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Research Article

Chromatographic and mass spectrometric characterization of essential oils and extracts from *Lippia* (Verbenaceae) aromatic plants

Analytical methodologies based on GC and HPLC were developed for the separation and quantification of carnosic acid, ursolic acid, caffeic acid, *p*-coumaric acid, rosmarinic acid, apigenin, luteolin, quercetin, kaempferol, naringenin, and pinocembrin. These methods were used to characterize essential oils and extracts obtained by solvent (methanol) and by supercritical fluid (CO_2) extraction from stems and leaves of *Lippia* (Verbenaceae family) aromatic plants (*Lippia alba*, *Lippia origanoides*, *Lippia micromera*, *Lippia americana*, *Lippia graveolens*, and *Lippia citriodora*). Supercritical CO_2 extraction isolated solely pinocembrin and narigenin from three *L. origanoides* chemotypes. Solvent extracts possessed a more varied composition that additionally included apigenin, quercetin, and luteolin. Solvent extraction afforded higher overall flavonoid yields from all species in comparison with supercritical CO_2 extraction. Pinocembrin was determined in *L. origanoides* extract at a concentration of 30 mg/g of plant material, which is more than ten times higher than the amount at which polyphenols are regularly found in aromatic plant extracts.

Keywords: Essential oil / Flavonoid / *Lippia* / Origanoides / Supercritical fluid extraction
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1 Introduction

Genus *Lippia* belongs to the Verbenaceae family and contains around 250 species, among them, herbs and shrubs distributed throughout Central and South America and tropical Africa. These species have economic value due to their use as sources of essential oils and extracts with various interesting properties, such as antimicrobial, antibacterial, antifungal, antioxidant, larvicidal, and insecticidal activities, among many more [1]. Despite the relatively large number of pharmacological reports on their properties, very few of these plants have been included in European or North American pharmacopeias [2]. Most of the studies on composition and biological activities of *Lippia* species have focused on their essential oils [3–5]. However, there have been comparatively much fewer publications on the composition of their extracts, which constitutes a big deficit that needs to be corrected because these species are used in popular medicine mostly in the form of infusion [6–8].

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Abbreviations: ACF, caffeic acid; **ACR**, carnosic acid; **APC**, *p*-coumaric acid; **AR**, rosmarinic acid; **AU**, ursolic acid; **DAD**, diode-array detector; **FID**, flame ionization detection; **K**, kaempferol; **L**, luteolin; **LRI**, linear retention index; **N**, naringenin; **P**, pinocembrin; **Q**, quercetin; **SFE**, supercritical fluid extraction

In this work, the volatile secondary metabolite profiles of several *Lippia* aromatic plants commonly used in traditional folk medicine in Colombia was determined. Polyphenol contents in their extracts, obtained with methanol/water and with supercritical fluid (CO_2) were quantified as well. For this determination, chromatographic methodologies were developed for the simultaneous determination of 11 well-known natural antioxidants (six flavonoids, four phenolic acids, and a triterpene, Fig. 1), using either GC/flame ionization detection (FID) or LC/diode-array detector (DAD) as separation and detection techniques.

2 Materials and methods

2.1 Standard substances and vegetal material

Borneol, camphene, camphor, carvacrol, carvone, *trans*-β-caryophyllene, caryophyllene oxide, 1,8-cineole, citronellal, α-copaene, β-copaene, *p*-cymene, α-eudesmol, γ-eudesmol, eugenol, geranial, geraniol, geranyl acetate, α-humulene, limonene, *cis*-limonene oxide, *trans*-limonene oxide, linalool, 6-methyl-5-hepten-2-one, β-myrcene, neral, nerol, *trans*-nerolidol, 1-octen-3-ol, α-phellandrene, α-pinene, β-pinene, α-terpinene, γterpinene, terpinolene, thymol, caffeic, carnosic, *p*-coumaric, rosmarinic and ursolic acids, apigenin, kaempferol, luteolin, naringenin, pinocembrin and quercetin, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Leaves and stems of the studied species were obtained from experimental plots maintained at the Pilot Agroindustrial Complex of the National Center for

