Volatile profiling of Arnicão (Lychnophora salicifolia mart.), a wild medicinal species from Brazilian Cerrado

Volatile Profiling of Arnicão (*Lychnophora salicifolia* Mart.), A Wild Medicinal Species From Brazilian Cerrado

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Abstract

The Cerrado is a diverse Brazilian savanna with more than 12,000 species. Arnicão (*Lychnophora salicifolia* Mart – Compositae) is an endemic species occurring in central and southeast Brazil, at higher altitudes, in sandstone and quartzite soils. *Lychnophora* species have been reported for anti-inflammatory, antioxidant and UV protectant effects. Local communities use it to prepare traditional remedies (i.e. ointments or creams). The aim of this work was 1) to characterize the qualitative composition of *L. salicifolia* volatile fraction; 2) to evaluate the influence of the environmental conditions on the volatile fraction composition, by investigating plants harvested both in areas under stress conditions and protected from human interference; 3) optimizing a non-separative method for routine in-field analyses. Separative (HS-SPME-GC-MS) and non separative (HS-SPME-MS) approach combined with PCA are proposed to discriminate between plant populations of *L. salicifolia* from two distinct areas. Forty-eight individuals were randomly collected from four populations. A clear separation between populations from protected and non-protected areas was observed, with an important presence of caryophyllene derivatives in populations from non-protected areas, usually with compounds associated to plant defense. Both analytical approaches gave the same results, confirming HS-SPME a useful approach for rapid in-field analysis of samples of wild populations.

Keywords

Lychnophora salicifolia, Arnica of Cerrado, volatile fraction, HS-SPME, Non separative analysis

Introduction

Brazilian flora comprises around 55,000 species, divided into important biomes, such as the Amazon rainforest, the Atlantic Forest and a savanna area in Central Brazil, known as Cerrado. This last is the second largest Brazilian biome, which covers nearly 22% of the Brazilian territory (2.5 million km^2). Cerrado is a diverse savanna with a flora with more than 12,000 species (44% are endemic) and has been recognized as a global biodiversity hotspot (Mendonça et al. 2008). It is bordered by a diverse array of biomes, and the proximity of these diverse vegetation types may have played a role in its striking species richness (Simon et al., 2009).

Contrary to the other Brazilian biomes, Cerrado is the largest area of advanced agriculture plantations of soybean, cotton, corn, sugarcane, eucalyptus, and cattle, which cause intense impact on wild species preservation. However, up to date only a small percentage of these spontaneous species were chemically investigated, making this biome a very promising source for prospecting secondary metabolites of potential interest.

Many Cerrado plant species are used in the local traditional medicine (Corrêa, 1984; Lorenzi & Mattos, 2008). Surprisingly, several Brazilian species are popularly named as Arnica, a worldwide European medicinal plant (*Arnica montana* L.), largely used for its anti-inflammatory properties. This is probably due to the influence of European immigrants in Brazil over the last centuries, who found plants of the Brazilian traditional medicine with uses similar to those of the European tradition (Pavarini et al., 2013).

Among the reported species endemic of the Brazilian savannah (Cerrado), two species of genus *Lychnophora* (Compositae), *Lychnophora salicifolia* Mart. and *L. ericoides* Mart., are known in Brazil as "Arnica" (Arnicão and Arnica do Cerrado, respectively).

Lychnophora is an endemic Brazilian genus consisting of 35 species (Loeuille 2014), restricted to the regions of central Brazilian Cerrado (Coile & Jones 1981). It occurs in a specific

Cerrado physiognomy, named “Cerrado rupestre”, which is characterized by high number of endemic species, with a high percentage of Compositae species (Mansanares et al., 2002; 2007; Collevatti et al. 2009; Simon et al. 2009). Habitats of *Lychnophora* species are also highly unstable with high level of disturbance caused by occasional fire and human pressure, which can affect population size and genetic balance (Colevatti et al. 2009).

The main chemical constituent reported for *Lychnophora* species are sesquiterpenes lactones (Borella et al. 1998; Sakamoto et al. 2003), flavonoids derivatives (Gobbo Neto and Lopes 2008), triterpenes (Borella et al. 1998), lignans and chlorogenic acids (Borsato et al. 2000; Santos et al. 2005), and essential oils (Lyra et al. 2008; Curado et al., 2006). *Lychnophora* species have shown anti-inflammatory, antioxidant, and UV protectant effects (Gobbo-Neto et al. 2005; Chicaro et al. 2004). Other biological activities have been reported for *Lychnophora* species, such as trypanocide (Grael et al. 2000, 2005; Jordão et al. 2004; Takeara et al. 2003; Oliveira et al. 1996), cytotoxic (Lequesne et al. 1979), analgesic (Santos et al. 2005; Borsato et al. 2000), antifungic and antibacteria (Miguel et al. 1996).

Although *L. ericoides* is more popular and studied, *L. salicifolia* has a larger distribution and variability (Coile & Jones, 1981). Local communities use the leaves and branches of *L. salicifolia* in the preparation of traditional remedies that are sold as either ointments or creams mostly for their anti-inflammatory properties (Borsato et al. 2000).

