methyl-5-hepten-2-one, 0.043% (w/w); hernandulcin, 0.154%(w/w). Since none of the 6-methyl-5-hepten-2-one should have arisen by degradation of hernandulcin, it follows that 6-methyl-5-hepten-2-one is a naturally occurring component of *Lippia dulcis* oil.

The ratio of 6-methyl-5-hepten-1-one / hernandulcin may be an indicator of the severity of degradation of hernandulcin. The degradation ratio was essentially equal for pentane (0.86) and steam distillation (0.87), indicating that the same amount of degradation occurred in each extraction. Because of

the low temperature during pentane extraction, it seems unlikely the hernandulcin degraded during the extraction phase, but rather in GC analysis phase. Because their degradation ratios are the same, it appears that no degradation of hernandulcin occurred during steam distillation. So either pentane or steam distillation could be used to extract *L. dulcis* oils (but a study should use only one method).

To further examine the effects of inlet injector temperature on the degradation of hernandulcin, a series of analyses were made, using one 4h distilled oil sample, by increasing the injector temperature (Table 4). 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.

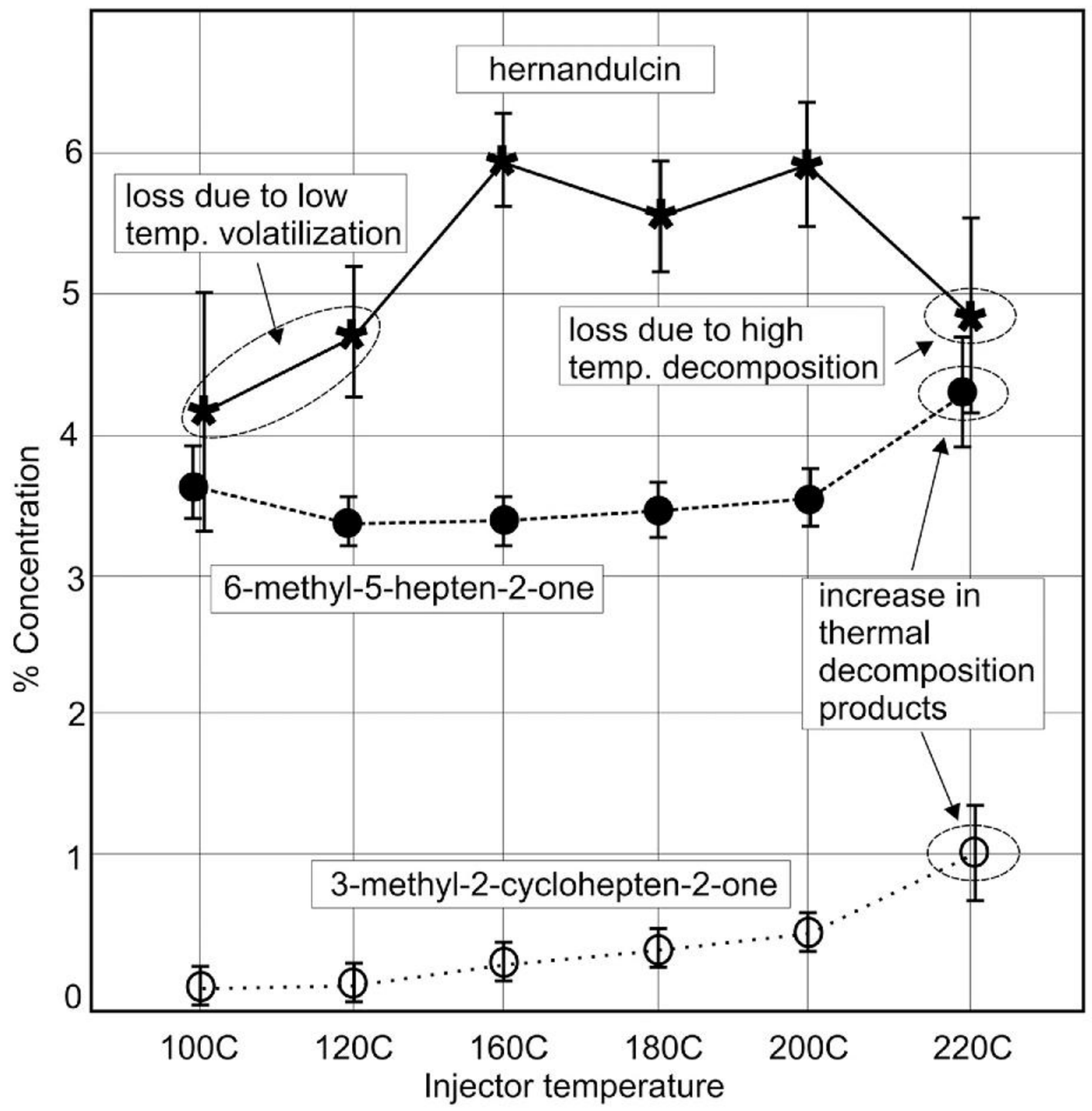

Figure 1. Plots of hernandulcin versus putative decomposition products: 6-methyl-5-hepten-2-one and 3-methyl-2-cyclohepten-2-one with changes in the injector temperature for GC analyses.