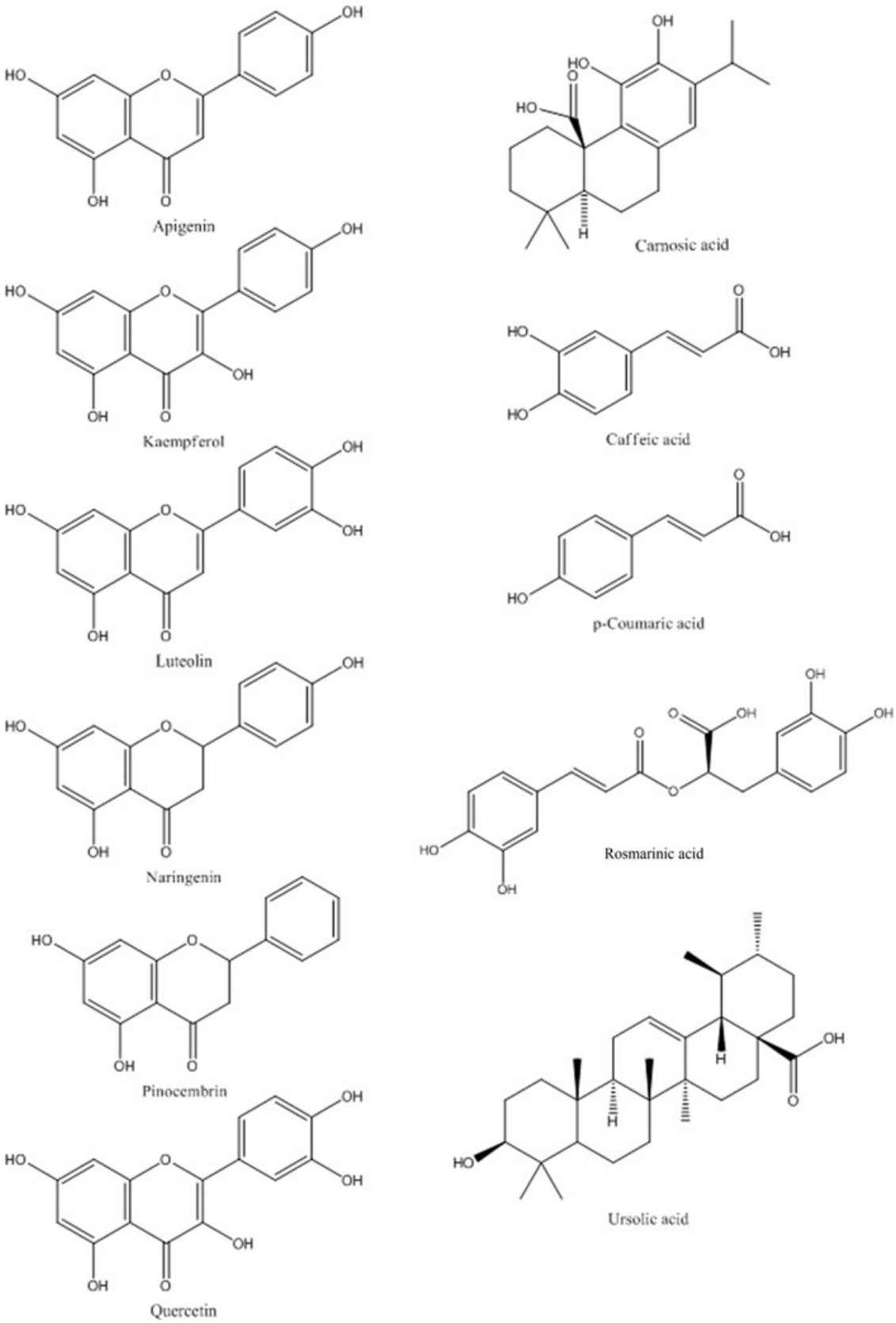

Figure 1. Chemical structures of the 11 natural antioxidants determined in solvent (methanol) and supercritical fluid (CO_2) extracts of *Lippia* aromatic plants, by means of LC-DAD and GC-FID methods.

Agroindustrialization of Aromatic and Medicinal Tropical Vegetal Species, CENIVAM, at Universidad Industrial de Santander, in Bucaramanga, Colombia. The following ten species and chemotypes were studied, whose voucher specimens were deposited at the Colombian National Herbarium (COL; voucher number in parenthesis): *Lippia alba*, carvone-rich chemotype (512078); *Lippia alba*, citral-rich chemotype (512077); *Lippia alba*, carvone-citral chemotype (512084); *Lippia origanoides*, caryophyllene-rich chemotype (519798); *Lippia origanoides*, carvacrol-rich chemotype (512075); *Lippia origanoides*, thymol-rich chemotype (519799); *Lippia graveolens* (555839); *Lippia citriodora* (517189), *Lippia micromera* (516924), and *Lippia americana* (516287). These experimental plots were started with plant material gathered during botanical expeditions for which an official collection permit was granted by the Colombian Ministry of Housing and Environment.

2.2 Essential oils and plant extracts

Essential oils were obtained by microwave-assisted hydrodistillation from chopped leaves and stems of the studied plants [9]. After their separation by decantation, all essential oils were dried with anhydrous sodium sulfate. For their chromatographic analysis, essential oil (50 μL) samples were mixed with *n*-tetradecane (2 μL) and diluted with dichloromethane to 1.0 mL.

Solvent extraction was performed according to the procedure described by Hertog et al. [10]. Chopped and dried plant material (0.5 g) was mixed with an aqueous methanol solution (62.5%, 40 mL), which contained *p*-hydroxybenzoic acid (1 g/L), and with hydrochloric acid (6 M, 10 mL). The mixture was sonicated (15 min) and brought to reflux (2 h). The extract was separated by filtration over a polyvinylidene fluoride membrane (0.45 μm). One portion was dedicated to HPLC analysis and the remnant was devoted to derivatization in order to perform GC analysis. For the latter, extraction with ethyl acetate (3 × 10 mL) afforded an organic phase that was concentrated to 10 mL by rotoevaporation, dried with anhydrous sodium sulfate, filtered, and brought to dryness with a nitrogen flow, before dissolving in acetone. Dimethyl sulfate was used as methylation reagent to obtain the corresponding derivatives of hydroxyl-bearing compounds in this extract, following the procedures proposed by Hergert et al. [11] and Forsyth et al. [12].

Finely grounded dry plant material (200 g) was placed in the 2 L extraction chamber of a Thar SFE-2000–2-FMC50 (Thar Instruments, Pittsburgh, PA, USA) and extracted with supercritical CO_2 at 50 MPa and 333 K. An initial static period (20 min) was followed by a continuous CO_2 flow (40 g/min, 100 min), which was conducted to a vortex collection chamber, in which the pressure was reduced to 0.1 MPa.

