UNIVERSITY OF NEW SOUTH WALES

PhD Thesis Proposal

Development of process for purification of α and β-vetivone from Vetiver essential oil
&
Investigation of effects of heavy metals on quality and quantity of extracted Vetiver oil

Thai Danh Luu
{B. Agric. Sc. (Qld University, Australia), M. Sc. (VUB, Belgium)}

School of Chemical Sciences and Engineering
Unversity of New South Wales, Sydney

Supervisor: Prof Neil Foster [1]
Co-supervisors: Prof Tam Tran [2] and Dr Paul Truong [3]
[1] School of Chemical Sciences and Engineering, UNSW
[2] Division of Engineering, Science and Technology, UNSW-Asia
[3] Veticon Consulting and International Vetiver Network, Brisbane

I. INTRODUCTION

In recent years, there is an increasing trend in research of essential oils extracted from various herbs and aromatic plants due to the continuous discoveries of their multifunctional properties other than their classical roles as food additives and/or fragrances. New properties of many essential oils, such as antibacterial, antifungal, antioxidant, and anti-inflammatory activities have been found and confirmed (Aruoma et al, 1996; Hammer et al, 1999; Güllüce et al, 2003). The pharmacological properties of essential oils extracted from plants have received the greatest interest of academic institutes and pharmaceutical companies (Ryman, 1992; Loza-Tavera, 1999; Courreges et al, 2000; Carnesecchi et al, 2002 and Salim et al, 2003). On the other hand, the insecticidal activities of essential oils are more favored by agricultural scientists and agri-business. Consequently, many investigation and new findings have significantly prompted and expanded novel applications of essential oils which are now been widely used as natural insecticides, cosmeceuticals, and aroma therapeutic agents.

One such plant that is extensively used in perfumery industry is Vetiver (*Vetiveria zizanioides* L.) originating from India, is a tall tufted perennial scented grass with a straight stem, long narrow leaves and a lacework root system that is abundant, complex, and extensive. Since the ancient time, Vetiver grass has been used as a fragrant material and in traditional medicine because its roots contain essential oils that have aromatic and biological properties. The oil and its constituents are used extensively for blending oriental types of perfumes and floral compounds, as well as in other cosmetic and aromatherapy applications. It is very persistent and one of the finest fixatives known. Vetiver oil is a main ingredient in 36% of all western quality perfumes and 20% of all men's fragrances.

Currently, the methods used for extraction of Vetiver are mainly hydro-distillation, steam distillation and solvent extraction. However, the hydro and steam distillation have several drawbacks, such as operating at high temperatures leading to break-down of thermally-labile components, promoting hydration reaction of chemical constituents, requiring a post-extraction process to remove water and incomplete extraction of essential oils from plant materials. The solvent extraction does not have the same drawbacks as of distillation, but it has one major disadvantage that makes it less favourable for essential oil extraction: solvent residue in extracted essential oil.

Recently, an advanced method is used for extraction of flavours and fragrances from natural materials, namely supercritical fluid extraction (Caredda et al, 2002). Supercritical fluid extraction exploits the unique properties of gases above their critical points to extract soluble components from a raw material. Recently, there has been increased interest in supercritical and subcritical extraction which use carbon dioxide as a solvent. Carbon dioxide is an ideal solvent for the extraction of natural products because it is non-toxic, non-explosive, readily available and easy to remove from extracted product. The supercritical CO_2 extraction (SCE) has several advantages over hydro-distillation, steam distillation and solvent extraction including: elimination of problem of toxic residual solvent in the products, operation at lower temperatures leading to less deterioration of the thermally-labile components in the extract. Furthermore, the supercritical CO_2 extraction retains organoleptic characteristics of the starting plant materials in extracts that do not occur in the traditional extraction methods.

Vetiver essential oil (VO) consists of more than 100 compounds that are mainly sesquiterpenes and their derivatives. Among the odorous components in vetiver oils, three constituents (khusimol, α-vetivone and β-vetivone) are the major constituents, and their presence is often considered as the fingerprint of the oil. α-vetivone and β-vetivone were found to be repellent to insects, as they are the least volatile so the most effective repellents (Zhu et al, 2001 b).

Recently, the antioxidant activity of VO and its components was studied and showed that VO possessed a strong free radical scavenging activity as compared to standard antioxidants such as α-tocopherol and butylated hydroxytoluence (BHT). The presence of natural antioxidants from various aromatic and medicinal plants is closely related to the reduction of chronic diseases such as DNA damage, mutagenesis, and carcinogenesis (Briskin, 2000; Wargovich et al, 2001 and Reddy, 2003). Essentially, antioxidants inhibit free radical propagation in biological systems. Interestingly, α-vetivone and β-vetivone were found to be responsible for such type of activity. Therefore, α-vetivone and β-vetivone could be considered as novel natural antioxidants, which might have alternative potential applications such as effective termiticides and cosmoceuticals. The effective and cheap purification of α-vetivone and β-vetivone will be a key factor in extending the applications of such compounds in near future.

As commercial extraction and research of Vetiver essential oil are based on the commercial variety of Vetiver, *Vetiveria zizanioides*, no studies are carried out on essential oils extracted from other Vetiver species, such as native Vetiver species. Among seven native Vetiver species recorded, four of them (*Vetiveria elongata, Vetiveria pauciflora*, *Vetiveria intermedia* and *Vetiveria filipes*) have been found in Australia, mainly in Queensland and Northern Territory. These native species may be alternative sources for extraction of Vetiver essential oil that have high yield and high content of α-vetivone and β-vetivone.

Vetiveria zizanioides L. is not only grown for extracting essential oils applied in pest control, cosmeceuticals and aroma therapeutic field as mentioned previously, but also grown for phytoremediation of heavy metals in soils, such as lead, zinc, iron, cadmium, copper (Chiu et al, 2006; Xia, 2004; Lai, 2003 and Yang et al, 2003). This is due to the facts that Vetiver grass (Vetiver zizanioides) is a fast-growing plant that tolerates various environments, including soil pH values between 3.0 and 10.5 and temperatures from 14 to 55 °C. In addition, high concentrations of heavy metals in soils contaminated with multiple elements do not affect the plant's growth (Roongtanakiat and Chairoj, 2002). Furthermore, Vetiver grass has an ability to accumulate high concentrations of heavy metals in roots, especially Pb (1094 mg/kg of dry root weight), Fe (24737 mg/kg of dry root weight) (Wilde, et al, 2005) and Zn (1162 mg/kg of dry root weight) (Yang et al, 2003). The high absorbability of Vetiver grass raises the concern about Vetiver essential oil that is extracted from roots is contaminated with heavy metals as Vetiver grass is unintentionally grown on heavy metal contaminated soils.

II. AIMS

The aims of this study is to develop method that efficiently purify α-vetivone and β-vetivone from essential oil extracted from Vetiveria zizanioides and three Australian native species **(*Vetiveria elongata, Vetiveria rigidus* and *Vetiveria filipes*)** and to investigate the effect of Pb, Zn and Cu on Vetiveria zizanioides essential oil. To obtain these goals, the following works will be carried out:

- **Optimize supercritical CO_2 extraction for yield of Vetiver essential oil and α-vetivone and β-vetivone**: efficiency of supercritical fluid extraction depends on 4 main variables: extraction temperature, pressure, time and particle size of plant materials. To optimize this process, serial combinations of such variables at 3 levels (3^4 = 81 treatments) will be tested for oil yield and relative concentrations of α-vetivone and β-vetivone. The yield and chemical compositions obtained from each treatment will be compared to those obtained from conventional extraction methods (hydro distillation, steam distillation and solvent extraction).

- **Purify α-vetivone and β-vetivone by using supercritical CO_2 fractionation:** chemical constituents of essential oil extracted from plant materials at every combination of extraction pressure, temperature, time and particle size of plant materials will be characterized. Therefore, the information obtained from optimization of SCF extraction in previous experiment will be very useful in this section. The identified optimal operating conditions are used to obtain essential oil with high yield and high content of α-vetivone and β-vetivone. Then the extract will be subjected to further treatments that fractionate the extract to obtain an oil fraction rich of α-vetivone and β-vetivone.

