CHAPTER II

EXPERIMENTAL

2.1 Study on the highly volatile constituents of *Vetiveria zizanioides* roots grown under different cultivation conditions

2.1.1 Apparatus and Chemicals

2.1.1.1 Apparatus

1. Solid phase microextraction (SPME) with a polydimethylsiloxane (PDMS) fiber, Supelco, Bellefonte, PA, USA
2. Likens-Nickerson Simultaneous distillation and extraction (SDE) apparatus, manufactured by Chrompack, Netherlands
3. Magnetic stirrer and magnetic bar
4. Vacuum rotary evaporator, model B-169, manufactured by BUCHI, Switzerland, consisting of
    a. Water bath, model B-480, USA
    b. Air pump, KNF laboport, USA
    c. Cooling device, NESLAB, USA
5. Blender, manufactured by Maulinex, Ireland
6. Autopipette, manufactured by BRAND, Germany
7. Hand crimper, 20 mm cap, manufactured by Supelco, USA
8. Headspace vial 22 mL, Hewlett Packard, USA
9. Septa (PTFE faced silicone) 22 mm, Supelco, USA

10. Gas chromatographic systems, details are depicted in Table 2.1.

Table 2.1 Three systems of gas chromatographic instrument

	Apparatus		
	GC	**GC-MS**	**GC×GC**
Manufacture	Hewlett Packard USA		
Model	Agilent 6890 Series GC system	Agilent 6890 Series GC system coupled to HP 5973 Mass Selective detector	Agilent 6890 Series GC system
Injector	Autoinjector model Agilent 7683 Series		Autosampler model G2913A Series
Capillary column	- 60 m × 0.25 mm × 1.00 µm film thickness, HP-5MS Agilent Technologies, USA - 30 m × 0.25 mm × 0.25 µm film thickness, HP-5MS, Agilent Technologies, USA		30 m × 0.25 mm × 0.25 µm film thickness DB-5, Agilent Technologies, USA coupled with a 1.0 m × 0.1 mm × 0.1 µm film thickness BP-20, SGE International, Ringwood, Australia
detector	Flame ionization detector	Mass spectrometer	Flame ionization detector
Data processing system	Hewlett Packard USA		Chemstation software, Agilent Technologies, USA
Accessory	-	-	Longitudinally modulated cryogenic system (LMCS), Everest model, Chromatography Concepts, Doncaster, Australia

2.1.1.2 Chemicals

1. Dichloromethane, AR grade, Merck, Germany
2. Sodium sulphate anhydrous, Lab grade, Merck, Germany
3. Nitrogen gas, 99.99% (HP grade), TIG, Thailand
4. Air, Air Zero grade, TIG, Thailand
5. Hydrogen gas, 99.99% (HP grade), TIG, Thailand
6. Helium gas, 99.99% (HP grade), TIG, Thailand
7. Carbondioxide gas, dip tube, Chiangmai N&N, Thailand
8. Standard alkanes (C_8-C_{22}), Fluka, Switzerland

2.1.2 Materials

Mae Hae, an ecotype of *Vetiveria zizanioides* Nash. from Northern Thailand was grown by three different cultivation systems. Bare root tillers of vetiver grass were employed as planting material for all systems. In the first and second systems, the plants were grown in a potting mixture of sandy soil, rice husks and cow dung at the ratio 2:2:1, respectively. Rock phosphate and feldspar were also added to the potting mixture for nutrient supplement at the concentration of 400 g and 2 kg per ton, respectively. No microbial inoculation was used in the first system. In the second system, the vetiver grass was inoculated with arbuscular mycorrhizal fungi, azospirillum, and azetobacter N_2-fixing bacteria and potassium containing silicate mineral dissolving bacteria. Microbial inoculation was done at the time the planting materials were transplanted by placing each type of microbial inoculums below the base of planting materials at the following

