Quantitation of Selected Volatiles from Leaves and Rhizomes of Shell Ginger (*Alpinia zerumbet*)

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Special graduation project presented as partial requirement to obtain a Bachelor of Science degree in Food Science and Technology.

Presented by:

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Abstract. Shell ginger (*Alpinia zerumbet*) is a species native of southeastern Asia which is used as a culinary spice and as a medicinal plant. To characterize the volatiles differentially present in the leaves and rhizomes of shell ginger, the two plant materials were individually extracted using solvent and the extracts were subsequently distilled by Solvent Assisted Flavor Evaporation (SAFE). The resulting volatile isolates were then analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). As a result of the GC-MS experiments, 35 volatiles where identified. To aid in the identification experiments and separate volatiles based on their polarity, SAFE isolates were fractionated into six fractions by Solid-Phase Extraction (SPE) using different solvent mixtures. Eighteen compounds were present in the non-polar eluent, five were present in the medium polarity eluent (non-polar and polar association), and eleven volatiles were resent in the polar eluent. Five volatiles including 1,8-cineole, linalool, fenchyl acetate, bornyl acetate, and *trans*-methyl cinnamate were selected and quantitated by GC-MS using isotopically labelled $^{2}H_{32}$-pentadecane as an internal standard. *Trans*-methyl cinnamate (87.20 ppm), 1,8-cineole (70.50 ppm), and linalool (21.60 ppm) were the highest in the leaves. Fenchyl acetate (431.50 ppm) and bornyl acetate (253.40 ppm) were the highest in the rhizomes.

Key words: Aromatic compounds, GC-MS, SAFE, SPE.

Resumen. Azucena de Porcelana (*Alpinia zerumbet*) es una especie nativa del sudeste asiático que es utilizada como especia culinaria y como planta medicinal. Para caracterizar los volátiles presentes diferencialmente en las hojas y los rizomas del jengibre de concha, los dos materiales vegetales se extrajeron individualmente usando solventes y los extractos se destilaron posteriormente por Evaporación de Sabores Asistida por Solventes (SAFE). Los aislados volátiles se analizaron por Cromatografía de Gases-Espectrometría de Masas (GC-MS). Como resultado de GC-MS, se identificaron 35 volátiles. Para ayudar en los experimentos de identificación y separar los volátiles en función de su polaridad, los aislados SAFE se diluyeron en seis fracciones mediante Extracción en Fase Sólida (SPE) utilizando diferentes mezclas de solventes. Dieciocho compuestos volátiles se identificaron en el eluyente no polar, cinco en el eluyente de polaridad media (asociación no polar y polar), y once volátiles en el eluyente polar. Cinco volátiles que incluyen 1,8-cineol, linalol, acetato de fenilo, acetato de bornilo y *trans*-cinamato de metilo se seleccionaron y cuantificaron por GC-MS usando $^{2}H_{32}$-pentadecano marcado isotópicamente como un estándar de referencia. El *trans*-cinamato de metilo (87.20 ppm), 1,8-cineol (70.50 ppm) y linalol (21.60 ppm) fueron los más altos en hojas. El acetato de fenilo (431.50 ppm) y el acetato de bornilo (253.40 ppm) fueron los más altos en rizomas.

Palabras clave: Compuestos aromáticos, CG-MS, SAFE, SPE.

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1. INTRODUCTION

Alpinia zerumbet (Pers.) B.L. Burtt and R.M. Smith, known commonly as "shell ginger", is a perennial plant belonging to the Zingiberaceae family. Shell ginger is native to southeastern Asia regions including Japan, Taiwan, South China, and Malaysia (Govaert *et al*. 2010). It grows from 2.4 to 3.0 meters, with leaves measuring up to 600 and 200 mm in length and width, respectively (Lim 2016). While shell ginger can tolerate cool temperatures (10 °C); it needs warm temperatures between 20-25 °C and constant humidity (Mahr 2018). Shell ginger could reproduce sexually *via* seed and asexually by spreading rhizomes (Rodger 2011). Shell ginger is commonly used in Asia as a culinary spice and is also consumed for its purported medicinal properties (Victório *et al*. 2009). Shell ginger leaves are used to make tea (Chan 2009), and as a flavor in various foods such as noodles, desserts, candies, bread, and juice (Lim 2016). Traditionally, rhizomes are used to treat nausea, stimulate digestion, and treat traumatism; however, the efficacy of these uses remains unknown (Lete and Allué 2016). Shell ginger have been used in folk medicine for its anti-inflammatory, bacteriostatic and fungi static properties (Zoghbi *et al*. 1999).

The investigation of shell ginger started in Honduras in 1996 by the Honduran Agriculture Research Foundation (FHIA), related to its agro ecological conditions that have a direct impact on the production quality. In 2001, twenty-five farmers located in Yoro department cultivated 21 hectares of shell ginger resulting in the production of 36,000 pounds (FHIA 2001). The exportation of shell ginger from Honduras in the last few years has produced around USD 46,000, annually (FIDE 2017). Honduras´s exports shell ginger primarily to the United States of America (USA) representing 99% of the total exportation, and the rest is exported to Nicaragua (FIDE 2017). Shell ginger was introduced in the USA and grown in Florida, California, Arizona, and Texas (Gilman and University of Florida 1999). In 2020, the FHIA established that the production yields in Honduras were around 11,000 - 17,000 kg/ha with a production cycle of 10 months (FHIA 2020).

Previous studies have identified numerous volatiles in both the leaves and rhizomes of shell ginger. According to Elzaawely *et al*. (2006); Elzaawely *et al*. (2007); Pimentel *et al*. (2009); Victório *et al*. (2009b); Murakami *et al*. (2009b); Padalia *et al*. (2010); Victório *et al*. (2011); and Kuraya *et al*. (2017), five volatiles were reported in ginger leaves, 1,8-cineole, linalool, limonene were the volatiles with higher concentration. Sixteen volatiles were reported in leaf essential oil being α-pinene, *α*-terpinene, *β*-pinene, borneol, camphene, camphor, methyl cinnamate the ones with higher concentration. Others have reported 20 volatiles in rhizomes such as, Murakami *et al*. (2009); Padalia *et al*. (2010); and Victório *et al*. (2011b), being *α*-pinene, *β*-pinene, frenchyl acetate, linalool, methyl cinnamate, and 1,8-cineol the compounds with higher concentration.

Shell ginger rhizomes are commonly used as a food seasoning due to the presence of aroma-active volatiles. Aroma-active compounds are perceived by olfactory tissue of the nasal cavity (Grosch *et al*. 2008 & Tranchida 2019) and they can be measured by Gas Chromatography-Mass Spectrometry (Nigam and Levi 1963 & Delahunty 2006). Although some volatiles have been identified in shell ginger such as esters, lactones, terpenes, carbonyl compounds, and ionones (Adlard 2013). The odorants responsible for shell ginger´s unique aroma has not been determined (Bogacz and Pietkiewicz 2008).

To determine the volatiles deferentially present in the leaves and rhizomes, the current study was aimed at quantitating selected volatiles in the different plant tissues by employing modern instrumentation. This study used innovative technology as Solvent Assisted Flavor Evaporation (SAFE), Solid-Phase Extraction (SPE), and Gas Chromatography-Mass Spectrometry (GC-MS) to produce a clean volatile extract while avoiding the loss of labile aroma compounds and preventing the formation of thermally generated artifacts during distillation (Schieberle and Grosch 1987 & Wenqi *et al*. 2019).

This research was aimed to quantify selected volatiles present in the leaves and rhizomes of *Alpinia zerumbet* through the following objectives:

- Determine various volatile compounds in ginger leaves and rhizomes by Gas Chromatography-Mass Spectrometry (GC-MS)
- Identify various volatile compounds in ginger leaves and rhizomes depending on their polarity.
- Quantify selected volatile compounds in ginger leaves and rhizomes isolates after fractionation.

2. MATERIALS AND METHODS

Location

Both cultivation of shell ginger and all chemical analysis were done in Dr. John P. Munafo´s Functional Food Laboratory located in the Food Science Department at The University of Tennessee, Knoxville 37996.