- **Investigate the essential oil yield and chemical composition of 3 Australian native Vetiver species:** different Vetiver species will have different essential yield content and different composition of chemical constituents. The identification of species that has the highest essential oil yield with high concentration of interested constituents (α-vetivone and β-vetivone) is one factor determining the effectiveness of extraction. Roots of three Australian native vetiver species (*Vetiveria elongata* 1, *Veteveria elongata* 2 and *Vetiveria filippes*) and *Vetiveria zizanioides* grown on same conditions will be collected and subjected to extraction using supercritical CO_2 extraction (SCE)...

- **Investigate the effects of heavy metals on quality and yield of *Vetiveria zizanioides* grown on heavy metal contaminated soils:** lead, zinc and iron are the heavy metals that are accumulated in roots of *Vetiveria zizanioides* at very high concentrations. This study is carried out to determine whether high concentrations of heavy metals accumulated in roots can affect yield, chemical compositions as well as contaminate Vetiver essential oil or not. Each heavy metal is tested at 3 concentrations: low, medium and high, with 3 replicates (3 plants) for each level. To test the interaction of three heavy metals, factorial design with 3 factors (3 heavy metals) at 2 concentration level (low and high) is used (2^3 = 8 treatment), each treatment has 3 replicates (3 plants).

III. LITERATURE REVIEW

3.1. Vetiver species

Vetiver belongs to family of Graminae (Poaceae), subfamily of Panicoideae. There are ten Vetiver species that distribute throughout tropical Africa, Asia, Australia; common to flood plains and stream banks (Watson and Dallwitz, 1989).

Among Vetier species, *Vetiveria zizanioides* has the most important values in terms of economics and environment. It is the fact that essential oil extracted from its roots has been used for a long time in perfumery industry, medicines and other uses. Recently, *Vetiveria zizanioides* has been applied as "green technology" to control soil erosion and to remove pollutants in soils and water. Consequently, all of researches or studies related to Vetiver species are implemented on *Vetiveria zizanioides.* It results in no information on production and chemical composition of essential oil extracted from other Vetiver species found in the literature, especially from Australian native Vetiver species. Therefore the term Vetiver oil mentioned in this study refer to the oil extracted form *Vetiveria zizanioides.*

3.1.1. Vetiveria zizanioides

Vetiveria zizanioides is a tall, tufted, perennial, scented grass, with a straight stem, long narrow leaves and a lacework root system that is abundant, complex, and extensive (Chomchalow, 2001). It offers an inexpensive yet effective and eco-friendly tool to combat soil erosion. The roots have been used in Asia for centuries for their fragrance, and are woven into aromatic matting and screens. The plant also contains active ingredients used in traditional medicine and as botanical pesticide (Chomchalow, 2001). Erect culms are up to 2m tall and over 2.5m with flower head. It flowers but setting no seeds (Figure 3.1 a). The root system can reach around 5 m in length in 18 months (Figure 3.1 b).

The roots of some cultivars and ecotypes possess essential oil that has been utilized as fragrant material since ancient times

a **b**

Figure 3.1: a) *Vetiveria zizanioides* – commercial variety used for essential oil extraction
b) Root system of *Vetiveria zizanioides*
Source: http://www.vetiver.org

3.1.2.. Vetiveria elongata

Vetiveria elongata is found around (and even within) freshwater lagoons, damp depressions, seashores, dunes, edges of marsh, moist Melaleuca stands and rivers in the Northern Territory and Queensland. It is also found in New Guinea (National Research Council (U.S.). Board on Science and Technology for International Development, 1993). The grass is freely grazed by cattle.

The Australian native grass is perennial; it has erect culms of 150-250 cm long (Figure 3.3). Leaf-blade margins are scabrous. Leaf-blades are conduplicate and stiff with 25–50 cm long and 4–8 mm wide. Inflorescence is a panicle with branches tipped by a raceme. Panicle is 15-30 cm long and 2-3 cm wide, it has pale yellow. Panicle branches are straight. Racemes are 1.5-2.5 cm long, bearing few fertile 3-6 fertile spikelets on each. Pedicelled spikelets are 6.75-9.75 mm long. Callus hairs are 3.75-5.6 mm long. Column is glabrous. Awn is exserted to

enclosed, straight to geniculate with contorted column and straight arista, 1.9-6.5(-8.25) mm long (http://www.kew.org/data/grasses-db).

Figure 3.3. *Vetiveria elongata.* Source: http://grassland.argon.utu.edu.tw

3.1.3. *Vetiveria filipes*

Vetiveria filipes is found from Northern Territory to New South Wales, it is also found in Malesia ((Papua New Guinea: Western Province). It is characteristic of the vegetation often growing on riverbanks, sandbanks, riverine plains, high ground near creeks, Melaleuca swamps, dry creek beds, Depressions in open forests, Savannah (Eucalypt) forest on alluvial flat of creek, wet gully in savanna grassland, locally common; at 0 to 30 m altitude. It is eaten freely by cattle. This species is of special botanical interest because its morphology is intermediate between vetiver and lemongrass (National Research Council (U.S.). Board on Science and Technology for International Development, 1993).

Figure 3.4. *Vetiveria filipes*. Source: http://grassland.argon.utu.edu.tw

The grass is perennial and grows into caespitose. It has short rhizomes. Culms are erect with 70–100 cm long; and each culm has 3–6 nodes. Ligule is 0.2-0.75 mm long and has a fringe of hairs. Leaf-blades are stiff with length of 15–45 cm and width of 3–6 mm. Leaf-blade surface is scaberulous and rough adaxially. Leaf-blade margins are scabrous. Leaf-blade apex is obtuse. Inflorescence is a panicle with branches tipped by a raceme. Panicle is contracted and lanceolate: 15–30 cm long; 3–7 cm wide. Primary panicle branches are whorled at most nodes; simple. Racemes are 3.5–9 cm long; bearing 3–7 fertile spikelets on each. Rhachis are fragile at the nodes. Rhachis internodes are filiform with 7–15 mm long. Rhachis internode tip is oblique. Spikelets are appressed in pairs. Fertile spikelets are sessile; only 1 in the cluster. Companion sterile spikelets are pedicelled; 1 in the cluster. Pedicels are filiform; 3–8 mm long (http://www.kew.org/data/grasses-db).

3.1.4. *Vetiveria rigidus*

This Australian species has only recently been described (National Research Council (U.S.). Board on Science and Technology for International Development, 1993). It is found near waterhole in *Corymbia confertiflora* woodland, Queensland.

National Research Council (U.S.). Board on Science and Technology for International Development (1993). *Vetiver Grass: A Thin Green Line Against Erosion*. Washington, D.C. : National Academy Press.

Vetiveria rigidus is perennial and clumps densely. It has erect culms with 130-150 cm long (Figure 3.5). Leaf-blades are 30-50 cm long and 3-5 cm wide. Ligule is a fringe of hairs. Inflorescence is a panicle with branches tipped by a raceme. Panicle is open; 20–27 cm long and 8–10 cm wide. Primary panicle branches are whorled at most nodes; each branch is 0.5–1 cm long. Racemes are 3–4.5 cm long; bearing 3–4 fertile spikelets on each. Rhachis is fragile

at the nodes and scabrous on surface. Rhachis internodes is filiform; 4–11 mm long. Rhachis internode tip is oblique (http://www.kew.org/data/grasses-db).

Figure 3.5. *Vetiveria rigidus*

.

Production of Vetiver essential oil

The vetiver oil is traditionally known as "vetivert oil" in trade, and is obtained from the aromatic roots of vetiver. The annual world trade in 'vetivert oil" is estimated around 250 tons, with Haiti, Indonesia (Java), China, Japan, India, Brazil being the main producers, and USA, Europe, India and Japan being the main consumers (Lavania, 2003). In terms of volume, vetiver is the leading essential oil exported from Indonesia and is said to be competitive with the one produced in Haiti. However, the typical Indonesian product is sometimes discounted in the international market due to its having a 'smoky burnt' character. The best type of vetiver oil is believed to come from Reunion Islands (Chomachalow, 2001).

