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Comparison of different extraction methods for the analysis of volatile secondary metabolites of *Lippia alba* (Mill.) N.E. Brown, grown in Colombia, and evaluation of its in vitro antioxidant activity

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Abstract

Hydrodistillation (HD), simultaneous distillation solvent extraction (SDE), microwave-assisted hydrodistillation (MWHD), and supercritical fluid (CO_2) extraction (SFE) were employed to isolate volatile secondary metabolites from fresh leaves and stems of Colombian *Lippia alba* (Mill.) N.E. Brown. Kovàts indices, mass spectra or standard compounds were used to identify around 40 components in the various volatile fractions. Carvone (40–57%) was the most abundant component, followed by limonene (24–37%), bicyclosesquiphellandrene (5–22%), piperitenone (1–2%), piperitone (ca. 1.0%), and β-bourbonene (0.6–1.5%), in the HD, SDE, MWHD, and SFE volatile fractions. Static headspace (S-HS), simultaneous purge and trap in solvent (CH_2Cl_2) (P&T), and headspace solid-phase microextraction (HS-SPME) were used to sample volatiles from fresh *L. alba* stems and leaves. The main components isolated from the headspace of the fresh plant material were limonene (27–77%), carvone (14–30%), piperitone (0.3–0.5%), piperitenone (ca. 0.4%), and β-bourbonene (0.5–6.5%). The in vitro antioxidant activity of *L. alba* essential oil, obtained by hydrodistillation was evaluated by determination of hexanal, the main carbonyl compound released by linoleic acid subjected to peroxidation (1 mm Fe^{2+}, 37 °C, 12 h), and by quantification of this acid as its methyl ester. Under the same conditions, *L. alba* HD-essential oil and Vitamin E exhibited similar antioxidant effects.

Keywords: *Lippia alba*; Extraction methods; Headspace analysis; Antioxidants; Volatile organic compounds; Carvone

1. Introduction

Lippia alba (Mill.) N.E. Brown, also known as *Lippia geniculata HBK* or *Lantana alba* (Mill.), is a shrub about 0.8 m tall that belongs to the Verbenaceae family [1]. Genus *Lippia* encompasses about 200 species, which grow wild in Central and South America and in Africa. *L. alba* possesses sedative properties and has also been used in folk medicine against intestinal and gastric illnesses [2]. Large variations have been observed in the composition of *L. alba* essential oils, depending on the part of the plant employed in the distillation, on the plant's state of development and on the geographic location, the characteristics of the soil, climate, and other local conditions [3–5]. In order to achieve a thorough characterization of the volatile secondary metabolites present in Colombian *L. alba*, high-resolution gas chromatographic (HRGC) techniques were employed to analyse plant volatile metabolites obtained by hydrodistillation (HD), microwave-assisted hydrodistillation (MWHD), simultaneous distillation solvent extraction (SDE), and supercritical fluid (CO_2) extraction (SFE). Different headspace techniques, such as static headspace (S-HS), simultaneous purge and trap (P&T) in solvent (CH_2Cl_2) extraction, and solid-phase microextraction in the vapour phase (HS-SPME) were used to isolate volatile fraction compounds from fresh leaves. The antioxidant activity of the essential oil obtained by HD was evaluated by means of a previously developed methodology based on the determination of carbonyl compounds, mainly hexanal, released by a model lipid system (linoleic acid emulsion) with and without the essential oil added [6,7].

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2. Experimental

2.1. Plant material and reagents

All plant material was collected from the same garden in Bucaramanga (Colombia) during June–September 2000 (N° COL 480747, Dr. J.L. Fernández, National Herbarium, UN, Bogotá, Colombia). Only fresh, undamaged *L. alba* stems and leaves were utilised in the extractions. *n*-Tetradecane, 2-(*tert*-butyl)-4-methoxyphenol (BHA), ferrous sulfate, tris, potassium chloride and dichloromethane were purchased from Merck (Darmstadt, Germany). Vitamin E, linoleic acid, methyl linoleate, 2,6-di(*tert*-butyl)-*p*-cresol (BHT), BF_3-methanol reagent, and pentafluorophenylhydrazine (PFPH) were purchased from Aldrich (Milwaukee, WI, USA). Poly(dimethylsiloxane) (PDMS, 100 μm) and poly(dimethylsiloxane)-divinylbenzene (PDMS-DVB, 65 μm)-coated SPME fibres were acquired from Supelco (Bellefonte, PA, USA). High-purity gases for chromatography were obtained from AGA-Fano (Bucaramanga, Colombia).

2.2. Extractions

MWHD used a domestic microwave oven (Kendo, 2.45 GHz, 800 W) with a side orifice through which an external glass condenser joined a round flask with the plant material (100 g) and water (1 l), inside the oven. The oven was operated for 30 min at full power, which caused water to boil and reflux. Essential oil was decanted from the condensate. HD was carried out in a similar manner, but without microwave irradiation, using an electrical heater (boiling water) for 2 h and after that, decanting the essential oil from the condensate previously saturated with NaCl. For GC analysis, 30 μl of essential oil were added to 1.0 ml of dichloromethane and 0.5 μl *n*-tetradecane, used as IS.

Already-published procedures were employed to perform dynamic headspace with simultaneous P&T in solvent (dichloromethane) [8], SDE in the micro-version apparatus [9,10], and HS SPME [11]. SFE (CO_2) used a J&W Scientific (Folsom, CA, USA) high-pressure Soxhlet extractor, following a procedure described elsewhere [10,12]. Each type of plant extraction was repeated five times.

2.3. Sampling of fresh leaves and stems

A headspace sampler (Hewlett-Packard (HP) 7694E, Palo Alto, CA, USA) connected to a gas chromatograph (HP 5890 A Series II) was used to sample the vapour phase above 5 g of chopped fresh *L. alba* plant material contained in a 20 ml-vial at 40° C. The sample loop and transfer line temperatures were 100 and 110° C, respectively. The experimentally determined equilibration time was 15 min.