Plant material

Shell ginger seedlings were obtained from Well-Sweep Herb Farm and grown in a climate-controlled Venlo-style greenhouse located at the University of Tennessee. Seedlings were transplanted into pots and placed on propagation trays. Plants had 12 hours of light per day, kept at 25 $\pm$ 3 °C, and irrigated daily for 15 minutes at 10 mL/minute. Plants were treated biweekly with 20-20-20 water-soluble fertilizer. Leaves and rhizomes were harvested, dried for three days at room temperature and stored in plastic bags before analyzes.

Experimental design

The majority of the analysis performed were classified as qualitative. The quantitation of selected volatiles (1,8-cineole, linalool, fenchyl acetate, bornyl acetate and methyl cinnamate*)* of leaves and rhizomes identified by Gas Chromatography-Mass Spectrometry (GC-MS) were compared by using a Completely Randomize Design. A T-test was performed to evaluated significant differences between plant material ($P \leq 0.05$) using Microsoft Excel Version 16.0 from Office 365 (Microsoft Corporation, Redman, WA, USA). To determine significant differences beyond volatiles a Duncan test was used ($P \leq 0.05$) using Statistical Analysis System (SAS version 9.1®).

Solvent Assisted Flavor Evaporation (SAFE)

Leaves and rhizomes of shell ginger were analyzed separately following Dr. Munafo´s laboratory manual as in the following manner: First, dried plant material were weighted to obtain 10 ± 0.5 g and grounded to a fine powder using a coffee grinder. Freshly distilled pentane (100 mL) from Fisher Scientific (Waltham, MA, USA) and $^{2}H_{32}$-pentadecane (50 µL) were combined with the ground samples and manually shaken for 5 minutes. The sample were centrifuged at 3,857 ×g (5000 rpm) for 10 min. A second extraction of the residual material was completed by adding 50 mL of pentane and centrifuged as described above. The organic layers were combined, filtered using No. 41 Whatman filter paper (North Bend, OH, USA), and dried over using anhydrous sodium sulfate obtained from Fisher Scientific (Waltham, MA, USA). The final extract was submitted to SAFE; a high vacuum distillation operated at 41 °C. The isolates were defrosted at room temperature and transferred to a pear bottom flash with boiling chips. The samples were condensed at 45 °C on the Vigreux column to a final volume of ~200µL and stored at -80 °C (Cheriyedath, 2019).

Gas Chromatography-Mass Spectrometry (GC-MS)

Isolates were analyzed following the mythology of Chiang N, Tang C & Munafo JP (2018) by an Agilent Technologies 78204 GC system (Santa Clara, CA). A Zebron™ ZB-FFAP GC capillary column (30m ×, 0.32 mm OD × 0.25 µm film thickness) from Phenomenex (Torrance, CA, USA) was connect to an Agilent Technology 5977B mass spectrometry detector (Santa Clara, CA, USA). The sample was injected on column (1 µL) and transported with a carrier gas (1 mL/minute) at an initial temperature of 35 °C, which will be held. To identify the compounds present in isolates, a total ion chromatogram was analyzed using the National Institute of Standards (NIST) library in combination with retention indices and authentic reference standards.

Solid-Phase Extraction (SPE)

A silica SPE cartridge (Strata® SI-1 Silica (55 µm, 70 Å), 2 g/12 mL) from Phenomenex (Torrance, CA, USA) was affixed to an SPE manifold and fractionation of the volatile isolate were performed under a vacuum, following Dr. Munafo´s laboratory manual. During the process, both distilled pentane and ether were used to generate different fractions based on the polarity of the compounds in isolates (Table 1). First, three wash tubes (P, E, P) and 6 sample tubes (0, 2, 5, 10, 50, 100) were placed inside the manifold. The manifold was connected with vacuum tubing, to reach a pressure of -60,000 Pa. Once the vacuum was stable, the cartridge was placed over the first wash tube and pentane was dispensed (10 mL). Then, the cartridge was moved to the second wash tube and was rinsed with ether (10 mL). For the third and final wash, the cartridge was moved to be washed with pentane (10 mL). Once the cartridge was conditioned, the sample (0.5 mL) was pipetted into the middle of the cartridge and pulled into the column. To begin the fractionation, the columns were kept in place and 5 mL of F1 was dispensed. Then for each subsequent fraction, the cartridge was moved and fractionated with its corresponding solvent wash (Figure 1). Once the process was completed, vacuum was released, fraction tubes capped, and stored at 4 °C. The fractions were analyzed using GC-MS.

Table 1. Concentration of ether and pentane for SPE fractions.

	Concentration	Relation pentane:ether	mL of pentane	mL of ether
F1	100%	100:00	25.00	0.00
F2	2%	98:20	24.50	0.50
F3	5%	95:00	23.75	1.25
F4	10%	90:10	22.50	2.50
F5	50%	50:50	12.50	12.50
F6	100%	00:100	0.00	25.00

Pentane (C_5H_{12}) is a non-polar solvent with a boiling point of 36.1 °C and it relative polarity is 0.009 (Murov 2020).

Ether ($C_4H_{10}O$) is a non-polar solvent with a boiling point of 34.6°C and it relative polarity is 0.117 (Murov 2020).

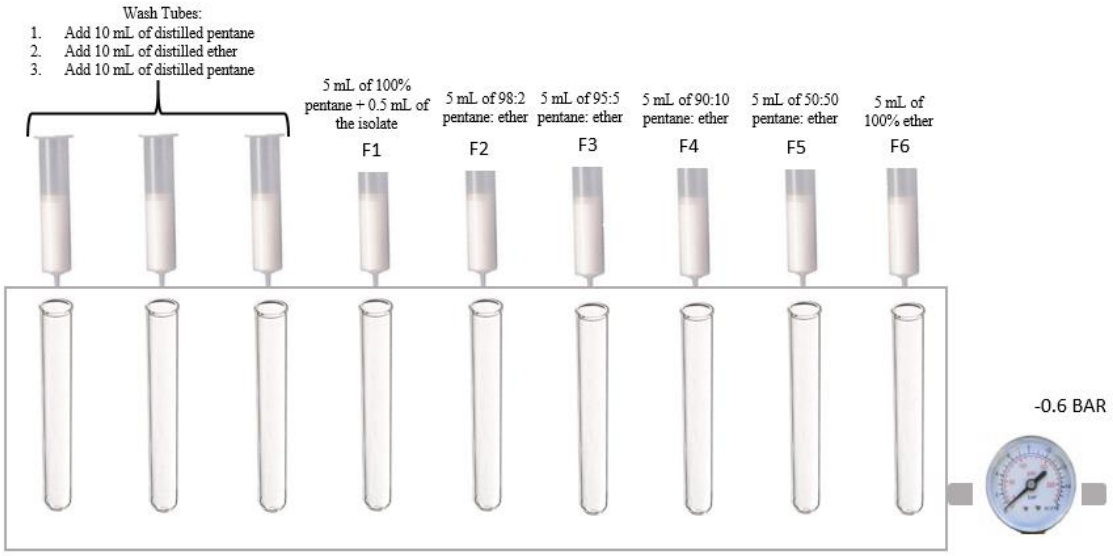


Figure 1. SPE methodology

Preparation of leaves and rhizomes for GC-MS analysis

Dried plant material (200 mg) was ground using a coffee grinder and added to a 10 mL test tube with a PTFE cap. Next, 4 mL of pentane and 50 µL of $^{2}H_{32}$-pentadecane were added to the test tube and manually shaken for 5 minutes. The samples were extracted for 30 minutes and then filtered. The filtered extract was transferred to an amber vial (1.5 µL) and analyzed by GC-MS. Dried leaves (n = 3) and rhizomes (n = 3) were analyzed separately. The concentrations of volatiles were calculated based on the concentration of the $^{2}H_{32}$-pentadecane as an internal standard.

