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Environmentally Benign Extraction Processes in Analytical Separation of Essential Oils

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ENVIRONMENTALLY BENIGN EXTRACTION PROCESSES IN ANALYTICAL SEPARATION OF ESSENTIAL OILS

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the doctor of philosophy in chemistry degree and is acceptable for the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This dissertation is dedicated to my wife Florence Mukami, my daughter Alicia Wanjiru Mburu who have been my inspiration and dealt with my absence during my graduate studies. My parents Joseph Kiratu and Alice Kiratu, who have given me the opportunity of an education and support throughout my life. My siblings Naomi, Mary, Daniel, Gideon, Loise, and Solomon for their moral support and encouragement.

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ABBREVIATIONS

ANOVA: Analysis of variance

ASES: Aerosol solvent extraction system

BBD: Box-Behnken design

CCD: Central-composite design

CN: *Chrysothamnus nauseousus*

DOE: Design of experiment

GC-MS: Gas chromatography-mass spectrometry

HD: Hydro distillled

KA: Kangari

KIBW: Kibwezi

m/z: Mass-to-charge ratio

MAE: Microwave assisted exraction

MS: Media of square mean

NIST: National institute of standards and technology

NJA: Njabini

P-B: Plackett-Burman

P_c: Critical pressure

PGSS: Particles from gas-saturated solutions

ppm: parts per million

PSE: Pressure solvent extraction

Res III: Resolution three

RESS: Rapid expansion of supercritical solutions

RSM: Response surface methodology

SAS: Supercritical antisolvent

SC-CO_2: Supercritical carbon dioxide

SEDS: Solution-enhansed dispersion by supercritical fluids

SF: Supercritical fluid

SFE: Supercritical fluid extraction

SFE-GC: Supercritical fluid extraction-Gas chromatography

SFE-SFC: Supercritical fluid extraction-Supercritical fluid chromatography

SFE-SPE-GC: Supercritical fluid extraction-Solid phase extraction-Gas chromatography

SFs: Supercritical fluids

T_c: Critical temperature

UAE: Ultra assisted extraction

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ABSTRACT

ENVIRONMENTALLY BENIGN EXTRACTION PROCESSES IN ANALYTICAL SEPARATION OF ESSENTIAL OILS

JOHN KIRATU

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Supercritical fluid extraction (SFE) technology has been well received as an environmentally friendly processing technique. Over the last two decades, its use in many processing industries has tremendously advanced. This is as a result of pressure from regulating bodies aimed at reducing the wide-scale use of organic solvents due to negative environmental impacts. Supercritical carbon dioxide (SC-CO_2) is considered to be environmentally benign and has been used in the development of a wide-range of alternative processes in various industries to totally or partially eliminate the use of organic solvents. Conventional processes for essential oil extraction involve steam distillation and organic solvent extraction. Steam distillation involves high heat, which can cause sample hydrolysis and thermal degradation of heat-sensitive compounds, whereas in organic solvent extraction, polluting solvents and expensive post-processing of the extract for solvent elimination is involved.

SFE can be divided into two major stages, the extraction of the analyte of interest from the bulk matrix and the collection of the analyte. There has been a lot of research on the optimization of analyte extraction. However, researchers have largely ignored the collection stage. To achieve high analyte recovery and extraction efficiency in SFE, the extraction step and subsequent collection step should be considered integrated.

This dissertation focuses on a comprehensive study, using the response-surface methodology experimental design approach, of the collection of volatile compounds following supercritical fluid extraction (SFE) and application to the extraction of essential oils from selected plants found in the Great Plains region which are of interest to ethnobotany colleagues.

Parameters that influence the collection of the extract after SFE by trapping with a small volume of an organic solvent were investigated. Time, depressurization flow rate, cooling temperature, solvent type, and analyte type were found to be the most important factors affecting trapping. The optimal collection conditions for the three solvents considered in the study were isopropanol (25.58 min, 2.07 ^{o}C, and 0.3 L/min), acetonitrile (28.30 min, -8.20 ^{o}C, and 0.3 L/min), and dichloromethane (26.8 min, 3.21 ^{o}C, and 0.3 L/min). The amount of solvent was found to be significant in less viscous solvents and insignificant in viscous solvents. Cooling position and restrictor position were found to be insignificant.

In the extraction of essential oils from *Chrysothamnus nauseosus* (rabbit brush), *Rhus aromatic* (skunk brush), and *Matricaria chamomilla* L (chamomile), pressure, time, and temperature were found to be the most significant extraction parameters. In *Chrysothamnus nauseous* (rabbit brush) the major compounds identified by GC-MS were limonene (35.77%), trans-β-ocimene (27.41), camphor (11.57%), β-phellandrene (4.64%), β-pinene (4.13%), eucalyptol (2.20%), β-cis-ocimene (2.66%), camphene (1.96%), and artemiseole (1.61%). In *Rhus aromatic* (skunk brush) the main compounds were limonene (20.48%), linalool (37.31%), caryophyllene (12.5%) eucalyptol (9.14%), α-phellandrene (5.5%), and geraniol (1.2%). In chamomile samples from three different

regions in Kenya were α-bisabolol, α-bisabolol oxide B, α-bisabolol oxide B, matricine, dicycloether, and β-cis-farnesene. The optimal extraction conditions (temperature, pressure and time) for chamomile, rabbit brush, and skunk brush oils were (47 ^{o}C, 6620 psi, 45 min), (37 ^{o}C, 1720 psi, 43 min), and (35 ^{o}C, 3570 psi, 40 min) respectively. Selected major essential oils identified in the different samples were quantified. α - Bisabolol concentrations in Kangari, Kibwezi, and Njabini chamomile sample were 1.03±0.006 mg/g, 0.759±0.092 mg/g, and 0.90±0.011 mg/g respectively. Limonene and camphor concentrations in rabbit brush were 2.052±0.020 mg/g and 0.652±0.010 mg/g respectively. Limonene, linalool, and caryophyllene concentrations in skunk brush were 1.448±0.027 mg/g, 2.28±0.014 mg/g, and 0.956±0.018 mg/g.