2.3 GC

Compound identification was based on chromatographic (retention times and linear retention indices, linear retention index (LRI), use of standard compounds) and spectroscopic (mass spectra interpretation, comparison with databases and standard compounds) criteria. The spectroscopic and chromatographic data were obtained with a GC 6890 Plus (Agilent Technologies, Palo Alto, CA, USA) equipped with a mass selective detector 5973 (Electron impact ionization, EI, 70 eV; Agilent Technologies), split/splitless injector (1:30 split ratio), and a MS-ChemStation G1701-DA data system, that included the spectral libraries WILEY, NIST, and QUADLIB 2007. A fused-silica capillary column DB-5MS (J&W Scientific, Folsom, CA, USA) of 60 m × 0.25 mm id, coated with 5% phenyl poly(methylsiloxane) (0.25-μm film thickness) was used. The GC oven temperature was programmed from 45°C (5 min) to 150°C (2 min) at 4°C per min, then to 250°C (5 min) at 5°C per min, and finally, to 275°C (15 min) at 10°C per min. The temperatures of the injection port, ionization chamber, and of the transfer line were set at 250, 230, and 285°C, respectively. Helium (99.995%, Linde, Bucaramanga, Colombia) was used as carrier gas, with 155 kPa column head pressure and 27 cm/s linear velocity (1 mL/min, at constant flow). Mass spectra and reconstructed (total) ion chromatograms were obtained by automatic scanning in the mass range of *m/z* 30–300 at 5.1 scan/s for essential oil analysis and *m/z* 40–500 for flavonoid analysis. A gas chromatograph GC 7890 (Agilent Technologies), equipped with FID, split/splitless injector (1:30 split ratio), and a data system (ChemStation Rev. B.03.02 [341]) was used for GC analysis and quantification of essential oil components and flavonoid derivatives. The detector and the injector temperatures were set at 250°C. A DB-5 (J&W Scientific) 60 m × 0.25 mm id capillary column coated with 5%-phenyl poly(methylsiloxane) (0.25-μm film thickness) and a DB-WAX (J&W Scientific) 60 m × 0.25 mm id capillary column coated with poly(ethyleneglycol) (0.25-μm film thickness) were used for quantification and linear retention indices determination. The oven temperature for both columns was programmed identically as for the GC-MS analysis described above.

2.4 LC

An Agilent Technologies 1200 LC, equipped with a quaternary pump (AT G1353A), a manual injector (G1328B), and a UV-Vis DAD (G1315B) was employed. A ZORBAX Eclipse XDB-C18 (4.6 × 150 mm, 5 μm) column was used. Chromatographic data were processed with an Agilent Chemstation LC data system, version B.04.01. Binary mixtures of solvents A (water with acetic, *o*-phosphoric or trifluoroacetic acid as modifier) and B (methanol and ACN as organic modifiers) in various proportions were used to achieve peak resolution.

3 Results and discussion

3.1 Essential oil composition

A total of 91 compounds, which represented between 84 and 99% of the total chromatographic area, were identified in the ten essential oils studied (Table 1). Limonene

Table 1. Relative amounts (%) of volatile and semivolatile secondary metabolites in essential oils of *Lippia* aromatic plants

Number	Compound	LRI		*L. alba*, CTa1	*L. alba*, CTa2	*L. alba*, CTa3	*L. origanoides*, CTo1	*L. origanoides*, CTo2	*L. origanoides*, CTo3	*L. citriodora*	*L. micromera*	*L. americana*	*L. graveolens*
		DB5MS	DBWAX										
1	α-Thujene	925	1026	–	–	–	1.0	1.5	0.7	–	2.8	0.8	0.9
2	α-Pinene[a)]	933	1022	–	0.2	0.1	0.5	0.4	2.3	0.7	1.0	–	3.5
3	Camphene[a)]	946	1054	0.1	–	0.3	0.1	0.1	2.6	–	0.2	0.1	1.0
4	Sabinene	969	1122	–	–	–	0.1	0.1	0.5	1.9	0.6	7.4	0.7
5	1-Octen-3-ol[a)]	974	1350	–	–	0.1	0.3	0.2	0.9	1.1	–	–	–
6	β-Pinene[a)]	982	1108	–	–	–	–	–	–	–	0.3	0.5	1.6
7	6-Methyl-5-hepten-2-one[a)]	986	1241	–	5.2	1.9	–	–	–	1.5	–	–	–
8	β-Myrcene[a)]	988	1165	0.8	0.1	0.7	2.8	2.5	1.5	0.3	3.1	0.6	3.4
9	6-Methyl-5-hepten-2-ol	992	1365	–	0.2	–	–	–	–	0.2	–	–	–
10	α-Phellandrene[a)]	1006	1164	–	0.1	0.5	0.9	0.3	9.9	–	0.5	0.6	4.5
11	δ-3-Carene	1013	1149	–	–	–	0.2	0.2	–	–	0.3	0.3	4.5
12	α-Terpinene[a)]	1022	1181	–	–	–	1.6	2.7	0.6	–	4.0	–	2.6
13	*p*-Cymene[a)]	1029	1250	0.1	0.1	–	10.0	12.0	11.2	–	13.1	1.2	12.8
14	Limonene[a)]	1034	1200	30.2	3.2	22.4	1.2	0.4	7.2	10.7	1.1	1.7	0.5
15	β-Phellandrene	1036	1210	–	0.1	–	1.0	0.8	6.5	–	5.0	2.6	–
16	1.8-Cineole[a)]	1040	1225	–	–	–	–	–	–	5.0	–	–	18.5
17	*trans*-β-Ocimene	1048	1253	0.3	0.3	0.5	0.2	0.4	0.6	1.8	0.2	0.5	0.2
18	γ-Terpinene[a)]	1065	1250	–	–	–	5.0	9.5	1.5	–	12.5	2.6	4.6
19	*cis*-Sabinene hydrate	1075	1450	–	–	–	0.4	0.4	0.2	0.8	–	0.3	0.4
20	Terpinolene[a)]	1090	1286	–	–	0.1	0.1	0.1	0.6	–	0.2	0.2	3.2
21	Linalool[a)]	1101	1553	0.3	2.0	0.6	0.7	0.3	0.9	0.7	0.2	0.2	2.8
22	*trans*-Sabinene hydrate	1106	1160	–	–	–	0.1	0.1	0.3	–	–	–	0.5
23	*trans-p*-Mentha-2.8-dien-1-ol	1127	1580	0.4	–	0.1	0.1	–	0.4	0.4	–	–	0.8
24	*cis*-Limonene oxide[a)]	1138	1350	0.1	–	0.1	–	–	–	0.4	–	–	–
25	*trans*-Limonene oxide[a)]	1143	1352	–	–	–	–	–	–	0.5	–	–	–
26	Citronellal[a)]	1154	1381	–	0.7	0.3	–	–	–	–	–	–	–
27	Camphor[a)]	1157	1496	–	0.2	–	–	–	0.1	–	–	–	–
28	*cis*-Chrysanthenol	1163	1449	–	0.6	0.1	–	–	–	–	–	–	–
29	Rosefuran epoxide	1172	1509	–	1.0	0.1	–	–	–	–	–	–	–
30	Borneol[a)]	1182	1709	0.3	0.8	0.6	–	0.1	3.1	–	0.8	0.1	0.2

