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Evaluation of the volatile components and the seasonal variation of the methyl salicylate from *Stifftia chrysantha* Mikan by HS-SPME/GC-MS

[Evaluación de los componentes volátiles y la variación estacional del salicilato de metilo en *Stifftia chrysantha* Mikan por HS-SPME/GC-MS]

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Abstract
The volatile components from roots and aerial parts of *Stifftia chrysantha* Mikan, a Brazilian endangered native shrub of the Rain Forest, were extracted monthly and analyzed by solid phase microextraction coupled and gas chromatography/ mass spectrometry techniques. Among the 25 identified constituents, methyl salicylate was present at high concentrations mainly in the volatile fractions from fruits (85.75%).

Keywords: SPME, *Stifftia chrysantha* Mikan, methyl salicylate, essential oils.

Resumen
Los componentes volátiles de diferentes partes del *Stifftia chrysantha* Mikan, un arbusto brasileño nativo en peligro de extinción de la Selva Tropical, se extrajeron mensualmente por microextracción en fase sólida y analizaron, por cromatografía de gases acoplada a espectrometría masas. De los 25 componentes identificados, el salicilato de metilo fue el componente principal particularmente en las fracciones volátiles de las frutas (85.75%).

Palabras Clave: SPME, *Stifftia chrysantha* Mikan, salicilato de metilo, aceites esenciales.

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INTRODUCTION

The genus *Stifftia* (Asteraceae) (Corrêa, 1984) includes 17 species. In this genus, *Stifftia chrysantha*, "The Golden Flowered Stifftia" is a beautiful shrub attaining 4 meters in height. The flower heads are solitary at the branch ends, each head containing about twenty five closely set florets of tubular blossoms nearly 5 cm long, of bright orange colour. In Brazil, this species is popularly known as "Gold Rain" or "Fox Tail" due to the orange colour and its shape (Robinson *et al.,* 1991). *S. chrysantha,* a native Brazilian species, is widespread in the southern tropical forests. Unfortunately, nowadays this species is endangered, being found mainly in National Parks, Botanical Gardens and in very high inaccessible areas (Prefeitura Municipal do Rio de Janeiro, 2007). In Rio de Janeiro, *S. chrysantha* is used as an ornamental plant, often visited by humming birds (Mendonça *et al*., 2005). The only reported study on *S. chrysantha* mentions the presence of flavonoids and glycosylsterol besides its antitumoral activity (Oliveira *et al.*, 1999). This work aims to show the season variation of methyl salicylate from different parts of *S. chrysantha* using solid phase microextraction (SPME) coupled to GC/MS. SPME has been successfully used for the chemical characterization of flavor and fragrances and it is now favored over other procedures due to its simplicity, speed, low cost and solventless operation (Dias-Maroto *et al.,* 2002; Vázquez *et al*., 2011). SPME has also been proposed for analysis related to biogenic organic compounds released *in situ* from live parts of plants (Loi *et al*., 2008).

MATERIAL AND METHODS

Plant Material and Sanmple Preparation

Aerial parts and roots of *S. chrysantha* were harvested monthly in the garden of the National Museum, Rio de Janeiro. The botanical vouchers were identified by Dr. Roberto L. Esteves and kept at the Herbarium (HB) of the Rio de Janeiro National Museum under number R208153. The plant parts were collected early in the morning from the same chosen specimen. Plant material was taken immediately to the laboratory and leaves, roots, fruits and flowers were cut in small pieces.

Headspace solid-phase microextraction

Leaves, flowers, roots and fruits of *S. chrysantha* (500mg) were reduced to small pieces and powdered one by one, separately. For SPME sampling, each plant material was placed in a 10 mL glass vial, weighted and the vial closed by a septum. The SPME headspace volatiles were collected using a Supelco divinylbenzene-carboxen 75μm fiber (CAR-DVB) for 15 min at 80^{o}C. After sampling, the SPME device was placed in the injector of the GC–MS chromatograph manually, where desorption takes place for 5 min ate 250^{o}C.

GC-MS Analysis

Quantitative and qualitative analysis were carried out on a GC-MS QP 5000 Shimadzu with a ZB-5MS fused silica analytical column (30m x 0,25mm x 0,25μm film thikness), consisting of Crossbond (5% diphenyl, 95% dimethyl polysiloxane). The temperature programme for the gas chromatography was as follows: initial temperature, 60°C held for 1 min, linear gradient of 3°C/min to 290°C, then isocratic gradient was performed, the hold time was 30 min. The injector temperature was 260°C, and injection was performed in the splitless mode (splitless time, 30s). The carrier gas was helium (99.999% purity, 1.0 mL/min). Identification of the detected analytes was accomplished by matching their mass spectra with the available data base and confirmed by comparing their retention indices with literature data (Adams, 2004).