1 CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 INTRODUCTION

The Pollution Prevention Act of 1990 established a new policy for environmental protection.[1] Its focus was on reducing the amount of pollution at the source through cost-effective changes in production, operation, and raw-materials use. Source reduction is more desirable than waste management or pollution control, as was shown by the studies involving 14 chemical plants.[2] It showed that plants were able to save $21.8 million from source-reduction activities. Source reduction refers to practices that reduce hazardous substances from being generated. These practices may incorporate technology or equipment modification, process or procedure modification, reformulation or redesign of products, substitution of raw materials, and improvement in housekeeping, maintenance, training, or inventory control. It also includes the practices that increase efficiency in the use of energy, water, or other natural resources.

The concept of green chemistry was developed as one of the initiatives of the Pollution Prevention Act of 1990. It entails the design of chemical products and processes that reduces or eliminates the use or generation of hazardous substances. Anastas and Warner originally published the 12 Principles of Green Chemistry in 1998,[3] to provide a roadmap for scientists and engineers in designing new materials, products, processes, and systems to achieve sustainability. Although it is not realistic to apply all 12 principles at the same time, as many principles as possible should be accommodated to realize the full benefit. Tradeoffs are made in order to match the cost involved compared to the returns.

Separation of substances is a key step in many chemical production and it is indispensable for qualitative and quantitative analysis. Extraction of natural products from plants dates back at least 5000 years to the Sumerians.[4] Plant products have been used for centuries for medicinally beneficial purposes. Essential oils are a complex liquid mixture consisting of volatile hydrocarbon compounds, which define the essence of the plant. They are widely used as raw materials in many industries, including pharmaceutical, food, perfumery, aromatherapy, and cosmetic, among others.[5] Traditionally, essential oils have been extracted by hydro distillation, soxhlet extraction, percolation, turbo-extraction (high-speed mixing), and sonication. These techniques are time-consuming, energy inefficient and require relatively large quantities of polluting solvents.[6] In relation to green chemistry, green extraction of natural product has been based on discovery of extraction processes which reduce energy consumption, allow use of alternative solvents, and ensure a safe and high quality extract. To that respect, nonconventional extraction techniques, which are fast, energy efficient, and use minimally polluting solvents, have been developed. These techniques include microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized-solvent extraction (PSE), and supercritical fluid extraction (SFE). The main advantages of the MAE, PSE, and UAE are the large reduction of extraction time, higher yields, improved selectivity, higher stability, and organoleptic quality of the extract. Apart from the innovative extraction techniques, there has been a lot of research to develop alternate solvents to replace organic solvents.[7-11] The alternative solvents suitable for green chemistry are those that have low toxicity, are inert, easy to recycle, nonflammable, cheap, and do not contaminate the product.[12] There is no perfect green

solvent that can apply to all situations and therefore decisions have to be made. Among the alternative solvents considered, supercritical fluids are the most widely sought solvent.

1.2 BACKGROUND

1.2.1 Supercritical Fluids

In the phase diagram, Figure 1.1, three phases can be distinguished at the triple point. As the temperature and pressure is increased, the liquid becomes less dense due to thermal expansion and the vapor becomes denser due to increasing pressure. This causes the phase to be less distinguishable and eventually the density of the two phases become identical and the distinction between them disappears due to the establishment of dynamic equilibrium. This point is known as the critical point and the new phase is called the supercritical fluid phase. The temperature and pressure at this point are referred as critical temperature (T_c) and critical pressure (P_c).

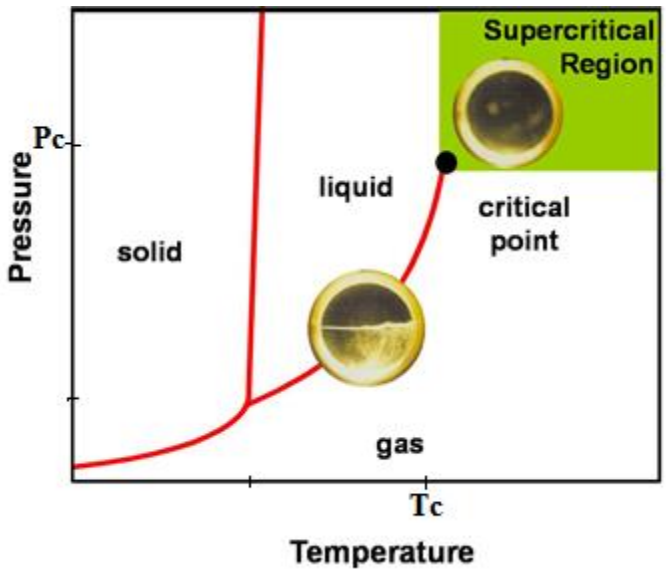

Figure 1.1. Phase diagram illustrating the formation of supercritical phase. As the pressure and temperature increases the boundary between liquid and gas disappear, and supercritical phase is reached beyond critical pressure and critical temperature (Pc, Tc).[9]

The occurrence of the supercritical phase was first reported by Baron Cagniard de la Tour in 1822[13]. He visually observed that the gas-liquid boundary disappeared when liquid ethanol was heated inside a sealed gun barrel.