A home-made dynamic P&T apparatus, similar to that described by Umano and Shibamoto [13] was used to collect volatile compounds from fresh *L. alba* stems and leaves. Nitrogen (99.995%) was employed as purge gas and dichloromethane as trapping solvent for 2 h extractions of chopped fresh plant material (100 g, ca. 5 mm-wide chips). The dichloromethane solution (ca. 50 ml) was concentrated in a Kuderna Danish apparatus, followed by evaporation with dry N_2 to 1.0 ml. *n*-Tetradecane (0.5 μl) was added to the final extract as IS.

A PDMS-coated (100 μm) SPME fibre was exposed for 60 min at 22 ± 1° C to the vapour phase above 10 g of chopped fresh plant material contained in a 50 ml-vial. Preliminary experiments using fibre exposition times between 5 and 120 min were used to determine the optimal fibre exposition time. The absorbed substances were thermally desorbed (260° C, 5 min) from the SPME fibre into the injection port of a gas chromatograph (HP 5890 A Series II), using an SPME-liner in the injection port. The experimental

Table 1
Experimental parameters used for isolation and GC analysis of *Lippia alba* (Mill.) N.E. Brown volatile fractions

Isolation methods	Parameters		
	Plant material (g)	Extraction time (min)	Sample preparation for GC analysis
Extractive techniques			
HD	100	120	30 μl essential oil + 0.5 μl IS dissolved in CH_2Cl_2 to 1.0 ml. Injection volume: 1 μl. Split 1:30.
MWHD	100	30	30 μl essential oil + 0.5 μl IS dissolved in CH_2Cl_2 to 1.0 ml. Injection volume: 1 μl. Split 1:30.
SDE	10	120	1 ml extract CH_2CL_2 solution with 0.5 μl IS. Injection volume: 1 μl. Split 1:30.
SFE	10	120	30 μl extract + 0.5 μl IS dissolved in CH_2Cl_2 to 1.0 ml. Injection volume: 1 μl. Split 1:30.
HS techniques			
S-HS	5 g in 20 ml-vial	15 (equilibration time at 40 °C)	Injection volume: 1 ml. Split 1:30.
P&T	100	120	1 ml concentrated CH_2Cl_2 extraction solution with 0.5 μl IS. Injection volume: 1 μl. Split 1:30.
HS-SPME	10 g in 50 ml-vial	60 (fibre exposure time)	Thermal desorption in GC injection port (260 °C, 5 min). Split 1:30.

parameters of plant extraction and chromatographic analysis, used in this work, are summarised in Table 1.

2.4. Antioxidant activity determination

Linoleic acid oxidation was induced according to Tamura and co-workers [14,15]. The assay buffer was prepared by mixing 2 ml of a 2.5 g/l aqueous solution of linoleic acid with 3 ml of a tris buffer (pH 7.4), containing 0.75 mM KCl, 2 g/l sodium dodecyl sulfate and 2.01 mM ferrous sulfate. *L. alba* HD-essential oil, Vitamin E, or BHA was added to the assay buffer to obtain concentrations of 1.0, 2.5, 5.0, 10.0, and 20.0 (except for BHA) g/l. The resulting solution was incubated at 37° C for 12 h. At the end of this period, 100 μl of a 20 g/l BHT methanolic solution were added to stop the radical-mediated chain reactions and the tube was cooled down for 10 min in an ice-water bath. 0.5 ml of this solution was transferred to a 2 ml vial with a PTFE/silicone seal for SPME sampling.

The SPME with simultaneous on-fibre derivatisation followed the procedure described elsewhere [6,7]. Briefly, a PDMS-DVB (65 μm)-coated SPME fibre was previously saturated with pentafluorophenylhydrazine (PFPH) and immediately exposed for 40 min at room temperature to the vapour phase above the incubated solution placed in the 2 ml-vial with Teflon/silicone septum. The hexanal-PFPH derivative (C_6-PFPH) thus formed in situ on the SPME fibre was thermally desorbed (260° C, 5 min) in the injection port of a gas chromatograph. Hexanal has also been measured by its derivatisation with PFPH directly in the linoleic acid emulsion, subjected to peroxidation, followed by liquid–liquid extraction of the C_6-PFPH with hexane, as described elsewhere [16,17]. All assays were run in triplicate.

The decrease in linoleic acid concentration was used as another indicator of lipid peroxidation. The procedure described above for Fe^{2+}-lipid peroxidation induction in linoleic acid emulsions with and without *L. alba* HD-essential oil added was followed by derivatisation of linoleic acid with BF_3-methanol. The resulting solutions were analysed by HRGC-flame ionization detection (FID) to quantify linoleic acid as its methyl ester, following a procedure described elsewhere [17].

2.5. Chromatographic analysis

Compound identification was based on mass spectra (EI, 70 eV) obtained with a gas chromatograph (HP 5890 A Series II) equipped with a mass selective detector (MSD HP 5972), split/splitless injector (1:30 split ratio), and a data system (HP ChemStation 1.05) with NBS75K and Wiley 138K mass spectra libraries. An HP-5MS 50 m × 0.25 mm i.d. capillary column 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 270 °C (15 min) at 4.5 °C min^{-1} for the analysis of volatile fractions, essential oils, and SDE and SFE extracts, except for analysis of the volatile compounds isolated by HS-SPME, when the GC oven was programmed from 40 °C (5 min) to 200 °C (5 min) at 5 °C min^{-1}, and then to 250 °C (10 min) at 10 °C min^{-1}. The temperatures of the ionisation chamber and of the transfer line were 185 and 285 °C, respectively. Mass spectra and reconstructed chromatograms were obtained by automatic scanning in the mass range *m/z* 40–350 at 3.5 scan s^{-1}. Chromatographic peaks were checked for homogeneity with the aid of the mass chromatograms for the characteristic fragment ions.