Lychnophora salicifolia occurs in a rocky savanna type (Cerrado rupestre), in central and southeast Brazil, at higher altitudes, ranging from 950 to 1800 m above sea level, in sandstone and quartzite soils (Coyle & Jones 1981). It is a 3-m diploid shrub species with 36 chromosomes (2n=18) (Mansanares et al. 2002), and presents large leaves, purple flowers and its small seeds are wind dispersed. Besides the preserved areas of occurrence in the Brazilian Cerrado, *L. salicifolia* can also be found in areas with high human impact. Plants such as *Lychnophora* species are endemic and subjected to habitat vulnerability. The knowledge of

volatiles compounds of a species such *L. salicifolia* can be helpful to study the effect of human impact on natural populations.

Lychnophora shows an aggregate distribution forming spatially defined populations, in well-delimited patches, with adaptations to Cerrado environment and climate, and the effect of periodic fires. The dense tomentum present in this species appears to protect their stems from fire damage (Coile & Jones 1981). *L. ericoides* presents a high genetic diversity and genetic differentiation among populations and gene flow between populations is unlikely (Collevatti et al. 2009). Since *L. salicifolia* has a distribution and genesis very similar to *L. ericoides*, the two plants are expected to behave similarly.

Population variability and areas of occurrence of *Lychnophora* species could have contributed to a chemical diversity in secondary metabolite formation. For instance, Lyra et al. (2008) and Curado et al. (2006) have reported that *L. ericoides* showed different essential oil profiles among and within populations, which could be due to environmental factors, such as insect pressure, damage, or abiotic factors, but also to genetic variation inside the population. Gouvea et al. (2012) reported qualitative and quantitative statistical correlation of geographic localization and decreased similarity of polar metabolites profile, such as flavonoid C-glucosides, chlorogenic acid derivatives and sesquiterpenoids. Specimens of *L. salicifolia* collected from the same location have varied quantitatively, but on average the individuals are chemically quite similar.

To the best of the authors' knowledge, there are no reports concerning the essential oil composition of *L. salicifolia*, and only two articles for *L. ericoides* (Curado et al. 2006; Lyra et al. 2008). The same is true for the volatile fraction of *Lychnophora* species.

In general, analytical methods require long time for sample preparation, and a significant mass of plant material. These aspects limit the number of samples to be analyzed. In this respect, high concentration capacity headspace methods are simpler, less laborious,

quicker, solvent-free and easy to automate (Kataoka et al. 2000). The most popular of them is headspace-solid phase microextraction (HS-SPME) (Belliardo et al. 2006). The same authors reported several applications of HS-SPME, among them, the discrimination of different species of the same genus, and intraspecific discrimination of cultivars, chemotypes and populations of native plants. Another possible use, although less common, is its application to selection and breeding of plant accessions, and germplasm conservation.

This work aimed at 1) characterizing the composition of the volatile fraction (through essential oil and headspace analysis) of different *Lychnophora* species; 2) evaluating the influence of the environmental conditions on volatile fraction composition of different populations of *L. salicifolia,* by considering plants harvested in areas both in stress conditions and protected from human interference; 3) optimizing a non-separative method to be used in routine in-field analysis to discriminate between samples.

Material and Methods

Plant Material

Forty-eight dried leaf samples of *Lychnophora salicifolia* were randomly collected from four populations (Table 1). Samples were harvested from individual plants located at National Park Chapada dos Veadeiros (Ls-60 and Ls-67), São João d'Aliança (Ls-85) and São Gabriel (Ls-86), in the state of Goiás, Brazil.

Samples were dried at 38°C up to constant weight. A voucher of each specimen and population were deposited at the Embrapa Genetic Resources and Biotechnology herbarium (CEN). Collections and analysis were authorized by the Brazilian Ministry of Environment (process IBAMA 02001.003166/2013-26).

HS-SPME Sampling

Five hundred mg of dried leaves were ground in a coffee mill and placed in a 20 mL headspace vial and sampled by HS-SPME with a CAR/PDMS/DVB fused silica fiber (2 , 50/30 μm) for 60 min at 80 °C, after 15 min of equilibration time. 2μL of an internal standard (Nonadecane 2mg/mL in dibutylphthalate) were placed in a similar vial and preloaded into the fiber prior sampling according to in-fiber standardization method (Wang et al., 2005). After sampling the accumulated analytes were thermally desorbed by introducing the fiber into the GC injector (desorption time: 5 minutes). The SPME device and the fused silica fibers were purchased from Supelco (Bellafonte, PA, USA). Before use, all fibers were conditioned as recommended by the manufacturer. A series of experiments was carried out to select amount of sample, sampling temperature, equilibration and sampling times.

Essential Oil Distillation

Twelve g of a mixture of a proportional amount of dried leaves from all individuals of each population were subjected to hydrodistillation in a Clevenger microdistillator apparatus (Bicchi et al. 1983) for 2 hours; the essential oil was recovered with 300μL of cyclohexane for further analysis.

Analysis Conditions

Separative approach - Analyses were carried out with an MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr, Germany) installed on an Agilent 7890A GC unit coupled to an Agilent 5975C MSD (Agilent, Little Falls, DE, USA).

GC-MS analyses - chromatographic conditions: injector temperature, 230°C; injection mode: split; split ratio: 1/20; carrier gas: helium at constant flow rate, 1 mL/min. Column: a) Mega SE52 (stationary phase polydimethylsiloxane with 5% of phenyl, df = 0.25 μm, dc = 0.25 mm,

length = 30 m) (Mega, Legnano (Milan), Italy); b) SGE SolGelwax (stationary phase 100% polyethylene glycol) (df = 0.25 μm, dc = 0.25 mm, length = 30 m) (SGE, Melbourne, Australia). Oven temperature program: from 50°C (1 min) to 250°C at 3 °C/min.