Vetiver roots do not yield their oils easily because the essential oils are located inside hard-to-reach root tissues. To be extracted, these oils must diffuse (which is a relatively slow physical process) from inside fibrous rot tissues outward to the surface. Furthermore, vetiver oil consists of a high percentage of sesquiterpenes (which have high molecule weights with low vapor pressures), which also contribute to the long extraction times needed. The most valuable fractions of vetive oil have the highest boiling points and constitute the high specific gravity oil portion, and characteristically pass through the condenser in greatest volume late in the distillation. These fractions are rich in vetivones and vetiverol (Chomchalow, 2001).

Spongy root mass of certain cultivars of *Vetiveria zizanioides* contains trace amount of essential or volatile oil, known as vetiver oil or 'khus oil', which can be extracted by distillation. Distillation is long and involves high pressure using water and steam distillation method. The process of distillation consists of soaking the root mass prior to packing in the distillation unit, and steam is allowed to pass through for a period of six hours or more. The steam distillation produces around 0.3-1.0% of oil although higher percentages can also be obtained. A resinoid is also produced by solvent extraction for perfumery work (Chomchalow, 2001).

Age, quality and stage of root harvest, and processing for distillation are vital components for efficient processing of essential oil distillation. Essential oil could be distilled both from fresh and dried roots. Recovery of essential is very high from fresh roots, but leaving the roots in open for 2-3 days after harvesting with a day temperature of around 25° C yields high quality essential oil, since the undesirable non-polar low boiling components of the oil are naturally evaporated off, although oil recovery is somewhat reduced. Both freshly harvested roots and semidried roots soaked overnight in water could be used for essential oil extraction (Lavania, 2003). The dried roots after washing and drying can be stored for 12-24 months so enzymatic process can increase oil yield (Dowthwaite and Rajani 2000).

In order to improve quality and increase shelf life, the freshly distilled oil need to be dehydrated to remove water either by anhydrous sodium sulphate or natural evaporation by air drying, and then allowed to mature by natural oxidation for about six months in amber color glass bottles with a bit of air trapped inside the container till it develops green coloration. Excessive oxidation is to be avoided as this may lead to malodor formation. However, to obtain vetiver oil truly representative of its occurrence *in planta* modern methods of liquid carbon dioxide extraction may be done. The vetiver 'oleoresin' thus obtained is a very stable more mobile golden liquid, free from residue, enriched with polar compounds and quality odor. Absence of residue makes such oil more soluble in alcohol and improves its miscibility suitable for blending with other perfume materials (Lavania, 2003).

3.3. Properties of Vetiver Oil

Vetiver oil is a light to dark brown, olive, or amber viscous oil having a deep smoky, earthy-woody odor with a sweet persistent undertone. The color and scent can vary according to the source. Poorer grades with darker color and have smoky back notes are also produced in China and Java by subsistent farmers with primitive equipment (Dowthwaite and Rajani 2000).

Vetiver oil has a rather powerful smell but is very pleasant when diluted (Curtis 1996). It blends well with oils of sandalwood, rose, violet, jasmine, opopanax, patchouli, oakmoss, lavender, clary sage, mimosa, cassia, and ylang ylang (Lawless, 1995) . It is a high-priced oil as it is used extensively in fine perfumery and cosmetic products. In dilute state, it smells like sandalwood oil (Georgi, 1924). It is used exclusively in the preparation of compound perfumes, in which the oil, on account of its low volatility, is normally used as a base to fix other high-value volatile oils like rose oil, lavender oil, and jasmine oil (Chomchalow, 2001).

3.4. Chemical composition of Vetiver essential oil

Chemical composition of vetiver oil is extremely complex, containing over 100 sesquiterpene-type compounds and their derivatives, belonging to 11 structural classes (Akhila and Rani, 2002). The main constituents of vetiver oil comprise of: sesquiterpene hydrocarbons (γ-cadenene, clovene, α-amorphine, aromadendrine, junipene and their alcohol derivatives), vetiverols (khusimol, epiglobulol, spathulenol, khusinol; carbonyl derivatives), vetivones (α-vetivone, β-vetivone, khusimone; and ester derivative). Among of them, three carbonyl compounds, α-vetivone, β-vetivone and khusimone, are considered the primary odor-influencing components; β-vetivone has the better odor, and is considered the most important, while its major isomer nordihydro β-vetivone has a strong, rich, woody-peppery note(Lavania, 2003). All these components individually and collectively contribute to the characteristic odor of the vetiver. Therefore, they, α-vetivone, β-vetivone and khusimol, can be considered as the 'finger print' of vetver oil (Demole et al. 1995).

There are distinct geographical differences in quality and perfumery note of essential oil obtained from different geographic regions of the world. In a broad sense, the essential oil of vetiver having high specific gravity, negative optical rotation, high vetiverol concentration and higher ester value is considered superior from perfumery view point (Lavania, 2003). Reunion oil with roseate note is highly regarded in perfumery industry, but the vetiver oil (khus oil) obtained from the roots occurring in wild state in north Indian plains, commonly known as 'khus' is considered to be the best for its balsamic woody note. Lately, vetiver genotypes producing vetiver oil with roseate and saffron note have also been identified from north Indian plains (Lal et al. 1998).

3.5. Uses of Vetiver essential oils

Vetiver oil has been utilized as raw materils for various fragrant products such as perfumes, deodorants, lotions, soaps, etc. In addition, vetiver oil plays an important role in aromatherapy. Furthermore, vetiver oil is shown to have insecticidal activity. Currently, vetiver oil is proved to have antioxidant and anticancer activities

3.5.1. Perfumery

Vetiver is known for its perfumery value since ancient times. On account of its pleasing aroma and slow evaporation rate falling under the category of lower 'base note' vetiver oil as such is a 'perfume in its own right' for which no synthetic substitute is yet available. Vetiver oil is the basis of the Indian perfume 'Majmua' and is the major ingredient in some 36 % of all western perfumes, such as Caleche, Chanel, Dioressence, Parure, Opium, Guerlain, Christian Dior, Givenchy (Dowthwaite and Rajani 2000) and 20 % of all men's fragrances. A 15 – 30 % dilution of vetiver oil in alcohol is good enough to make true vetiver perfume, and its further dilutions have value as vetiver 'eau de cologne' and 'eau de toilette'. 'Vetiver pour Homme' by Carven 1957, and 'Vetivert' by Guerlain 1961, are the two famous 'eau de toilette' for men prepared from vetiver oil (Groom 1992).

Furthermore, the vetiver oil is one of the finest fixatives known (Lavania, 2003). Its complex chemical composition and oil odor, high solubility in alcohol that improves its miscibility with other perfumery material, makes it a unique perfumery resource for which no synthetic substitute is yet available. In addition to its own perfumery value on account of vetiver hydrocarbons and carbonyl compounds, their alcohol derivatives i.e. vetiverols lend unique position to vetiver oil for perfumery applications as a valuable resource. Because of clear-cut differences in boiling point of the various constituents of vetiver oil, its vetiverol fraction could be easily separated by fractional distillation of oil under high vacuum. Also, vetiverol could be acetylated with acetic anhydride to produce vetiveryl acetate. Both vetiverols and acetates have softer odors and fixative qualities, and are used as blender with high-class perfumery products. They blend well with ionone, linalool, cinnamic alcohol, oak-moss, vanila, sandalwood, patchouli and rose bases, and are frequently used in western 6 type of fragrances having chypre, fougere, rose, violet and amber aldehyde base, and oriental fragrances and floral compounds (Lavania, 2003).

In addition to its direct perfumery applications, vetiver oil in its diluted form is extensively used in after-shave lotions, air freshners and bathing purposes, as well as flavoring syrups, ice cream, cosmetic and food preservation. Khus essence is used in cool drinks, and for reducing pungency of chewing tobacco preparations, providing sweet note to other masticatories and incense sticks (National Research Council 1993).