Quantitation and comparison of selected volatiles in ginger leaves and rhizomes

The ion chromatogram for the three replicates for both leaves and rhizomes were analyzed by NIST-library with a comparison of retention time and standards. The area under the peak for each volatile was measured and transformed to part per million (ppm) values [1]. Then, a comparison of the values beyond plant material and within volatiles was conducted by Student T-test and Duncan.

$$ppm = \frac{\frac{\left(0.325\,\frac{\mu g}{\mu}\,\text{standars}\right)(50\mu L\ \text{standards})(\text{compound integrated area})}{(\text{standars integrated area})}}{\text{sample (g)}} \quad [1]$$

3. RESULTS AND DISCUSSION

Identification of compounds using SAFE isolates by GC-MS

To generate an identification of volatiles in ginger, SAFE leaves and rhizomes isolates were analyzed by through GC-MS; as a result, a database with compounds was obtained to further analysis. GC-MS has been a method and technique useful for the detection of the low concentrated compounds and to separate mixtures at a molecular level (Huhtaniemi *et al*. 2012). To confirm the compounds an ion monitoring mass spectra and relative abundance were compared between references standards and SAFE isolates.

To identify volatiles presence in both ginger leaves and rhizomes isolates, the ion chromatogram was analyzed by NITS-library, which compare ions of references standards and the volatiles ions. As many volatiles share common ions, they must be fragmented first chromatographically and then enter to the mass-spectrometer (Cook-Botelho *et al.* 2017). Volatiles are composed by carbon and hydrogen atoms that forms cyclic compounds with double bonds.

Results from this analysis were two chromatograms representing the peaks generated from ginger leaves and rhizomes SAFE isolates (Figure 2) obtain by GC-MS. The retention time reflected the relation between the sample injection, time needed to run through the column, and its volatilization allowing compounds differentiation. Another aspect measured was peak area, which reflected compounds abundance and classification in SAFE isolates allowing the identification of it aroma. For both ginger isolates, 58 peaks were identified initially and sorted to obtain a final list of 35 compounds, due to impurities and non-active compounds were eliminated. These selected compounds were classified based on previous studies (Dein *et al*. 2019) made in the Munafo´s Laboratory that determined their contribute to aroma of different plant.

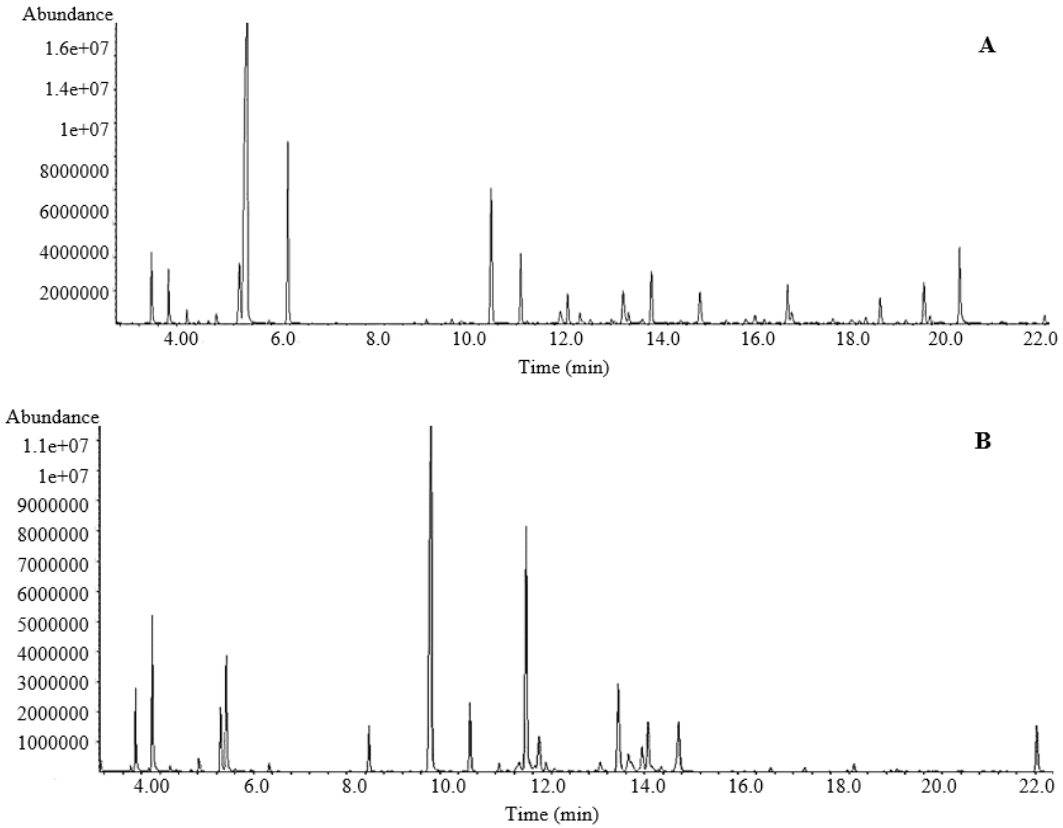


Figure 2. Chromatogram of leaves (A) and rhizomes (B) SAFE isolates.

The compounds that reflected highest concentration in ginger leaves and rhizomes isolates were *β*-myrcene, limonene, 1,8-cineole, *p*-cymene, fenchyl acetate, camphor, and *trans*-methyl cinnamate (Table 2). This compounds are also found in other products such as, myrcene in mango (Munafo P *et al.* 2014), limonene in Propolis and Hoary Mountain mint (Tomaszewski *et al.* & Dein *et al*. 2019), 1,8-cineole in Cardamom seeds (Paul *et al.* 2020), *p*-myrcene and camphor in oregano (Gong and Ren & Nutrizio *et al*. 2020), and *trans*-methyl cinnamate in the genus Ocimum. (Zahran *et al*. 2020). Every volatile was confirmed by breakage ion and retention time based on previous studies in Dr. Munafo´s laboratory (Munafo 2014 & Dein 2019), described as *β*-myrcene (4.93 min), 1,8-cineole (5.09 min), fenchyl acetate (9.42 min), camphor (10.27 min), and *trans*-methyl cinnamate (20.19 min). An aromatic ring was determined to be in *p*-cymene compound structure (Figure 3), however the other volatiles could be able to provide aroma as well.