In recent years, there has been an increased interest of supercritical fluids due to their versatility for application in various fields. Supercritical fluids exhibit a dual characteristic. The motion of fluid molecules resembles that of gas while, on the other hand, dissolving power is similar to that of a liquid. According to the empirical correlation developed by Chrastil, Equation 1.1[14], solubility of a solute in a solvent is related to density and temperature

$$\boldsymbol{s = \rho^{a} \exp[\left(\frac{b}{T}\right) + c]} \qquad \textbf{(1.1)}$$

Where, *s* is solute solubility, ρ is solvent density, T is absolute temperature, and *a, b,* and *c* are correlating parameters calculated by regression from experimental data. *a* is an

association number of the solvato-complex formed between solute and SCF, *b* is a function of the enthalpy of solvation and enthalpy of vaporization, and *c* is a function of association number and molecular weights of the solute and supercritical fluid. The suitability of using supercritical fluids (SFs) as an extraction solvent is connected to the density and the possibility of varying density, which renders different solvating powers. Some of examples of substances used as supercritical solvents and their critical temperature and pressure are given in Table 1.1.

Table 1.1. Examples of substances used as supercritical solvents and their corresponding critical temperature and pressure.[7, 15]

Gases	**Critical Temperature (K)**	**Critical Pressure (MPa)**
Carbon dioxide	304.17	7.38
Fluoroform	298.85	4.82
Ethane	305.34	4.87
Methane	190.55	4.59
Ethylene	282.35	5.04
Propane	369.85	4.24
Nitrous oxide	309.15	7.28
Acetylene	308.70	6.24
Ammonia	405.5	11.3
Water	647.10	22.06
Argon	150.66	4.86
Xenon	289.70	5.87

Many of the fluids listed in Table 1.1 would not be suitable for practical extractions due to their unfavorable physical properties, costs, or reactivity. For instance, ethylene has a sub ambient critical temperature. However, its flammability limits its

application. Most polar fluids have high critical temperatures, which can be destructive to both the analyte and the extraction system. Nitrous oxide is considered as an isoelectronic analog of carbon dioxide. However, it exhibits a high reactivity towards many compounds and can cause physiological effects.[16] Fluoroform has the ability to solubilize solutes through intermolecular hydrogen bonding in the supercritical state[16], but its high cost limits its use for SFE. Carbon dioxide is the most commonly used supercritical fluid in industry[17] due to its immediate advantages discussed below.

1.2.1.1 Major Advantages of SFE

Due to the unique properties of SFs, SFE is regarded as a promising alternative technique to conventional extraction methods. Some of its major advantages are: (i) SFs have dual characteristics, where the fluid properties lie between those of gas and liquids. It has density similar to that of liquids and have viscosities and diffusivities that are closer to that of gases. Thus, SFs can diffuse faster into a solid matrix than liquids and yet possess solvent strength similar to that of a liquid. SFs diffusivity is $\sim 10^{-4}$ cm^2 s^{-1} while of a liquid is $\sim 10^{-5}$ cm^2 s^{-1} therefore, penetration into solid material is more effective than with liquid solvents. This renders much faster mass transfer, resulting in faster extractions.[18] It is possible to reduce extraction time from hours or days using liquid-solid (L-S) extraction to minutes using SFE.[19, 20] (ii) Selectivity can be achieved by controlling solvation power of the fluid through manipulation of temperature and pressure. For example, Song et al., were able to selectively extract vindoline from among 100 alkaloid compounds from the leaves of *Catharanthus roseus*.[21] (iii) Extract recovery is easy as it is achieved by depressurization, allowing the supercritical fluid to return to the gas phase, leaving no or little solvent residue. This eliminates post-extraction processes, which are

costly and time consuming and often results in loss of volatile components.[22] (iv) The fluid flows continuously during dynamic extraction, hence fresh fluid is always available resulting in complete extraction.[23] (v) Small sample size can be used. Typically, 20-100 g of sample is needed for L-S methods while as little as 0.5-1.5 g is needed for SFE method.[18] It has been demonstrated that from only 1.5 g of plant samples, 100 volatile and semi-volatile compounds were extracted and quantified by gas chromatography (GC).[20] (vi) Compared with L-S methods, which requires tens to hundreds of milliliters of organic solvent, SFE requires no or significantly less organic solvents which are not environment benign.[20, 23, 24] (vii) The operating temperature can be low, therefore undesired reactions such as hydrolysis, oxidation, and degradation can be avoided. This makes it desirable for extraction of thermally labile compounds. [25] (vii) Coupling with chromatographic method is possible, minimizing loss of highly volatile compounds.[26] (ix) In large-scale supercritical CO_2 applications, the solvent can be recycled, minimizing waste generation. (x) SFE provides a well-defined extraction process and mechanisms making it easier to quantitatively assess and evaluate. The process can be then optimized accordingly.[11]

1.2.2 Supercritical Carbon Dioxide (SC-CO_2)

Carbon dioxide is the most commonly used supercritical fluid. It is an ideal supercritical fluid as it is environmentally benign. It has a low critical temperature (31.1°C) and pressure (72.8 atm). This low critical temperature enables extraction to be carried out at comparative low temperature (often as low as 40-50°C), decreasing the risk of damaging of thermally labile compounds. It is nontoxic and nonflammable, so its use in a laboratory environment can eliminate the cost problem associated with solvent

disposal, as well as long-term exposure of personnel to potential toxic vapors. It is cheap and readily available, about 40 percent of CO_2 is sourced from ethanol plants.[27] Therefore no additional greenhouse effect results, as it is already present in the environment system and its use as an extracting solvent does not cause any further increase CO_2 in the atmosphere. It is inert, it is gaseous at room temperature and therefore easily removed, it can be recycled when used in large scale, it does not leave any solvent residue making it desirable for extracting natural flavors, fatty oils, essential oils, and anti-oxidants to be used in products for human consumption. The main drawback of SC-CO_2 is its low polarity, this problem can be overcome by employing polar modifiers (co-solvents) to change the polarity and increase solvating power towards polar analytes.[28]