MSD conditions - ionization mode: electron impact (70 eV); acquisition mode: scan, scan range: 35−350 amu; ion source temperature, 230°C; quadrupole temperature, 150°C; transfer line temperature, 250°C.

Non separative MS method: injector temperature: 250°C, injection mode: split, ratio: 1/20; carrier gas: helium, flow rate: 0.4 mL min^{-1}; fiber desorption time and reconditioning: 5 min; transfer column: deactivated fused silica tubing (dc 0.10 mm, length 6.70 m) (Mega, Legnano, MI, Italy); GC oven temperature:250°C.

Compound Identification

The compounds detected in both essential oil and headspace were identified by comparison of their mass spectra to those of authentic standards or with data already available in in-house or commercial mass spectral libraries (Adams and Wiley Mass Spectral Data) and from literature and by comparing their calculated linear retention indices using a C_9-C_{25} series of alkanes (100 ppm in cyclohexane).

Statistical Analysis

Sixty-three selected compounds were used to perform the PCA analysis. The data set from HS-SPME GC-MS analyses were submitted to PCA using the XLStat software Version 2014.3.02 (Addinsoft, Paris, France).

Results and Discussion

Essential Oil Composition of L. salicifolia

The question to be answered in this study was whether leaf volatile profiles of *L. salicifolia* harvested from a disturbed and stressful condition have different composition compared to that of an area protected from human interference. A field description of all harvesting areas, considering two close populations from a protected area (Chapada dos Veadeiros National Park - CVNP, Alto Paraíso, Brazil), and two others located in disturbed areas, is shown in **Table 1**. The CVNP is a federal Brazilian protected area, created in 1961, and covers 64,796 ha. The areas of occurrence of *L. salicifolia* populations at CVNP (Ls-60 and Ls-67) showed no signs of damage or harvesting activities. On the other hands, the two other colleting sites, S. Gabriel (Ls-86) and S. João d'Aliança (Ls-85) (**Table 1**), presented high level of disturbance, been close to agriculture fields and secondary roads, as well as to livestock and pasture.

The habitats of *L. salicifolia* populations are under similar soil type and altitude, as well as it is the temperature and humidity of this region, also because the plants were harvested in the dry season. However, the two unprotected areas (Ls-85 and Ls-86) showed some differences: Ls-85 is in between two agricultural fields, while Ls-86 is a little more protected in a hill area, although still close to agricultural, pasture and urban areas. Since these populations do not seem to be over harvested for traditional uses, it is expected that biotic factors, such as insect pressure, is one of the most important aspects differentiating these areas. The high chemical diversity among populations observed can be hypothesize to be associated to a few genes, then can be activated by the stress conditions, considering the isolation and unexpected gene flow among populations (Collevatti et al. 2008).

Sixty-two peaks were detected in the GC-MS profile of the *L. salicifolia* leaves essential oil obtained by microdistillation, accounting for about 65 to 95% of total essential oil (**Table 2**). The most abundant compounds were myrcene (0.6-17.7%), γ-cadinene (3.95-6.71%), *epi*-α-

cadinol (7.7-13.4%), 14-hydroxy-9-epi-(*E*-)-caryophyllene (1.88-37.7%), 14-hydroxy-humulene (1.68-5.70%) (Table 2).

The essential oil of *L. salicifolia* showed a predominance of mono- and sesquiterpenes hydrocarbons, with a high percentage of oxygenated sesquiterpenes (27.1-57.9%), that predominate in population Ls-86 (Table 2).

Volatile Fraction as Tool to Evaluate the Influence of Environmental Conditions on L. salicifolia

The above method to characterize the volatile fraction of *L. salicifolia* through its E.O. is well established (hydrodistillation + GC analysis), but it is rather time consuming since, in agreement with the Pharmacopoeia, it takes at least 2 hours for hydrodistillation and about 50 minutes for the GC analysis. Moreover, it requires also a relatively high amount of plant material i.e.at least 100 grams for distillation and 10 grams for microdistillation. The first issue (i.e. time-consumption) is a sort of bottleneck, in particular when a large number of samples has to be analyzed, since it affects the total time needed for quality control analysis. The second aspect (i.e. the amount of plant material) is critical for both species that are either preserved or species whose harvesting is subjected to limitations.

The volatile fraction when analyzed by Headspace Solid-Phase Microextraction (HS-SPME) combined with GC-MS can successfully be used as an alternative (or a complement) to essential oil to screen, characterize, and/or discriminate plant samples (Bicchi et al. 2006 and related references).

Forty-eight samples of *L. salicifolia* leaves were analyzed and sixty-three compounds detected in their volatile fractions. **Table 3** reports the normalized HS-SPME-GC-MS average areas. Twenty-six compounds (average > 1%, in bold in Table 3) were selected and their normalized areas used as variables for multivariate statistical analysis (Principal Component

Analysis - PCA). **Figure 1** shows the scores plot (A) on the first and the second principal components (F1-F2 plane) and the loading plots (B). The variance explained from the first two principal components (F1 and F2) were 26.49% and 23.58% respectively.

PCA provided two informative results. First, it clearly shows a discrimination between plant populations harvested in the protected area CVNP (Environment-P) and those harvested in unprotected and disturbed areas (Environment-N). Compounds such as 14-hydroxy-9-epi-(E)-caryophyllene and 14-hydroxy-α-humulene showed the highest discrimination value for populations harvested in the more disturbed areas, while 2,4-heptadienal, butyl benzoate, and β-bisabolene had the highest positive correlation between variables in protected areas.

