CHAPTER II

EXPERIMENTAL

2.1 Apparatus chemicals and samples

2.1.1 Apparatus

1. Gas chromatograph, Agilent 6890 Series GC system, Hewlett Packard, U.S.A., consisting of
   - Flame ionization detector (FID)
   - Data processing system, Dell OptiPlex GX 1
   - Capillary column: HP-5 (5% phenyl dimethylsiloxane), 2 x 30 m x 0.25 mm I.D., 0.25 μm film thickness, Hewlett Packard, U.S.A.
   - Column connector assembly for column 0.25 mm I.D., Agilent, U.S.A.
2. Gas chromatograph-mass spectrometer, Hewlett Packard 5973 Mass Selective Detector, Hewlett Packard, U.S.A., consisting of
   - Gas chromatograph, Agilent 6890 Series GC system manufactured by Hewlett Packard
   - Data processing system, Hewlett Packard, U.S.A.
   - Capillary column: HP-5 (5% phenyl dimethylsiloxane), 2 x 30 m x 0.25 mm I.D., 0.25 μm film thickness, Hewlett Packard, U.S.A.

- Column connector assembly for column 0.25 mm I.D., Agilent, U.S.A.

3. Gas chromatograph, Varian 3700 Series GC system, Varian export corporation, U.S.A., consisting of
   - Thermal conductivity detector (TCD)
   - Column: Stainless steel, 5 m x 0.50 mm I.D., packed with 3%OV-1 (100% methylsilicone), Alltech, U.S.A.
   - Chromjet integrator
4. Solid phase microextraction fiber holder, Supelco, U.S.A.
5. Solid phase microextraction fiber, 100 μm polydimethylsiloxane, Supelco, U.S.A.
6. Vacuum rotary evaporator, Buchi Rotavapor model R-124, Buchi Labortechnik AG, Switzerland
7. Blender, Moulinex, Ireland
8. Hand crimper, 20 mm cap, Supelco, U.S.A.
9. Headspace vial 22 mL, Hewlett Packard, U.S.A.
10. Septa (PTFE/silicone) 22 mm, Supelco, U.S.A.
11. TLC alumina sheet silica gel 60 F_{254}, Merck, Germany
12. UV lamp, Vilber Lourmat, France

2.1.2 Chemicals

1. Dichloromethane, AR grade, J. T. Baker Inc., U.S.A.
2. Toluene, AR grade, BDH, England
3. Ethyl acetate, AR grade, J. T. Baker Inc., U.S.A.
4. Ethanol, AR grade, Merck, Germany
5. Hexane, AR grade, Merck, Germany
6. Diethyl ether, AR grade, Merck, Germany
7. Silica gel 60 (230-400 mesh ASTM), 0.063 – 0.200 mm, Merck, Germany
8. Sodium sulfate anhydrous, AR grade, Fluka, Switzerland
9. Helium gas, HP grade, TIG, Thailand
10. Nitrogen, HP grade, TIG, Thailand
11. Air, Air-zero grade, TIG, Thailand
12. Hydrogen, HP grade, TIG, Thailand

2.1.3 Samples

Scented vetiver grass (*Vetiveria zizanioides* Nash) grown in Mae Hae district, Chiang Mai province located in the Northern part of Thailand with aged in a range of 16 to 18 months. The roots were collected and air dried at room temperature for 1 week. Then, the root samples were baked in an oven at 70 °C for 24 hrs before blending.

2.2 Investigation of the aroma components in scented vetiver root by solid phase microextraction-gas chromatograph-mass spectrometry (SPME-GC-MS)

2.2.1 SPME conditioning

In the study, a manual SPME holder was used to perform the experiments. A fused silica fiber of 10 mm in length, 100 μm in diameter, and with 100 μm thickness of polydimethylsiloxane (PDMS) was chosen to extract the volatile components in the dried scented vetiver roots samples. A new PDMS microextraction fiber must be thermally conditioned prior to adsorption at 250 °C in a injection port of GC under a flow of helium for 1 h to reduce bleeding before use. This can be done by inserting the SPME syringe needle into a splitless injector of GC while the purge is open. After conditioning, a blank fiber was run to ensure that no contaminant was in the fiber coating prior to exposure of the fiber to the sample of interest. Normally, the conditioned fibers were used immediately to prevent contamination. Between each use, fibers were kept sealed from ambient air by piercing the tip of SPME needle into a small pierce of septum to prevent accidental contamination. Prior to each use, all used fibers were preconditioned by thermally desorption at 250 °C for 30 min in injection port of GC. If any carry over was observed by GC, the 30 min thermal preconditioning was repeated.

2.2.2 Root sample preparation

First, 5 g of blended scented vetiver root sample was transferred to a 22 mL glass vial via filter funnel. Then, the vial was sealed rapidly with PTFE silicone septum and aluminium cap. The vial was allowed to stand at room temperature for 20 min before being subjected to extraction.