1.2.2.1 Solubility in Supercritical Carbon Dioxide

Solubility has a direct impact on the rate, yield, design, and economy of the process. It is therefore considered as the most vital criterion that dictates the efficacy of most of supercritical fluid processes. Either high solubility or low solubility is desired depending on the process of interest. For example, in supercritical extraction, high solubility is desired conversely, low solubility is desired in supercritical anti-solvent precipitation processes to manufacture particles. The variation of solvent strength can be described in terms of density parameter as described by modified version of the Hildebrand solubility equation 1.2. [29]

$$\boldsymbol{\delta = 1.25 P_c^{1/2} (\rho_{sf}/\rho_l)} \qquad \mathbf{1.2}$$

It relates the solvent strength (Hildebrand parameter, δ) of the reduced density of the supercritical fluid (ρ_{sf}) relative to the reduced density of the fluid in its liquid state (ρ_l)

and the critical pressure of the fluid (P_c). Maximum solubility is achieved when SFs density approaches that of target analyte.

Carbon dioxide (O=C=O) is a nonpolar, but polarizable, molecule. However, it has small polarity due to the presence of quadrupole moment.[30, 31] It can dissolve non-polar and slightly polar compounds. Its solvent power for low molecular-weight compounds is high and it decreases with increasing molecular weight. It has high affinity with oxygenated organic compounds of medium molecular weight.[31] The solubility increases with increasing pressure at fixed temperature due to the greater attractive forces between the solute and carbon dioxide, hence enhancing solvation.

1.2.3 SC-CO_2 Applications

1.2.3.1 Food Industry Applications

Besides increasing environmental concerns and government measures, consumer health consciousness has increased. This has been one of the major driving forces for manufacturers to adopt green technology in food processing. SC-CO_2 has been widely used in refining, adding value to byproducts, extraction of bioactive compounds, extrusion processes, and fractionation and purification of food products. The major advantages for its application in food industry is nontoxicity, no residual solvent, minimal coextraction of natural antioxidants, and, hence, better shelf life of products, no thermal degradation with minimal effect on nutritional value, and cost effective due to fewer processing steps.

SC-CO_2 has been used in edible-oil refining whereby undesirable compounds have been selectively removed without the loss of valuable compounds. For instance, in refining of wheat germ oil, SC-CO_2 was used and the extracted oil had a higher

tocopherol (vitamin E) content than that of commercial hexane extraction.[32] It has been used in refining of green coffee oil obtained by mechanical pressing.[9] Caffeine, chlorogenic acid and waxes were removed without affecting triglyceride content. Free fatty acids were removed from rice bran oil with 97.8% efficiency using SC-CO_2.[33] It has also been used in the selective removal of caffeine from green tea while avoiding the extraction of antioxidants.[34] SC-CO_2 has been widely used in adding value to byproducts in the food industry by removal of valuable compounds. Some of the examples are the removal of polyphenols from wine lees, which is a byproduct of wine production,[35] extraction of phenolic compounds from pomegranate seeds and buckthorn pomace, which are byproducts of juice production,[36] and extraction of carotenoids from tomato skins, which are byproducts of tomato processing.[35] It has also been used in getting fractions of omega-3-enriched fish oils from fish byproducts.[37] SC-CO_2 has also been used in producing a range of puffed food products like pasta, ready-to-eat cereals and confectionery with improved texture, color and taste.[38]

1.2.3.2 Pharmaceutics Industry Applications

In recent decades, there has been an increase in the application of supercritical fluid technology in the pharmaceutical industry. This is as a result of a continuous effort of pharmaceutical industry to move from the use of potentially harmful solvents to environmentally friendly processes. The main use of SC-CO_2 is in drug extraction and analysis, drug particle and polymorph engineering, purification and recrystallization, coating, micronization and preparation of drug delivery systems, and conversion of highly brittle crystalline incipients to amorphous.[39] There are several SC-CO_2 particle formation processes, which include rapid expansion of supercritical solutions (RESS),

supercritical anti-solvent (SAS) precipitation, aerosol solvent extraction system (ASES), solution-enhanced dispersion by supercritical fluids (SEDS), and particles from gas-saturated solutions (PGSS).[39] RESS involves atomizing a product solution in a supercritical fluid into a low-pressure vessel to produce polymeric microparticles.[40] In SAS SC-CO_2 is used as anti-solvent to cause precipitation of the substrate dissolved in a liquid solvent. ASES involves spraying of a solution through atomization nozzle into compressed carbon dioxide. Dissolution of SC-CO_2 into liquid droplets causes large volume expansion and supersaturation within the liquid mixture resulting in the formation of small, uniform particles.[41] SC-CO_2 has been used in coating. Souto et al used SFC to develop a microparticle coated with bovine serum albumin.[42]

1.2.3.3 Natural Product Applications

In recent decades, there has been enhanced concern for the quality and safety of foods and medicine, and there has been a strict regulation on nutritive and toxicity levels. Also, there has been consumer preference of natural, as opposed to synthetic, substances. Natural products have been a focus of many researchers due to their rich source of bioactive compounds with a range of potential applications mainly in the food, pharmaceutical, and cosmetic industries. Since these active compounds are usually present in low concentration, research has been aimed at developing more effective and selective extraction methods for recovery of these compounds, which also comply with regulations on the use of hazardous and toxic solvents. Traditionally used methods such as steam distillation and solvent extraction have few adjustable parameters to control to achieve selectivity, high energy cost, and hazardous solvent usage among other disadvantages. Therefore, there has been a need to develop alternative techniques. SFE

with CO_2 has been considered as an alternative extraction technique, which is more selective, efficient and environmental friendly than conventional methods.