A gas chromatograph (HP 5890 A Series II) equipped with flame ionisation detection (FID), split/splitless injector (1:30 split ratio), and a data system (HP ChemStation HP Rev. A.06.03 [509]) was used for GC analysis of essential oils. The detector and injector temperatures were set at 250 °C. A 50 m × 0.2 mm i.d. capillary column coated with 5%-phenyl poly(methylsiloxane) (0.20 μm film thickness) (HP-5) was used. The oven temperature was programmed from 35 °C (15 min) to 250 °C (40 min) at 3 °C min^{-1}. Helium (99.995%) was used as carrier gas, with 152 kPa column head pressure and 35.7 cm s^{-1} linear velocity. Hydrogen and air at 30 and 300 ml min^{-1}, respectively, were used in the FID, with nitrogen (30 ml min^{-1}) as a make-up gas. The various compounds were identified by Kovàts retention indices [18], determined utilizing a non-logarithmic scale on the HP-5 capillary column and by comparison of mass spectra with those of standards and reported data [19–21].

The determination of hexanal, as PFPH-derivative, was performed by capillary GC with electron-capture detection (ECD), as described elsewhere [6,7,16]. Analysis of linoleic acid methyl ester was carried out by GC-FID using an *INNOWAX* (HP) fused-silica capillary column (60 m, 0.25 mm i.d.), coated with cross-linked poly(ethylene glycol) (0.25 μm phase thickness). The GC oven temperature was programmed from 80 °C (5 min) to 270 °C, at 2.5 °C min^{-1}. The injector (1:30 split ratio) and detector temperatures were maintained at 265 and 270 °C, respectively.

3. Results and discussion

3.1. Lippia alba (Mill.) N.E. Brown volatile fraction extraction and analysis

Fig. 1 presents the chromatographic profile of the volatile fraction isolated by SDE from *L. alba*. The oils obtained by HD and MWHD were similar to the SDE extracts, but the extracts isolated by SFE contained higher amounts of sesquiterpenoids and heavier hydrocarbons (Cn > 25). Peak identification for the chromatogram on Fig. 1 appears in Table 2. More than 30 volatile secondary metabolites were found at relative concentrations above 0.05%. The same number of components was found in the HD (2 h extraction) and MWHD (just 30 min extraction) essential oils with very similar yields (0.70 and 0.69%, respectively). In this

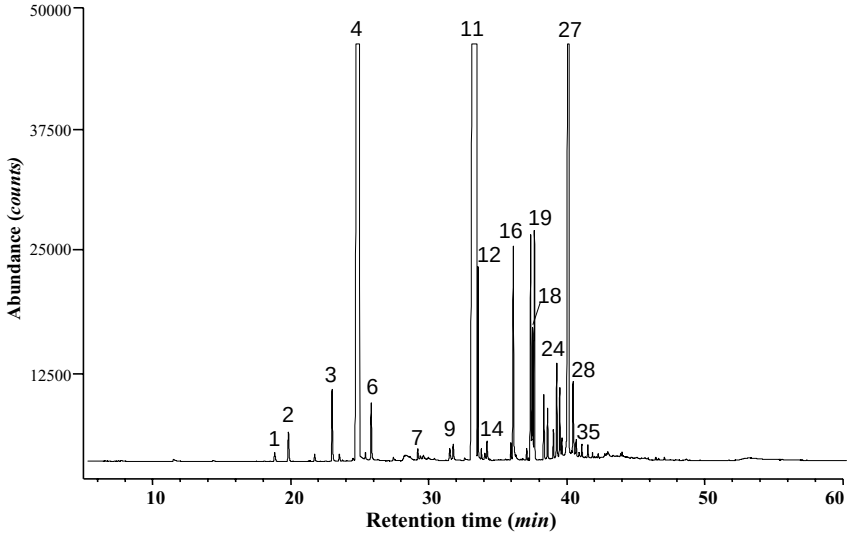

Fig. 1. Typical chromatogram of the *Lippia alba* (Mill.) N.E. Brown extract obtained by SDE. Peak assignments and identification appear in Table 2.

application, microwave irradiation highly accelerated the extraction process, but without causing considerable changes in the volatile oil composition, phenomenon which was already described by Paré et al. [22]. Oil yields of 0.22% (dry mass basis) and of 0.6–0.8% on fresh weight basis were obtained by hydrodistillation from *L. alba*, grown in Guatemala [23] and in India [24], respectively.

Carvone was the main component (40–57%) in all volatile fractions, followed by limonene (24–37%), bicyclosesquiphellandrene (5–22%), piperitenone (1–2%), piperitone (0.8–1.2%), and β-bourbonene (0.6–1.5%). Previous work [25] established, using bidimensional chromatography with a chiral stationary phase in the second column ("heart-cutting" technique), that carvone in Colombian *L. alba* was present as the S-isomer.

Table 3 compares the main components found in the Colombian HD-essential oils with those obtained by hydrodistillation (3 h) from *L. alba* grown in Cuba [4], and by steam distillation (4 h) from plants grown in three different municipalities in Brazil [3]. These data illustrate the large compositional variations that different geo-botanical conditions can cause on the volatile secondary metabolite fractions from plants belonging to the same species, but different chemotypes. For instance, the major components found in HD-essential oil from *L. alba* grown in Guatemala were limonene (44%) and piperitone (31%) [23]. Linalool was the main compound in oils hydrodistilled from *L. alba*, cultivated in Indian plains (65%) [24,26], in Uruguay (55%) [27], and in Valinhos, São Paulo State, Brazil (67–83%) [28,29]. Colombian *L. alba* HD-essential oil is distinguished by its high content of limonene (24–37%) and S-carvone (40–57%); the latter is a high-value substance for the perfume industry and cosmetics.

HD and MWHD-essential oils, SDE and SFE extracts consisted of monoterpene hydrocarbons, $C_{10}H_{16}$ (33.3, 30.2, 37.9 and 24.6%), monoterpenones and other oxygenated monoterpenes (54.8, 61.6, 48.2 and 44.7%), sesquiterpene hydrocarbons, $C_{15}H_{24}$ (11.3, 19.0, 13.9 and 29.4%) and oxygenated sesquiterpenes (0.9, 0.5, 0.4 and 1.1%), respectively (Table 2). Among the four extractive methods employed, SDE isolated monoterpene hydrocarbons in higher relative amount (37.9 ± 0.13%); these substances possess the highest volatility in the extract. The SFE technique isolated a larger relative quantity of heavier compounds (29.37 ± 0.09%), including sesquiterpenoids and hydrocarbons (Cn > 25), and thus is the method of choice for the sesquiterpene hydrocarbon extraction. A similar trend was observed in our previous works, where the chemical compositions of essential oils and extracts, obtained by HD, SDE and SFE from *Lepechinia schiedeana* (*Labiatae* family) [17] and *Cananga odorata* flowers (*Annonaceae* family) [10], were compared. MWHD had not only the advantage of being much faster than conventional HD, but also enjoyed the highest reproducibility (s = 0.03–0.07, $n = 5$), in comparison with HD (s = 0.11–0.41), SDE (s = 0.13–0.56) and SFE (s = 0.09–0.84).