proportions: 100 infective propagule for arbuscular mycorrhizal fungi and 5 x 10^8 cells for each bacterium per plant. In the first month, the vetiver plants were grown in the nursery and small plastic bags containing 500 g of potting mixture per bag were used as container. After that, the plants were transferred to 2500 mL plastic tubes containing the same potting mixture for outdoor cultivation. In the third system, the plants from the first system were separated and taken out of the plastic bags while still in the nursery. Most of the potting mixtures were removed from the root systems and then the plants were transplanted into the mixture of rice husks and compost in plastic baskets. Some roots of the vetiver grass passed through the basket and extended into the lower part of the container in which they were fed with nutrient solution. This cultivation is shown in Figure 2.1. After five months, the roots of vetiver grass from each system were taken out from the whole plants and they were washed and dried at room temperature for 2 weeks. Then, the root samples were placed in an oven at 70 ºC for 24 h before extraction.

Figure 2.1 Hydroponic cultivation

2.1.3 Extraction of vetiver root essential oils

The extraction was carried out in a modified Likens-Nickerson simultaneous distillation and extraction (SDE) apparatus illustrated in Figure 2.2. Fifty grams of each blended vetiver root sample and 200 mL of distilled water was added to a 500-mL round-bottom flask. Both flasks were connected to the apparatus, and more dichloromethane and distilled water were added into the central arm. The flask containing dichloromethane was heated by using a water bath at 50 ºC and the flask containing vetiver root and distilled water was heated by using a paraffin oil bath at 200 ºC. Extraction of each vetiver root sample was performed for 5 h in triplicate. The solution of the extracted oil obtained was dried over a layer of anhydrous sodium sulphate and evaporated before being kept in a vial.

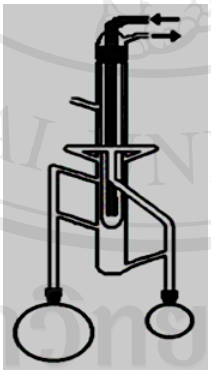

Figure 2.2 Likens-Nickerson apparatus

2.1.4 Analysis of volatile constituents in vetiver root essential oils

2.1.4.1 GC×GC analysis

The GC×GC instrumental system used in this study is shown in Figure 1.5. It was equipped with FID and fitted with LMCS operated at 100 Hz data acquisition using a Chemstation software. The column set comprised a 30 m × 0.25 mm × 0.25 µm film thickness HP-5 coupled with a 1.0 m × 0.1 mm × 0.1 µm film thickness BP-20. The column was anchored with retaining nuts to the support frame so that the trap can move back and forth along the column (see Figure 1.5 page 33) without the column moving. A small internal flow of nitrogen was introduced to prevent ice from grasping the column. The trap movement was achieved by use of a suitable A. C. motor drive. The HP Chemstation programmable event table is used to initiate the electronic controller which controled the motor operation and hence modulation of the trap. A modulation frequency was operated at 6 s per cycle. The thermostatically controlled cryogenic trap was maintained at about -20 ∀C for the duration of each analysis. The optimum condition of column temperature was programmed from 120 °C to 180 ∀C at a rate of 2 °C/min. The split (10:1) injection and detector temperature were 250 and 260 °C, respectively. Hydrogen gas was used as carrier gas with a flow rate of 1.5 mL/min. The GC was operated in constant flow mode. Only vetiver oil sample obtained by normal soil cultivation was employed in this experiment. The obtained GC×GC chromatogram of vetiver essential oil cultivated by normal soil is illustrated in Figure 3.1.

2.1.4.2 SPME-GC∆GC analysis

∉ SPME conditioning

A manual SPME holder was used. A fused silica fiber of 10 mm in length, 100 µm in diameter, and with 100 µm thickness of PDMS was chosen to extract the volatile constituents in the vetiver oils obtained from SDE method. A PDMS microextraction fiber was thermally conditioned prior to adsorption at 250 °C in an injection port of GC 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 opened. 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. 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 an injection port of GC. If any carry over was observed by GC, the 30 min thermal preconditioning was repeated.