Table 1. Continued

Number	Compound	LRI		*L. alba*, CTa1	*L. alba*, CTa2	*L. alba*, CTa3	*L. origanoides*, CTo1	*L. origanoides*, CTo2	*L. origanoides*, CTo3	*L. citriodora*	*L. micromera*	*L. americana*	*L. graveolens*
		DB5MS	DBWAX										
31	Umbelulone	1188	1652	–	–	–	–	–	–	–	0.8	–	–
32	Terpinen-4-ol	1189	1577	–	–	–	0.7	1.1	1.3	–	0.4	0.3	2.2
33	α-Terpineol	1203	1705	–	–	–	0.1	–	1.1	1.7	0.1	–	4.0
34	*cis*-Dihydrocarvone	1204	1517	0.3	–	0.4	–	–	–	1.0	–	–	–
35	*trans*-Dihydrocarvone	1212	1537	0.5	–	0.3	–	–	–	–	–	–	–
36	Nerol[a)]	1230	1708	–	3.0	0.7	–	–	–	2.0	–	–	–
37	Thymyl methyl ether	1232	1599	–	–	–	1.9	1.0	–	–	14.9	–	0.4
38	Neral[a)]	1247	1589	–	21.0	10.4	–	–	–	15.6	–	–	–
39	Carvone[a)]	1253	1732	50.3	–	25.3	–	–	–	–	–	0.7	–
40	Geraniol[a)]	1254	1755	–	6.0	–	–	–	–	2.7	–	–	–
41	Piperitone	1264	1641	3.1	0.1	1.1	–	–	–	–	–	–	–
42	Geranial[a)]	1277	1741	–	27.0	10.4	–	–	–	18.9	0.5	–	–
43	Thymol[a)]	1290	2193	–	–	0.1	54.5	9.9	0.5	–	29.1	–	0.7
44	Carvacrol[a)]	1304	2172	–	–	–	1.7	46.2	–	–	1.0	0.2	–
45	δ-Elemene	1345	1481	–	–	–	–	–	–	–	–	0.7	–
46	Piperitenone	1350	1842	6.1	–	2.2	–	–	–	–	–	–	–
47	Thymyl acetate	1350	1858	–	–	–	4.8	0.1	–	–	2.7	–	–
48	Eugenol[a)]	1358	2080	–	0.3	0.2	0.1	0.3	0.6	0.3	–	–	0.2
49	Geranyl acetate[a)]	1379	1662	–	0.6	0.4	–	–	–	1.3	–	–	–
50	α-Copaene[a)]	1387	1450	–	–	0.1	0.1	–	0.9	0.5	–	0.8	0.3
51	β-Cubebene	1394	1466	–	–	–	–	–	0.1	–	–	2.2	–
52	β-Bourbonene	1396	1517	1.0	–	–	–	–	0.2	0.8	–	1.3	–
53	β-Elemene	1398	1496	–	0.8	1.7	–	–	0.3	–	–	–	0.3
54	*trans*-β-Caryophyllene[a)]	1436	1606	–	5.4	2.4	2.4	2.0	11.3	2.3	1.9	12.2	6.5
55	β-Gurjunene	1444	1447	0.1	–	0.2	–	–	1.0	–	–	–	–
56	β-Copaene[a)]	1446	1607	–	–	–	–	–	–	–	–	0.6	–
57	α-Guaiene	1448	1498	–	1.4	1.3	–	–	–	–	–	–	0.1
58	γ-Muurolene	1464	1709	–	–	–	–	–	–	–	–	0.5	–
59	α-Humulene[a)]	1472	1674	–	1.6	0.5	1.5	1.5	6.0	–	0.2	2.6	4.0
60	γ-Gurjunene	1476	2210	0.1	0.1	0.3	0.1	0.1	1.0	0.5	–	1.2	–
61	γ-Muurolene	1486	1697	–	–	0.1	0.2	0.1	1.3	–	0.2	–	0.6