2.2.3 SPME sampling and analysis procedure

After the root samples were prepared, extractions by SPME were performed in direct SPME sampling mode. All samplings were conducted at different conditions. The sampling time for direct SPME was designated as extraction time, and was achieved by optimization of the SPME extraction. The experiments were carried out according to the scheme in **Fig. 2.1** and **Fig. 2.2** that showed SPME extraction at room temperature and higher temperature, respectively. **Table 2.1** showed the GC-MS conditions.

The sampling vial was held 20 min for
equilibrium at room temperature
⇓
Pierce the septum of the vial with needle of
SPME device and fasten the device with a clamp
⇓
Expose the fiber to the headspace above the sample
by depressing the plunger and lock it in the bottom position
⇓
Allow the SPME fiber to extract the volatiles
from sample headspace for 10 min
⇓
Withdraw the fiber back into the needle sheath
and pull the device out of the sample vial
⇓
Introduce the needle sheath of the SPME device into the injector of GC-MS
⇓
Start the analysis by depressing the plunger and lock it
in the lowed position so that the fiber was exposed to
the GC-MS injection port and allowed desorption of the volatiles
⇓
After exactly 5 min, withdraw the fiber back into
the needle and pull the needle out of the injector
⇓
Start collecting GC-MS data
⇓
Repeat the analysis again but change
the extraction time to 20, 30 and 60 min.

Fig. 2.1 SPME sampling and analysis procedure at room temperature (30 °C).

The sampling vial was held 20 min
for equilibrium at 50 °C
⇓
Pierce the septum of the vial with needle of
SPME device and fasten the device with a clamp
⇓
Expose the fiber to the headspace above the sample
by depressing the plunger and lock it in the bottom position
⇓
Allow the SPME fiber to extract the volatiles
from sample headspace for 30 min
⇓
Withdraw the fiber back into the needle sheath
and pull the device out of the sample vial
⇓
Introduce the needle sheath of the SPME device into the injector of GC-MS
⇓
Start the analysis by depressing the plunger and lock it
in the lowed position so that the fiber was exposed to
the GC-MS injection port and allowed desorption of the volatiles
⇓
After exactly 5 min, withdraw the fiber back into
the needle and pull the needle out of the injector
⇓
Start collecting GC-MS data
⇓
Repeat the analysis again but change the temperature
to 70 and 90 °C.

Fig. 2.2 SPME sampling and analysis procedure at higher temperature

Table 2.1 Conditions of GC-MS

Operation	Conditions
1. Instrument	GC-MS, Hewlett Packard 5973
2. Capillary column	Phase HP-5, 2 x 30 m x 0.25 mm I.D., 0.25 μm film thickness, connected with column connector assembly
3. Temperature program	90 °C (1 min) – 150 °C at rate 5 °C/min 150 °C (1 min) – 195 °C at rate 0.5 °C/min 195 °C (1 min)
4. Injector temperature	250 °C
5. Injection	Splitless mode with SPME insert
6. Carrier gas	Helium at 1.0 mL/min
7. Transfer-line temperature	280 °C
8. Ion source temperature	230 °C
9. Acquired mass range	29 – 550 amu
10. Ionization mode	Electron Impact (EI)
11. Electron energy	70 eV
12. MS quadrupole temperature	150 °C

The comparison of results obtained from this experiment were summarized in **Table 3.1** and **Table 3.2**. The representative chromatograms obtained by SPME-GC-MS at room temperature were shown in **Fig. 3.1A, B, C** and **D**. The chromatograms obtained by SPME-GC-MS at 50, 70 and 90 °C were shown in **Fig. 3.2A, B** and **C**, respectively. The chemical structure of identified components were shown in **Fig. 3.3**.

2.3 Separation and Isolation of the aroma components in scented vetiver roots

2.3.1 Extraction

Air dried root of scented vetiver (*Vetiveria zizanioides* Nash) weighed 350.51 g was ground and macerated in dichloromethane for 3 days at room temperature (~ 30 °C), followed by filtration. The water in the filtrate was eliminated by addition of sodium sulfate anhydrous. Then, the solvent was removed under reduced pressure using the rotary evaporator followed by evacuation using the vacuum pump to remove the last traces of solvents. The obtained filtration was evaporated to dryness to give dark brown yellow viscous liquid as crude extract 19.16 g (5.47% yield by weight from dried material). The crude extract was analyzed by gas chromatograph-mass spectrometry (GC-MS) technique. GC-MS conditions used were shown in **Table 2.1**. The obtained GC-MS chromatogram of the dichloromethane crude extract was shown in **Fig. 3.4** and **Fig. 3.5**, respectively. The chemical structure of identified components were shown in **Fig. 3.3**. The mass spectral data of all volatile components were summarized in **Table 3.3**.

2.3.2 Isolation and Purification of the aroma components

2.3.2.1 The dichloromethane crude extract was subjected to classical liquid column chromatography (CC) having conditions as follows:

Stationary phase	:	silica gel 90g
Mobile phase	:	C_7H_8 : EtOAc (4:1) then with EtOAc and EtOH subsequently
Diameter of column	:	1.5 inches

The column was packed by slurry method. Silica gel 60 for column chromatography, mesh 0.063 – 0.200 mm, was used as absorbent. The crude extract was dissolved in a small volume of dichloromethane and then the solution was added to the top of column and eluted with mobile phase subsequently. The fraction collecting was approximately 10 mL/fraction.