∉ **Sample preparation and SPME sampling**

Ten microliters of essential oil obtained from normal soil cultivation were placed in a 22 mL headspace bottle sealed with a PTFE/silicone septum and an aluminium cap. The sample bottle was incubated in a water bath at 70 ºC for 15 min before being subjected to extraction by SPME. A SPME fiber of 1 cm long and coated with PDMS 100 µm thick was mounted in the manual SPME holder and preconditioned for 2 h in a GC injection port set at 250 ºC. By insertion through the septum of the sample bottle, the fiber was then exposed to the sample headspace for 20 min prior to desorption of the volatiles into the splitless injection port of the GCΔGC instrument in which all conditions were the same as that used for GCΔGC analysis. The obtained SPME-GC×GC chromatogram of highly volatile constituents of the essential oil obtained from normal soil cultivation is depicted in Figure 3.1.

2.1.4.3 SPME-GC-MS analysis

Before extraction of the volatile constituents, SPME fiber must be thermally conditioned. The sample preparation was the same as in the item 2.1.4.2. The volatile constituents from SPME of vetiver essential oil were analyzed using an HP model 6890 gas chromatograph equipped with an HP-5MS (5% phenylmethylpolysiloxane) capillary column (60 m × 0.25 mm i.d., film thickness of 1.00 µm) interfaced to an HP model 5973 mass-selective detector. The oven temperature was initially held at 110 ºC for 1 min and then increased by 1 ºC/min to 150 ºC with a hold time of 1 min. Then the temperature was increased at a rate of 0.5 ºC/min to 200ºC with a final hold time of 1 min. The

injector and detector temperatures were 250 and 280 ºC, respectively. Purified helium was used as the carrier gas at a flow rate 1 mL/min. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 29-300. The electron multiplier voltage was 1150 V. The ion source and quadrupole temperatures were set at 230 ºC and 150 ºC, respectively. Identification of volatile components was performed by comparison of their Kovát retention indices, relative to C_8-C_{22} n-alkanes, and by comparison of the mass spectra of individual components with the reference mass spectra in the Wiley 275 and NIST 98 database as well as in those of the previous reports.[22,25,30,31] All SPME-GC-MS chromatograms of volatile constituents of all vetiever oil samples and the identified volatile components, as well as their relative peak area percents, are shown in Figure 3.2 and Table 3.1, respectively.

2.1.5 Method for calculation of the Kovát retention indices[171-174]

A solution of each n-alkane from C_8 to C_{22} was injected with the sample in the GC-MS instrument. A HP-5MS (5% phenyl-polymethylsiloxane) capillary column (30 m × 0.25 mm i.d., film thickness of 0.25 μm) was interfaced to an HP model 5973 mass-selective detector. The oven temperature was initially held at 60 ºC and then increased by 3 ºC/min to 246 ºC. The injector and detector temperatures were 250 and 280 ºC, respectively. Purified helium was used as the carrier gas at a flow rate 1 mL/min. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 29-300. The electron multiplier voltage was 1150 V. The ion source and quadrupole temperatures were set at 230 ºC and 150 ºC, respectively. The retention times of the n-alkane peaks

were recorded. The retention times of the peak of interests were also recorded. The value was derived by allotting the alkanes a value of 100 times their carbon number and giving the sample a value equivalent to a hypothetical alkane eluting at the same time. From temperature-programming, non-isothermal Kovát retention indices (I_x) of all individual components were calculated by using a definition of Van den Dool and Kratz. The value of I_x was achieved from the isotherm and temperature program method is called Kovát and Van den Dool retention indices which both methods use same equation to calculate the retention indices showed below.

$$I_x = 100n + 100(t_x - t_n) / (t_{n+1} - t_n)$$

Where t_n and t_{n+1} are retention times of the reference n-alkane hydrocarbons eluting immediately before and after chemical compound X; t_x is the retention time of compound X. The obtained I_x was comparative to those obtained in the Adams's reference[175] which retains the I_x of most components in various essential oils.