3.5.2. Aromatherapy

The main action of vetiver oil is on the nervous system and it is both sedating and strengthening in effect (Wilson 1995). It is excellent in the treatment of depression, nervous tension, *debility*, insomnia* and many stress-related diseases, and acts as an *aphrodisiac* where there is a clear connection between impotence or frigidity and stress (Wilson 1995). It may be used in massage blends and the bath; it has a rather powerful smell but is very pleasant when diluted. It stimulates the circulatory system and makes a useful massage oil for elderly or debilitated people with poor circulation (Chomchalow, 2001). It also helps to stimulate the production of red blood cells and is thus beneficial for *anemia*. It makes a useful warming and pain-relieving rubbing oil, suitable for deep massage of muscular aches and pains, sprains, stiffness, rheumatism and arthritis (Chomchalow, 2001). It may be added to sports oil blends and massaged into muscles before and after sports. In skin care, it helps to balance the secretion of sebum. It is used in lotions, compresses and baths for the treatment of oily skin, acne and weeping sores (Curtis 1996).

Vetiver oil revitalizes the body by fortifying the red blood corpuscles crucial in transporting oxygen to all parts of the system. Increased blood flow could alleviate muscular aches and pains and said to be useful in cases of *rheumatism* and *arthritis* (Sellar 1992). Shealy (1998) advocates that vetiver oil is particularly useful for jet lag, and for grounding and clarity while traveling.

3.5.3. Insecticides

Vetiver oil is known to repel insects; people in India and elsewhere have placed vetiver root among their clothes to keep insects away (Lavania, 2003). It also repels flies and

cockroaches and may make a useful ingredient in insect repellents (National Research Council 1993). It has been used to repel moths (Sealy 1998). The two tricyclics esquiterpenoids – zizanal and epizizanal – isolated from vetiver oil show insect repelling activity (Jain *et al.* 1982).

Jain et al. (1982) reported that at least six compounds (α, β-vetivone, khusimone, zizanal, epizizanal and (C)-(1S, 10R)-1,10-dimethylbicyclo[4,4,0]-dec-6-en-3-one) were repellent to insects. Recently, studies of Zhu et al (2001 a and b) found three other vetiver oil compounds that were repellent to the Formosan subterranean termite including: nootkatone, zizanol and bicyclovetivenol. Zhu et al (2001 b) discovered that compound volatility was inversely proportional to repellent effectiveness. Vetivones (α and β) were the least volatile and the most effective repellents. The volatility of nootkatone is similar to α and β vetivone.

3.5.4. Antioxidant

Many studies have shown that the presence of natural antioxidants from various aromatic and medicinal plants is closely related to the reduction of chronic diseases such as DNA damage, mutagenesis, and carcinogenesis (Craig, 1990; Zhu et al, 2002; Briskin, 2000; Reddy et al, 2003). Essentially, antioxidants inhibit free radical propagation in biological systems.

The study of Hyun-Jin Kim et al (2006) showed that the vetiver oil (VO) possessed a strong free radical scavenging activity (antioxidant activity) when compared to standard antioxidants such as butylated hydroxytoluene (BHT) and α-tocopherol. Among the complex constituents in the crude VO, β-vetivenene, β-vetivone, and α-vetivone, which were identified to be responsible for strong antioxidant activities. These results show that VO and some of its inherent components can be potential alternative natural antioxidants.

3.5.5. Anticancer

The study of Chen Feng et al (2003) showed that vetiver essential possessed anticancer activity. At 100ppm in cancer cell lines, vetiver oil inhibited the growth up to 89% of SiHa cervical cells, 88% of CaSki cervical cells and 89% of MCF-7 breast cancer cells. However, the results were preliminary, therefore it needs to be confirmed by further study.

3.6. Supercritical fluid extraction and fractionation

Supercritical fluid extraction (SFE) is a separation method that exploits the unique properties of gases above their critical points to extract soluble components from a raw material (Simandi, 1998). In comparison with conventional, liquid organic solvents, supercritical fluids have a higher diffusivity and lower density, viscosity, and surface tension. On the other hand, the properties of supercritical fluids can be varied over a wide range by changing the operational conditions (Reinoso et al, 2006).

Recently, there has been increased interest in application of super-critical and subcritical fluid extraction of essential oils from plant materials (McHugh and Krukonis, 1986; Stahl et al., 1987; Perrut, 1991; King and Bott, 1993; Brunner, 1994; Reverchon, 1997). Several compounds have been examined as SFE solvents. For example, hydrocarbons such as hexane, pentane and butane, nitrous oxide, sulphur hexafluoride and fluorinated hydrocarbons (Smith, 1999). However, carbon dioxide is the most frequently used solvent for SFE, because of its practical advantages including its nontoxic and nonflammable character, environmental safety, huge availability, low cost at high purity, and suitability for extracting heat labile, natural compounds with low volatility and polarity and easy to remove from extracted products (Reinoso et al, 2006).

Supercritical fluid extraction using carbon dioxide as solvent allows operations at relatively low pressures and at near-room temperatures (Reverchon, 2006). Therefore, using SFE have several advantages over traditional extraction methods (namely steam distillation, hydro distillation and solvent extraction) including: elimination of the problem of toxic residual solvent in the products, the ability to use lower temperatures leading to less deterioration of the thermally-labile components in the extract (Simandi, 1998). SFE is more selective than extraction with commonly used solvents which extract unwanted components (e.g. tannins, chlorophyll, minerals). In addition, SFE using carbon dioxide helps retain the organoleptic characteristics of the starting plant materials (Moyler and Heath, 1988; Reverchon and Senatore, 1992; Simandi et al., 1993; Oszagyan et al., 1996). The only serious drawback of SFE is the higher investment costs in comparison with traditional extraction techniques. However, operation cost is relatively cheap and the process is very simple to be scaled up to industrial scale (Reverchon, 2006).

3.6.1. Supercritical fluid fractionation

Fractional separation of the extracts is the process used to improve the selectivity of SFE**.** The principle of this operation is to induce the selective precipitation of different compound families as a function of their different saturation conditions in the SFE (Reverchen and Macro, 2006).

The basic extraction and fractionation scheme consists of an extraction vessel charged with the raw matter to be extracted. As a rule, the starting material is dried and grinded to favor the extraction process. It is loaded in a basket located inside the extractor that allows fast charge and discharge of the extraction vessel. The SFE product at the exit of the extractor flows through a depressurization valve to a separator in which, due to the lower pressure, the extracts are released from the gaseous medium and collected (Reverchon and Macro, 2006).

The more sophisticated extraction schemes contain two or more separators. In this case, it is possible to fractionate the extract in two or more fractions of different composition by setting opportune temperatures and pressures in the separators (Dauksas et al, 2002; Vagi et al, 2002; Marongiu et al, 2001; Esquivel et al, 1999). This strategy can be used when it is required the extraction of several compound families from the same matrix and they show different solubility in SC-CO2. It takes advantage of the fact that SC-CO2 solvent power can be continuously varied with pressure and temperature. For example, it is possible to perform a first extraction operating at low CO2 density (e.g., 0.29 g/cm^3, 90bar, 50◦C) followed by a

second extraction step at high CO2 density (e.g., 0.87 g/cm^3, 300 bar, 50 ◦C). The most soluble compounds are extracted during the first step (for example, essential oils) and the less soluble in the second one (e.g., antioxidants) (Grigonis et al, 2005; Ramirez et al, 2004; Senorans et al, 2000 and Ilbanez et al, 1999).

3.6.2. Selection of the operating parameters

The selection of the operating conditions depends on the specific compound or compound family to be extracted. There are five crucial parameters that need to be taken into account as conducting SFE of essential oils including: extraction temperature, extraction pressure, extraction time, CO_2 flow rate and particle size.

3.6.2.1. Extraction temperature

The increase of temperature reduces the density of SC-CO2 (for a fixed pressure) thus reducing the solvent power of the supercritical solvent; but it increases the vapor pressure of the compounds to be extracted (Reverchon and Marco, 2006). Therefore, the tendency of these compounds to pass in the fluid phase is increased. For the extraction of thermo labile compounds, extraction temperature has to be fixed between 35 and 60^{0}C; e.g., in the vicinity of the critical point and as low as possible to avoid degradation (Reverchon and Marco, 2006).

