

SUPPLEMENTARY MATERIAL

Development and validation of Headspace Solid-Phase Microextraction coupled with Gas Chromatography (HS-SPME-GC) method for the analysis of *Zingiber zerumbet* L.

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ABSTRACT

Headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC) was explored to determine the fingerprinting characteristic of *Zingiber zerumbet* L. volatiles to differentiate between different ginger species. The effect of different fibers [polydimethylsiloxane (PDMS, 7 μ m), polyacrylate (PA, 85 μ m)], different temperature and time on the HS-SPME-GC was investigated by using response surface methodology coupled with full factorial experimental design. The area percentage of the major sesquiterpenes (Zerumbone and α -humulene) were $56.3 \pm 4.7\%$ and $47.5 \pm 27.2\%$ with PA fiber, respectively at optimum condition of 70 °C and 30 min. Validation of the developed HS-SPME-GC method with limits of detection and quantification for zerumbone was 0.09 and 0.28 μ g/g, respectively, demonstrating the suitable sensitivity of HS-SPME-GC method for the quantification of sesquiterpenes. Therefore, the simplicity of HS-SPME-GC makes it a convenient tool for qualitative and quantitative comparison of different ginger species targeting at the marker sesquiterpene molecule, zerumbone.

Keywords: Headspace solid-phase microextraction; *Zingiber zerumbet*; Zerumbone; Gas chromatography; Optimization

Experimental

Materials and chemicals

Authentic, fresh *Z. zerumbet* rhizomes [IC 348571 (Acc. 302)] were collected from Indian Institute of Spices Research (ICAR Institute) Calicut, Kerala, India. Washed rhizomes were pre-treated with metabisulphite (1000 ppm) overnight, and then air-dried. The air dried ginger sample was sliced and dried in a conventional cross-flow dryer (Alpha, India) at 45 ± 2 °C for 6-8 hours. The dried ginger was ground using Comminuting Mill (CADMILL, USA) having a mesh diameter of 200 μ m, operating at 1440 rpm and then the powder was stored in the refrigeration condition until further use. Zerumbone standard (98% purity) was procured from Sigma Aldrich, the manual HS-SPME holder and silica fibers were procured from Supelco (Bellefonte, PA, USA).

HS-SPME fiber screening

Fiber screening was carried out based on the polarity of the compound to be extracted. Two fibers with coating (1 cm long), polydimethylsiloxane (PDMS, 7 μ m), and polyacrylate (PA, 85 μ m) were evaluated and compared. Prior to GC analysis, each SPME fiber was conditioned in the injector of the GC system, according to the instructions provided by the manufacturer.

Extraction of analytes using HS-SPME

One gram of ginger rhizome powder was hermetically sealed in 15 ml screw top vial with a polypropylene hole cap and PTFE/silicone septa (Supelco, Bellefonte, PA, USA) and was placed in a thermostatic bath at the desired temperature. Further, SPME device was inserted into the sealed vial by manually penetrating the septum. Then, the fiber was exposed to the sample HS during the extraction period. After sampling, the SPME apparatus was immediately injected into the GC injector and the fiber was thermally desorbed with desorption time of 2 min at 250 °C. Before sampling, each SPME fiber was reconditioned for 4 min in the GC injector port at 250 °C. As the fiber was "cleaned" by heating in the injector, it can be reused. The optimization experiments were conducted using HS-SPME for the extraction of analytes at 50, 60, and 70 °C and 15, 30 and 45 min using the two fibers.

Analysis of SPME extracts by using GC-FID and GC-MS

The analysis of SPME analytes was carried out using GC- FISIONS- 8000 with flame ionization detector (FID) and an HP- 5 column (cross-linked 5% Ph Me silicone- 30m x 0.53mm x 0.88 μ m) was used. Operating conditions for GC were as follows: Nitrogen (99.99%) was used as the carrier gas at a constant flow rate of 5 ml/min; temperature of injector 250 °C. Temperature programming was: 60 °C, 2 min; 60-220 °C, 5 °C/min; 220 °C,

10 min. GC–MS analysis was also carried out for HS-SPME and volatile oil extracted by hydro-distillation, using a GC (PERKLIN ELMER AUTOSYSTEM XL) with electron impact ionization mode and Capillary ELITE-1 column (100% dimethyl polysiloxane- 30m x 0.32mm x 0.25 μ m). Operating conditions for GC-MS were as follows: Helium (99.9999%) was used as the carrier gas at a constant flow rate of 1 ml/min; temperature of injector 250 °C, interface 160 °C and split 1:20 and temperature programming was as mentioned above. The mass spectral analyses (PERKLIN ELMER TURBOMASS GOLD MASS SPECTROMETER) were performed at 70 eV and ion source with temperature 150 °C. The Kovats indices were calculated by co-chromatography using a mixture of n-alkanes as internal standards. The compound identification was made by computer library search by matching mass fragments and Kovats indices (Kovats, 1958).