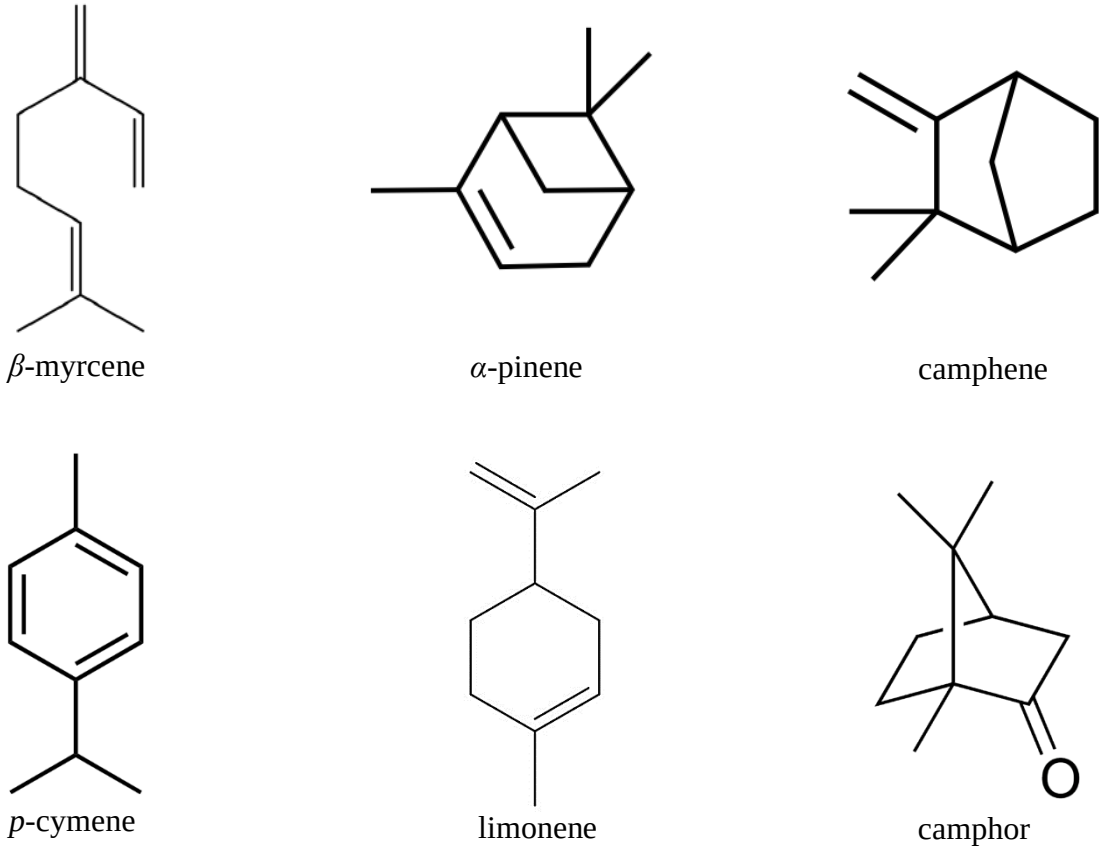


Figure 3. Major volatile compounds structure of ginger leaves and rhizomes.
Source: NIST Chemistry WebBook – the NIST WebBook (Linstrom 1997).

Ginger leaves isolate reflected a more prominent volatiles than rhizomes isolate due to their variety of compounds and its abundance. Monoterpenes were the most predominant compounds in both profiles (Table 2) which agree with other authors such as Murkamia *et al*. (2008). The major compounds found in leaves SAFE isolate were *β*-myrcene (5%), limonene (5%), 1,8-cineole (38%), *p*-cymene (11%), ylangene (4%), camphor (9%), linalool (4%), *trans*-methyl cinnamate (6%). In contrast, the quantitatively significant compounds of rhizomes SAFE isolate were *α*-pinene (6%), camphene (12%), limonene (6%), *α*-thujone (4%), fenchyl acetate (52%), camphor (7%), and *β*-caryophyllene (5%). The results well also observed by other authors in ginger isolates, such as Elzaawely *et al*. (2006); Pimentel *et al*. (2009); Padalia *et al*. (2010); Victório *et al*. (2011); Kuraya *et al*. (2014); Kuraya *et al*. (2017); and Houng *et al*. (2017).

Table 2. Volatiles identified in ginger leaves and rhizomes by GC-MS using SAFE isolates.

Peak [a]	Compound[b]	[M-H]-[c] (m/z)	MS[d] (m/z)	RT[e]	Leaves PA[f]/10[6] Abundance	Rhizomes PA[f]/10[6] Abundance
1	*α*-pinene	136	93	3.06	71	48
2	*α*-fenchene	136	79	3.35	1	<1
3	camphene	136	121	3.43	58	99
4	*β*-pinene	136	93	3.81	16	4
5	sabinene	136	91	3.95	1	2
6	*γ*-terpinene	136	93	4.06	4	2
7	Δ-carene	136	77	4.27	4	2
8	*β*-myrcene	136	41	4.93	118	10
9	limonene	136	68	4.93	116	47
10	1,8-cineole	154	154	5.09	879	<1
11	*γ*-terpinene	136	93	5.55	6	3
12	*p*-cymene	134	119	5.96	248	<1
13	*α*-terpinolene	136	121	6.12	<1	1
14	ylangene	204	105	6.63	92	<1
15	*α*-pinene	152	93	7.68	<1	2
16	*α*-thujone	152	110	8.08	<1	35
17	benzaldehyde	106	77	8.58	<1	1
18	linalool	170	55	8.89	<1	<1
19	fenchyl acetate	196	81	9.42	<1	415
20	camphor	152	95	10.27	220	56
21	benzadehyde	106	77	10.57	3	<1
22	linalool	154	93	10.89	94	8
23	linalyl acetate	196	43	11.07	<1	2
24	*β*-caryophyllene	204	133	11.73	30	43
25	4-terpineol	154	59	11.89	42	<1
26	*α*-selinene	204	189	12.07	<1	4
27	bornyl acetate	196	95	13.40	67	2
28	*α*-humulene	204	93	13.06	67	13
29	*α*-terpineol	154	59	13.66	79	<1
30	methyl 3-phenylpropionate	164	104	16.32	3	<1
31	2-phenylethanol	122	91	17.51	11	<1
32	*cis*-methyl cinnamate	162	131	18.21	10	<1
33	nerolidol	222	69	19.57	12	<1
34	*trans*-methyl cinnamate	162	131	20.19	130	<1
35	vanillin	152	151	3.06	3	3

[a]Volatiles numbered by their retention time. [b]Identified by comparing retention indices on a FFAP column, mass spectra, retention time, in comparison to data from authentic references standards. Atomic mass (g/mol). [d]Ion breakage. [e]Retention time in minutes. [f]Peak Area (PA) reflected in abundance and reduced to $10^{6.}$

Identification of compounds using SAFE isolates and diluted in six fractions by SPE

SPE is a technique where the compounds are dissolved and suspended in a mixture (Lehotay & Schenck 2000). This procedure was based on the separation of compounds by their chemical properties like polarity using solvents (pentene and diethyl ether). The mobile phase is the dissolved or suspended solute and the stationary phase is the solid that transported the sample (Moldoveanu and David 2015). The main objective is to separate the interested compounds from impurities or unwanted compounds which star in the stationary phase. Pentane and diethyl ether (Figure 4) are commonly used since their affinity with non-polar compounds. However, diethyl ether is more polar than pentane due to its oxygen in its structure that allows the separation of compounds with polar and non-polar association based on their structure.

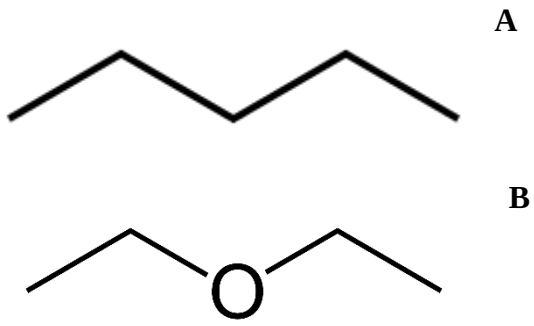


Figure 4. Solvents used for SPE fractions, pentane (A) and diethyl ether (B)
Source: NIST Chemistry WebBook – the NIST WebBook (Linstrom 1997)

Results based in this analysis presented six fractions generated from SAFE both SAFE isolates. The first fraction of ginger leaves and rhizomes isolates reflected 15 and 9 peaks respectively which were considered volatiles (Figure 5). Ginger leaves and rhizomes volatiles were compared based on their retention time and peak area to determine abundance. After further analysis, eight volatiles show similarities in both isolates (Table 3) some of them were α-pinene, camphene, β-myrcene with highest abundance. Leaves reflected more variation of compound since it had eight volatiles additional than rhizomes such as β-pinene and limonene. Fraction 1 was washed by a solution made-up with 100% pentane solution, which classified volatiles with the most non-polar association. All the compounds presented a similar structure ($C_{10}H_{16}$), and atomic mass (136 g/mol) which allows the affinity with pentane.

Table 3. Common volatiles identified in leaves and rhizomes SPE fraction 1.