3.6.2.2. Extraction pressure

The extraction pressure is a very important parameter of SFE, since it determines both CO_2 density and solvent power (Reinoso et al, 2006). The solvent power is frequently described in terms of the SC-CO2 density at the given operating conditions. The density of CO_2 can vary from about 0.15 to 1.0 g/cm^3 and is inter-connected to both pressure and temperature. Variation of CO_2 density is strongly non-linear, therefore for proper selection the accurate tables of CO2 properties should be used (Span and Wagner, 1996). In addition, extraction pressure is the most relevant process parameter that can be used to tune the selectivity of the SCF. The general rule is: the higher is the pressure, the larger is the solvent power and the smaller is the extraction selectivity (Reverchon and Marco, 2006).

3.6.2.3. CO_2 flow rate

CO2 flow rate is a relevant parameter if the process is controlled by an external mass transfer resistance or by equilibrium: the amount of supercritical solvent feed to the extraction vessel, in this case, determines the extraction rate (Reverchon and Marco, 2006). CO_2 flow rate affects the extraction rate of the easily accessible solute. During the initial process stages, increased solvent flow rates are expected to favor the extraction (Reinoso et al, 2006). Depending on the situation, higher solvent flow rates may cause negative (Louli et al, 2004), negligible, or little (Roy et al, 1996) or weak positive effects (Rozzi et al, 2002) on the extraction process.

3.6.2.4. Particle size

Particle size plays a determining role in extraction processes controlled by internal mass transfer resistances, since a smaller mean particle size reduces the length of diffusion of the solvent (Reverchon and Marco, 2006). However, if particles are too small, they can give problems of channeling inside the extraction bed. Part of the solvent flows through channels formed inside the extraction bed and does not contact the material to be extracted thus causing a loss of efficiency and yield of the process (Reverchon and Marco, 2006). As a rule, particles with mean diameters ranging approximately between 0.25 and 2.0 mm are used. Moreover, the production of very small particles by grinding could produce the loss of volatile compounds. Process duration is interconnected with CO2 flow rate and particle size and has to be properly selected to maximize the yield of the extraction process (Reverchon and Marco, 2006).

3.6.2.5. Extraction time

Extraction time is varied depending on the type of solutes to be extracted and on the operational conditions, such as CO_2 flow rate and particle size, and it has to be properly selected to maximize the yield of the extraction process. Commonly, a static extraction period from 1 min up to 2 h can be maintained before the dynamic extraction is hold from 15 min up to 5 h (Ramirez et al, 2004; Dean and Liu, 2000 and Scalia et al, 1999). Alternatively, a single extraction step for 30 min to 4 h has been employed (Damjanovic et al, 2005; Poli et al, 2003; Vilegas et al, 1997).

3.7. Application of Vetiver grass for heavy metal phytoextraction

Vetiver grass, *Vetiveria zizanioides*, is highly tolerant to extreme soil conditions (including heavy metal contamination such as that of Pb, Zn, and Cu) and can produce high biomass even when growing in contaminated areas (Truong and Baker, 1998). In Australia, vetiver has been used to stabilize mining overburden and highly saline, sodic, and alkaline (pH 9.5) tailings of coal mines and higher acidic (pH 2.7) tailings of gold mines with high As contents (Truong and Baker, 1998 and Truong, 1999). Recently, there are several studies to investigate its ability to remove heavy metals in contaminated soils (Chiu et al, 2006; Wilde et al, 2005; Chen et al, 2004; Lai and Chen, 2004). All of these studies confirmed that Vetiver grass has a high ability in accumulation of heavy metals in roots, particularly Pb, Zn and Cu.

IV. METHODOLOGY

4.1. Materials

Roots of *Vetiveria zizanioides* and 3 Australian native Vetiver species as well as young plants *Vetiveria zizanioides* used in heavy metal study are supplied by Vetigrass Company, Brisbane Queensland.

4.2. Plant cultivation for heavy metal study

Each Vetiver plant is grown in one pot (diameter: 50 cm and height: 60 cm) filled with sandy soil. Each pot is placed on a basket in order to collect excess irrigated water (that may contain heavy metals).

Two weeks after planting, solutions of heavy metals are applied onto plant pots in 1 week interval.

Water is daily irrigated

After 6 months, leaves, stems, roots and soils are collected for further processing and analysis.

4.3. Plant material treatment

- Roots are washed to remove soils or sands, and then they are air-dried at room temperature for 48-72 hours. The dried roots are milled by a knife mill. The particles of milled roots are separated according to their particle sizes by using a vibratory sieve system. The root particle can be stored in -20^0C freezer before extraction (Julian Martinez et al, 2004).

4.4. Extraction

4.4.1. Supercritical fluid extraction

Water bath is adjusted to the specified temperature; the extraction vessel loaded with 30 grams of dry root is connected to the system as presented in Figure 1. Air is purged from the system by pressing CO_2 at low pressure (<20 bar). The system is then isolated until reaches certain pressure, then changed into dynamic stage, finally depressurized. The oil is accumulated in collecting vessel.

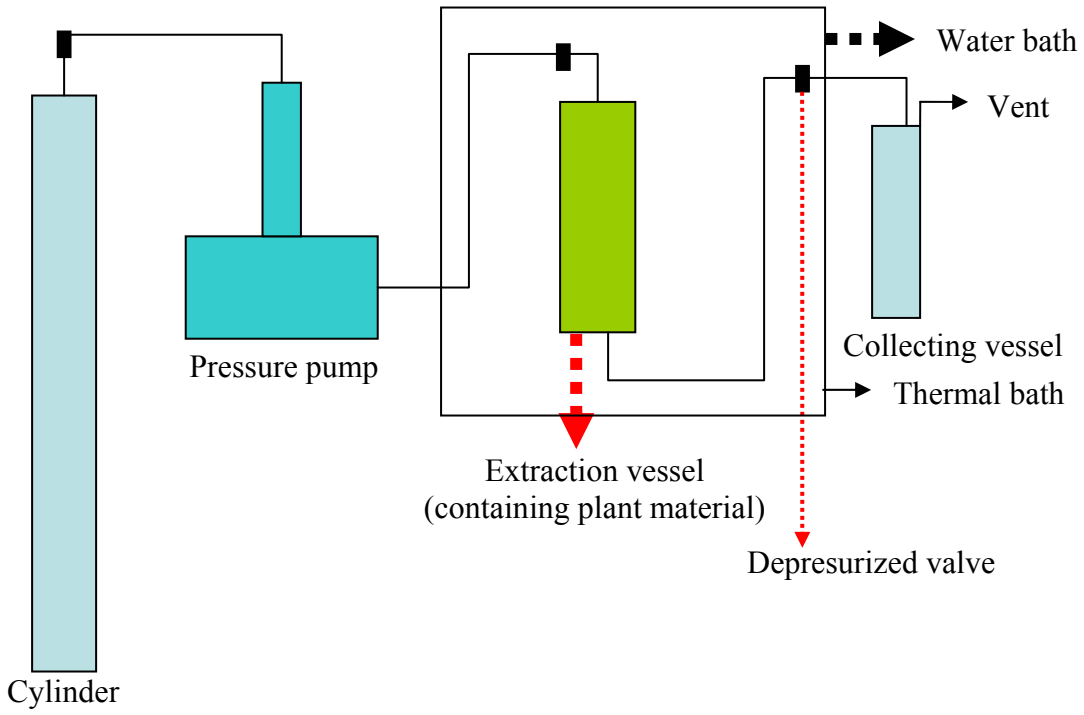

Figure 4.1: Schematic diagram of SCF extraction

Respone surface methodology is most common statistical method used in optimization experiments. In this study, it is employed in order to find optimal operating condition of supercritical fluid extraction that achieve the highest oil yield and content of α and β-vetivone.

There were three variable parameters studied in this work including: processing pressure ξ_1, extraction temperature ξ_2 and extraction time ξ_3. The responses (yield, content of α and β-vetivone) were assumed to be affected by the three independent variables ξ_i . It was also assumed that the independent variables (referred to as responses), y, which were experimentally measured, defined the system.