Method Validation

Calibration standards with concentrations of 100, 200, 400, 600, 800 and 1000 μ g/g were prepared by adding the zerumbone standard directly in the starch matrix and also used for the validation study at different concentrations (100–800 μ g/g). The proposed HS-SPME method was validated for selectivity, linearity, accuracy, limit of detection (LOD) and limit of quantification (LOQ) (FAO/WHO, 2011). The linearity study was performed by injecting the spiked solid carrier mixture in triplicates corresponding to each point in the calibration curve of total peak area vs. standard marker mass (0.1-1 mg of the standard sesquiterpene, zerumbone). The regression equation was calculated by linear least squares regression. The coefficient of variation of Horwitz was used for evaluating the precision (FAO/WHO 2011). The standard deviation of the y-intercept (S_a) of the regression line was calculated for the standard zerumbone. The S_a parameter was further used to estimate the LOD and LOQ with the following equations:

$LOD = 3.3(S_a/b)$ and $LOQ = 10(S_a/b)$, where b is the slope of the calibration curve.

Experimental design and statistical analysis

Optimization of HS-SPME was performed using the response surface methodology (RSM), two independent variables, viz. temperature and time were chosen for this study and optimized by employing central composite rotatable design (CCRD) experimental design (Table S1). Each factor in the design was investigated at five levels ($-\alpha$, -1 , 0 , $+1$, $+\alpha$). A set of 13 experiments were carried out, and all variables were taken at a central coded value as zero. The minimum and maximum limits of variables were investigated, and the full experimental design with respect to their values in actual and coded form is listed in Table S5. After completing all the 13 experiments, the average maximum zerumbone content was

taken as the dependent variable (Y). A second order polynomial equation was then fitted to the data by a multiple regression procedures, and an empirical model was developed which related the response measured by the independent variables. The regression analysis - ANOVA, and experimental and predicted values were performed using statistical software (Statistica v5.5, StatSoft, Tulsa, USA). The response surface graphs for the variables against the response values were plotted using the same software. All the experiments were conducted in triplicates, and relative standard deviation (RSD %) was calculated.

Table S1 Comparison of extraction efficacy between PDMS and PA at 70°C and 30min

Peak No.*	Retention (min)	Time	Area \pm RSD (%)	
			PDMS (7 μ m)	PA (85 μ m)
1	10.833		1.2 \pm 0%	3.0 \pm 6.9%
2	18.570		2.9 \pm 34.1%	2.1 \pm 3.2%
3	19.590		39.5 \pm 29.3%	28.7 \pm 4.2%
4	22.840		1.6 \pm 12.8%	1.8 \pm 7.8%
5	23.240		4.4 \pm 3.2%	4.9 \pm 11.5%
6	23.490		2.8 \pm 0%	3.1 \pm 13.6%
7	26.753		47.5 \pm 27.2%	56.3 \pm 4.7%

* Peak no. can be visualized from fig. 1B

Table S2 Optimisation analysis by RSM

a. Regression coefficient					
R-sqr=0.899	Regression coefficients				
Mean/Interaction	70.960				
Temp. (L)	-1.1 37				
Temp. (Q)	0.0 13				
Time (L)	0.6 73				
Time (Q)	-0.0				
1L by 2L	-0.0 07				
b. ANOVA results					
	SS	Df	M SS	F –ratio	p
Temp. (L)	19. 85	1	19. 85	45. 43	0. 00
Temp. (Q)	3. 06	1	3. 06	7. 01	0. 03
Time (L)	0. 97	1	0. 97	2. 22	0. 18
Time (Q)	1. 43	1	1. 43	3. 28	0. 11
1L by 2L	1. 34	1	1. 34	3. 08	0. 12
Error	3. 06	7	0. 43		
Total SS	30. 36	12			
c. Optimized values					
Predicted value at solution: 56.6749 (70°, 30min)					
	Observed	Critical	Observed		
	Minimum	Values	Maximum		
TEMP	-1.41400	0.29311	1.414000		
TIME	-1.41400	-1.06214	1.414000		