Table 1. Continued

Number	Compound	LRI		*L. alba*, CTa1	*L. alba*, CTa2	*L. alba*, CTa3	*L. origanoides*, CTo1	*L. origanoides*, CTo2	*L. origanoides*, CTo3	*L. citriodora*	*L. micromera*	*L. americana*	*L. graveolens*
		DB5MS	DBWAX										
62	*ar*-Curcumene	1488	1775	–	–	–	–	–	0.2	3.9	–	–	–
63	Bicyclosesquiphellandrene	1494	1624	3.5	1.5	8.0	–	–	–	–	–	–	–
64	Germacrene D	1495	1719	–	–	0.1	0.1	0.1	0.7	1.4	0.4	16.3	–
65	α-Zingiberene	1501	1719	–	–	–	–	–	0.3	0.3	–	–	
66	Geranyl isobutanoate	1508	1633	–	0.6	–	–	–	–	–	–	–	–
67	α-Muurolene	1508	1736	–	–	–	–	–	0.7	–	0.2	–	–
68	β-Selinene	1509	1744	–	–	–	–	–	1.2	–	–	1.7	1.1
69	Bicyclogermacrene	1510	1750	0.2	–	0.7	0.1	0.1	–	1.3	–	5.6	–
70	α-Bulnesene	1515	1627	–	0.8	0.8	–	–	–	–	–	–	–
71	β-Bisabolene	1515	1745	–	–	–	0.6	–	–	–	–	–	0.9
72	γ-Cadinene	1526	1789	–	0.3	–	0.1	–	0.8	–	–	0.5	0.2
73	Cubebol	1529	1855	0.3	0.2	0.4	–	–	–	1.0	–	–	–
74	δ-Cadinene	1530	1783	–	–	–	0.1	–	1.6	–	–	1.5	0.3
75	*trans*-Calamenene	1534	1844	–	–	–	–	–	1.1	–	0.3	–	–
76	*cis*-Calamenene	1536	1845	–	–	–	–	–	0.4	–	–	0.4	0.3
77	*trans*-Cadin-1.4-diene	1547	1783	–	–	–	–	–	–	–	–	0.6	–
78	α-Cadinene	1551	1799	–	–	–	–	–	–	–	–	0.6	–
79	*trans*-Nerolidol[a)]	1566	1946	–	0.1	–	–	–	0.2	1.7	–	–	–
80	Germacrene B	1581	1840	–	–	–	–	–	–	–	–	3.0	–
81	Germacren-4-ol	1593	2123	–	0.1	0.2	–	–	–	–	–	2.8	–
82	Spathulenol	1595	2043	–	–	0.1	–	–	–	4.7	–	–	0.1
83	Caryophyllene oxide[a)]	1600	2005	–	2.2	0.4	0.4	–	2.2	5.0	0.5	2.0	3.5
84	*cis*-Bisabolol	1617	1626	–	1.0	–	–	–	–	–	–	–	–
85	Humulene II epoxide	1629	1964	–	0.5	–	–	–	–	–	–	–	0.9
86	Cubenol	1636	2057	–	–	–	–	–	0.2	0.4	–	3.6	0.1
87	γ-Eudesmol[a)]	1647	2184	–	–	–	–	–	0.6	–	–	–	0.5
88	*trans*-Asarone	1648	2314	–	1.0	–	–	–	–	–	–	–	–
89	α-Muurolol	1658	1660	–	–	–	–	–	0.3	–	–	0.4	–
90	α-Cadinol	1672	2219	–	–	–	–	–	0.3	–	–	1.9	–
91	α-Eudesmol[a)]	1674	2116	–	–	–	–	–	1.4	–	–	–	3.2

a) Identification based on LRI and comparison with mass spectra of standard compound.

(0.4–30.2%), β-myrcene (0.1–3.4%), *trans*-β-ocimene (0.2–1.8%), and linalool (0.3–2.8%), were the only substances common to all oils. The relative amount of the most abundant component varied between 11.3% (*trans*-β-caryophyllene, in CTo3, the *L. origanoides*, caryophyllene-rich chemotype), and 54.5% (thymol, in CTo1, the *L. origanoides*, thymol-rich chemotype). The number of constituents with relative amount above 1% varied between six (*L. alba*, carvone chemotype, CTa1) and 21 (*L. citriodora* and *L. origanoides*, caryophyllene-rich chemotype, CTo3). Ten essential oils of six *Lippia* species were studied, which included three chemotypes of both *L. alba* and *L. origanoides*. Two of the *L. alba* chemotypes, rich in carvone (CTa1) or citral (CTa2), had a very different composition, with only nine substances in common, while the third chemotype contained many constituents found in either CTa1 or CTa2. On the other hand, the three *L. origanoides* chemotypes had 25 constituents in common, but differed in their major components. This fact was also observed by Stashenko et al. [13, 14]. The thymol (CTo1)- and carvacrol (CTo2)-rich *L. origanoides* chemotypes had similar composition, although with contrasting differences in the relative amounts of thymol (54.5 and 9.9%, respectively) and carvacrol (1.7 and 46.2%, respectively). The main components of the third *L. origanoides* chemotype (CTo3) were *trans*-β-caryophyllene (11.3%) and *p*-cymene (11.2%), which were also present in the other two oils.

In order to search for composition-based similarities among the essential oils, their composition table was subjected to principal component analysis based on the covariance matrix. Figure 2 contains the representation of the ten essential oils in the space formed by the first three principal components, which correspond to 81.03% of the information. As can be seen in Fig. 2, the most closely related compositions were those of *L. americana*, *L. graveolens*, and *L. origanoides*, caryophyllene-rich chemotype (CTo3), essential oils. Their composition contained 23 substances in common, the most abundant of which was *trans*-β-caryophyllene (12.2, 6.5, and 11.3% in *L. americana*, *L. graveolens* and *L. origanoides* CTo3 oils, respectively), followed by *p*-cymene (1.2, 12.8, and 11.2%, respectively). Other compounds in common, with relative abundances above 1%, were γ-terpinene, α-humulene, β-selinene, and caryophyllene oxide. The oils of *L. micromera* and *L. origanoides*, thymol-rich chemotype (CTo1), appear close to this group. There were 13 compounds common to these five oils, the most abundant of which was *p*-cymene (13.1 and 10.0% in *L. micromera* and *L. origanoides*, thymol-rich chemotype (CTo1), respectively).