The plants harvested in the unprotected area include a) Ls-86 population individuals whose volatile fraction contains 14-hydroxy-α-humulene, 14-hydroxy-9-epi-(E-)-caryophyllene, and myrcene as main components, and b) Ls-85 population individuals, with a predominance of δ- and α-cadinene, *trans*-β-caryophyllene, α-terpineol, and β-pinene.

The plant harvested in the protected area include a) Ls-60 population containing compound such as butyl benzoate, 2,4-heptadienal, β-bisabolene, (Z)-3-hexenyl benzoate, p-cymene, sabinene, tricyclene, *epi*-α-cadinol, γ-cadinene, limonene, linalool, α- humulene, and b) Ls-67 population characterized by triquinane sesquiterpenes, such as presilphiperfol-7-ene, α-isocomene, β-maalinene, and silphiperfol-5-ene<7-epi>, and caryophyllene oxide, and α-copaene. Although both populations were placed by PCA in a common group, triquinane sesquiterpenes and fatty acids derivatives contribute to their discrimination, with the exception of some outlier individuals from Ls-60 mixed with Ls-67. A hypothesis may be that these populations are isolated and not subjected to disturbance. It could be expected that the gene flow among these individuals is more intense/evident, opening the possibility of more diversity of biosynthetic pathways and secondary metabolite formation.

Compounds with the triquinane skeleton-type were previously reported from more than

60 plant genera, almost always in the Compositae family (Radulović & Denić, 2013), with few rare exceptions as reported by Pinto et al. (2013) in *Anemia tomentosa* var. *anthriscifolia* (Pteridophyta). Curado et al. (2006) have also reported some triquinane sesquiterpenes in the essential oil of *L. ericoides*, such as cameroonan-7-α-ol and silphiperfolan-7β-ol.

Coincidently, the essential oil of *L. salicifolia* bulked leaves also showed a predominance of triquinane sesquiterpenes in populations Ls-60 and Ls-67, which also showed some compounds not found in the volatile fraction, such as prenopsan-8-ol, cameroonan-7-α-ol, modheph-2-ene, and silphiperfol-6-ene. The biological activity of triquinane sesquiterpenes is not clear in the literature. The biosynthetic pathway of these compounds origins from a caryophyllyl cation, which could lead through distinct paths to cameroonan-7-α-ol or silphiperfol-5-ene, presilphiperfol-7-ene followed by α-isocomene, or modheph-2-ene (Radulović & Denić 2013). In any case, their precursor is a humulyl cation followed by caryophyllyl cation, which can also forms β-caryophyllene, and probably most caryophyllene derivatives (Dewick 2002). The interesting aspect in this study is that these compounds are mostly observed in populations of protected areas (Ls-60 and Ls-67), in both the volatile fraction and essential oil.

On the other hand, a higher concentration of caryophyllene derivatives was observed in populations from non-protected areas (Ls-85 and 86), usually with compounds associated to plant defense. Probably, the biosynthetic pathway from caryophyllyl cation to the caryophyllene derivatives prevails and blocks the path to the triquinane sesquiterpenes.

The second informative data from PCA was that populations from the same location could also be discriminated based on volatiles, as it can be seen on the first axis (PC1=26.5%). A clear discrimination between Ls-67 from Ls-60 was observed mostly based on monoterpenes, triquinane sesquiterpenes and cadinol derivatives. On the other hand, Ls-86 was differentiated from Ls-85 because of the higher amount of oxygenated sesquiterpenes, mainly 14-hydroxy-9-

epi-(E-) caryophyllene.

Plant volatiles have an important role in plant stress and defense responses. Although a correlation between the reported data and the stress conditions in each area is quite difficult to establish, it is evident that population Ls-86 corresponding to the area under more intense human impact, showed a high concentration of caryophyllene derivatives.

Since β-caryophyllene is one of the most common sesquiterpenes elicited by herbivore damage (Holopainen 2004), it would be expected that plants from more disturbed areas have a more herbivore impact. For example, *trans*-β-caryophyllene is emitted by leaves in several wild relatives of maize in response to attack by lepidopteran larvae (Köllner et al. 2008).

The PCA from *L. salicifolia* essential oil was quite similar to that obtained from the volatile fraction sampled by HS-SPME, both showing a similar discrimination of the investigated populations (data not reported).

Non-Separative HS-SPME-MS Approach as Alternative Tool to Discriminate the Geographical Origin of L. salicifolia Samples

This study also aimed to develop a non-separative headspace solid phase microextraction−mass spectrometry method (HS-SPME-MS), in view of its possible application to an in-field monitoring of plants biodiversity. Non-separative MS methods were introduced by Marsili (1999) to study off-flavors in milk and provide a diagnostic and representative mass spectrometric fingerprint of the volatile fraction of a sample, analyzed directly by MS in combination with a suitable chemometric elaboration without a preliminary chromatographic separation. These methods can successfully be used to characterize and discriminate quickly samples within a set.

The volatile fraction of 48 of *L. salicifolia* leaves was analyzed with HS-SPME-MS in combination with PCA. **Figure 2** reports the HS-SPME-MS-TIC profile, together with the mass

spectral fingerprint of a *L. salicifolia* sample. The absolute intensity of all diagnostic ions of the markers present in the volatile fraction MS fingerprint of each *L. salicifolia* sample were considered for PCA. **Figure 3** shows the PCA plot of HS-SPME–MS patterns of the same samples processed in Figure 1. The PCA results were similar to that obtained with the separative approach: the plant populations harvested in the protected area are separated from those from unprotected and disturbed areas.