Table S3 Validation of HS-SPME-GC for Zerumbone quantitation

Parameter	Value
Accuracy	98.424±14.24
Slope	844.8
Intercept	2.319
Linearity range	100-800 µg/g
Correlation co-efficient (r^2)	0.953
SE of intercept	52.856
SD of intercept	23.638
LOD	0.0923 µg/g
LOQ	0.2798 µg/g

Table S4 Identification of Flavour Compounds in *Z. Zerumbet* rhizomes by GC-MS

Rt (min)	Compound		Kovats Index	Area (%)	
	<i>Z. zerumbet</i> oil	HS-SPME		<i>Z. zerumbet</i> oil	HS-SPME
4.76	Tricyclene	-	922 ^{a,b,c}	0.14	-
4.99	α -Pinene	α -Pinene	933 ^{a,b,c}	1.65	0.20
5.47	Camphene	Camphene	954 ^{a,b,c}	6.76	2.27
6.36	α -Myrcene	α -Myrcene	990 ^{a,b,c}	0.23	0.24
6.85	3-Carene	3-carene	1010 ^{a,b,c}	0.58	0.16
7.02	o-Cymene	o-Cymene	1018 ^{a,b,c}	0.21	0.26
7.11	Eucalyptol	Eucalyptol	1022 ^{a,b,c}	2.04	1.83
8.38	L-Fenchone	-	1071 ^{a,b,c}	0.05	-
8.90	Linalool	Linalool	1088 ^{a,b,c}	0.31	0.33
9.86	Camphor	Camphor	1125 ^{a,b,c}	1.60	1.84
10.92	Terpinen-4-ol	Terpinen-4-ol	1164 ^{a,b}	0.09	0.15
11.29	α -Terpineol	α -Terpineol	1177 ^{a,b}	0.11	0.17
13.89	Bornyl acetate	Bornyl acetate	1272 ^a	0.08	0.07
17.60	β -Caryophyllene	β -Caryophyllene	1414 ^{a,b,c}	0.23	1.46
18.60	Humulene	Humulene	1455 ^{a,b,c}	4.09	20.76
20.05	β -Elemene	β -Elemene	1514 ^a	0.30	0.61
20.76	2-Tridecenal, (E)-	-	1545 ^{a,b}	0.31	-
20.91	Ethyl linalool	-	1551 ^a	0.21	-
21.64	Caryophyllene oxide	Caryophyllene oxide	1582 ^{a,b,c}	3.12	2.73
21.90	Humulene oxide II	Humulene oxide II	1593 ^{a,b}	2.32	2.64
22.36	1,5-Cycloundecadiene, 8,8-dimethyl-9- methylene-	1,5-Cycloundecadiene, 8,8-dimethyl-9- methylene-	1613 ^a	0.29	0.38
22.87	cis-Z- α -Bisabolene epoxide	cis-Z- α -Bisabolene epoxide	1637 ^a	1.38	1.31

23.11	2-Dodecenal	2-Dodecenal	1648 ^a	0.23	0.06
24.43	Zerumbone	Zerumbone	1707 ^a	39.44	43.16
24.76	α -trans-Bergamotene	-	1723 ^a	0.37	-
25.36	Tetradecanoic acid	Tetradecanoic acid	1752 ^{a,b,c}	2.39	0.87
29.41	n-Hexadecanoic acid	n-Hexadecanoic acid	1952 ^{a,b}	7.38	1.05
31.44	10-Methyldodecan-4-olide	10-Methyldodecan-4-olide	2060 ^a	0.09	0.07
32.41	11,14-Eicosadienoic acid, methyl ester	11,14-Eicosadienoic acid, methyl ester	2113 ^a	0.84	0.02
32.60	cis-Vaccenic acid	cis-Vaccenic acid	2124 ^a	12.16	1.50
32.89	3,6,6-Trimethylundecane-2,5,10-trione	3,6,6-Trimethylundecane-2,5,10-trione	2141 ^a	0.11	0.03
33.10	Octadecanoic acid	Octadecanoic acid	2153 ^{a,b,c}	2.51	0.29
33.89	17-Octadecynoic acid	17-Octadecynoic acid	2153 ^a	0.21	0.05
Terpenes (%)				11.3	5.04
Sesquiterpenes (%)				51.25	72.67
Terpene alcohols (%)				2.76	2.48
Acids (%)				25.49	3.78
Aldehydes (%)				0.54	0.06
Others (%)				0.49	0.48
Total (%)				91.83	84.51