Compound[a]	**RT**[b]	**Leaves PA**[c]**/10**[6]	**Rhizomes PA/10**[6]
α-pinene	3.06	178	516
α-fenchene	3.35	3	48
camphene	3.43	142	64
sabinene	3.95	7	22
Δ-carene	4.27	11	28
β-myrcene	4.93	40	132
β-phellandrene	5.00	10	17
γ-terpinene	5.55	17	33

[a]Identified by comparing retention indices on a FFAP column, mass spectra, retention time, in comparison to data from authentic references standards. [b]Retention time (RT) in minutes. [c]Peak Area (PA) reflected in abundance and reduced to $10^{6.}$

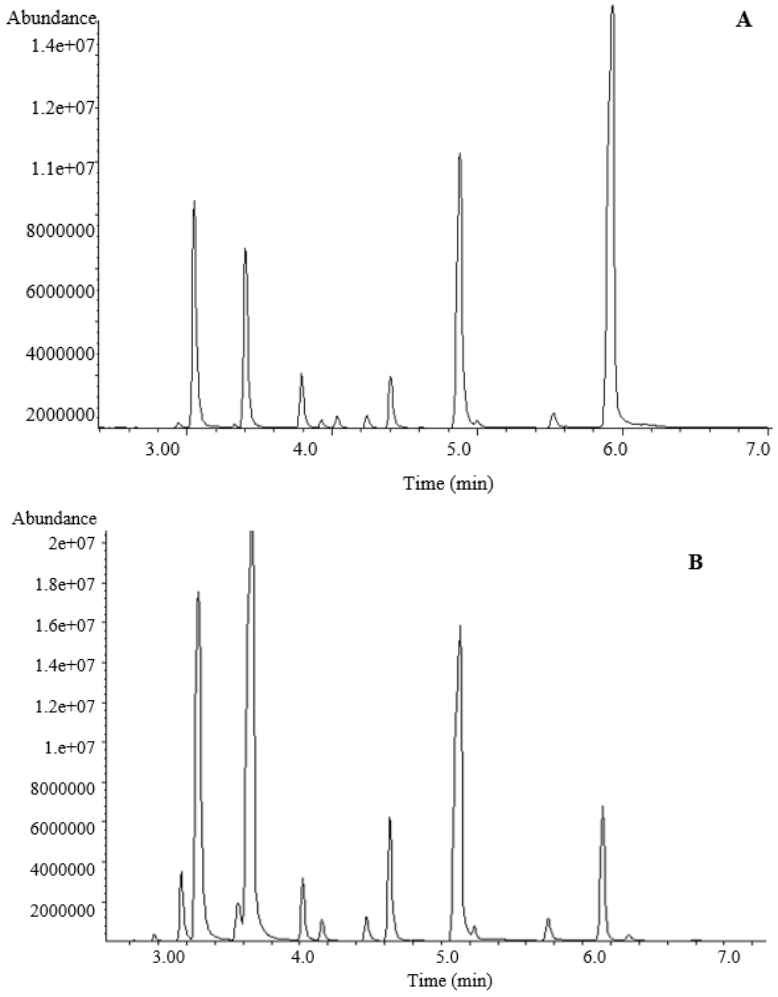


Figure 5. Chronogram for SPE fraction 1 of leaves (A) and rhizomes (B).

The second fraction of ginger leaves and rhizomes isolates reflected 5 and 7 peaks, respectively, which were considered volatiles (Figure 6). Ginger leaves and rhizomes volatiles were compared based on their retention time and peak area to determine abundance. After further analysis, five volatiles show similarities in both isolates (Table 4) which were camphene and limonene; highest abundance volatile reported was camphene. Rhizomes reflected more variation of compound since it had two volatiles (α-fenchene and β-myrcene) additional than leaves. Fraction 2 was washed by a solution made-up with 98% of pentane and 2% of diethyl ether, which classified the volatiles with a high non-polar association. Compound reflected a similar structure ($C_{10}H_{16}$), atomic mass (136 g/mol), expect for α-humulene ($C_{15}H_{24}$, 204 g/mol). Due to a "carry-over" process, when a compound is in a high concentration in the sample it can bleed into several fractions as α-pinene.

Table 4. Common volatiles identified in leaves and rhizomes SPE fraction 2.

Compound[a]	**RT**[b]	**Leaves PA**[c]**/10**[6]	**Rhizomes PA/10**[6]
α-pinene	3.06	1.44	10.88
camphene	3.73	1.08	25.37
β-pinene	3.81	0.49	1.09
limonene	4.93	1.92	13.21
α-humulene	13.06	1.35	2.74

[a]Identified by comparing retention indices on a FFAP column, mass spectra, retention time, in comparison to data from authentic references standards. [b]Retention time (RT) in minutes. [c]Peak Area (PA) reflected in abundance and reduced to $10^{6.}$

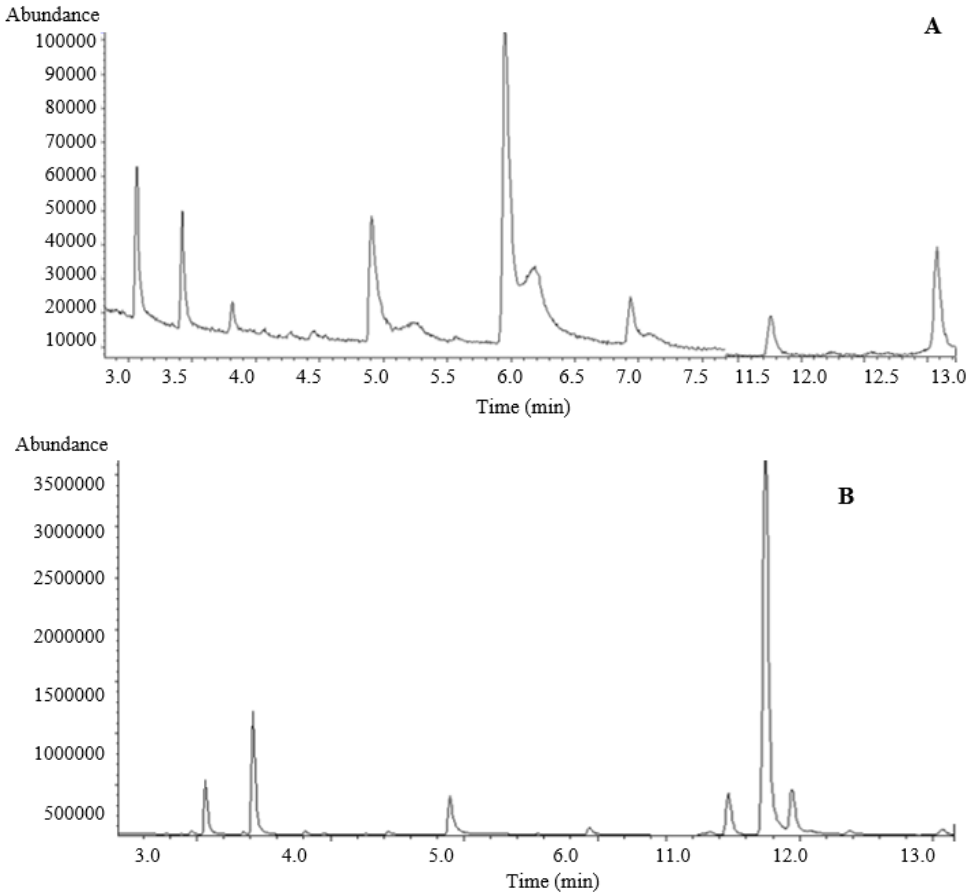


Figure 6. Chronogram for SPE fraction 2 of leaves (A) and rhizomes (B).

The third fraction of ginger leaves and rhizomes isolates reflected 5 and 6 peaks, respectively, which were considered volatiles (Figure 7). Ginger leaves and rhizomes volatiles were compared based on their retention time and peak area to determine abundance. After further analysis, two compounds show similarities in both isolates (Table 5), which were fenchyl acetate and 1,8-cineole; highest abundance volatile reported was fenchyl acetate. Rhizomes reflected more variation of compound since it had four volatiles (α-pinene, camphene, dl-limonene, and b-salinene) additional than leaves. Fraction 3 was washed by a solution made-up with 95% of pentane and 5% of diethyl ether, which classified the leftover of non-polar compounds. 1,8-cineole structure is $C_{10}H_{18}O$ and an atomic mass of 154 g/mol, while fenchyl acetate structure is $C_{12}H_{20}O_2$ and an atomic mass of 196 g/mol.

Table 5. Common volatiles identified in leaves and rhizomes SPE fraction 3.