Lipids from plants and animals have been extracted both on a commercial scale and in laboratory analysis. Higher selectivity has been achieved using SFE, where valuable minor substances such as tocopherols and carotenes accompanying the oil have been selectively extracted.[43] Some lipids, like glycolipids and phospholipids, are not easily extracted and require a solvent modifier like methanol. SFE give cleaner extracts with less minerals and proteins. Comparable results were obtained for SFE-CO_2 versus dichloromethane soxhlet for lanolin extraction from wool, but the product for SFE-CO_2 was cleaner.[44]

Essential oil extraction via SFE is probably the area that has received most attention in recent years. Comparing the essential oils obtained from other traditional extraction methods, the extract from SFE-CO_2 is superior and is less costly. The composition and odor of SFE extracts is different as compared to extract of steam distillation.[7, 8] SFE removes monoterpenes and sesquiterpenes together as compared to extract of steam distillation, which extracts monoterpenes but leaves most sesquiterpenes.[45] Higher yields are achieved due to the absence of hydrolysis. SF-CO_2 extraction studies of essential oils from lavender showed three times higher linalyl acetate content as compared to steam distillate, presumably due to hydrolysis.[46]

1.2.3.4 Additional Applications

SC-CO_2 has been used as a blowing agent in polymer foaming, replacing the hazardous chlorofluorocarbons, hydro chlorofluorocarbons and volatile organic solvents traditionally used.[47] It has also been used in textile dyeing and cleaning processes. It has

been used in chemical and biochemical reactions as a solvent, replacing volatile organic solvents. The same desirable qualities exploited in extraction makes SF solvents a superior medium for chemical reactions offering higher selectivity and higher reaction rates.

1.2.4 Supercritical Fluid Extraction (SFE)

Supercritical extraction has been well studied and models explaining the thermodynamic, kinetic behavior, and effect of processing parameters, like temperature and pressure, have been formulated.[48] The extraction process can be roughly divided into three steps. The first step is the release of the analytes from the sample matrix into the supercritical fluid. This process depends on mass transfer kinetics, solubility and the analyte/matrix interactions. The second step is sweeping of the analyte from the vessel to the collection system, and the last step is the collection of the analyte by depressurizing the supercritical fluid into collection device. All the three steps are equally important and should be considered as integrated.

1.2.4.1 Extraction Mechanism

The SFE process occurs as a continuous process, which can be separated into four main steps.[49] SC-CO_2 diffuses into the solid sample matrix to reach the analyte. It adsorbs to the particle surface to form an external fluid film around the solid particles via solvent-solid interaction. The analyte is then released reversibly from the matrix and dissolved into SC-CO_2. The dissolved analyte diffuses to the edge of the sample particle, and then bulk SC-CO_2 solvates the analyte for final removal. These processes are similar to conventional extraction including reversible adsorption/desorption processes that involve mass transport operations between solid and fluids. Equations governing the SFE process

include differential mass balance for the solute, equation 1.3, which describes the transport of solute in a fluid flowing through continuous contact, and kinetic equations, equation 1.3-1.5 that describe the rate of solute transfer between two phases.[48, 50]

$$\frac{\partial c}{\partial t} + U\frac{\partial c}{\partial x} - D_a\frac{\partial^2 c}{\partial x^2} = -\frac{(1-\varepsilon)}{\varepsilon}aN_A \qquad \mathbf{1.3}$$

Where c is the concentration of analyte in the bulk of SF phase, U is the superficial velocity of SF through the vessel, D_a is the axial dispersion coefficient, x is the linear position in the vessel measured from SF inlet, ε is the volume fraction of SF in the vessel, t is the time, a is the specific surface area of the solid, and N_A is the flux of analyte towards SF.

The conventional mass transfer of the analyte from the interface with concentration C_i into the bulk of SF with concentration C, referred as external mass transfer, can be described by equation 1.4,

$$N_A = K_C(C_i - C) \quad \mathbf{1.4}$$

where K_C is the mass transfer coefficient.

The diffusion of the analyte inside the solid particle, referred to as internal mass transfer, can be described as the mass fraction q_s of the analyte in the solid at the interface with SF, which is in equilibrium with C_i when the desorption kinetic is negligible and mass fraction of analyte in the solid q, equation 1.5

$$N_A = K_s(q - q_s) \quad \mathbf{1.5}$$

The analyte desorption kinetics that involve dissolution of the analyte from the solid at the solid-SF interface can be described by, equation 1.6,

$$N_A = k_d q - k_a c\left(1 - \frac{q}{q_{max}}\right) \quad \mathbf{1.6}$$

where k_d and k_a are desorption and adsorption coefficients, respectively and are dependent on temperature and molecular energy. The rate of desorption onto the solid surface is proportional to the rate of molecular collision with the surface, which is proportional to the analyte concentration (c) in the SF phase. q_{max} is surface capacity, and q is mass fraction of analyte in the solid.