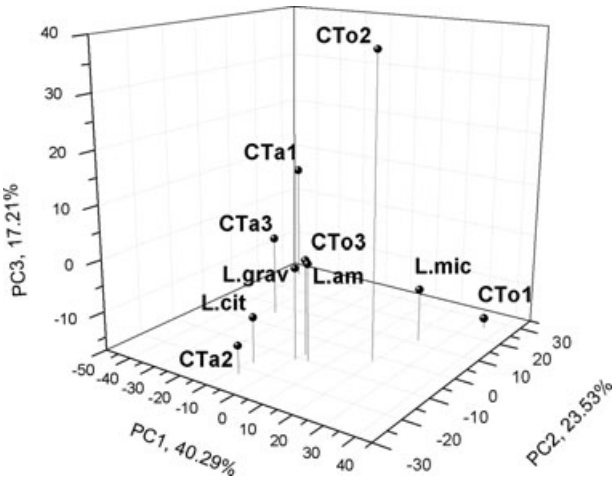

Figure 2. Representation of ten essential oils from *Lippia* aromatic plants in the space formed by the first three principal components (81.03% of the information), based on the covariance of their content of 91 terpenes identified by GC-MS analysis. CTa1, *Lippia alba*, carvone-rich chemotype; CTa2, *Lippia alba*, citral-rich chemotype; CTa3, *Lippia alba*, carvone-citral chemotype; CTo1, *Lippia origanoides*, thymol-rich chemotype; CTo2, *Lippia origanoides*, carvacrol-rich chemotype; CTo3, *Lippia origanoides*, caryophyllene-rich chemotype; *L. am*, *Lippia americana*; *L. cit*, *Lippia citriodora*; *L. grav*, *Lippia graveolens*; *L. mic*, *Lippia micromera*.

3.2 LC method for flavonoids

Figure 3 contains a typical chromatographic profile obtained after the study of operating conditions that afforded good resolution and sensitivity for a mixture of caffeic acid (ACF), carnosic acid (ACR), ursolic acid (AU), *p*-coumaric acid (APC), rosmarinic acid (AR), quercetin (Q), naringenin (N), luteolin (L), kaempferol (K), apigenin (A), and pinocembrin (P). An *o*-phosphoric acid aqueous solution (0.1%), solvent A, was mixed with methanol, solvent B, according to the following gradient elution program, at 1.0 mL/min, with a column (ZORBAX Eclipse XDB-C18) temperature of 35°C: 0–6 min: 40% B, 10 min: 48% B, 10–23 min: 48% B, 30 min: 100% B, 30–40 min 100% B. The absorbance registered at the following wavelengths was associated to the antioxidants indicated in parenthesis: 210 nm (carnosic

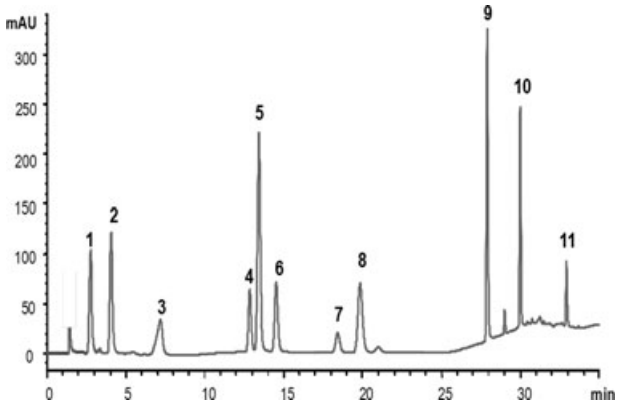

Figure 3. Typical chromatographic profile obtained from a mixture of flavonoids and phenolic acids with the HPLC-DAD method developed. Nonpolar ZORBAX Eclipse XDB-C18 column, wavelength: 210 nm. 1, caffeic acid (ACF); 2, *p*-coumaric acid (APC); 3, rosmarinic acid (AR); 4, quercetin (Q); 5, naringenin (N); 6, luteolin (L); 7, kaempferol (K); 8, apigenin (A); 9, pinocembrin (P), 10, carnosic acid (ACR); 11, ursolic acid (AU).

acid, ACR; ursolic acid, AU; naringenin, N; pinocembrin, P), 280 nm (caffeic acid, ACF), 325 nm (rosmarinic acid, AR; *p*-coumaric acid, APC; apigenin, A) and 370 nm (luteolin, L; kaempferol, K; quercetin, Q). The injected sample volume was 20 μL. The chromatographic profiles obtained with six consecutive injections of a 40 mg/kg solution of the 11 standard compounds of flavonoids and phenolic acids showed variation coefficients between 0.04 and 1.1% for retention times and between 1.8 and 4.3% for chromatographic areas. Linear calibration curves with slopes between 8.3 and 116.5 counts kg/mg and determination coefficients R^2 between 0.9982 and 0.9998 were obtained. Detection limits down to sub-mg/kg were obtained for seven of the 11 antioxidants examined: ACF (0.28), ACR (0.24), AU (2.36), APC (0.48), AR (1.37), Q (0.73), N (0.66), L (0.80), K (1.69), A (1.13), P (0.36).