2.2 A comparative study on volatile constituents of Thai vetiver root essential oils subjected to different extraction methods and analyzed by comprehensive two-dimensional gas chromatography-mass spectrometry

2.2.1 Apparatus and Chemicals

2.2.1.1 Apparatus

1. Likens-Nickerson Simultaneous distillation and extraction (SDE) apparatus, manufactured by Chrompack, Netherland
2. SFX^{TM} 220 supercritical fluid extractor, ISCO, USA consisting of
   a. Supercritical fluid syringe pump, model 100DX, ISCO, USA
   b. Controller, model 100DX, ISCO, USA
   c. Modifier syringe pump, model 100DX, ISCO, USA
   d. Adjustable restrictor, model 5738D, ISCO, USA
   e. Restrictor temperature controller, model 260D, ISCO, USA
3. Microwave-assisted solvent extractor, ETHOS SEL, MILESTONE Ltd., Germany, consisting of
   a. Twelve position rotor, model SK-12, MILESTONE Ltd., Germany
   b. Twelve 100 mL microsampling Teflon vessels
   c. Medium rotor, model SK-12, MILESTONE Ltd., Germany
   d. Control software, model NOVA-10, MILESTONE Ltd., Germany

e. Magnetic stirring for homogenous mixing in every vessel, model ASM-400, MILESTONE Ltd., Germany

f. Temperature Control, model ATC-400 FO Fiber-Optic, MILESTONE Ltd., Germany

g. Focused Infrared contactless temperature monitor, model IRTC-500, MILESTONE Ltd., Germany

h. Solvent Sensor, model QPS-3000, MILESTONE Ltd., Germany

i. System controller, model 640 Terminal, MILESTONE Ltd., Germany

4. Magnetic stirrer and magnetic bar

5. Soxhlet extractor, Organomation Associates Inc., USA

6. Vacuum rotary evaporator, model B-169, manufactured by BUCHI, Switzerland, consisting of

a. Water bath, model B-480, BUCHI, Switzerland

b. Air pump, KNF laboport, BUCHI, Switzerland

c. Cooling device, BUCHI, Switzerland

7. Blender, manufactured by Maulinex, Ireland

8. Autopipette, manufactured by BRAND, Germany

9. Gas chromatographic systems, details are depicted in Table 2.2

Table 2.2 Three systems of gas chromatographic instrument

	Instrument		
	GC-MS	**GC×GC-FID**	**GC×GC-qMS**
Manufacture	Hewlett Packard USA		
Model	Agilent 6890 Series GC system coupled to HP 5973 Mass Selective detector	Agilent 6890 Series GC system	
Injector	Autosampler model HP6873 series		
Column set	BPX-5 × BP-20*	BPX-5 × BP-20* Solgel wax × BP-1* EtTBS-βCD × BP-20*	BPX-5 × BP-20*
detector	Flame ionization detector		Quadrupole mass spectrometer
Data processing system	Hewlett Packard USA	Chemstation software, Agilent Technologies, USA	
Accessory	-	Longitudinally modulated cryogenic system (LMCS), Everest model, Chromatography Concepts, Doncaster, Australia	

* BPX-5 × BP-20: BPX-5 (30 m × 0.25 mm × 0.25 µm film thickness), Agilent Technologies, U.S.A coupled with BP-20 (0.8 m × 0.1 mm × 0.1 µm film thickness), SGE International, Ringwood, Australia, Solgel wax × BP-1: Solgel wax (30 m × 0.25 mm × 0.25 µm film thickness), Agilent Technologies, U.S.A coupled with BP-1 (1 m × 0.15 × 0.25 µm film thickness), Agilent Technologies, USA, EtTBS-βCD × BP-20: EtTBS-ηCD (20 m × 0.25 mm × 0.25 µm film thickness), MeGA, Italy coupled with BP-20 (1 m × 0.1 mm × 0.1 µm film thickness), SGE International, Ringwood, Australia.