The whole separation resulted in eight separated fractions (code: A1 – A8). All fractions were analyzed by GC-MS having conditions shown in **Table 2.1**. Comparison of each chromatogram obtained was shown in **Fig. 3.6**. Chromatogram of separated fraction was shown in **Fig. 3.7-3.14**. The mass spectral data of all separated fraction was shown in **Table 3.4**. Comparison of detail of each fraction was shown in **Table. 3.5**. Each separated fraction was evaporated for elimination of the solvent and was then subjected to sensory evaluation. The sensory evaluation for odor quality of each fraction was performed by asking the description from 12 judges. It was found that all the judges evaluated fraction A6 as having the best aroma quality among all fractions.

2.3.2.2 Fraction A6 which had been evaluated for aroma character was subjected to further separation using thin layer chromatography (TLC) having conditions as follows:

TLC plate	:	10 cm x 10 cm
Stationary phase	:	TLC alumina sheet silica gel 60 F_{254}
Mobile phase	:	hexane : diethylether (1:1)

In this work, fraction A6 was spotted onto the TLC plate. After that the components in fraction A6 were separated on TLC plate after developing in a tank of mobile phase. The TLC plate was left to dryness and was examined for the separated fractions using ultraviolet light (UV) at 365 nm.

The results yielded three separated bands (code: B1-B3). Each separated band was dissolved in ethanol and was analyzed by GC-FID having conditions shown in **Table 2.2.** All GC chromatograms were shown in **Fig. 3.15A, B** and **C,** respectively. The solvent was eliminated by rotary evaporator before all the fractions were subjected to sensory evaluation by 12 judges. The results revealed more than 80% of the judges agreed that fraction B3 possessed the best aroma quality of all fractions.

Table 2.2 Conditions of GC-FID

Operation	Conditions
1. Instrument	GC Agilent 6890 series
2. Capillary column	Phase HP-5, 2 x 30 m x 0.25 mm I.D., 0.25 µm film thickness, connected with column connector assembly
3. Temperature program	90 °C (1 min) – 150 °C at rate 5 °C/min 150 °C (1 min) – 195 °C at rate 0.5 °C/min 195 °C (1 min)
4. Injector temperature	250 °C
5. Detector temperature	250 °C
6. Injection	Split mode
7. Carrier gas	Helium at 1.0 mL/min
8. Detector make up	Nitrogen at 35 mL/min
9. Hydrogen	40 mL/min
10. Air	400 mL/min

2.3.2.3 The fraction B3 that having aroma character was further separated by the use of preparative gas chromatograph. The column was prepared by packing the materials into the stainless steel column. The conditions of preparative gas chromatograph are listed as follows:

Instrument	:	GC Varain 3700
Packing material	:	1.1 g of 3% OV-1 (100% methylsilicone)
Column	:	Stainless steel, 5 m x 0.50 mm I.D.
Temperature program	:	95 °C (5 min) – 190 °C (2 °C/min)
		190 °C (40 min)
Injector temperature	:	250 °C
Injection	:	Splitless
Carrier gas	:	Helium at 1.0 mL/min
Detector	:	TCD
Filament temperature	:	270 °C

There were 12 separated components (code: C1-C12) obtained from the method above. Each component was collected in ethanol when the component flowed pass through an outlet of a preparative gas chromatograph. After that, all components were evaporated for elimination of the solvent. The odor quality of these components were evaluated in this study. 12 judges were asked to describe the odor of these components using as many descriptions as they could think of. It was found that more than 80% of the judges preferred the aroma quality of component C6 while the other components were evaluated as not having aroma quality. The component C6 was

further analyzed by GC-MS in order to confirm its purity. GC-MS conditions were shown in **Table 2.1**. The obtained chromatogram and mass spectral data were shown in **Fig. 3.16** and **Fig. 3.17**, respectively.

The scheme of total procedures in item 2.3 was shown in **Fig 2.3**.

Scented vetiver roots macerated in CH_2Cl_2

⇓

CC of dichloromethane crude extract using silica gel 90 g

[eluent, first with C_7H_8 : EtOAc (4:1) then EtOAc and EtOH, subsequently]

⇓

8 fractions

(A1 – A8)

⇓

Elimination of the solvent

⇓

Aroma quality evaluation

⇓

Fraction A6

⇓

TLC of fraction A6 using TLC alumina sheet silica gel 60 F_{254}

[eluent, C_6H_{14} : C_4H_{10} (1:1) and examine with UV light λ 365 nm]

⇓

3 separated bands

(B1 – B3)

⇓

Dissolve in ethanol and then eliminate the solvent

Fig. 2.3 Extraction separation and isolation of the aroma components in scented vetiver root.

⇓

Aroma quality evaluation

⇓

Component B3

⇓

Separation by preparative GC

⇓

12 Components

(C1-C12)

⇓

Collect each component in ethanol

⇓

Eliminate the solvent and aroma quality evaluation

⇓

Aroma component (C6)

⇓

Confirm the purity by GC-MS

Fig. 2.3 (continued)