$Y = f(\xi_1, \xi_2, \xi_3)$

The expected form of the model can be expressed as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_j$$

where β_0 β_i β_{ii} and β_{ij} are regression coefficients, and x_i are the coded variables linearly related to ξ_1. The coding of ξ_1 into x_i is expressed by the following equation:

$x_i = 2(\xi_i - \xi_i^*)/d_i$

where ξ_i = actual value in original units; ξ_i^* = mean of high and low levels of ni; and d_i = difference between the low and high levels of ξ_i.

Table 4.1: Coded and uncoded independent variables

Experiment	Coded variables			Uncoded variables		
	Pressure	Temperature	Time	Pressure (bar)	Temperature (ºC)	Time (min)
1	-1	-1	-1	100	40	60
2	1	-1	-1	180	40	60
3	-1	1	-1	100	60	60
4	1	1	-1	180	60	60
5	-1	-1	1	100	40	120
6	1	-1	1	180	40	120
7	-1	1	1	100	60	120
8	1	1	1	180	60	120
9	-1.68	0	0	207.2	50	90
10	1.68	0	0	72.8	50	90
11	0	-1.68	0	140	33.2	90
12	0	1.68	0	140	66.8	90
13	0	0	-1.68	140	50	39.6
14	0	0	1.68	140	50	140.4
15	0	0	0	140	50	90
16	0	0	0	140	50	90
17	0	0	0	140	50	90
18	0	0	0	140	50	90
19	0	0	0	140	50	90
20	0	0	0	140	50	90

4.4.2. Solvent extraction

- Apparatus: Soxhlet extractor (4 sets)
- Solvent: hexane
- Extraction time: 6 h
- Replications: 3
- Weight of each replication: 30 g of dried roots

30 gram of dried roots is loaded in extraction thimble that is then placed in Soxhlet chamber. Solvent in the round bottomed flask is heated then turned into vapors rising through the outer chamber and into the condenser. The vapors condense into liquid and fall back into the Soxhlet chamber where the solvent extracts the compounds of interest and leaves the solid mass behind. As the mixture of solvent and essential oil rises to certain level, the chamber is flushed due to a siphoning effect. The solvent is redistilled from the solution in the flask and condenses in the chamber, repeating the extraction with fresh solvent. The Soxhlet Extraction is usually completed when the solution in the Soxhlet chamber is the same color as the pure solvent.

After extraction, solvent is removed from mixture by vaporization.

4.4.3. Hydro distillation (Julian Martinez et al, 2004)

- Apparatus: Clevenger
- Solvent: water
- Extraction time: 8 h
- Replications: 3
- Weight of each replication: 30 g of dried roots

30 g fraction of Vetiver roots were put into a 1 L flask with 0.5 L of distilled water, the mixture is heated to form the vapor that contains essential oils. The vapor condenses in the condenser and drops into the collecting tube. The essential oil is lighter than water, so it floats on water surface. After extraction completes, water in collecting tube is removed first, then essential oil is collected.

The collected essential oil is dehydrated by Sodium sulphate anhydrous

4.4.4. Steam distillation

- Apparatus: modified Clevenger
- Solvent: water
- Extraction time: 10 h
- Replications: 3
- Weight of each replication: 30 g of dried roots

30 grams of dried roots are loaded into extraction chamber and steam is forced over the material. The hot steam helps to release the aromatic molecules from the plant material since the steam forces open the pockets in which the oils are held in the plant material. The molecules of these volatile oils then escape from the plant material and evaporate into the steam. The steam which then contains the essential oil, is passed through a cooling system (to condense the steam), condensing to a liquid form. Essential oil is then separated from the water (Fatemeh, et al 2004).

The collected essential oil is dehydrated by Sodium sulphate anhydrous.

4.5. Chemical analysis

4.5.1. Gas chromatography (GC) and Gas chromatography – Mass spectrophotemetry (GC-MS)

The constituents of essential oils extracted by SCF, hydro distillation, steam distillation and solvent extraction are qualitatively and quantitatively analyzed by using gas chromatography (GC) and gas chromatography - mass spectrometry (GC-MS). α-vetivone and β-vetivone as well as other constituents are identified by comparison of their GC retention indices with those of literature. The retention indices are determined in relation to a

homologous series of n-alkanes (C_8 – C_{24}) under the same operating conditions. Further identification is made by comparison of their mass spectra from literature. Relative concentrations of components were computed using the normalization method from the GC peak areas without correction factors.

4.5.2. Heavy metal analysis

4.5.2.1. Sample preparation

Soil, leave and root samples: the sample (1 g dry material) is slowly dissolved in 10 ml of concentrated HNO_3 overnight after which the mixture is incubated at 150^0C for 8 h. Then the solution is filtered through Watman filters No. 5 and diluted with double distilled water in 50 volumetric flasks (Zheljazkov et al, 1996).

Oil sample: the oil (05 ml) is reduced to ash at 400^0C for 4 hours. After cooling, 2 ml of HNO_3 is poured over the residue and the mixture was heated for 1 h in the muffle furnace. The procedure is repeated until the acid solution obtained is colourless. Once this is done, solution is transferred into a 10 ml flask with 2 ml of 20% hydrochloric acid. Heavy metal concentrations in the growth media and plant tissues were determined by digesting the tissue and media in concentrated nitric acid (Zarcinas et al, 1987).

4.5.2.2. Determination of heavy metals

The prepared samples are analysed for Pb, Zn and Fe using an Inductive Coupled Plasma Atomic Emission Spectrometer (ICP-AES).

4.6. Purification of α-vetivone and β-vetivone

Essential oil is extracted from 1.5 kg of dried Vetiver root using the optimal operating conditions determined in SCF extraction optimizing experiment.

The extract is analyzed by using GC and GC-MS to determine the relative concentration of α-vetivone and β-vetivone.

Then the extract is subjected to 2^{nd} and 3^{rd} round of SCF extraction with operating conditions based on information from previous experiment in order to remove most of chemical constituents except α-vetivone and β-vetivone from extract or remove α-vetivone together with β-vetivone from extract. There are 3 replications at each round of SCF extraction, each replication uses 5 g of essential oil.

4.7. Confirmation of purified α-vetivone and β-vetivone

The purified α-vetivone and β-vetivone are confirmed by GC-MS and NMR.

4.8. Antioxidant test of purified α-vetivone and β-vetivone

After purification, antioxidant activity of α-vetivone and β-vetivone are tested by using the $DPPH^{\bullet}$ free radical scavenging assay (Kim et al, 2005).

4.9. Statistic analysis

Using Minitab software

V. PUTATIVE WORKING PLAN

Activities	2006				2007				2008				2009			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Literature review																
Chemical analysis training																
Plant material preparation and treatment																
Essential oil extraction																
Chemical analysis																
Purification of α-vetivone and β-vetivone																
Antioxidant test of purified α and β-vetivone																
Plant cultivation																
Statistic analysis																
Writing draft thesis																
Correction of draft thesis																
Submission of thesis																

Note: each year is divided into 4 quarters

VI. EXPECTED RESULTS

1. The optimal operating conditions of supercritical fluid extraction will be determined in order to maximize the Vetiver essential oil yield and α-vetivone and β-vetivone

2. The efficent process of fractionating α-vetivone and β-vetivone will be developed

3. The native Vetiver species could have high yield and high content of α-vetivone and β-vetivone will be identified.

4. Heavy metals in contaminated soils will be determined whether affecting quality and quantity of Vetiver essential oil or not.

VII. REFERENCES

Akhila, A. and Rani, M. 2002. Chemical constitutents and essential oil biogenesis in *Vetiveria zizanioides*. *In* : Maffei, M. ed. Vetiveria. Pp. 73-109, Taylor and Francis, London.

Aruoma, O. I.; Spencer, J. P. E.; Rossi, R.; Aeschbach, R.; Khan, A.; Mahmood, N.; Munoz, A.; Murcia, A.; Butler, J.; Halliwell, B. An evaluation of the antioxidant and antiviral action of extracts of rosemary and provençal herbs. Food Chem. Toxicol. 1996, 34, 449-456.

Briskin, D. P. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant Physiol*. **2000**, *124*, 507-514.

Briskin, D. P. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant Physiol*. **2000**, *124*, 507-514.