Fig. 2 contains typical chromatograms of the volatile fractions isolated from fresh *L. alba* leaves, using static (S) and dynamic (P&T) HS techniques, and HS-SPME. Peak identification for these chromatograms appears in Table 4. Thirteen, twenty-nine and twenty-four secondary metabolites were detected at concentrations above 0.05% in the volatile fractions obtained by S-HS, P&T and HS-SPME, respectively. The main components were limonene (77.3, 62.7 and 26.7%), carvone (13.9, 29.8 and 15.5%), bicyclosesquiphellandrene (2.2, 1.1 and 32.8%), and β-bourbonene (0.5, 0.5 and 6.5%), respectively.

Fig. 3 shows the dependence of the main volatile compounds absorbed, upon fibre exposition time (5–120 min). This process is controlled both by diffusion rates, and distribution coefficients, K_D, of the various components, with very different volatilities and chemical nature. We chose 60 min of fibre exposition time, in order to attain a compromise

Table 2
Chemical composition of the *Lippia alba* (Mill.) N.E. Brown volatile fractions, obtained by different extraction techniques

Peak no.[a]	Compound	I_k[b]	Relative peak area[c] (%)			
			HD	MWHD	SDE	SFE
1	α-Pinene	935	0.05 ± 0.025	0.050 ± 0.001	0.1 ± 0.16	0.070 ± 1.0E-3
2	Camphene	949	0.14 ± 0.025	0.10 ± 0.050	0.26 ± 0.074	0.15 ± 0.087
3	β-Myrcene	993	0.40 ± 0.025	0.35 ± 0.012	0.53 ± 0.050	0.3 ± 0.11
4	Limonene	1033	32.60 ± 0.025	29.58 ± 0.050	36.90 ± 0.087	23.9 ± 0.11
5	(Z)-β-Ocimene	1043	0.1 ± 0.36	0.16 ± 0.037	0.11 ± 0.074	0.12 ± 0.012
6	Pinene oxide	1052	0.37 ± 0.025	0.37 ± 0.012	0.4 ± 0.14	0.3 ± 1.0
7	*cis*-Thujone	1106	0.18 ± 0.037	0.21 ± 0.062	0.1 ± 0.48	0.23 ± 0.087
8	Limonene oxide	1133	0.16 ± 0.025	0.19 ± 0.025	0.11 ± 0.087	0.19 ± 0.012
9	*trans*-Dihydrocarvone	1197	0.12 ± 0.050	0.08 ± 0.087	0.1 ± 0.12	0.18 ± 0.037
10	Pulegone	1235	0.15 ± 0.012	0.11 ± 0.025	0.2 ± 0.14	0.17 ± 0.074
11	Carvone	1245	51.0 ± 0.15	57.21 ± 0.025	45.1 ± 0.40	40.5 ± 0.10
12	Piperitone	1255	0.93 ± 0.012	1.23 ± 0.025	0.8 ± 0.36	1.00 ± 0.087
13	Geranial	1270	0.09 ± 0.012	0.12 ± 0.025	0.1 ± 0.30	0.41 ± 0.012
14	*trans*-Carvone oxide	1279	0.1 ± 0.14	0.19 ± 0.025	0.1 ± 0.42	0.11 ± 0.074
15	*trans*-Carvyl acetate	1339	0.09 ± 0.012	0.10 ± 0.037	0.1 ± 0.47	0.07 ± 0.074
16	Piperitenone	1347	1.47 ± 0.074	1.81 ± 0.025	1.1 ± 0.57	1.6 ± 0.87
17	β-Bourbonene	1384	0.7 ± 0.25	0.60 ± 0.012	1.0 ± 0.33	1.5 ± 0.10
18	α-Copaene	1388	0.40 ± 0.062	0.22 ± 0.037	0.5 ± 0.26	0.56 ± 0.037
19	β-Cubebene	1390	0.59 ± 0.087	0.35 ± 0.025	0.1 ± 0.60	0.51 ± 0.074
20	γ-Elemene	1418	0.3 ± 0.51	0.22 ± 0.025	0.4 ± 0.39	0.30 ± 0.074
21	β-Cedrene	1420	0.2 ± 0.20	0.2 ± 0.025	0.3 ± 0.35	0.37 ± 0.087
22	β-Caryophyllene	1424	0.2 ± 0.22	0.13 ± 0.025	0.2 ± 0.25	0.3 ± 0.10
23	α-Humulene	1452	0.4 ± 0.20	0.19 ± 0.012	0.5 ± 0.41	1.0 ± 0.11
24	β-Farnesene	1460	0.3 ± 0.25	0.24 ± 0.012	0.4 ± 0.37	0.78 ± 0.087
25	*allo*-Aromadendrene	1465	0.1 ± 0.17	0.12 ± 0.025	0.2 ± 0.35	0.3 ± 0.10
26	Germacrene D	1480	0.1 ± 0.20	0.12 ± 0.037	0.2 ± 0.40	0.15 ± 0.012
27	Bicyclosesquiphellandrene	1488	7.3 ± 0.25	4.7 ± 0.11	8.9 ± 0.47	21.75 ± 0.087
28	Germacrene A	1504	0.4 ± 0.17	0.28 ± 0.025	0.5 ± 0.41	0.95 ± 0.087
29	β-Bisabolene	1509	tr	tr	tr	0.11 ± 0.037
30	Cubebol	1514	0.2 ± 0.30	0.11 ± 0.050	0.1 ± 0.70	0.35 ± 0.087
31	δ-Cadinene	1524	tr	tr	tr	0.12 ± 0.025
32	(E)-α-Bisabolene	1530	tr	tr	tr	0.14 ± 0.074
33	Caryophyllene oxide	1581	tr	tr	tr	0.22 ± 0.050
34	Germacrene B	1584	0.3 ± 0.12	0.17 ± 0.74	0.1 ± 0.80	0.20 ± 0.025
35	α-Humulene oxide	1605	0.3 ± 0.19	0.19 ± 0.037	0.2 ± 0.44	0.22 ± 0.087
36	Cedranone	1617	0.1 ± 0.17	0.19 ± 0.050	0.1 ± 0.52	0.19 ± 0.062
37	*epi*-α-Muurolool	1641	–	–	–	0.11 ± 0.037
38	Hydrocarbon, C_{25}	2453	–	–	–	0.10 ± 0.025
39	Hydrocarbon, C_{26}	2576	–	–	–	0.12 ± 0.012
	Monoterpene hydrocarbons		33.3 ± 0.36	30.24 ± 0.050	37.9 ± 0.16	24.6 ± 0.11
	Monoterpenones		54.0 ± 0.15	60.65 ± 0.087	47.4 ± 0.57	43.7 ± 0.87
	Oxygenated monoterpenes[d]		0.8 ± 0.14	0.97 ± 0.037	0.8 ± 0.47	1 ± 1.0
	Sesquiterpene hydrocarbons		11.3 ± 0.51	19.03 ± 0.037	13.9 ± 0.70	29.4 ± 0.11
	Oxygenated sesquiterpenes[e]		0.9 ± 0.17	0.49 ± 0.050	0.4 ± 0.70	1.09 ± 0.087