Conclusions

Present results show that HS-SPME, combined with both separative (HS-SPME-GC–MS) and non-separative (HS-SPME-MS) approaches, is an effective tool for a reliable qualitative characterization of *L. salicifolia* samples in alternative to essential oil analysis. Both separative and non-separative methods afford to discriminate samples in function of their habitat (i.e. protected and non-protected) since they provide diagnostic sample fingerprinting in combination with PCA. The area characterized by a great diversity of volatiles resulted to be the most stressed as expected (i.e. Ls-86), since it is surrounded by intense agriculture practices.

These results confirm that HS-SPME can be an effective sample preparation technique for rapid analysis of a large number of plant samples from wild populations to be associated to biological in-field studies.

Disclosure statement

The authors do not report any potential conflict of interest.

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Table 1. Description of collection sites of *L. salicifolia* samples[1].

Sample	Collecting site description, latitude, longitude and altitude.
Ls-60	These samples were collected in the Chapada dos Veadeiros National Park, in a trail named Mulungu, a protected area with minor human influence. Most plants have1-1.5m high, occurring in a sloped area under rocky and sandy soil, in a population with approximately 50 individuals. Latitude 14° 06' 52" S; Longitude 47° 38' 46" O; Altitude1246 m.
Ls-67	Plants on this population have the same characteristics of the Ls-60, in the same area, but distant each other from 350 m, approximately. This is a more dense population with over 100 individuals. Latitude 14° 06' 43" S; Longitude 47° 38' 53" O; Altitude 1254 m.
Ls-85	This population is located in São João d'Aliança, at Goiás state, in a disturbed area, close to the main road and an intense agriculture field. Also, plants are in a more sloped area, with plants occurring in both sides of the secondary road. Individuals are in general higher than those of Ls-60 and Ls-67, with 1,5 to 2m high, approximately. Latitude 14° 42' 11" S; Longitude 47° 26' 48" O; Altitude 1168 m.
Ls-86	This population is located in São Gabriel, at Goiás state. This population occurs in disturbed area, with plants close to the secondary road, and to an area of cattle pasture. This population has a lower number of individuals compared to the others. Latitude 15° 09' 10" S; Longitude 47° 37' 44" O; Altitude 1016m.

[1]Samples acronym code refers to the initials of each species and collecting number in the herbarium vouchers, collected by R.F. Vieira.

Table 2. Essential oil composition of the four populations of *L. salicifolia* collected in Brazil. Data are expressed as percentage normalized areas calculated on three replicates.

I^T_S[1]	$I^T_{S\ tab}$[2]	Compounds	Populations			
			Ls-60	**Ls-67**	**Ls-85**	**Ls-86**
921	926	Tricyclene	0.21	0.40	0.6	0.43
930	931	α-Thujene				0.08
937	939	α-Pinene			0.5	0.14
976	975	Sabinene	0.34	0.69		0.08
979	975	β-Pinene				0.42
992	991	Myrcene	0.60	0.77	1.50	17.7
1005	1005	α-Phellandrene			0.10	0.15
1018	1018	α-Terpinene			0.10	0.09
1027	1027	*p*-Cymene			0.10	0.22
1031	1031	Limonene	0.31		0.20	1.30
1062	1062	γ-Terpinene			0.10	0.11
1089	1089	α-Terpinolene			0.10	0.12
1100	1097	Linalool	1.17	0.55	0.34	0.61
1105	1105	Nonanal	0.54	0.64	0.20	0.40
1179	1177	Terpinen-4-ol	0.27		0.22	0.27
1191	1189	α-Terpineol	1.15	0.56	0.40	1.70
1295	1290	Thymol	0.80	0.67	0.18	0.18
1334	1337	Presilphiperfol-7-ene	0.30	0.65		0.12
1378	1379	Silphiperfol-6-ene	0.50	1.08	0.10	0.33
1386	1385	trans-β-Damascenone	0.80	1.40	0.20	0.26
1420	1418	β-Caryophyllene	0.37	0.67	0.40	0.29
1455	1455	Geranyl acetone + α-humulene	0.60	0.72	0.34	0.31
1499	1492	*(Z)*-Methyl isoeugenol	1.30	1.00	0.30	0.41
1515	1514	γ-Cadinene	6.71	3.95	6.40	4.10
1525	1524	δ-Cadinene			0.50	0.49
1572	1570	*(Z)*-3-Hexenyl benzoate	1.80	3.50	0.50	0.19
1580	1578	Spathulenol	1.15	1.80	0.60	0.34
1584	1583	Caryophyllene oxide	2.11	3.16	1.60	1.50
1610	1608	Humulene epoxy II	0.34		0.50	0.35
1643	1640	epi-α-Cadinol	13.0	12.9	13.4	7.70
1653	/	MW=218	2.80	4.70	3.70	3.46
1669	1670	14-Hydroxy-9-epi-(E-)-Caryophyllene	1.88	2.25	9.09	37.7
1686	1686	α-Bisabolol	0.89	2.50	1.60	0.84
1714	1714	14-Hydroxy-α-Humulene	1.68	2.50	2.90	5.70
1791	/	MW=234	10.2		22.0	0.58
1801	/	MW=222	4.36	2.10		0.63
1831	/	MW=234	3.40	0.80	5.40	1.80
2098	/	MW=272	2.50	1.40		0.29
2175	/	MW=324	3.00	3.30	5.20	1.60
2299	/	MW=324	0.5	0.7		0.18