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, Table 4). 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.

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Table 3. Comparison oil compositions of *Lippia dulcis* (Mexican *Lippia* or Orozuz) extracted in pentane (RT, overnight) vs. steam distilled (4h). t < 0.1%, NI = not integrated. GC injector run at 220°C.

KI	Compound	pentane ext.	steam distilled
	percent oil yield (DM basis)	0.13%	2.13%
846	(E)-2-hexenal	4.7	t
850	(Z)-3-hexenol	3.4	t
921	tricyclene	-	0.1
932	α-pinene	0.2	2.1
946	camphene	1.8	12.7
952	benzyl aldehyde	0.1	-
974	1-octen-3-ol	4.1	t
974	β-pinene	t	0.6
981	6-methyl-5-hepten-2-one	7.9	3.4
988	myrcene	0.2	1.4
1024	limonene	0.7	4.6
1026	benzyl alcohol	t	-
1036	benzene acetaldehyde	1.8	-
1046	3-me-2-cyclohexene-1-one	4.7	0.4
1086	terpinolene	0.3	1.7
1095	linalool	t	0.4
1100	n-nonanal	t	-
1106	phenyl ethyl alcohol	4.0	-
1141	camphor	21.2	33.9
1150	(2E,6Z)-nonoadienal	0.3	-
1165	borneol	0.4	0.5
1179	p-cymen-8-ol	0.2	0.1
1186	α-terpineol	0.5	0.1
1190	methyl salicylate	t	-
1345	α-cubebene	-	0.1
1356	eugenol	0.5	-
1374	α-copaene	1.5	4.0
1387	β-bourbonene	0.1	0.5
1387	β-cubebene	-	0.1
1409	α-gurjunene	-	0.1
1417	(E)-caryophyllene	2.2	6.0
1432	α-trans-bergamotene	-	0.3
1440	(Z)-β-farnesene	-	0.2
1452	α-humulene	-	0.3
1454	(E)-β-farnesene	0.8	2.3
1464	9-epi-(E)-caryophyllene	-	0.3
1469	dehydro-sesquicineole	-	0.3
1478	γ-muurolene	0.2	0.3
1480	germacrene D	0.3	1.1
1500	α-muurolene	0.4	0.6
1505	β-bisabolene	0.3	1.0
1514	cubebol	-	0.1
1522	δ-cadinene	2.2	3.8
1561	(E)-nerolidol	0.2	0.3
1582	caryophyllene oxide	1.3	0.6
1622	sesquiterp.,85,93,136,218	-	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
1660	cis-calamen-10-ol	0.3	-
1662	sesquiterp.,43,109,218,236	-	1.7
1667	isomer of 1662	-	1.0
1683	α-bisabolol	0.1	0.1

1685	epi-α-bisabolol	2.6	2.6
1722	methyl-tetradecanoate	1.4	-
1764	sesquiterp.,111,55,178,228	0.9	-
1783	sesquiterp.,43,111,135,236	1.7	-
1849	sesquiterp.,175,189,217,232	-	-
1851	(+) hernandulcin	9.2	5.9
1865	(-) epi-hernandulcin	2.7	0.7
1892	triene, hydrocarbon,79,67, 93,248	1.3	-
1921	methyl-hexadecanoate	1.0	-
1933	cyclohexadecanolide	1.0	-
1959	hexadecanoic acid	10.7	1.2
2113	hydrocarbon,71,123,55,296	0.6	0.8
2124	methyl octadecanoate	0.6	-
2132	linoleic acid	35.5	2.5
2158	octadecanoic acid	14.2	1.0
2200	docosane	1.1	t
2300	tricosane	0.5	t
2400	tetracosane	0.4	0.9
2500	pentacosane	0.2	0.1

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

Table 4. ANOVA for selected components of *Lippia dulcis* oil distilled 4h and injected at 100°C, 120°C, 160°C, 180°C, 200°C and 220°C. F ratio from ANOVA, Signif.: *** = P=0.001; ** =P = 0.05. nt = not tested by ANOVA.

KI	Compound	100°C	120°C	160°C	180°C	200°C	220°C	F ratio	Signif.
981	6-methyl-5-hepten-2-one	3.66	3.38	3.38	3.47	3.55	4.29	5.52	***
1046	3-me-2-cyclohexene-1-one	0.10	0.10	0.30	0.38	0.50	1.02	18.19	***
1851	(+) hernandulcin	4.18	4.78	5.86	5.51	5.85	4.79	5.22	*
1865	(-) epi-hernandulcin	0.59	0.47	0.41	0.36	0.43	0.68	nt	nt