RESULTS AND DISCUSSION

Plants release a large variety of volatile organic compounds into the environment as part of their interaction with other organisms. Volatile organic compounds have been reported to comply some important roles in plant communication with other organisms, but little is known about the biological functions of most of these substances (Abel *et al*. 2009). By providing information across distances, plant volatiles are implicated in multiple functions such as the attraction of pollinators or defense against herbivorous insects via direct deterrence or attraction of insect enemies (Rasmann *et al.* 2005). Methyl salicylate (MeSA) is a common plant stress signal elicited in response to abiotic and biotic factors such as damage by insect herbivores and pathogens and is also the airborne version of salicylic acid, used by plants as a signal to propagate systemic acquired resistance (Park *et al.* 2007). The aim of this work was to investigated the volatile fractions of one of the most endangered species in Rio de Janeiro during a 12 month period in 2006. The identified constituents in the volatile fractions from different parts of *Stifftia* *chrysantha* and their percentage values are summarized in Table **1** where all the 25 compounds are arranged according to their retention indices (RI). The detached compounds appear in bold font. Although some organs volatile profiles do not show great chemical composition diversity, the percentage of the identified constituents in each total volatile fractions was quite high, except the volatile fractions from roots.

Table 1
Volatile fraction composition for different organ from the harvested *S. chrysantha* Mikan.

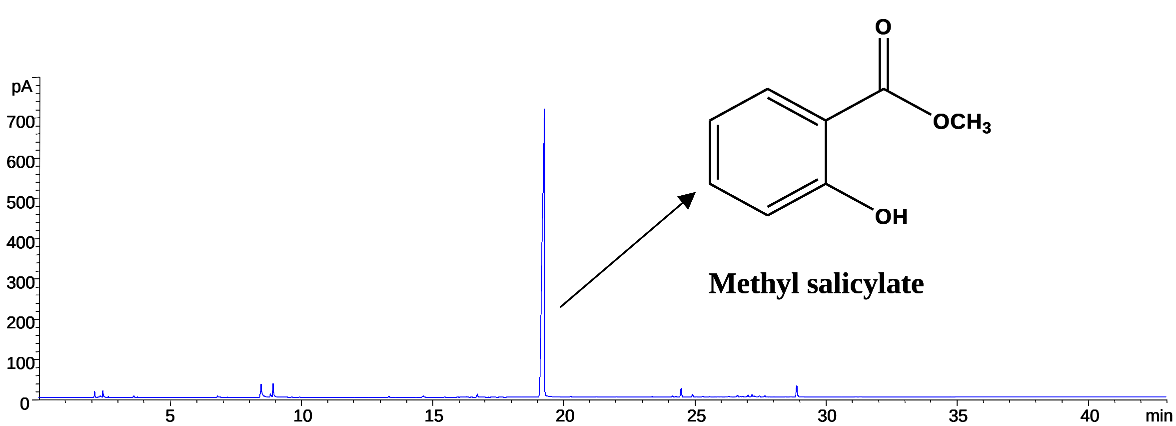
	Compounds	[a]*RI* [Lit]	[b]*RI*	*Leaves*	*Roots*	*Fruits*	*Flowers*
1	**Hexenal**	801	802	7.3	0.3	*	2.2
2	**(*Z*) 3-Hexen-1-ol**	858	858	36.3	*	4.7	10.7
3	**1-Hexanol**	871	868	17.9	*	2.0	39.5
4	**α-Pinene**	939	936	*	0.7	*	*
5	**Camphene**	953	950	*	1.9	*	*
6	**β-Pinene**	981	977	*	0.3	*	*
7	**6-Methyl-5-hepten-2-one**	985	985	*	*	*	2.7
8	***Cis*-3-hexenyl acetate**	1005	1006	19.5	*	0.6	10.2
9	**n-Hexyl acetate**	1009	1013	*	*	5.0	9.1
10	**Linalool**	1100	1099	*	*	*	1.2
11	**Borneol**	1162	1165	*	0.6	*	*
12	**Methyl salicylate**[b]	1188	1193	2.3	*	85.8	11.2
13	**Hexylvalerate**	1244	1241	*	*	*	1.6
14	***Cis*-3- hexyl isovalerate**	1245	1245	*	*	1.3	2.9
15	**α-Cubenene**	1345	1342	*	0.4	*	*
16	**Cicloisosativene**	1367	1365	*	4.7	*	*
17	**α-Ionone**	1422	1419	*	0.9	*	*
18	**β-Farnesene**	1445	1442	*	15.4	*	*
19	**γ-Muurolene**	1475	1474	*	4.7	*	*
20	**Germacrene-D**	1487	1481	*	24.8	*	*
21	**δ-Amorfene**	1512	1516	*	7.9	*	*
22	**γ-Cadienene**	1514	1518	*	3.2	*	*
23	**Spatulenol**	1578	1573	*	0.3	*	*
24	**Epi-α-cadinol**	1640	1638	*	0.4	*	*
25	**α-Cadinol**	1654	1652	*	0.5	*	*
	Sum of Component % for each plant organ			83.3	67.0	99.4	91.3