2.1.1.2 Chemicals

1. Dichloromethane, AR grade, Merck, Germany
2. Toluene, AR grade, Merck, Germany
3. Methanol, AR grade, Merck, Germany
4. Sodium sulphate anhydrous, Lab grade, Merck, Germany
5. Nitrogen gas, 99.99% (HP grade), TIG, Thailand
6. Air, Air Zero grade, TIG, Thailand
7. Hydrogen gas, 99.99% (HP grade), TIG, Thailand
8. Helium gas, 99.99% (HP grade), TIG, Thailand
9. Carbondioxide gas, dip tube, Chiangmai N&N, Thailand
10. Standard alkanes (C_8-C_{22}), Fluka, Switzerland

2.2.2 Materials

Roots of *Vetiveria zizanioides* Nash, Mae Hae ecotype, grown in Chiang Mai province located in the northern part of Thailand (altitude 310 m), were harvested at the age of 12 months in October 2004. The roots were washed and air dried at room temperature for 2 weeks before being placed in an oven at 70 ºC for 24 h. The dry roots were ground and blended well before being subjected to four different extraction methods.

2.2.3 Extraction procedures

2.2.3.1 Simultaneous distillation and extraction (SDE)

The extraction was carried out in a modified Likens-Nickerson SDE apparatus for 24 h to obtain the highest yield of essential oil. Fifty grams of dried vetiver root and 200 mL of distilled water were added to a 500 mL round-bottom flask. Dichloromethane (150 mL) was added to another 250 mL round-bottom flask. Both flasks were connected to the apparatus, and more dichloromethane and distilled water were added into the central arm of the apparatus. The flask containing dichloromethane was heated by using a water bath at 50 ºC and the flask containing vetiver root and distilled water was heated by using a paraffin oil bath at 200 ºC. After extraction, the solvent in the distillate was eliminated to dryness by using vacuum rotary evaporation.

2.2.3.2 Supercritical fluid extraction (SFE)

A SFX^{TM} 220 supercritical fluid extractor (ISCO, USA) was used for extraction of the same vetiver root samples. Three grams of blended vetiver roots were placed in 8 mL stainless steel vessels. The extraction was performed by using supercritical carbon dioxide as the extraction medium modified with a selection of three solvents: dichloromethane, toluene and methanol. The extraction conditions which gave the highest yield of vetiver oil for each type of modifier are shown in Table 2.3. After extraction, the obtained extracts were dried by blowing with N_2 gas for 20 min.

Table 2.3 The extraction conditions of SFE

Extraction modifier	% v/v	Temperature (∀C)	Pressure (psi)	Extraction time (min)
Dichloromethane	10	80	4000	15
Methanol	10	100	2000	10
Toluene	20	80	5000	10

2.2.3.3 Microwave-assisted extraction (MAE)

A microwave-assisted solvent extraction system (ETHOS SEL, Germany) was equipped with 12 closed-vessel microwave extraction cells. Microwave energy was produced by a 1000 W magnetron. The system allows maximum pressure of 30 bars and temperature of 260 ∀C. The blended vetiver root (1.5 g) was dispersed in 30 mL organic solvent: dichloromethane, methanol or toluene. Eleven mixtures were made for each solvent and placed in 11 extraction cells. The extraction conditions providing the highest yield for each solvent is demonstrated in Table 2.4. After extraction, the cells were allowed to cool to room temperature and all extracts obtained from each solvent were combined and filtered through 10 g of sodium sulfate anhydrous prior to being dried using vacuum rotary evaporation.

Table 2.4 Extraction conditions used in MAE extraction for three solvents

Extraction solvent	Extracted temperature (∀C)	Extracted time (min)
Dichloromethane	60	20
Methanol	80	15
Toluene	100	25

2.2.3.4 Soxhlet extraction (SE)

Fifty grams of ground vetiver roots were placed in a cellulose thimble and transferred to a Soxhlet apparatus. The vetiver oil was extracted with 500 mL of solvent for 24 h. Three solvents, viz. dichloromethane, methanol, and toluene, were investigated. The obtained extracts were dried by using a vacuum rotary evaporator.

All extraction methods were performed for triplication. All vetiver oil samples obtained from each extraction were diluted 1:10 v/v with n-hexane prior to injection into GC-MS, GC-FID, GCΔGC-qMS and GC×GC-FID, respectively. Physical properties of the vetiver root oils and their percentage yields (w/w) obtained by different extraction methods are summarized in Figure 3.3 and Table 3.2, respectively.