[1]experimental linear retention index
[2]tabulated linear retention index

#	Constituents	I^{T}_{S} [1]	$I^{T}_{S\ tab}$ [2]	Ls-60		Ls-67		Ls-85		Ls-86	
				Average	STD	Average	STD	Average	STD	Average	STD
1	**tricyclene**	921	926	4135	1671	2842	1467	7346	3323	10695	4152
2	α-thujene	930	931	244	259	162	74	465	441	2445	1180
3	α-pinene	937	939	478	391	365	150	8468	25402	6525	3950
4	benzaldehyde	963	961	818	224	740	101	1145	252	1077	231
5	**sabinene**	976	975	1762	747	1381	836	1666	962	4269	2828
6	**β-pinene**	979	979	303	83	230	77	3300	926	8440	2869
7	**myrcene**	992	991	32202	30020	61760	50682	65262	34247	372884	118523
8	α-phellandrene	1005	1005	438	207	337	63	450	167	1801	507
9	**2,4-heptadienal**	1012	1012	1472	341	1428	172	1575	218	1997	278
10	α-terpinene	1018	1017	79	79	12	39	97	110	545	236
11	***p*-cymene**	1027	1025	1925	1241	1334	213	1426	363	4161	1208
12	**limonene**	1031	1029	8570	5296	5367	2033	7360	1810	34682	9308
13	cis-β-ocimene	1041	1040	225	96	544	1016	399	255	1897	1020
14	benzene acetaldehyde	1047	1043	94	53	110	46	624	367	361	59
15	trans-β-ocimene	1052	1050	nd		58	193	nd		54	187
16	γ-terpinene	1062	1060	203	57	156	64	293	153	952	438
17	*cis*-sabinene hydrate	1070	/	216	246	116	130	617	644	1442	785
18	α-terpinolene	1089	1089	343	223	236	75	294	55	2063	769
19	2-nonanone	1094	/	1351	949	1852	1805	813	364	2238	2623
20	**linalool**	1100	1097	1868	1098	1185	677	2465	900	4164	1742
21	nonanal	1105	1101	1614	643	1243	670	1307	606	2212	2642
22	α-campholenal	1128	1125	nd		nd		40	141	nd	
23	*trans*-sabinol	1141	1140	18	71	nd		81	282	42	
24	MW=152	1160	/	481	374	939	1491	660	396	2238	1081
25	pinocarvone	1164	1162	0	0	0	0	56	195	25	0
26	terpinen-4-ol	1179	1177	144	250	40	133	282	212	1281	542
27	p-cymen-8-ol	1186	1183	671	517	137	312	344	239	1026	266
28	α-terpineol	1191	1189	2795	2138	2078	1051	4027	825	14035	2387
29	**methyl salicylate**	1194	1192	83	121	35	117	nd		nd	

Table 3. Normalized HS-SPME-GC-MS average areas for each population together with standard deviation (in bold: marker compounds selected for PCA).

#	Constituents	I^{T}_{S}[1]	$I^{T}_{S\ tab}$[2]	Ls-60		Ls-67		Ls-85		Ls-86	
				Average	STD	Average	STD	Average	STD	Average	STD
31	Silphiperfol-5-ene	1326	1329	545	1014	491	452	nd		1582	602
32	**Presilphiperfol-7-ene**	1334	1337	6844	1294	7428	6274	nd		8926	3719
33	MW=204	1337	/	71	84	28	41	217	85	521	198
34	**7-epi-Silphiperfol-5-ene**	1345	1348	1737	3274	1294	1237	158	90	6636	2903
35	α-cubebene	1352	1351	65	109	39	66	174	35	367	64
36	MW = 204	1354	/	248	298	154	64	506	127	1763	445
37	MW=204	1363	/	331	748	393	359	458	260	844	465
38	MW = 204	1369	/	2701	5312	2824	2555	nd		3331	1379
39	**butyl benzoate**	1375	1376	13863	2467	13099	1917	12541	313	11515	555
40	**α-copaene**	1377	1376	2085	4600	1670	1599	nd		3460	1579
41	**β-maaliene**	1381	1380	2013	3786	1934	1552	336	134	7170	2544
42	MW=204	1384		302	338	183	179	813	139	1699	673
43	**α-isocomene**	1387	1386	1743	3382	1690	1461	nd		3973	1676
44	**trans-β-caryophyllene**	1420	1418	1728	1247	2176	2751	3978	2659	16877	9931
45	**α-humulene**	1455	1455	1827	449	1510	164	1901	420	11473	5399
46	Alloaromadendrene	1462	1461	494	504	464	221	1175	569	3560	1064
47	γ-muurolene	1478	1480	72	85	47	66	283	50	241	222
48	MW-204	1481		1079	1286	1094	1239	252	109	2448	1616
49	MW=204	1487		957	391	836	124	1449	301	1318	279
50	**β-bisabolene**	1510	1509	6270	3121	4611	987	8157	1316	5999	363
51	**γ-cadinene**	1515	1514	13263	8691	10238	2633	27158	4367	34195	9608
52	**δ-cadinene**	1525	1524	1770	1174	1828	910	3580	1161	12628	3432
53	**α-cadinene**	1540	1539	716	686	365	258	3244	907	3400	1040
54	**cis-3-hexenyl benzoate**	1572	1570	2350	1666	2027	899	1299	349	1541	330
55	**caryophyllene oxide**	1584	1583	7064	1043	4835	3336	3993	866	14246	3638
56	Humulene epoxy II	1610	1608	399	285	235	51	995	217	2484	557
57	MW = 222	1618		150	328	nd		721	494	3540	845