The data in Table **1** is related to the March harvest of 2006. * not detected < 0.1%; [b] Co-GC: co-injection with authentic compound; [a]RI [Lit] : Literature Retention Indices. [b] RI Experimental Retention Indices.

Among the identified compounds 3 monoterpene hydrocarbons (0.3-1.9 %); 2 oxygenated monoterpenes (0.6-1.2 %); 7 sesquiterpene hydrocarbons (0.4-24.8 %), 3 oxygenated sesquiterpenes (0.3-7.6 %) and 10 aliphatic and phenolcarboxilate compounds (0.3-85.8 %). The major volatile constituents identified were: methyl salicylate (2.3- 85.8 %); 1- hexanol (2.0-39.5 %); (*Z*) 3-hexen-1-ol (4.7-39.5 %); *β*-farnesene (15.4 %); *D*-germacrene (24.8 %) and cicloisosativene (4.7 %). Hexanal, 1- hexanol; *cis*-3-hexenyl acetate, (*Z*) 3-hexen-1-ol and methyl salicylate were found in high percentage in most of the analysed aerial parts volatile fractions. The major identified volatile constituent in the fruits of *S. chrysantha* was methyl salicylate, (Figure 1). The chromatography profiles of aerial parts show the remarkable presence of methyl salicylate as a major component of the fruit's volatile fraction. Methyl salicylate was found in expressive quantities in flowers and fruits. MeSA is a significant constituent of floral scents from various plant species and of volatile blends from herbivore-attacked vegetative plant parts, and it functions in pollinator attraction and defense against insects (Attaran *et al*., 2009; Du *et al*., 2009;). More recently, grafting experiments suggested that MeSA is a critical, phloem-mobile Systemic acquired resistance (SAR) long distance signal in tobacco (Park *et al.*, 2007). The aerial parts profiles were rich in monoterpenes and alcohols, while in the roots the sesquiterpenes are more representative compounds.

Figure 1

Typical chromatogram of *S. chrysantha* fruit volatile constituents obtained by SPME.



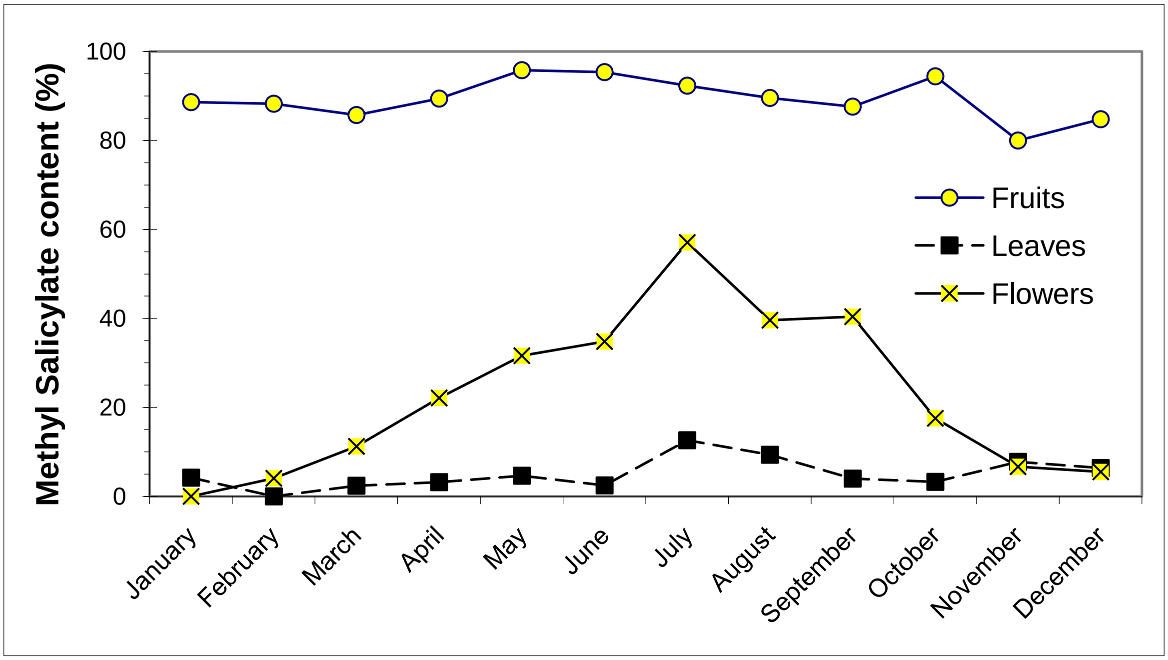
Seasonal influence on the volatile fractions of *S. chrysantha* aerial parts was observed for samples collected monthly throughout the year of 2006. Methyl salicylate was found in leaves, flowers and fruits. The fruit volatile fractions were found to be predominantly composed of MeSa. Considering all seasons through the year, the percentage of methyl salicylate in the fruit varied from 80.0 to 95.0 %, suggesting it is the place where this compound is stored. The highest concentration of this compound was verified in this organ during the whole year, reaching the highest amounts during the dry/cold season (May-June-July-