2.2.4 Investigation of volatile constituents in vetiver essential oils obtained from different extraction methods

2.2.4.1 GC-MS analysis

GC-FID and GC-MS analyses were performed using the same instruments as described for GCΔGC-FID and GCΔGC-qMS analysis. A two-column combination was used since it was set up for GC×GC, and effective GC-FID operation is achieved by simply not applying cryogen and keeping the modulator off. The primary capillary column comprised BPX5 phase with a dimension of 30 m Δ 0.25 mm i.d. Δ 0.25 µm film thickness serially coupled with a second capillary column (BP20 phase; dimension of 0.8 m Δ 0.1 mm i.d. Δ 0.1 µm film thickness). Both columns were obtained from SGE International. The GC-FID and GC-MS operated under the temperature program which was started at 60 ∀C and ramped to 240 ∀C at 3 ∀C $min.^{-1}$ The injection temperature was 250 ∀C with an injection volume of 0.1 µL in the split mode with a split ratio of 100:1. Helium was used as carrier gas and was maintained in a constant pressure mode. The conditions used in the mass spectrometer were the same as in GCΔGC-qMS instrument.

2.2.4.2 GCΔGC-FID and GCΔGC-qMS analysis

A gas chromatograph model HP6890 equipped with an FID detector and an HP 6890 series auto sampler was used for the GCΔGC-FID experiment. GCΔGC-qMS was operated on a gas chromatograph model HP6890 equipped with a model HP5973 mass selective detector and a model HP6873 auto sampler. The GCΔGC-FID system and

GC∆GC-qMS system were operated at 100 Hz and 20 Hz data acquisition, respectively. The GC was retrofitted with a longitudinally modulated cryogenic system (LMCS). CO_2 was employed as cryogen, which was thermostatically controlled at about -20 ∀C for the duration of each run. The column set for GC∆GC analysis consisted of two capillary columns which were serially coupled by a zero-dead-volume fitting. The column sets and operation conditions used in this experiment are listed in Table 2.5. The injection temperature was 250 ∀C with an injection volume of 1.0 µL in the split mode with a split ratio of 100:1. Helium carrier gas was maintained in constant pressure mode. The GC∆GC-MS transfer line temperature was 280 ∀C. A mass range of *m/z* 40-240 with a scan rate of ~ 20 Hz (cycle of 0.02 s), a scan speed of 10000 $amus^{-1}$ and a detector voltage of 1.8 kV were used. The volatile constituents were identified using the Adams 2001, NIST 98 and Wiley 275 database libraries.

Table 2.5 GCΔGC column sets and temperature programs

	Set 1		**Set 2**		**Set 3**	
	^{1}D	^{2}D	^{1}D	^{2}D	^{1}D	^{2}D
Stationary phase	BPX5	BP20	Solgel Wax	BP1	EtTBS-ηCD	BP20
Length (m)	30	0.8	30	1.0	20	1.0
Diameter (mm)	0.25	0.1	0.25	0.15	0.25	0.1
Film thickness (µm)	0.25	0.1	0.25	0.25	0.25	0.1
Modulation period	6 s		4 s		6 s	
Temperature program	120°C to 180°C at 2 Kmin^{-1}; hold 10 min		120°C to 260°C at 2 Kmin^{-1}; hold 10 min		120°C to 180°C at 2 Kmin^{-1}; hold 10 min	

^{1}D: first dimensional column

^{2}D: second dimensional column

BPX-5: 5% Phenyl polysilphenylene-siloxane

BP-20: Polyethylene glycol

Solgel Wax: Polyethylene glycol in a Sol-Gel matrix

BP-1: 100% Dimethyl polysiloxane

EtTBS-ηCD: 14% Cyanopropylphenyl-86% methylpolysiloxane

GCΔGC-FID and GCΔGC-qMS data were exported in ASCII file format (*csv files) and transformed as contour plots based on the modulation frequency and sampling rate by using the Agilent Chemstation software. For the data analysis of total peak height and area, the relevant files were exported as *csv integation files prior to analysis using a Matlab program, which summed peak pulses for each component to give the total area for that component.