3.3 GC method for flavonoid derivatives

The permethylation conditions for flavonoids were refined according to the results obtained with quercetin and rosmarinic acid (five hydroxyl groups each). Since having a large number of derivatives for each analyte hinders its quantitation, preference was given to those conditions that afforded reproducible permethylation, but not many families of partially methylated derivatives. The final procedure, which was applied to all extracts, consisted of mixing the sample (1 mg) with dimethyl sulfate and sodium carbonate in a 1:20:60 proportion in acetone (300 μL) and stirring for 2 h at 55–60°C. Distilled water (1 mL) was added to eliminate any dimethyl sulfate excess, followed by extraction with dichloromethane. The organic phase was dried with anhydrous sodium sulfate and concentrated to 100 μL, from where an aliquot was taken for chromatographic analysis. A typical chromatographic profile of a mixture of 19 derivatives of the 11 natural antioxidants subjected to this derivatization procedure appears in Fig. 4. Quercetin and ursolic acid produced their tetramethyl and monomethyl derivatives, respectively, while all other antioxidants afforded two derivatives each, which were either geometric isomers or derivatives with different methylation levels. Mass spectra were used to identify the various derivatives. The chromatographic conditions were a modification of those employed by Proestos et al. [15], which gave poor resolution between peaks 6 and 7 and 11 and 12 (Fig. 4). The improved separation was achieved with the DB-5 (60 m × 0.25 mm × 0.25 μm) column, an oven temperature program starting at 110°C (3 min) raised to 290°C (5 min) at 5°C per min then to 310°C (10 min) at 2.5°C per min. A split ratio of 1:10, a carrier gas flow of 1 mL/min, and a flame ionization detector temperature of 295°C were used. High separation efficiency was manifested by a calculated number of theoretical plates above 490 000. Resolution above 1.5 was achieved for all derivatives. Observed peak symmetry factors varied between 0.8 and 1.12, which indicated peak shapes close to the expected Gaussian character [16]. Linear calibration curves were obtained in the 10–1300 mg/kg concentration range, with determination coefficients R^2 between 0.9994 and 0.9999. Retention times and chromatographic areas had variation coefficients below 1% and between 2.2 and 9.1%, respectively. The following detection limits between 1.4 and 19.1 mg/kg were obtained for the 19 methyl-derivatives of the flavonoids and phenolic acids studied: *p*-coumaric acid methyl derivatives (2.1 mg/kg), caffeic acid methyl derivatives (1.9 mg/kg), carnosic acid methyl derivatives (2.6 mg/kg), rosmarinic acid methyl derivatives (4.9 mg/kg), ursolic acid methyl derivative (19.1 mg/kg), pinocembrin methyl derivatives (1.4 mg/kg), naringenin methyl derivatives (2.7 mg/kg), apigenin methyl derivatives (4.6 mg/kg), kaempferol methyl derivative (13.6 mg/kg), luteolin methyl derivatives (2.8 mg/kg), and quercetin methyl derivatives (7.3 mg/kg).

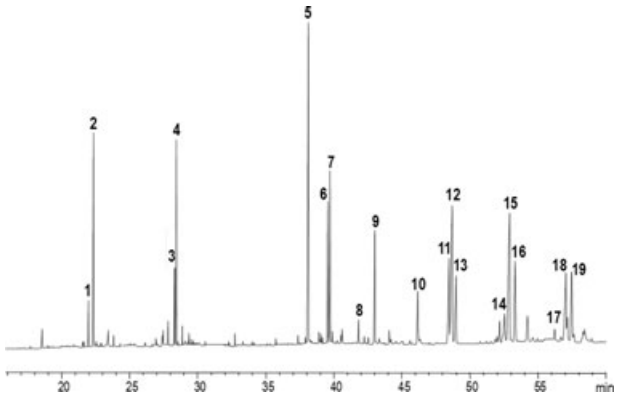

Figure 4. Typical chromatographic profile obtained from a mixture of methyl derivatives of flavonoids and phenolic acids, with the GC-FID method developed. DB-5 60 m × 0.25 mm id capillary column coated with 5%-phenyl poly(methylsiloxane, 0.25 μm film thickness. 1, *cis*-dimethyl-*p*-coumaric acid; 2, *trans*-dimethyl-*p*-coumaric acid; 3, *cis*-trimethyl caffeic acid; 4, *trans*-trimethyl caffeic acid; 5, monomethylpinocembrin; 6, dimethylcarnosic acid I; 7, dimethylcarnosic acid II; 8, dimethylpinocembrin; 9, dimethylnaringenin; 10, trimethylnaringenin; 11, dimethylapigenin; 12, trimethylapigenin; 13, trimetihylkaempferol; 14, tetramethylluteolin; 15, trimethyluteolin; 16, tetramethylquercetin; 17, *cis*-pentamethylrosmarinic acid; 18, *trans*-pentamethylrosmarínic acid; 19, monomethylursolic acid.

3.4 Extract composition

Supercritical fluid extraction (SFE) yields varied between 0.3 (*L. citriodora*, *L. alba* CTa1) and 4.3% (*L. graveolens*). None of the 11 natural antioxidants examined was found in *L. americana* extracts in concentration above their detection limits. Since flavonoids may be present in glycosylated form, acid hydrolysis was used to release them. HPLC analysis showed that indeed, there was a higher flavonoid amount in extracts subjected to acid hydrolysis. Table 2 presents the results of polyphenol quantification by means of the LC and GC methodologies developed, applied to solvent and SFE extracts after acid hydrolysis. Only five flavonoids (Q, N, L, A, and P) out of the 11 flavonoids and phenolic acids studied were found above their corresponding

Table 2. Flavonoid concentration (mg/g of plant material ± *S*, *n* = 3) in solvent and SFE extracts of *Lippia* aromatic plants, determined by GC and LC methodologies