August) (80-95%). Remarkable differences can be found in the amounts of MeSa throughout the year in the chemical composition of the different analyzed organs. The content of this compound was distributed unevenly among the seasons in leaves and specially in flowers. In this organ, the volatile composition difference between the dry/cold and wet/hot periods is very meaningful; the MeSa content can vary 57.1% during the year while in leaves the variation can reach 12.6%. In both cases the autumn/winter were found as the best seasons to obtain a higher MeSa content in the volatile fraction while in the summer was found the lowest content, reaching 0% in some months, probably due to the excess of rainfall. During the summer and spring, the rainfall usually is much higher than in the other seasons. Thus, the harvest of this plant should be in the flowering stage due to the highest volatile content of MeSa in all the aerial parts. The high volatility of methyl salicylate and the lower temperatures between May-September could explain the great amount of this constituent in these months. This is the flowering period for the *S. chrysantha*, been often visited by humming birds as reported by *Mendonça et al*, (Figure 2). This period corresponds to the Brazilian autumn-winter (may-aug). Shulaev *et al* have reported methyl salicylate could function as an airborne signal which activates disease resistance and the expression of defense related genes in neighboring plants and in the healthy tissues of the infected plant (Shulaev *et al.*, 1997). Systemic acquired resistance (SAR) in plants is a state of heightened defense that provides long-lasting, broad spectrum resistance to microbial pathogens and is activated systemically following a primary infection (Durrant *et al*., 2004). Another recent study extended this putative signaling function of MeSA to SAR in Arabidopsis MeSA production increases in leaves inoculated with the SAR-inducing bacterial pathogen *Pseudomonas syringae*; however, most MeSA is emitted into the atmosphere, and only small amounts are retained. It shows that in several Arabidopsis defense mutants, the abilities to produce MeSA and to establish SAR do not coincide. Thus, in this species, MeSA neither functions as a critical long-distance signal nor in any other SAR relevant process, including systemic SA accumulation (Vlot *et al.*, 2008). It seems that variations in the concentration of methyl salicilate is correlated not only to environmental conditions but to SAR in plants and it can be implicated in multiple functions such as the attraction of pollinators or defense against herbivorous insects. The results suggest the high content of methyl salicylate in this species could be correlated not only to environmental conditions as exposure to high concentrations of various air pollutants but to insect attack too. During the harvest, a black substance was observed on some plant leaves. Just healthy parts were used in the experiment, but black substance on plant leaves usually indicates the presence of a sucking insect excreting excess honeydew. Black sooty mould grows on the honeydew giving the leaves a blackened appearance. This 'sweet' honeydew on the leaves may attract bees as well as ants. Ants were also detected in the local of harvest and in the shrub barks and branches. Thus, the remarkable presence of methyl salicylate in the fruits and flowers of *Stifftia chrysantha* could be related to injuries caused by air pollution and insect/fungi attacks. As this particualar species is rate, no other area was found to compare in terms of the chromatographic volatile fractions profile.

CONCLUSIONS

This work established the previously unknown profile of volatile fractions from several organs of *S. chrysantha.* The presence of methyl salicylate was remarkable in the fruits and flowers of the studied plant. The results suggest that the high percentage of methyl salicylate found in aerial parts could be correlated to antiherbivore defense as observed in other plant species. It could be functioning as an airborne signal that activates the disease resistance and defense in this patricular plant species. Moreover, we have stablished the autumn/winter as the best period for havest of this species due to the higher concentration of methyl salicylate in all aerial parts.

Figure 2
Seasonal methyl salicylate profile of fruits, leaves and flowers from *S. chrysantha* obtained by SPME (CAR-DVB fiber) analysis.



** Suggested place to this figure: immediately after the reference of figure 2 in the results and discussion.*

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