Brunner, G. (1994) Gas Extraction: An Introduction to Fundamentals of Supercritical Fluids and the Application to Separation Processes. Steinkop, Darmstadt.

Caredda, A., Maringgiu, B., Porcedda, S., and Soro, C. (2002). Supercritical carbon dioxide extraction and characterization of *Laurus nobilis* essential oils. *Journal of Agricultural and Food Chemistry*, 50, 1492-1496.

Carnesecchi, S.; Langley, K.; Exinger, F.; Gosse, F.; Raul, F. Geraniol, a component of plant essential oils, sensitizes human colonic cancer cells to 5-fluorouracil treatment. *J. Pharmacol. Exp. Ther.* **2002**, *301* (2), 625-630.

Chen, Y., Shen, Z. and Li, X. (2004). The use of vetiver grass (Vetiveria zizanioides) in the phytoremediation of soils contaminated with heavy metals. Applied Geochemistry. 19: 1553–1565.

Chiu, K.K., Ye, Z.H. and Wong, M.H. (2006). Growth of *Vetiveria zizanioides* and *Phragmities australis* on Pb/Zn and Cu mine tailings amended with manure compost and sewage sludge: A greenhouse study. *Bioresource Technology*, 97, 158-170.

Chomchalow, N. 2001. The Utilization of Vetiver as Medicinal and Aromatic Plants with Special Reference to Thailand. PRVN Tech.Bull. No. 2001/1, ORDPB, Bangkok

Courreges, F.; Courreges, M. C. In vitro and in vivo activity of eugenol on human herpesvirus. *Phytother. Res.* **2000**, *14*, 495-500.

Craig, W. J. Health-promoting properties of common herbs. *Am. J. Clin. Nutr*. **1990**, *70* (Suppl.), 491S-499S.

Curtis, S. 1996. Essential Oils: Neal's Yard Remedies. Auriern Press.

Damjanovic, B.; Lepojevic, Zy .; Zy ivkovic, V.; Tolic, A. (2005). Extraction of fennel (Foeniculum Vulgare Mill.) seeds with supercritical CO2: Comparison with hydrodistillation. Food Chem. 9: 143-149.

Dauksas, E., Venskutonis, P.R., Sivik, B., Nillson, T. (2002). Effect of fast CO2 pressure changes on the yield of lovage (Levisticum officinale Koch.) and celery (Apium graveolens L.) extracts, J. Supercrit. Fluids. 22: 20–2101.

Dean, J. R.; Liu, B. (2000). Supercritical fluid extraction of Chinese herbal medicines: Investigation of extraction kinetics. Phytochem. Anal. 11: 1-6.

Demole, E.P., Holzner, G.W., Youssefi, M.J. 1995. Malodor formation in alcoholic perfumes containing vetiveryl acetate and vetiver oil. Perfum. Flav. 20: 35-40.

Dowthwaite, S.V.; and Rajani, S. (2000). Vetiver: Perfumer's liquid gold. *In:* Proceedings of ICV-2 held in Cha-am, Phethchaburi, Thailand, 18-22 Jan. 2000, pp. 478-81.

Esquivel, M.M,. Ribeiro, M.A., Bernardo-Gil, M.G. (1999). Supercritical extraction of savory oil: study of antioxidant activity and extract characterization, J. Supercrit. Fluids 14: 129–138.

Feng Chen, Xi Wang and Hyun-jin Kim (2003). Antioxidant, Anticarcinogenic and Termiticidal Activities of Vetiver Oil. Proceeding of third international Vetiver conference, Guangzhou, China, October 2003

Fuerer, H. 1970. Vetiver oil and vetiver fragrances in modern perfumery. Dragoco Rep.

Grigonis, D., Venskutonis, P.R., Sivik, B. Sandahl, M., Eskilsson, C.S. (2005). Comparison of different extraction techniques for isolation of antioxidants from sweet grass (Hierochloe odorata), J. Supercrit. Fluids. 33: 223–233.

Groom, N. 1992. The Perfume Book. Chapman & Hall, London.

Güllüce, M.; Sökmen, M.; Daferera, D.; Aar, G.; Özkan, H.; Kartal, N.; Polissiou, M.; Sökmen, A.; ahin, F. In vitro antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of Satureja hortensis L. J. *Agric. Food Chem*. 51, 3958-3965.

Hammer, K. A.; Carson, C. F.; Riley, T. V. Antimicrobial activity of essential oils and other plant extracts. J. Appl. Microbiol. 1999, 86, 985-990.

http://grassland.argon.utu.edu.tw

http://www.vetiver.org

http://www.kew.org/data/grasses-db

Hyun-Jin Kim, Feng Chen, Xi Wang, Hau Yin Chung and Zhengyu Jin (2005). Evaluation of Antioxidant Activity of Vetiver (*Vetiveria zizanioides* L.) Oil and Identification of Its Antioxidant Constituents. *J. Agric. Food Chem.,* **53** (20), 7691 -7695

Ibanez, E., Oca, A., Murga, G. D., Sebastian, S. L., Tabera, J., Reglero G. (1999). Supercritical fluid extraction and fractionation of different preprocessed rosemary plants. J. Agric. Food Chem. 47: 1400–1404.

Jain, S. C., Nowicki, S., Eisner, T., and Meinnald, J. 1982. Insect repellents from vetiver oil: 1.Zizanal and epizizanal. Tetra. Let. 23:4639–4642.

Kim H.J., Chen J.,Wang X., Chung H. Y., and Jin Z. (2005). Evaluation of Antioxidant Activity of Vetiver (*Vetiveria zizanioides L.*) Oil and Identification of Its Antioxidant Constituents. *Agric. Food Chem*, 53 (20), 7691 -7695

King, M. B. and Bott, T. R. (1993). Extraction of Natural Products Using Near-Critical Solvents. Blackie Academic & Professional, Glasgow.

Lai, H. Y. and Chen, Z. S. (2004). Effects of EDTA on solubility of cadmium, zinc, and lead and their uptake by rainbow pink and vetiver grass. Chemosphere, 55: 421–430

Lai, H.Y. and Chen Z.S. (2004). Effects of EDTA on solubility of cadmium, zinc, and lead and their uptake by rainbow pink and vetiver grass. *Chemosphere*, 55, 421-430.

Lal, R.K., Sharma, J.R., Naqvi, A.A. anf Misra, H.O.1998. Development of new varities – Dharani, Gulabi, Kesari of vetiver (Vetiveria zizanioides). J. Med. Arom. Plant Sci. 20 : 1067-1070.

Lavania, U.C. (2003). Vetiver Root – Oil and Its Utilization. PRVN Tech. Bull. No. 2003/1, ORDPB, Bangkok.

Louli, V.; Ragoussis, N.; Magoulas, K. (2004). Recovery of phenolic antioxidants from wine industry byproducts. *Biores. Technol. 92:* 201-208.

Loza-Tavera, H. Monoterpenes in essential oils. Biosynthesis and properties. *Adv. Exp. Med. Biol*. **1999**, *464*, 49-62.

Marongiu, B., Porcedda, S., Della Porta, G., Reverchon, E. (2001). Extraction and isolation of Salvia desoleana and Mentha spicata subsp. insularis essential oils by supercritical CO2, Flavour Fragr. J. 16: 384–388.

McHugh, M. A. and Krukonis, V. J. (1986) Supercritical Fluid Extraction, Principles and Practice. Butterworth Stoneham, Boston, MD.

Morris, E.T. 1983. Vetiver : Gift of India. Dragoco Report 6:158-165.

Moyler, D. A. and Heath, H. B. (1988) Liquid carbon dioxide extraction of essential oils. Dev. Food Sci. 18, 41-63.

National Research Council. 1993. Vetiver Grass: A Thin Green Line Against Erosion. National Academy Press, Washington, D.C.

Oszagyan, M., Simandi, B., Sawinsky, J., Kery, A., Lemberkovics, E. and Fekete, J. (1996) Supercritical fluid extraction of volatile compounds from lavandin and thyme. Flavour Fragrance J. 11, 157-165.