1.2.4.2 Extraction Profile

Mathematical models for SFE that are based on a heat transfer analogy, differential mass balance equation, and empirical models have been developed to explain the extraction mechanism. Some of these models include the hot-ball diffusion model, broken and intact cell model, shrinking core mode, and other models.[51] The extraction profile of analyte from solid matrix can be divided into three regions as illustrated in Figure 1.2.[34, 43, 48, 51]

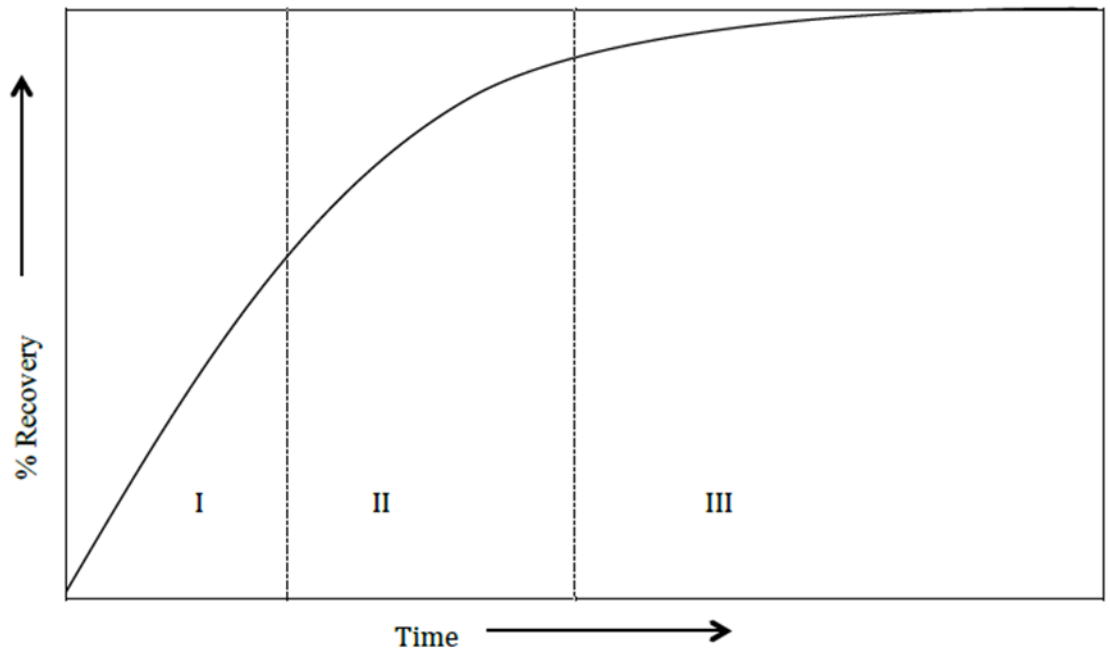

Figure 1.2. Dynamic extraction profile an analyte from a solid matrix.

The first region represents the initial stage of extraction where analytes adsorbed on the surface of the solid matrix are dissolved into the SF. In this region, solubility is the limiting factor and the process is a simple partitioning of the solute in a suitable solvent governed by quasi-equilibrium conditions. The initial extraction occurs rapidly as indicated by a steep slope. Factors that lead to efficient extraction in this region are high solubility of analyte, which can be enhanced by temperature, high flow of the SF, and minimal amount of dead volume in the extraction vessel. The second region illustrates the transition from the solubility-limited region to the diffusion-limited region. The rate of extraction is slower as the process is enthalpically controlled, where analyte-matrix interactions must be disrupted. In the third region, the diffusion-limited mobility of the particles from one phase to another is the major controlling factor. The lower rate is characterized by limited mobility of the analyte within the matrix and access of SF to the target analyte.

1.2.5 Parameters Governing Extraction

The extraction depends on the analyte solubility in the extraction SF, analyte-matrix interaction, analyte location within the matrix, and porosity of the matrix. Any thermodynamic, kinetic and physical parameters that can affect the above parameters influence the extraction.[23, 36, 52-54]

1.2.5.1 Effect of Pressure

The amount of solute that can be dissolved in a unit volume of SCO_2, solvent capacity, is a function of pressure related to the Hildebrand parameter.[55] Increasing pressure at a given temperature increases the density of SCO_2, increasing the amount of solute that can be dissolved in a unit volume of SCO_2. Pressure increases lead to a

decrease in intermolecular mean distance. Therefore, the specific interaction between the solute and the solvent molecules are increased, leading to higher solubility.[56] The fluid pressure is the main parameter that influences the extraction efficiency.[23] For a complex matrix, higher pressure is undesirable as selectivity is lost, owing to the presence of coextracted solutes due to higher solubility.

1.2.5.2 Effect of Temperature

The effect of temperature is difficult to predict, as it affects not only the density of the SC-CO_2, but also relates to the vapor pressure of the solute. Therefore, the impact of temperature on solubility of SC-CO_2 depends on both effects.[23], [52] The temperature effect is pressure dependent. At lower pressure an increase in temperature usually leads to a decrease in solubility. This is due to the stronger effect on the density of SC-CO_2. Increasing temperature leads to decrease of fluid density, which decreases the fluid solvent power and solubility.[57] At higher pressures an increase in temperature leads to increased solubility. This due to the effect of temperature on vapor pressure prevailing. The pressure at which the retrograde behavior is observed is referred to as the crossover pressure. Temperature effects viscosity and surface tension and this effect depends on the nature of the sample. For nonvolatile solutes, higher temperature results in lower recoveries owing to the decreased solubility. On the other hand, extraction of volatile solutes depends on competition between their solubility in CO_2 and their volatility. Depending on the pressure, temperature may cause increase, decrease, or have no effect on the SFE.[36, 57]