tr: Traces.
[a] Peak number in Fig. 1.
[b] Experimentally determined Kováts indices on the HP-5 column.
[c] Averages of five independent extractions ± ts/$\sqrt{n}$ ($n = 5$, 95% confidence).
[d] Oxides, aldehydes, alcohols, and acetates.
[e] Oxides, alcohols and ketones.

between the short saturation times of limonene and carvone, and the longer ones for β-bourbonene and bicyclosesquiphellandrene, the four constituents, which represent more than 80% of the *L. alba* volatile fraction.

The composition of volatile fractions isolated by different HS methods differed significantly. The relative amount of the most volatile compounds, monoterpenes, in the mixtures, dropped considerably from 80.53±0.05% (S-HS) to 64.26± 0.61% (P&T), and to 27.48±0.25% for the HS-SPME technique, whilst the sesquiterpenoid fraction (less volatile compounds) rose from 2.64 ± 0.05% (S-HS) and 2.98 ± 0.05% (P&T) to 54.86 ± 0.51% for HS-SPME (60 min fibre exposure) (Table 4). As S-HS is based on the equilibrium between plant volatiles and the surrounding atmosphere,

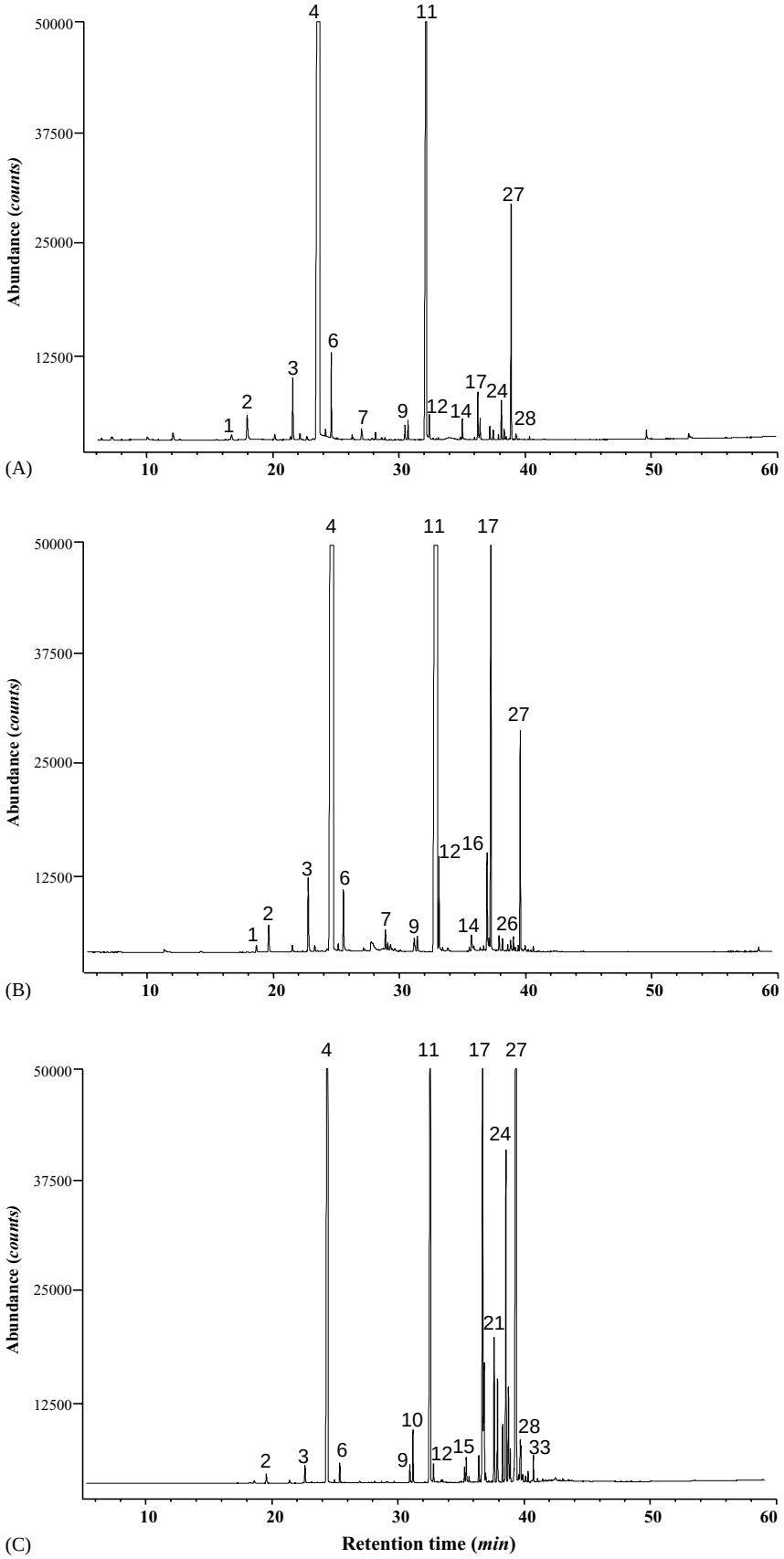

Fig. 2. Typical chromatograms of volatile fractions isolated from fresh *Lippia alba* (Mill.) N.E. Brown leaves by (A) static HS, (B) dynamic HS (P&T in solvent, CH_2Cl_2), and (C) HS-SPME. Peak assignments and identification are reported in Table 4.