Compound[a]	RT[b]	Leaves PA[c]/10[6]	Rhizomes PA/10[6]
1,8-cineole	5.09	10	5
fenchyl acetate	9.42	9	13

[a]Identified by comparing retention indices on a FFAP column, mass spectra, retention time, in comparison to data from authentic references standards. [b]Retention time (RT) in minutes. [c]Peak Area (PA) reflected in abundance and reduced to $10^{6.}$

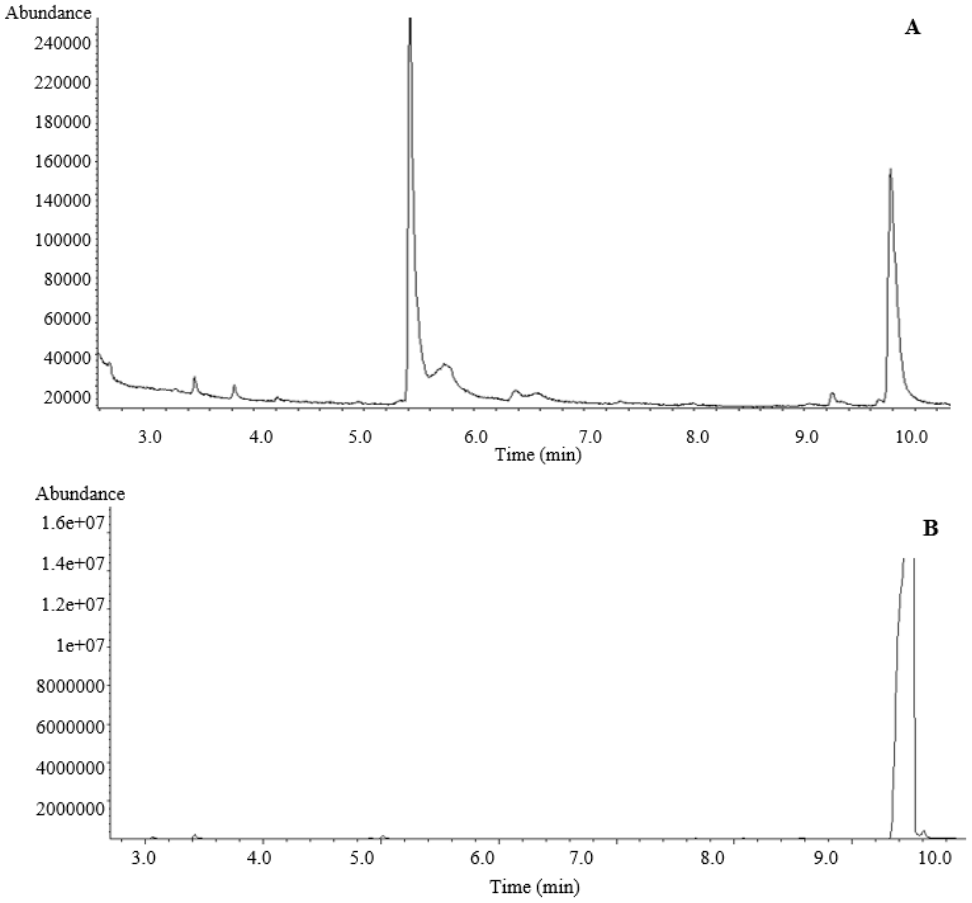


Figure 7. Chronogram for SPE fraction 3 of leaves (A) and rhizomes (B).

The fourth fraction of ginger leaves and rhizomes isolates reflected 8 and 6 peaks, respectively, which were considered volatiles (Figure 8). Ginger leaves and rhizomes volatiles were compared based on their retention time and peak area to determine abundance. After further analysis, three volatiles show similarities in both isolates (Table 6), 1,8-cineole, cyclohexanol, and camphor; highest abundance volatile reported was 1,8-cineole. Leaves reflected more variation of compound since it had two volatiles (bicyclo and α-terpinene) additional than rhizomes. Fraction 4 was washed by a solution made-up with 90% of pentane and 10% of diethyl ether. 1,8-cineole structure

is $C_{10}H_{18}O$ and an atomic mass of 154 g/mol, cyclohexanol structure is $C_6H_{12}O$ and an atomic mass of 100 g/mol and camphor structure is $C_{10}H_{16}O$ with an atomic mass of 152 g/mol.

Table 6. Common volatiles identified in leaves and rhizomes SPE fraction 4.

Compound[a]	**RT**[b]	**Leaves PA**[c]**/10**[6]	**Rhizomes PA/10**[6]
1,8-cineole	5.09	1566	838
cyclohexanol	7.20	6	44
camphor	10.27	19	52

[a]Identified by comparing retention indices on a FFAP column, mass spectra, retention time, in comparison to data from authentic references standards. [b]Retention time (RT) in minutes. [c]Peak Area (PA) reflected in abundance and reduced to $10^{6.}$

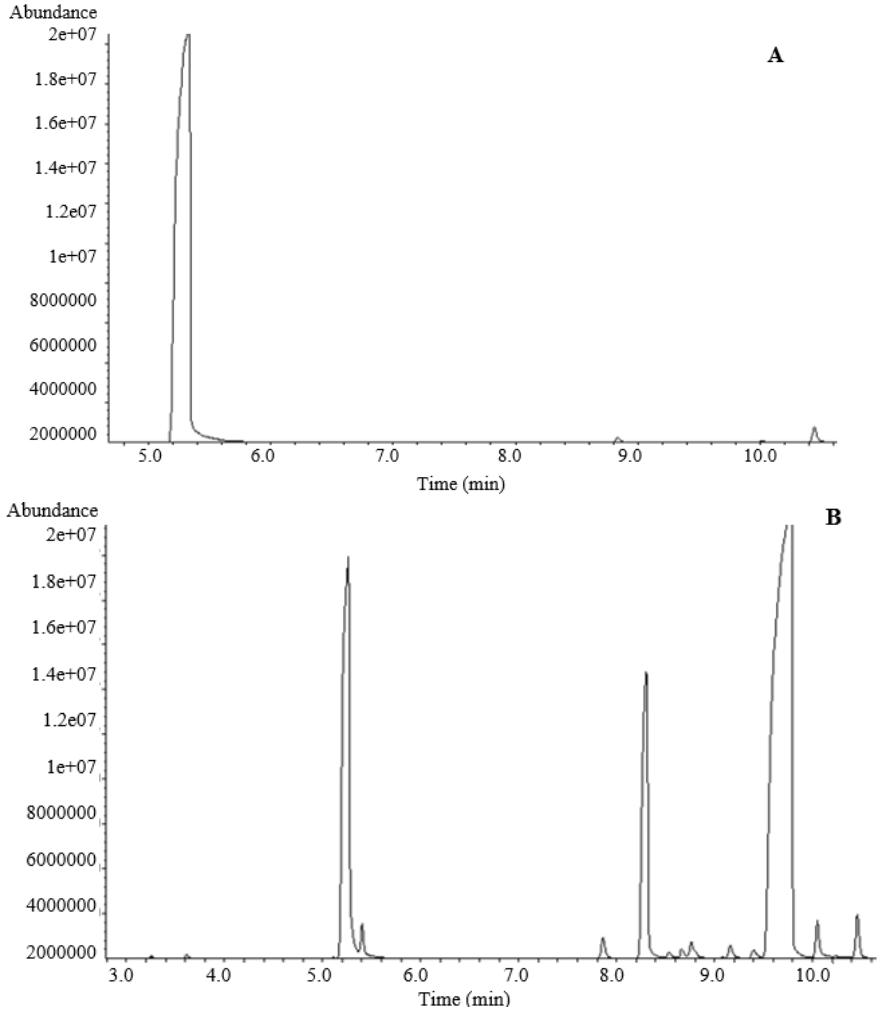


Figure 8. Chronogram for SPE fraction 4 of leaves (A) and rhizomes (B).