58	***epi*-α-cadinol**	1643	1640	12669	9514	12873	5709	24875	6205	39779	8687
59	MW = 218	1653		1461	1237	236	558	4253	1319	15044	4986
60	**14-hydroxy-9-epi-(*E*-)-Caryophyllene**	1669	1670	42	167	21	68	9021	5313	148072	89286
61	**14-hydroxy-α-Humulene**	1714	1714	60	239	nd		941	1222	12975	4425
62	MW=222	1725		nd		nd		406	132	1632	542
63	MW=222	1827		1018	882	758	511	4678	1492	5172	2851

[1]experimental linear retention index
[2]tabulated linear retention index

Figure 1

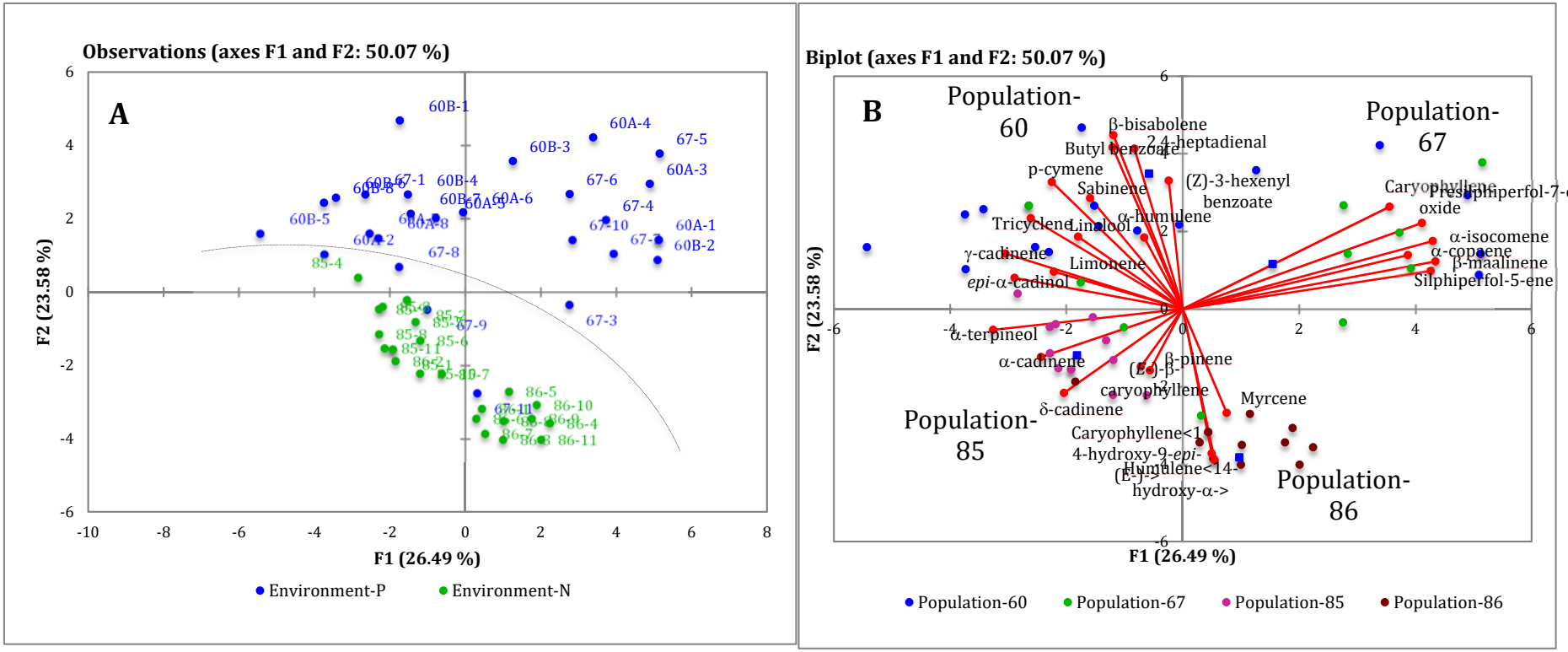

A
Observations (axes F1 and F2: 50.07 %)
F2 (23.58 %)
F1 (26.49 %)
Environment-P
Environment-N
B
Biplot (axes F1 and F2: 50.07 %)
Population-60
Population-67
Population-85
Population-86
β-bisabolene
2,4-heptadienal
Butyl benzoate
p-cymene
(Z)-3-hexenyl benzoate
Sabinene
Caryophyllene oxide
Presilphiperfol-7-ene
α-humulene
Tricyclene
Linalool
α-isocomene
α-copaene
γ-cadinene
Limonene
β-maalinene
Silphiperfol-5-ene
epi-α-cadinol
α-terpineol
α-cadinene
(E)-β-ocimene
β-pinene
caryophyllene
δ-cadinene
Myrcene
Caryophyllene<1
4-hydroxy-9-epi-(E)-
Humulene<14-hydroxy-α->
F2 (23.58 %)
F1 (26.49 %)
Population-60
Population-67
Population-85
Population-86