Table 3
Lippia alba (Mill.) N.E. Brown essential oil composition from plants of different geographical origins

Compound	Relative amount (%)				
	Brazil [3]			Cuba [4]	Bucaramanga, Colombia
	Santa Maria	Belterra	Chaves		
1,8-Cineol	34.9	–	3.1	–	–
Limonene	18.4	32.1	1.2	5.8	23.9–36.9
Carvone	8.6	31.8	–	40.0	40.4–57.2
Sabinene	8.2	0.7	1.0	0.01	–
β-Myrcene	37	11.0	–	0.3	0.33–0.53
Linalool	0.7	0.7	3.9	1.3	–
Neral	–	–	13.7	–	–
Geranial	–	–	22.5	–	0.09–0.41
Germacrene D	–	21.0	25.4	–	0.12–0.16
β-Caryophyllene	0.5	–	10.2	1.2	0.13–0.27
β-Guaiene	–	–	–	9.8	–
Piperitone	0.4	–	–	3.6	0.80–1.23
Piperitenone	–	–	–	8.3	1.1–1.8
Bicyclosesquiphellandrene	–	–	–	–	4.8–21.7
β-Bourbonene	0.5	–	–	3.0	0.6–1.5

probably this sampling method better imitates the genuine scent one could perceive from the fresh plant.

The compositions found for the various volatile fractions (Tables 2 and 4) were also subjected to principal component analysis (PCA) (STATISTICA, Version 6.0, StatSoft, Tulsa, OK, USA), in order to better appreciate the effect of extraction technique on the final composition (Fig. 4). The PCA results indicate that 92% of the information content of Tables 2 and 4 can be represented in a subspace formed by three coordinates (principal components) constructed from linear combinations of the various concentrations that describe each volatile fraction. Factor 1, which signifies 48.9% of the information, mainly represents sesquiterpene content (β-cubebene, germacrene D, bicyclosesquiphellandrene, *allo*-aromadendrene, etc.). Factor 2 corresponds to 34% of the information and consists of ketones (carvone, piperitone, piperitenone) and other oxygenated compounds (carvyl acetate, pinene and limonene oxides). Factor 3, with 9% of the information, is constructed mainly from contributions from geranial, cubebol, pinene oxide, and some other polar compounds. The close proximity of the points, related to MWHD, HD, and SDE volatile fractions

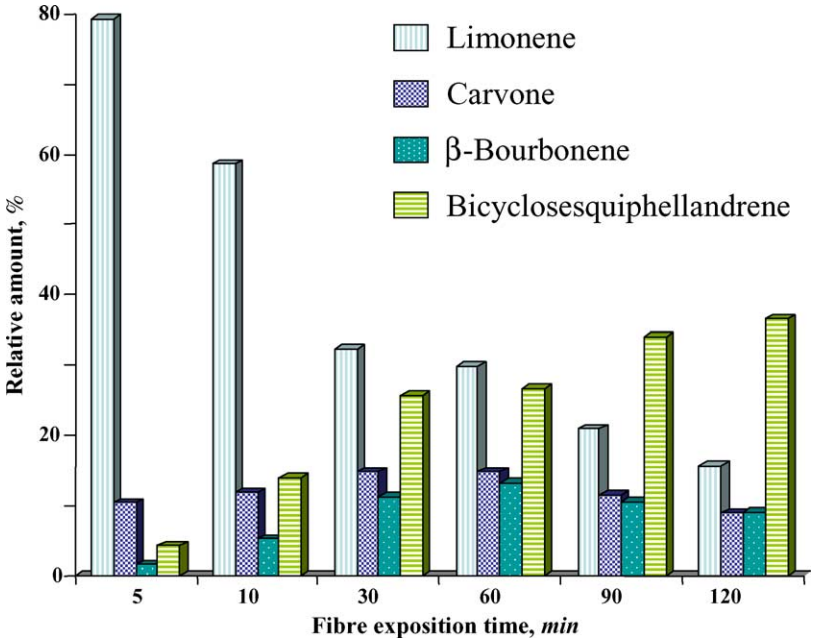

Fig. 3. Relative amount (%) in the headspace of main volatile components from *Lippia alba* (Mill.) N.E. Brown fresh leaves and stems (HS-SPME, PDMS/100 μm), as a function of fibre exposition time.

Table 4
Relative amount (%) and identification of the main components of *Lippia alba* (Mill.) N.E. Brown volatile fractions, obtained by S-HS, P&T and HS-SPME.