(a,b,c): Kovats indices on ELITE-1 column (relative to n-alkanes), NIST, literature, respectively; (-): Not detected

Table S5 CCRD experimental design with coded values

Sl. No	Temperature	Time	Zerumbone (%)±S.D
1	-1.000	-1.000	51.52 ±0.05
2	-1.000	1.000	55.83 ±0.07
3	1.000	-1.000	51.98 ±0.21
4	1.000	1.000	53.97 ±0.02
5	-1.414	0.000	52.70 ±0.02
6	1.414	0.000	51.72 ±0.06
7	0.000	-1.4 14	52.22 ±0.01
8	0.000	1.4 14	56.67 ±0.03
9	0.000	0.000	53.21±0.04
10	0.000	0.000	53.69±0.01
11	0.000	0.000	52.14±0.05
12	0.000	0.000	54.21±0.03
13	0.000	0.000	52.36±0.04

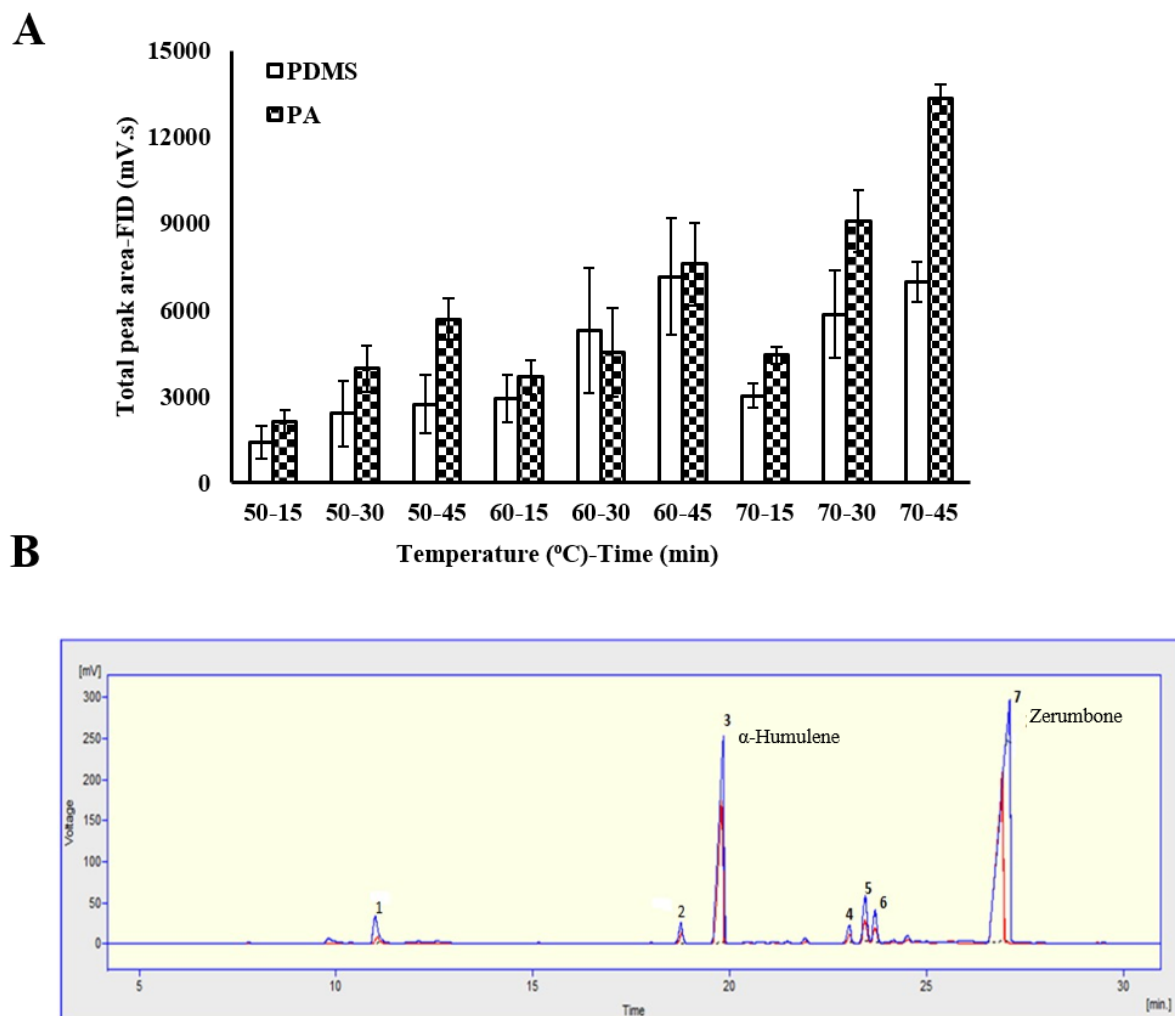


Fig. S1 (A) Effect of PA and PDMS fibers on the extraction efficiency of *Z. zerumbet* volatiles at different temperature and time. (B) GC profile of *Z. zerumbet* powder with HS-SPME (PDMS- red line and PA – blue line).