Figure 2

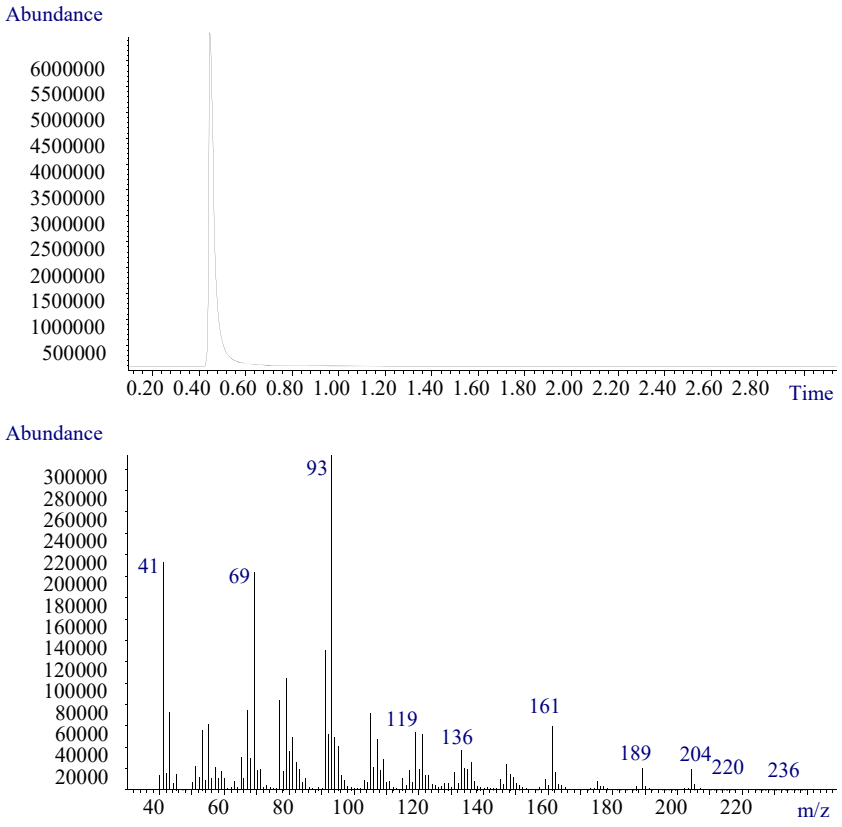
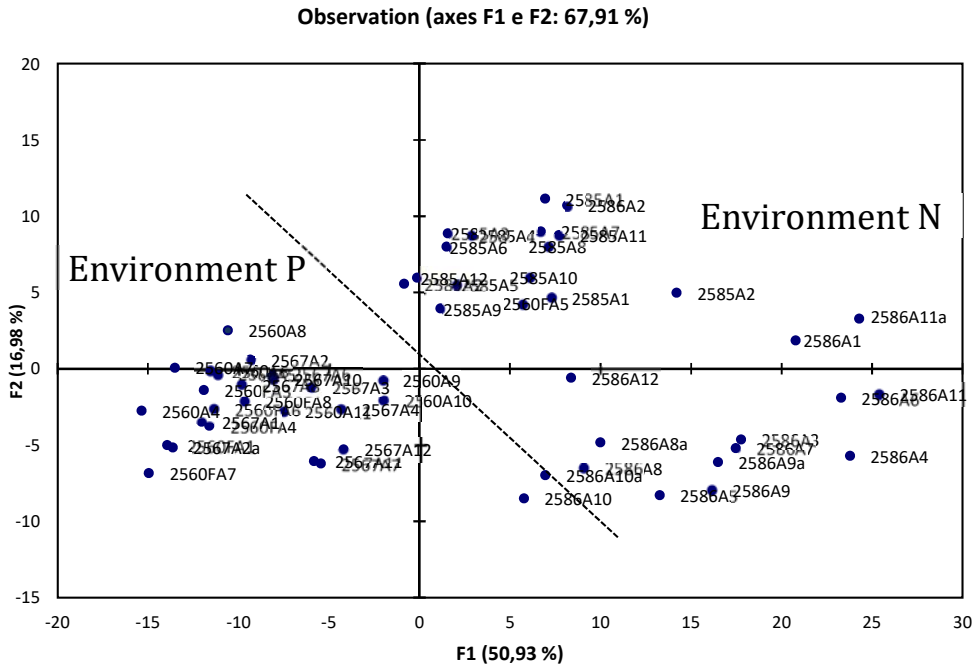

Abundance
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80
Time
Abundance
300000
280000
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
41
69
93
119
136
161
189
204
220
236
40 60 80 100 120 140 160 180 200 220
m/z

Figure 3


Observation (axes F1 e F2: 67,91 %)
Environment P
Environment N
F2 (16,98 %)
F1 (50,93 %)
20
15
10
5
0
-5
-10
-15
-20
-15
-10
-5
0
5
10
15
20
25
30
2586A2
2585A11
2585A6
2585A8
2585A10
2585A1
2585A2
2585A9
2560FA5
2586A11a
2586A1
2560A8
2560A9
2586A12
2586A11
2560A10
2586A8a
2586A4
2586A9a
2560FA7
2586A10a
2586A10
2586A9

Captions to the figures

Figure 1. Scores plot (A) on the first and the second principal components (F1-F2 plane) and loading plots (B).

Figure 2. HS-SPME-MS-TIC profile, together with the mass spectral fingerprint of a *L. salicifolia* sample.

Figure 3. Principal Component Analysis plot of HS-SPME–MS patterns.