Peak no[a]	Compound	Relative peak area[b] (%)		
		P&T	S-HS	HS-SPME
1	α-Pinene	0.16 ± 0.012	0.62 ± 0.012	tr
2	Camphene	0.37 ± 0.074	0.88 ± 0.050	0.20 ± 0.025
3	β-Myrcene	0.85 ± 0.074	1.06 ± 0.012	0.3 ± 0.31
4	Limonene	62.7 ± 0.76	77.34 ± 0.025	26.70 ± 0.074
5	(Z)-β-Ocimene	0.18 ± 0.025	0.63 ± 0.062	0.28 ± 0.012
6	Pinene oxide	0.58 ± 0.074	tr	tr
7	Thujone	0.38 ± 0.012	tr	tr
8	Limonene oxide	0.20 ± 0.099	tr	tr
9	*trans*-Dihydrocarvone	0.18 ± 0.074	tr	0.24 ± 0.099
10	Pulegone	0.21 ± 0.025	tr	0.8 ± 0.47
11	Carvone	30 ± 1.0	13.9 ± 0.58	15.45 ± 0.062
12	Piperitone	0.55 ± 0.074	0.39 ± 0.012	0.3 ± 0.12
13	Geranial	0.10 ± 0.037	0.35 ± 0.012	tr
14	*trans*-Carvone oxide	0.11 ± 0.025	0.30 ± 0.012	tr
15	*trans*-Carvyl acetate	0.08 ± 0.012	0.59 ± 0.012	0.21 ± 0.062
16	Piperitenone	0.43 ± 0.050	0.31 ± 0.012	0.40 ± 0.087
17	β-Bourbonene	0.5 ± 0.12	0.49 ± 0.025	6.5 ± 0.42
18	α-Copaene	0.05 ± 0.012	tr	0.66 ± 0.050
19	β-Cubebene	0.20 ± 0.012	tr	2.0 ± 0.63
20	γ-Elemene	0.18 ± 0.062	tr	0.23 ± 0.087
21	β-Cedrene	0.18 ± 0.012	tr	1.99 ± 0.074
22	β-Caryophyllene	0.11 ± 0.012	tr	1.8 ± 0.53
23	α-Humulene	0.14 ± 0.062	tr	0.72 ± 0.037
24	β-Farnesene	0.13 ± 0.074	tr	4.9 ± 0.31
25	*allo*-Aromadendrene	0.07 ± 0.012	tr	1.38 ± 0.025
26	Germacrene D	0.11 ± 0.062	tr	0.6 ± 0.43
27	Bicyclosesquiphellandrene	1.14 ± 0.012	2.15 ± 0.062	32.8 ± 0.56
28	Germacrene A	0.12 ± 0.062	tr	0.6 ± 0.15
29	β-Bisabolene	tr	tr	0.38 ± 0.050
30	Cubebol	0.05 ± 0.012	tr	tr
33	Caryophyllene oxide	tr	tr	0.3 ± 0.23
	Monoterpene hydrocarbons	64.3 ± 0.76	80.53 ± 0.062	27.5 ± 0.31
	Monoterpenones	31 ± 1.0	14.6 ± 0.58	17.2 ± 0.47
	Oxygenated monoterpenes[c]	1.07 ± 0.099	1.24 ± 0.012	0.21 ± 0.062
	Sesquiterpene hydrocarbons	2.93 ± 0.062	2.64 ± 0.062	54.6 ± 0.63
	Oxygenated sesquiterpenes[d]	0.05 ± 0.012	–	0.3 ± 0.24

tr: Traces.
[a] Peak number in Fig. 2.
[b] Averages of five independent extractions ± ts/$\sqrt{n}$ ($n = 5$, 95% confidence).
[c] Oxides, aldehydes, alcohols, and acetates.
[d] Oxides, alcohols and ketones.

confirms that they indeed have very similar compositions. On the other hand, Fig. 4 also shows that the HS techniques afford volatile fractions with different composition. The P&T and HS-SPME methods include concentration steps, and the relative chemical composition of the volatile fractions isolated by HS-SPME depends strongly upon the fibre exposure time (Fig. 3), among other variables.

3.2. *Lippia alba* (Mill.) N.E. Brown HD-essential oil in vitro antioxidative activity

One of the important areas of natural product research is related to the determination of the biological activity of plants and their extracts. Herbs provide foods with flavour and food-preserving power, including antiseptic and antioxidant activity. Natural antioxidants of plant origin are becoming more and more important, not only in food, but also in preventive medicine [30–32]. As many essential oils are incorporated in a large number of products (perfumes, soaps, creams, foods, drinks, and many others), it is important as well to evaluate their in vitro antioxidant properties.

In this work, the antioxidative activity of *L. alba* HD-essential oil was tested using its inhibitory (protective) effect toward oxidation of linoleic acid to carbonyl compounds, e.g. hexanal, the main product of this process [33–35]. Standards of BHA and α-tocopherol (Vitamin E) were also examined for their antioxidative activity using the same methodology. The protecting effects of *L. alba* essential oil, BHA and Vitamin E at various concentrations against the Fe^{2+}-induced oxidation of linoleic acid