Perrut, M. (2004). Supercritical fluid extraction/fractionation: Industrial development and economic issues. In State of the Art Book on Supercritical Fluids; Ainia: Spain, 2004; pp 171-178.

Perrut, M. (2000) Supercritical Fluid applications: Industrial developments and economic issues. Ind. Eng. Chem. Res. 2000, 39, 4531-4535.

Perrut, M. (1991) Les application des fluides supercritiques. In Deuxieme Colloque sur les Fluides Supercritiques, pp. 11-27. INPL, edite par Perrut, Paris.

Poli, F.; Muzzoli, M.; Sacchetti, G.; Tassinato, G.; Lazzarin, R.; Bruni, A. (2003). Antioxidant activity of supercritical CO2 extracts of *Helichrysum italicum*. Pharm. Biol. 41: 379-383.

Ramirez, P., Senorans, F.J. Ibanez, E. Reglero, G. (2004). Separation of rosemary antioxidant compounds by supercritical fluid chromatography on coated packed capillary columns, J. Chromatogr. A 1057: 241–245.

Reddy, L.; Odhav, B.; Bhoola, K. D. Natural products for cancer prevention: a global perspective. *Pharmacol. Ther*. **2003**, *99*, 1-13.

Reddy, L.; Odhav, B.; Bhoola, K. D. Natural products for cancer prevention: a global perspective. *Pharmacol. Ther*. **2003**, *99*, 1-13.

Reinoso, B. D., Moure, A., Dominguez, H. and Parajo, J. C. (2006). Supercritical CO2 extraction and purification of compounds with antioxidant activity. J. Agric. Food Chem **54**: 2441-2469.

Reverchon, E. and Marco, I. D. (2006). Supercritical fluid extraction and fractionation of natural matter. J. of Supercritical Fluids. In press.

Reverchon, E. (1997) Supercritical fluid extraction and fractionation of essential oils and related products. J. Supercrit. Fluids 10, 1-37.

Reverchon, E. and Senatore, F. (1992) Isolation of rosemary oil: comparison between hydrodistillation and supercritical CO2 extraction. Flavour Fragrance J. 7, 227-230.

Roongtanakiat, N., Chairoj, P., 2002. Vetiver grass for remedying soil contaminated with heavy metals. Paper no. 1962. In: Proceedings of the 17th World Congress of Soil Science, 14–21 August 2002, Bangkok, Thailand

Roy, B. C.; Goto, M.; Horise, T. (1996). Extraction of ginger oil with supercritical carbon dioxide: Experiments and modeling. Ind. Eng. Chem. Res. 35: 607-612.

Rozzi, N. L.; Phippen, W.; Simon, J. E.; Singh, R. K. (2002). Supercritical fluid extraction of essential oil components from lemonscented botanicals. Lebensm.-Wiss. Technol. 35, 319-324.

Ryman, D. *Aromatherapy, the Encyclopaedia of Plants and Oils and How They Help You*; PIATKUS Books, Judy Piatkus (Publishers): London, U.K., 1992; pp163-165.

Salim, E. I.; Fukushima, S. Chemopreventive potential of volatile oil from black cumin (*Nigella sativa* L.) seeds against rat colon carcinogenesis. *Nutr. Cancer*. **2003**, *45* (2), 195-202.

Scalia, S.; Giuffreda, L.; Pallado, P. (1999). Analytical and preparative supercritical fluid extraction of chamomile flowers and its comparison with conventional methods. J. Pharm. Biomed. Anal. 21: 549-558.
Sellar, W. 1992. The Directory of Essential Oils. C.W. Daniel Co. Ltd., Great Britain.

Senorans, F.J., Ibanez, E., Cavero, S., Tabera, J., Reglero, G. (2000). Liquid chromatographic-mass spectrometric analysis of supercritical fluid extracts of rosemary plants, J. Chromatogr. A 870: 491–499.

Shealy, C.N. 1998. The Illustrated Encyclopedia of Healing Remedies. Brideg Water Book Co.

Simandi, B., Sawinsky, J., Deak, A., Kemeny, S., Fekete, J., Kery, A., Then, M. and Lemberkovics, E. (1993) Fractionated extraction of essential and fatty oils from spices with carbon dioxide. In Solvent Extraction in the Process Industries, Vol. 2. Proceeding of ISEC '93, ed. D. H. Logsdail and M. J. Slater, pp. 676-683. New York.

Smith, R. M. (1999). Supercritical fluids in separation science—the dreams, the reality and the future, J. Chromatogr. A 856 (1999) 83–115.

Span, R. and Wagner, W. (1996). A new equation of state for carbon dioxide covering the fluid region from the triple-point temperature to 1100K at pressures up to 800MPa, J. Phys. Chem. Ref. Data. 25: 1509–1596.

Stahl, E., Quirin, K.-W. and Gerard, D. (1987) Dense Gases for Extraction and Re®ning. Springer-Verlag, Berlin.

Teja, A. S.; Eckert, C. A (2000). Commentary on supercritical fluids: Research and applications. Ind. Eng. Chem. Res. 39, 4442-4444.

Truong and Baker, 1998 Truong, P.N., Baker, D., 1998. Vetiver Grass System for Environmental Protection. Technical Bulletin No. 1998/1. Pacific Rim Vetiver Network, Bangkok, Thailand.

Truong, P.N., 1999. Vetiver Grass Technology for Mine Rehabilitation. Technical Bulletin No. 1999/2. Pacific Rim Vetiver Network, Bangkok, Thailand.

Vagi, E., Simandi, B., Daood, H.G., Deak, A., Sawinsky, J. (2002). Recovery of pigments from Origanum majorana L. by extraction with supercritical carbon dioxide, J. Agric. Food Chem. 50: 2297–2301.

Vilegas, J. H. Y.; De Marchi, E.; Lancas, F. M. (1997). Extraction of low-polarity compounds (with emphasis on coumarin and kaurenoic acid) from *Mikania glomerata* ("guaco") leaves. Phytochem. Anal. 8: 266-270.

Wargovich, M. J.; Woods, C.; Hollis, D. M.; Zander, M. E. Herbals, cancer prevention and health. Presented at the American Institute for Cancer Research 11th Annual Research Conference on diet, nutrition and cancer. *J. Nutr*. **2001**, *131*, 3034S-3036S.

Watson, L. and Dallwitz., M.J. (1989). *Grass Genera of the World*, (with microfiche and data disks). Australian National University Printing Service, Canberra.

Wilde, E.W., Brigmon, R.L., Dunn, D.L., Heitkamp, Dagnan, D.C. (2005). Phytoextraction of lead from firing range soil by Vetiver grass. Chemosphere, 61, 1451-1457.

Wilson, R. 1995. Aromatherapy for Vibrant Health and Beauty. Penguin Putnam Inc., New York.

Xia, H.P. (2004). Ecological rehabilitation and phytoremediation with four grasses in oil shale mined land. *Chemosphere*, 54, 345-353.
Yang, B., Shu, W.S., Ye, Z.H., Lan, C.Y. and Wong, M.H. (2003). Growth and metal accumulation in vetiver and two Sesbania species on lead/zinc mine tailings. *Chemosphere*, 52, 1593-1600.

Zheljazkov, V., Nielsen, N.E. (1996). Studies on the effect of heavy metals (Cd, Pb, Cu, Mn, Zn and Fe) uopn the growth, productivity and quality of lavender (*Lavandula angustifolia* Mill) production. Journal of essential oil research. 8, 259-274.

Zhu, B. C. R., Henderson, G., Chen, F., Fei, H. and Lain, R. A. (2001 b). Evaluation of Vetiver oil and seven insect-active essential oils against the formosan subterranean termite. J. Chem. Ecol. 27: 1617-1625.

Zhu, B. C. R., Henderson, G., Chen, F., Maistrello, L., and Laine, R. A. (2001 a). Nootkatone is a repellent for Formosan subterranean termite (Coptotermes formosanus). J. Chem. Ecol. 27:523–531.

Zhu, Q. Y.; Hackman, R. M.; Ensunsa, J. L.; Holt, R. R.; Keen, C. L. Antioxidative activities of oolong tea. *J. Agric. Food Chem.* **2002**, *50*, 6929-6934.