1.2.5.3 Effect of Modifier

SC-CO_2 is considered a nonpolar solvent. However, it has small polarity due to the presence of a quadrupole moment. It can dissolve nonpolar and slightly polar compounds. Its solvent power for low molecular-weight compounds is high and decreases with increasing molecular weight.[53] It has high affinity with oxygenated organic compounds of medium molecular weight. To widen the solubility range of SCO_2, co-solvents known as modifiers are used. Modifiers are added to adjust the polarity of SCO_2, hence enhancing the solubility of polar analytes.[23, 52] Modifiers can be introduced during extraction by pumping in modified CO_2 or by injecting the modifier liquid before extraction. The modifier of choice depends on the nature of analyte of interest. Methanol and ethanol are the most widely used modifiers.[23]

1.2.5.4 Effect of Flow Rate

The extraction efficiency is related to the speed of the SC-CO_2 flowing through the cell. Slow flow rates have been found to result to higher analyte recoveries.[58, 59] The lower the fluid velocity, the greater the contact time, facilitating partitioning and penetration of solvent into the matrix. However this is at expense of longer extraction time. Minimal time is realized during higher flow rates, but higher solvent volume is used. Equilibrium is hardly achieved, hence low recoveries. The solvent flow rate determines the amount of solvent to be used, total extraction time, and quality of the extract.[10] When choosing the best flow rate, time and solvent, cost should be considered apart from achieving higher analyte recoveries.

1.2.5.5 Effect of Particle Size

Particle size affects the extraction kinetics. Smaller particles create more surface area and a shorter diffusion path, which enhances the mass transfer.[60] The mass transfer depends on the location of the analyte in the matrix particle. When the analyte is on the surface of the particle, it is easily accessible and the limiting factor will be solubility.[54, 60] If it is embedded inside the particle, the solvent has to penetrate into the particle to access and dissolve the analyte. In this case particle size becomes very important and smaller particle size, which can be achieved by grinding, facilitates the exposure.

1.2.5.6 Effect of Time

Dynamic SFE

Dynamic SFE involves flushing the sample continuously with supercritical fluid. This technique is mostly used in both offline and online methods, where the aim is to exhaustively extract the analyte from the matrix. During the extraction, the sample is continuously swept with fresh SF. Selectivity can be achieved in dynamic SFE by changing the extraction parameters (pressure and temperature), which affect the density of the fluid.[61] Modifiers or derivatization reagent can also be introduced prior to flushing of the fluid to further enhance solubility toward the desired analyte.[54]

Static SFE

Static SFE involves pumping a fixed amount of supercritical fluid into an extraction vessel containing the sample. The SF is allowed to interact with the sample for a particular amount of time, and then the cell is decompressed into the trap. Since a fixed amount of SF is used, static SFE may not be exhaustive extraction technique. It is useful for solute solubility studies and studies of the effect of modifiers and derivatization since

known volumes can be directly added to the extraction cell. It rarely used for total extraction unless is combined with dynamic SFE. Extraction can also be done in a combined mode whereby a static extraction is performed for some period, followed by a dynamic extraction.[23]

1.2.6 Essential Oils

Essential oils are concentrated hydrophobic aroma compounds from plants. They are found in the bark, stems, roots, flowers, seeds, and other parts of plants. They usually consist of hydrocarbons, which exclusively consist of terpenes (monoterpenes, sesquiterpenes and diterpenes) and oxygenated compounds, which are mainly phenols, alcohols, oxides, ketones, esters, and aldehydes. Figure 1.3 illustrates different essential oils groups with selected examples.