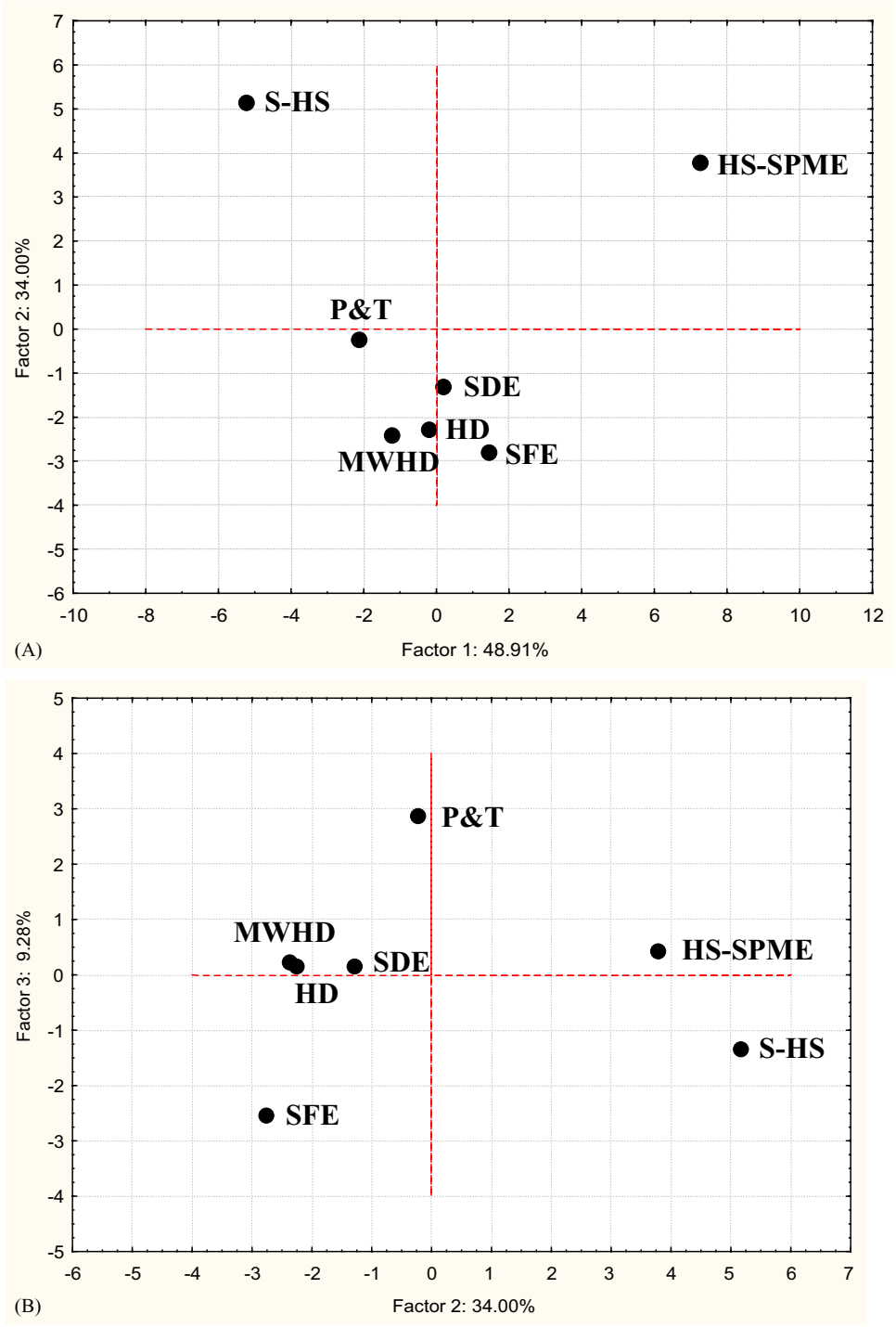

Fig. 4. Representation of the *Lippia alba* (Mill.) N.E. Brown volatile fractions in the coordinate system formed by the first three principal components. (A) Factor 1 vs. Factor 2. (B) Factor 2 vs. Factor 3. Factor 1 (48.9%) mainly represents sesquiterpene content (β-cubebene, germacrene D, bicyclosesquiphellandrene, *allo*-aromadendrene, etc.). Factor 2 (34%) consists of ketones (carvone, piperitone, piperitenone) and other oxygenated compounds (carvyl acetate, pinene and limonene oxides). Factor 3 (9%) is constructed mainly of geranial, cubebol, pinene oxide, and some other polar compounds contributions.

are shown in Table 5. The protecting effect was expressed using three different criteria. The first two approaches focused on the percent decrease in hexanal, released by the lipid system during 12 h incubation at 37 °C, both in the vapour phase (HS-SPME with on-fibre derivatisation) and in the linoleic acid emulsion (liquid–liquid extraction), according to methodologies established in [6,7] and [16,17], respectively. The third approach was based on the measurement of the loss of unsaturated fatty acids, one of the basic methods to determine lipid peroxidation progress [36].

Table 5
Protecting effect against lipid peroxidation observed for *Lippia alba* (Mill.) N.E. Brown HD-essential oil, Vitamin E and BHA.

Parameter measured	Antioxidant concentration (g/l)				
	1.0	2.5	5.0	10.0	20.0
HD-Essential oil[a]					
Hexanal concentration decrease (%)					
Headspace	19 ± 2.1	65 ± 1.0	71 ± 1.0	73.6 ± 0.5	71.6 ± 0.9
Solution	27 ± 1.9	44.9 ± 0.4	72 ± 1.3	78.0 ± 0.2	78.5 ± 0.6
LA[b] concentration (%)	32 ± 1.7	69 ± 5.0	72 ± 4.5	80 ± 23	85 ± 5.0
Vitamin E[a]					
Hexanal concentration decrease (%)					
Headspace	34 ± 0.51	46.0 ± 0.51	57.0 ± 0.63	62.4 ± 0.82	61 ± 3.2
Solution	36.1 ± 0.13	56.0 ± 0.51	64.4 ± 0.44	78.9 ± 0.9	75.5 ± 0.9
LA[b] concentration (%)	23 ± 1.7	42.2 ± 0.8	55 ± 2.1	85 ± 2.5	72 ± 1.6
BHA[a]					
Hexanal concentration decrease (%)					
Headspace	39 ± 6.3	56 ± 1.7	51 ± 2.1	49 ± 2.3	–
Solution	72 ± 1.5	75 ± 2.6	75.1 ± 0.8	80 ± 1.5	–
LA[b] concentration (%)	36.7 ± 0.9	39 ± 1.9	55.3 ± 0.7	63 ± 3.0	–

[a] Mean of three measurements ± s.
[b] LA: linoleic acid. LA relative concentration (%), with respect to its initial content in the test solution.

The protecting effect was expressed as the difference in linoleic acid concentration (determined as its methyl ester) between the blank and the test solutions without and with antioxidant, respectively, as described elsewhere [17]. The results from these different, but complementary analytical approaches to evaluate in vitro antioxidant activity, consistently showed that, within the 5–20.0 g/l concentration range, *L. alba* HD-essential oil showed antioxidant protecting effect, and exhibited an activity higher or similar to those of Vitamin E and BHA, both widely used as natural and synthetic additives.

4. Conclusion

A complete characterisation of a plant's volatile secondary metabolites requires the use of several extraction techniques. Plant material freshness also influences the volatile fraction profile, most of all, the HS fraction. Colombian *L. alba* (Mill.) N.E. Brown essential oils, obtained by HD and HDMW and extracts, isolated by SDE and SFE, respectively, were rich in S-carvone (41–57%), an important and high-value component for the perfume industry and as a starting material for fine organic synthesis. Headspace fractions were rich in limonene (27–77%), followed by carvone (14–30%). The high in vitro antioxidant activity exhibited by *L. alba* volatile oil makes this aromatic plant a promising source of natural antioxidants and justifies further research into the isolation of fractions and/or compounds responsible for the *L. alba* HD-essential oil antioxidant protecting effect in vitro.

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