Extract	Quercetin		Naringenin		Luteolin		Apigenin		Pinocembrin	
	LC	GC	LC	GC	LC	GC	LC	GC	LC	GC
L. alba, CTa1, MeOH extract					1.41 ± 0.03	1.2 ± 0.1				
L. alba, CTa2, MeOH extract					1.86 ± 0.06	1.69 ± 0.09				
L. origanoides, CTo1, MeOH extract	0.5 ± 0.3	0.21 ± 0.4	5.56 ± 0.07	3.85 ± 0.02	3.08 ± 0.04	2.87 ± 0.06			0.7 ± 0.1	0.5 ± 0.1
L. origanoides, CTo1, CO_2 extract			0.11 ± 0.02	0.11 ± 0.02					0.05 ± 0.02	
L. origanoides, CTo2, MeOH extract carvacrol	0.57 ± 0.02	0.38 ± 0.07	5.67 ± 0.05	6.7 ± 0.3	2.92 ± 0.07	2.13 ± 0.05			0.48 ± 0.03	0.31 ± 0.04
L. origanoides, CTo2, CO_2 extract			0.09 ± 0.02	0.06 ± 0.02					0.029 ± 0.004	
L. origanoides, CTo3, MeOH extract			1.26 ± 0.02	1.1 ± 0.8	3.2 ± 0.1	2.8 ± 0.5			30 ± 1	27.3 ± 0.5
L. origanoides, CTo3, CO_2 extract			0.032 ± 0.004						0.98 ± 0.05	0.74 ± 0.05
L. citriodora, MeOH extract					0.26 ± 0.03	0.26 ± 0.03				
L. graveolens, MeOH extract	0.18 ± 0.02	0.11 ± 0.05	1.06 ± 0.02	0.91 ± 0.04	2.9 ± 0.6	2.1 ± 0.3	0.51 ± 0.01	0.11 ± 0.09		
L. micromera, MeOH extract			0.24 ± 0.05	0.11 ± 0.03			1.03 ± 0.07	0.95 ± 0.08		

Table 3. Flavonoid concentrations found in solvent extracts of various common aromatic plants and *Lippia* species studied in this work

Species	Family	Flavonoid content, mg/g of plant material ± S ($n = 3$)					
		Quercetin	Naringenin	Pinocembrin	Luteolin	Apigenin	Reference
Ruta graveolens	Rutaceae	0.031 ± 0.001					[17]
Geranium purpureum	Geraniaceae	0.11 ± 0.02					
Castanea vulgaris	Fagaceae	0.036 ± 0.001	0.002 ± 0.002				
Jasminum officinalis	Oleaceae	0.035 ± 0.0002					
Origanum dictamnus	Lamiaceae	0.013 ± 0.002					
Nepeta cataria	Lamiaceae				0.036 ± 0.002		
Rosmarinus officinalis	Lamiaceae				0.016 ± 0.02		[18]
Salvia officinalis	Lamiaceae				0.002 ± 0.001	0.003 ± 0.001	
Thymus vulgaris	Lamiaceae				0.36 ± 0.03	0.040 ± 0.002	
Menta viridis	Lamiaceae					0.007 ± 0.001	
Menta pelugium	Lamiaceae		0.42 ± 0.02			1.10 ± 0.02	
Origanum majorana	Lamiaceae					0.008 ± 0.001	
Lippia graveolens	Verbenaceae	0.47 ± 0.02	3.35 ± 0.03	9.16 ± 0.02	0.51 ± 0.02		[6]
Verbena rigida	Verbenaceae				10.2 ± 0.64	4.4 ± 0.77	[19]
Verbena ternera	Verbenaceae				4.5 ± 0.36	6.4 ± 0.85	
Verbena venosa	Verbenaceae	–	–	–	4 ± 1.0	4.8 ± 0.62	
L. alba, CTa1	Verbenaceae				1.41 ± 0.03		Present work
L. alba, CTa2	Verbenaceae				1.86 ± 0.06		
L. origanoides, CTo1	Verbenaceae	0.5 ± 0.3	5.56 ± 0.07	0.7 ± 0.01	2.87 ± 0.06		
L. origanoides, CTo2	Verbenaceae	0.57 ± 0.02	5.67 ± 0.05	0.48 ± 0.03	2.92 ± 0.07		
L. origanoides, CTo3	Verbenaceae		1.26 ± 0.02	30 ± 1	3.2 ± 0.1		
L. citriodora	Verbenaceae				0.26 ± 0.03		
L. graveolens	Verbenaceae	0.18 ± 0.02	1.06 ± 0.02		2.9 ± 0.6	0.51 ± 0.01	
L. micromera	Verbenaceae		0.24 ± 0.05		1.03 ± 0.07		

detection limits in the extracts obtained by both techniques (solvent extraction and SFE). With the exception of the quantification of naringenin in the solvent extract from *L. origanoides*, carvacrol-rich chemotype (CTo2), the amount determined with the LC method was higher (about 10–15%) than that found with the GC method. Flavonoids Q, N, L, and P were found in the solvent extracts obtained from *L. graveolens* and the three *L. origanoides* chemotypes. The pinocembrin content found in the *L. origanoides*, caryophyllene-rich chemotype (CTo3) extract (30 mg/g of plant) was more than ten times the amount found in other extracts. Although the pressure employed during SFE was high enough (50 MPa) to favor extraction of polar substances without the use of a co-solvent, the number of polyphenols, and their concentration were lower in these extracts than in the solvent extracts obtained from the same plant material. In a separate project, we have optimized extraction conditions for *L. origanoides* chemotypes, using a Simplex method to enhance both yield and extract antioxidant activity. Flavonoid amounts found in other common aromatic plants (Table 3), compared to our results, indicate that the flavonoid contents found in the *Lippia* species studied are relatively high. *Lippia origanoides* should be considered an important source of bioactive substances, due to the variety of flavonoids and to their high concentration found in the extracts of all three chemotypes of this species found in Colombia.

4 Concluding remarks

The secondary metabolite profiles of *Lippia* aromatic plants are quite diverse. Even when chemotypes of the same species are compared, strong qualitative differences are found in their essential oil and extract compositions. The three *L. origanoides* chemotypes stand out as sources of bioactive essential oils and extracts due to their high content of well-known antioxidant and antimicrobial compounds such as thymol, carvacrol, quercetin, naringenin, pinocembrin, and luteolin [20–22].

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