The fifth fraction of ginger leaves and rhizomes isolates reflected 23 and 9 peaks, respectively, which were considered volatiles (Figure 9). Ginger leaves and rhizomes volatiles were compared based on their retention time and peak area to determine abundance. After further analysis, six volatiles show similarities in both isolates (Table 7), some of them were 1,8-cineole, linalool, and camphor; the highest abundance volatiles measured were camphor and linalool. Leaves reflected more variation of compound since it had 14 volatiles additional than rhizomes such as *β*-pinene and limonene. Fraction 5 was washed by a solution made-up with 50% of pentane and 50% of diethyl ether. Due to a carry-over process, when a compound is in a high concentration in the sample it can bleed into several fractions as 1,8-cineole. 1,8-cineole, linalool and α-terpineol structure is $C_{10}H_{18}O$ and an atomic mass of 154 g/mol, linalool oxide structure is $C_{10}H_{18}O_2$ with an atomic mass of 170 g/mol, camphor structure is $C_{10}H_{16}O$ with an atomic mass of 152 g/mol, and caryophyllene oxide structure is $C_{15}H_{24}O$ with an atomic mass of 220 g/mol.

Table 7. Common volatiles identified in leaves and rhizomes SPE fraction 5.

Compound[a]	**RT**[b]	**Leaves PA**[c]**/10**[6]	**Rhizomes PA/10**[6]
1,8-cineole	5.09	119	16
linalool oxide	8.89	6	3
camphor	10.27	551	679
linalool	10.89	239	123
α-terpineol	13.66	27	17
caryophyllene oxide	18.51	123	132

[a]Identified by comparing retention indices on a FFAP column, mass spectra, retention time, in comparison to data from authentic references standards. [b]Retention time (RT) in minutes. [c]Peak Area (PA) reflected in abundance and reduced to $10^{6.}$

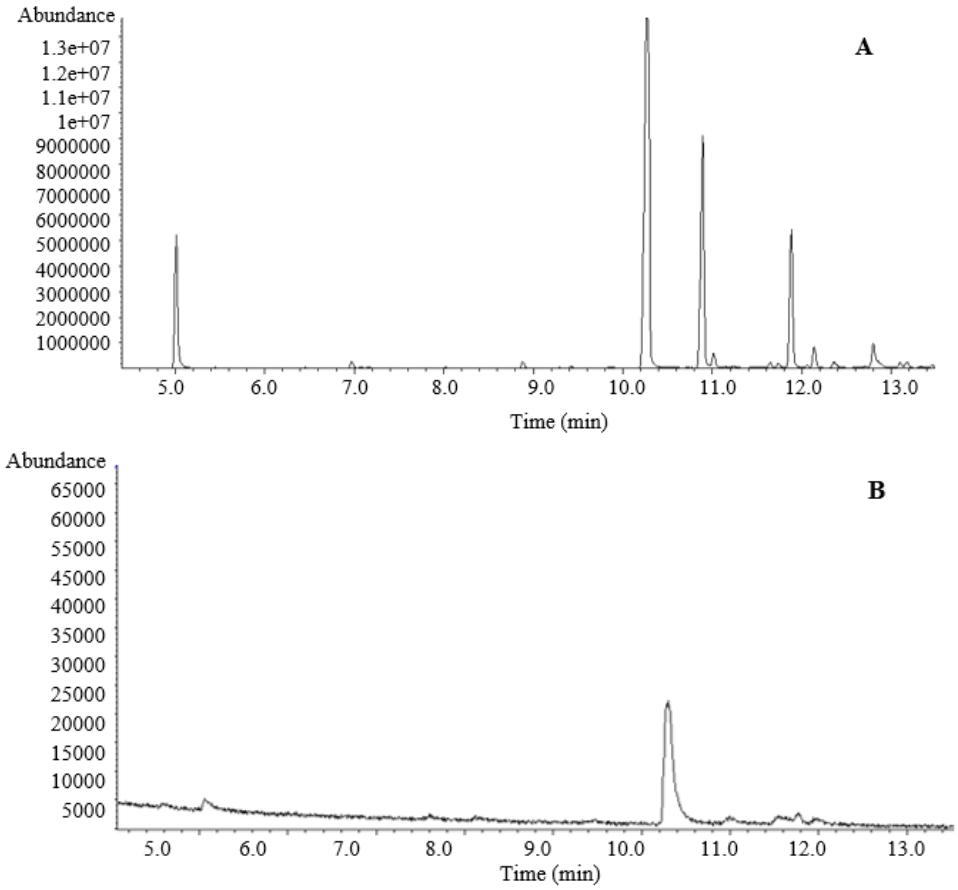


Figure 9. Chronogram for SPE fraction 5 of leaves (A) and rhizomes (B).

The sixth fraction of ginger leaves and rhizomes isolates reflected 18 and 12 peaks, respectively, which were considered volatiles (Figure 10). Ginger leaves and rhizomes volatiles were compared based on their retention time and peak area to determine abundance. After further analysis, eleven volatiles show similarities in both isolates (Table 8) where *α*-terpinol reported the highest concentration. Leaves reflected more variation of compound since it had six volatiles additional than rhizomes. Fraction 6 was washed by a solution made-up with 100% diethyl ether, which classified volatiles with a high polar association. 1,8-cineole, linalool, terpineole, α-terpineol structure is $C_{10}H_{18}O$ and an atomic mass of 154 g/mol, linalool oxide and epoxylinalool structure is $C_{10}H_{18}O_2$ with an atomic mass of 170 g/mol, camphor, *trans*-verbenol and *trans*-carveol structure is $C_{10}H_{16}O$ with an atomic mass of 152 g/mol, and pentanoic acid structure is $C_5H_{10}O_2$ with an atomic mass of 102 g/mol.

Table 8. Common volatiles identified in leaves and rhizomes SPE fraction 6.

Compound[a]	RT[b]	Leaves PA[c]/10[6]	Rhizomes PA/10[6]
1,8-cineole	5.01	4	4
linalool oxide	8.89	10	10
terpineol	9.29	3	3
pentanoic acid	10.20	1	2
camphor	10.24	3	3
linalool	10.87	17	17
α-terpinol	13.16	49	49
trans-verbenol	13.31	8	8
α-terpineol	13.66	221	217
epoxylinalool	14.86	6	6
trans-carveol	16.15	6	6

[a]Identified by comparing retention indices on a FFAP column, mass spectra, retention time, in comparison to data from authentic references standards. [b]Retention time (RT) in minutes. [c]Peak Area (PA) reflected in abundance and reduced to 10^6

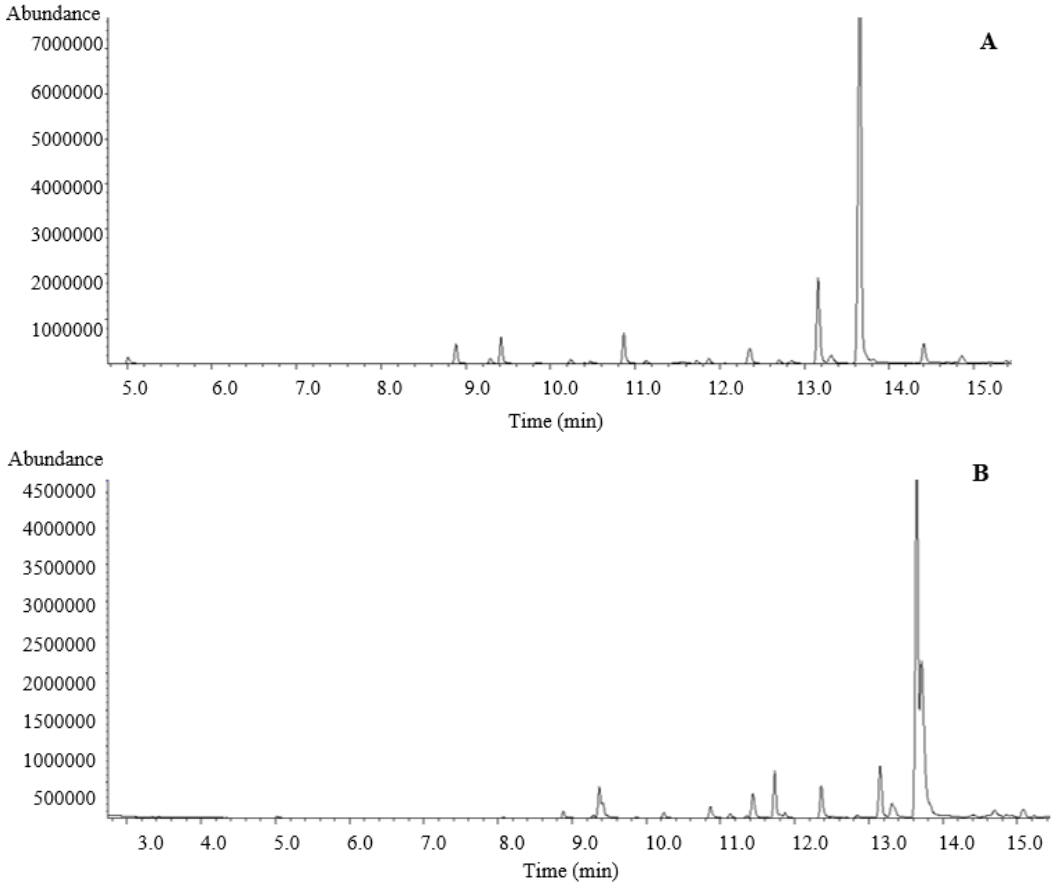


Figure 10. Chronogram for SPE fraction 6 of leaves (A) and rhizomes (B).