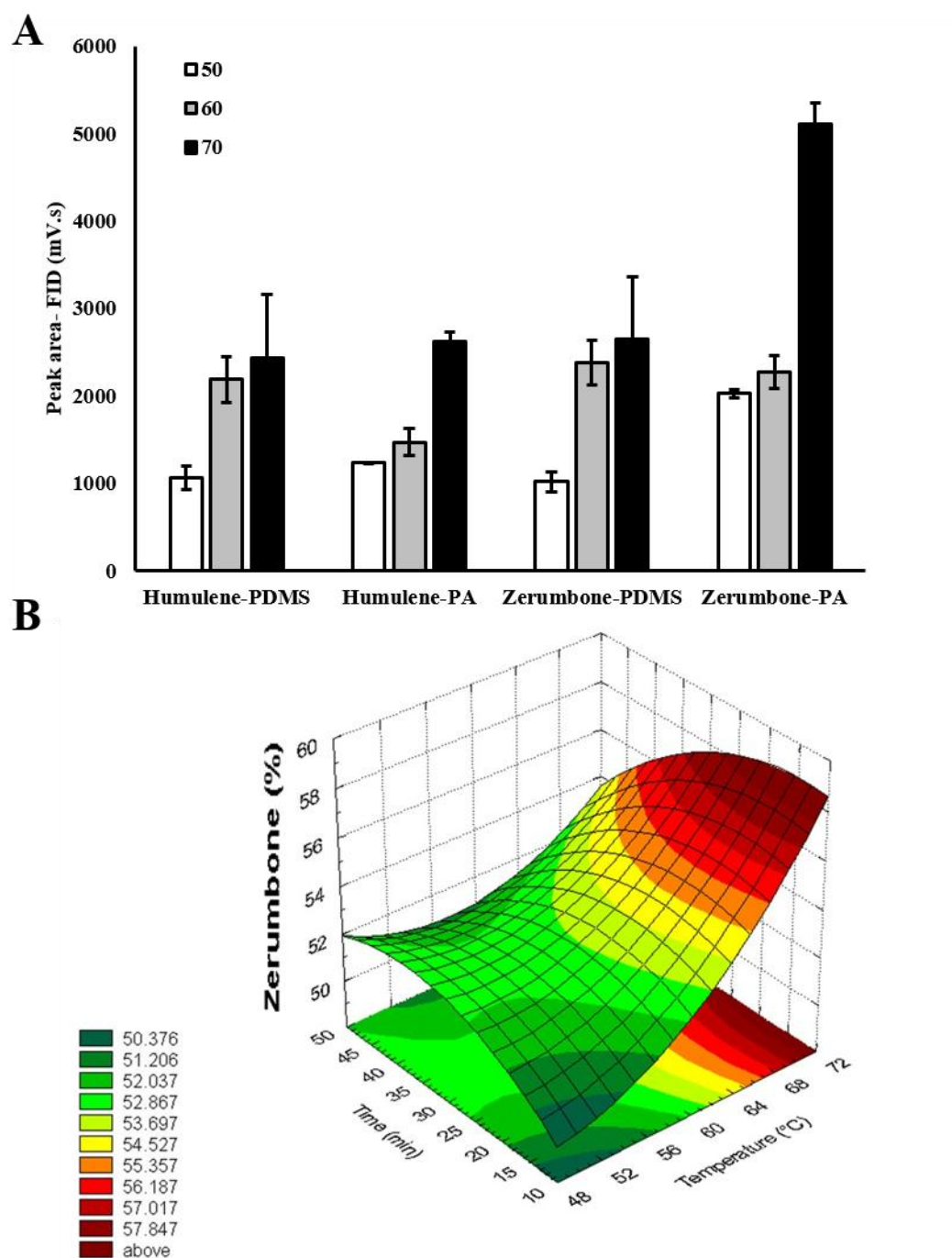


Fig. S2 (A) Effect of temperature on the extraction of monocyclic sesquiterpenes between the fibers (B) Response surfaces showing the effect of temperature and time against the zerumbone (%) extracted through HS-SPME-GC

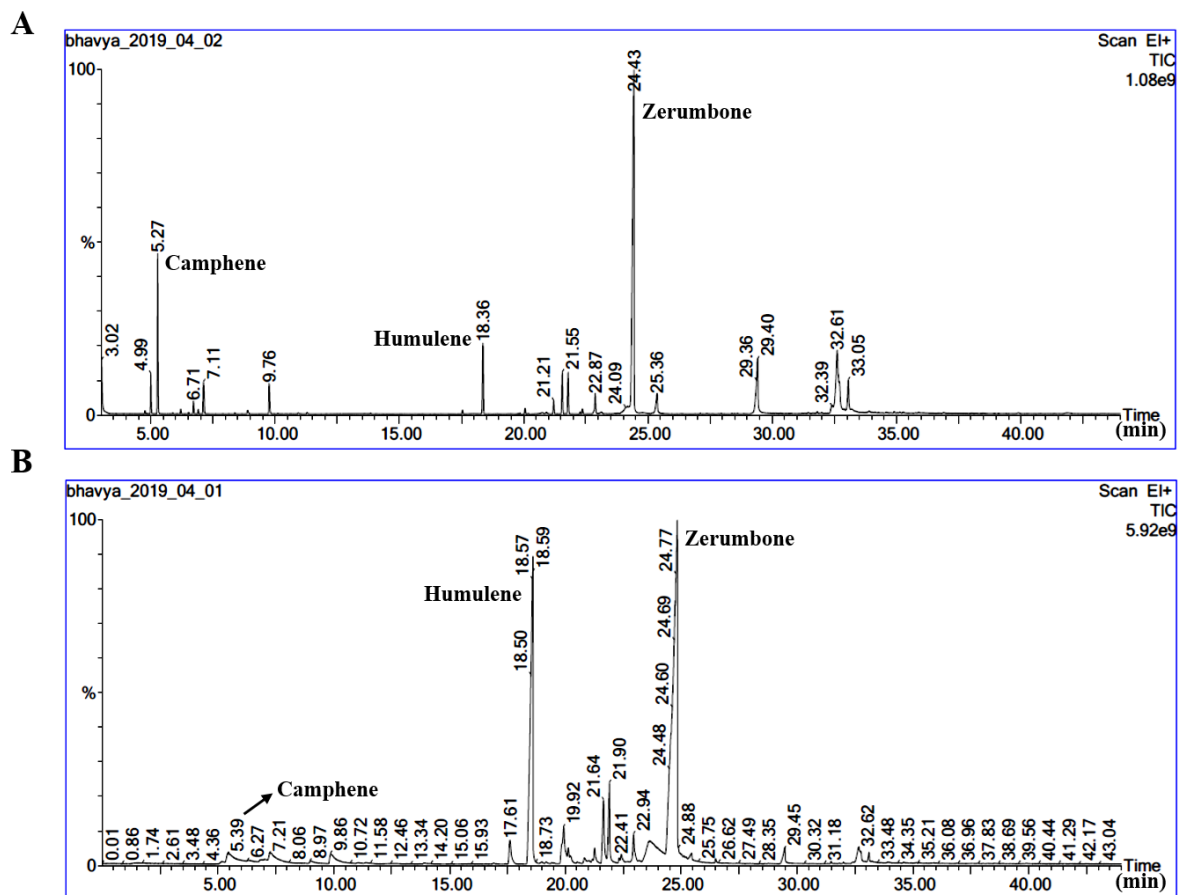


Fig. S3 GC-MS chromatogram of *Z. zerumbet* essential oil (A) and HS-SPME (B)

References

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