Quantitation and comparison of selected volatiles in ginger leaves and rhizomes

The selected volatiles were 1,8-cineole, linalool, fenchyl acetate, bornyl acetate, and methyl cinnamate based on the abundance and importance in ginger leaves and rhizomes (Figure 11). However, its importance to discuss that this study did not quantified other compounds presence in leaves that influence in a higher manner the ginger aroma. To analyze the data two statistical methods conducted with a significant level of ≤ 0.05. Student´s T-test was used to generate a comparison between the differences between each of the five selected volatiles in comparison of leaves and rhizomes. Then, Duncan´s was used to compare the compound levels (all 5) within volatiles of both plant materials.

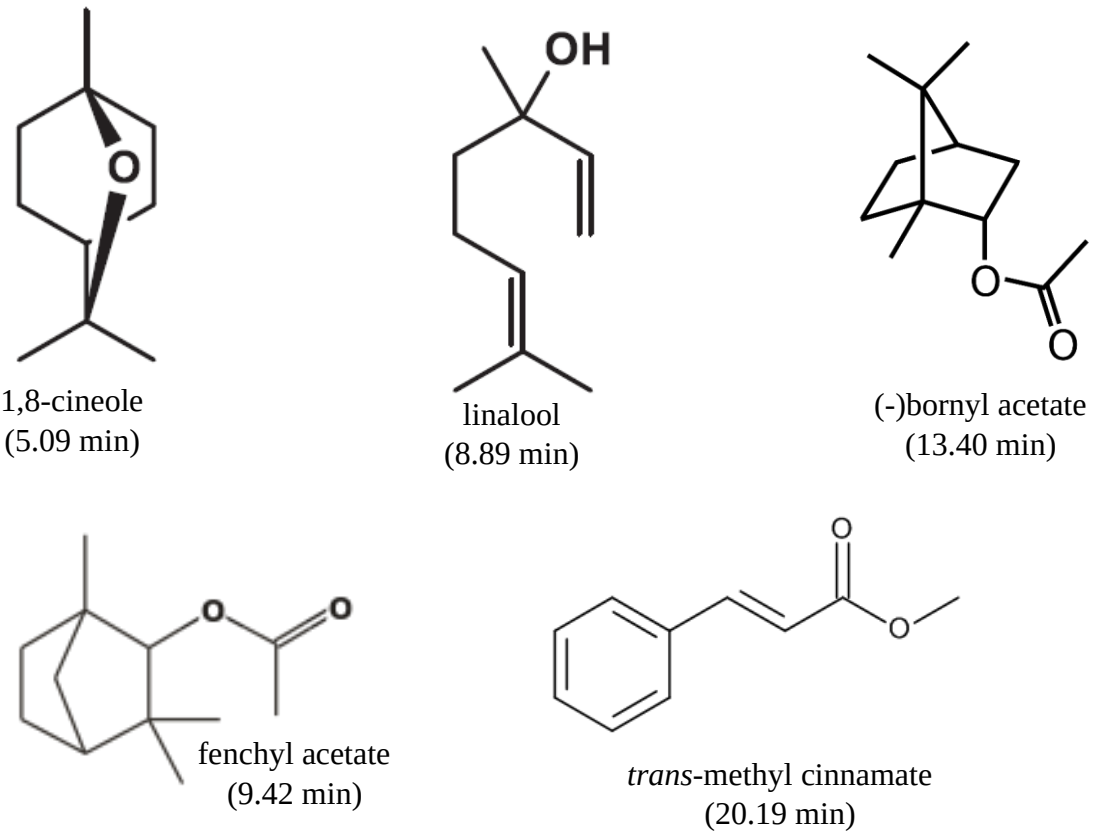
For the result of Student´s T-test a significantly difference was observed in the abundance of the selected volatiles within plant material isolates ($P \leq 0.05$). Leaves highest volatile compound was *trans*-methyl cinnamate with 87.2 ppm that was significantly higher than the second most abundance volatile (1,8-cineole). In contrast, rhizomes highest volatile was fenchyl acetate with an abundance of 431.5 ppm reflecting five times more ppm than the highest volatile compound in leaves (*trans*-methyl cinnamate).

To determine significant differences beyond volatiles a comparison of means by Duncan was conducted using SAS. As a result, of the quantitation for ginger leaves isolates 1,8-cineole, linalool, and fenchyl acetate had significant differences (Table 9); while bornyl acetate and *trans*-methyl cinnamate did not have significant differences among them. In contrast, for ginger rhizomes quantitation fenchyl acetate, bornyl acetate and methyl cinnamate were considered as a same group since a significant difference was not determined, while 1,8-cineole and linalool had significant differences.

Table 9. Average quantitation in ppm of selected volatiles identified in leaves and rhizomes of ginger.

Volatile	Leaves	Rhizomes	T-test
trans-methyl cinnamate	87.20 ± 7.00^{A}	0.08 ± 0.0^{C}	0.0021
1,8-cineole	70.50 ± 16.00^{B}	5.70 ± 2.00^{C}	0.0004
linalool	21.60 ± 3.00^{C}	2.90 ± 1.00^{C}	0.0002
bornyl acetate	0.01 ± 0.00^{D}	253.40 ± 17.00^{B}	<0.0001
fenchyl acetate	0.01 ± 0.00^{D}	431.50 ± 56.00^{A}	<0.0001

$*^{A-D}$ Mean separation in each column was performed by a Duncan Test ($P \leq 0.05$). Comparison of leaves and rhizomes in each row was done by using a T-test ($P \leq 0.05$).



1,8-cineole
(5.09 min)

linalool
(8.89 min)

(-)bornyl acetate
(13.40 min)

fenchyl acetate
(9.42 min)

trans-methyl cinnamate
(20.19 min)

Figure 11. Structures of quantified volatiles in ginger leaves and rhizomes.
Source: NIST Chemistry WebBook – the NIST WebBook (Linstrom 1997).

4. CONCLUSIONS

- Thirty-five volatiles were identified through GC-MS.
- Eighteen volatiles were classified with non-polar association between SPE fractions 1-4.
- Six volatiles were classified with non-polar and polar association in SPE fraction 5.
- Eleven volatiles were classified with polar association in SPE fraction 6.
- The major volatiles quantified were *trans*-methyl cinnamate and fenchyl acetate for leaves and rhizomes, respectively.
- Total volatile compounds quantified proved to be different from those in leaves and rhizomes, but when the volatile quantitation was analyzed individually, there was no differences.

5. RECOMMENDATIONS

- Generate solvent concentrations with a greater difference for SPE fractions to better evaluation of the affinity between volatiles and solvents or use different solvents.

- Quantify volatiles with higher intensity identify in GC-MS such as β-myrcene, limonene and camphor.

- Determine the aroma profile of volatiles compounds by Gas Chromatography-Olfactometry (GC-O).

- Evaluate different parameters in field production (temperature, light per day, harvest day, substrates, and fertilization and irrigation frequency) to determine if it influences the number of volatile compounds in leaves and rhizomes of shell ginger.

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