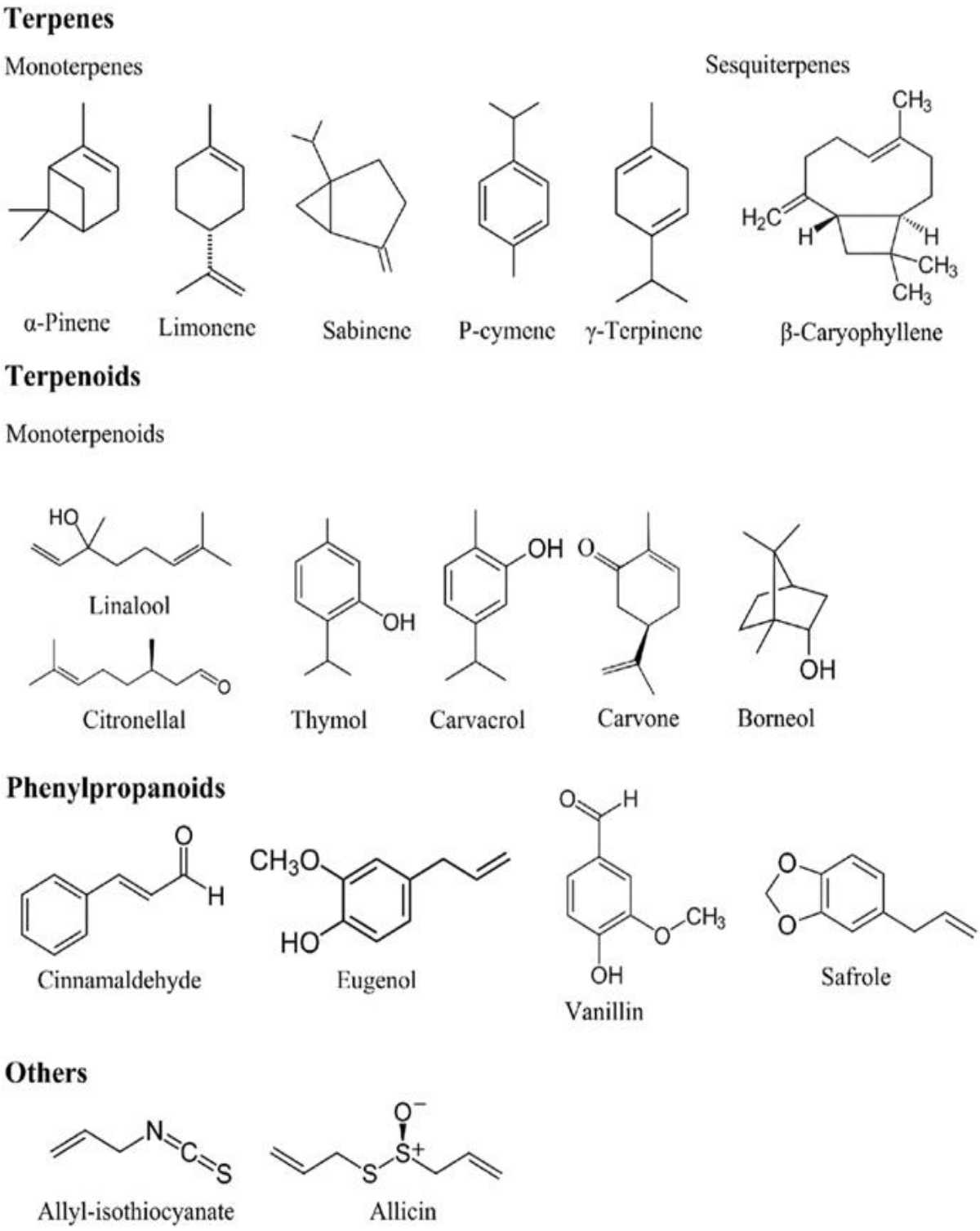

Figure 1.3. Chemical structures of some selected essential oil constituent.[62]

Essential oils represents less than 5% of the vegetal dry matter and vary according to the part of the plant employed as raw material.[63] The quality and composition of essential oils may be determined by factors such as climatic conditions, cultivation, soil, harvesting time, and others.[64] Essential oils have been used medicinally in history and their interest in recent decades has increased with the popularity of aromatherapy. Currently, most essential oils are used in the food, pharmaceutical, and cosmetic industries. Essential oils exhibit different biological properties depending on functional groups present. Oils in the same molecular class are likely to exhibit similar therapeutic properties as illustrated in Table 1.2.

Table 1.2. Properties of essential oil families.[65]

Compound	**Properties**
Monoterpene hydrocarbons	Stimulant, decongestant, antiviral, antitumor
Monoterpene alcohols	Antimicrobial, antiseptic, tonifying, spasmolytic
Sesquiterpene hydrocarbons	Anti-inflammatory, antiviral
Sesquiterpene alcohols	Anti-inflammatory, antiallergenic
Aldehydes	Spasmolytic, sedative, antiviral
Cyclic aldehydes	Spasmolytic
Ketones	Mucolytic, cell-regenerating, neurotoxic
Esters	Spasmolytic, sedative, antifungal
Oxides	Expectorant, stimulant
Phenols	Antimicrobial, irritant, immune stimulating

1.3 HYPOTHESIS AND AIM OF STUDY

Increase in the use of natural product has led to sustainability problem as large quantities of plant material is needed to produce a small quantity of essential oils. [4, 66] Many plants has been lost and some are in danger of extinction. Research has been aimed at finding alternatives to the use of threatened species with high concentrations of compounds of interest.[4, 32, 65, 67, 68] Therefore, methods that can be used in quantitative analysis of essential oils are needed.

Supercritical fluid extraction using carbon dioxide is a green extraction method that can be used in extraction of natural products. Essential oils are comprised of volatile and semi-volatile compounds which are lost during the collection step. This limits the use of SFE technique in extraction of volatile natural products.

It is hypothesized that if the collection step and extraction step in supercritical fluid extraction are modeled to understand how different parameters affect the collection and extraction of essential oils, the results can be used in developing a supercritical fluid extraction method that can be used as a sample preparation technique in analysis of essential oils.

The goal for this work is to develop a supercritical carbon dioxide extraction method that can be applied to essential oils from plant samples, with a minimal loss of essential oils during collection.

To support this goal, the first objective of this investigation, presented in chapter 2, is to use response-surface methodology (RSM) to model the parameters affecting the collection of essential oils to determine the conditions that can achieve >90% collection of extracted essential oils. These collections studies will be done using a set of standards

representative compound classes from essential oils. The next objective is to apply these collection conditions in the extraction of essential oils from chamomile, rabbit brush, and skunk brush. The extraction yield will be fit to a second-order polynomial model to determine how pressure, temperature, and time affect the extraction of essential oils using supercritical carbon dioxide. This is presented in Chapter 3.

2 CHAPTER 2: MODELING OF THE COLLECTION STEP AFTER SUPERCRITICAL FLUID EXTRACTION

2.1 Abstract

Supercritical fluid extraction (SFE) comprises of two major steps, extraction of the analyte from the sample matrix and subsequent collection of the analytes. To achieve quantitative results and make a proper conclusion on the efficiency of extraction step or the transfer of analytes from the extraction vessel to the collection system, both extraction and collection steps should be considered as integrated. The collection can be done either on-line into a chromatograph or off-line by depressurizing the supercritical fluid into a collection vessel. Off-line collection is the most widely used mode of collection due to its simplicity and cost efficiency. The collection vessel can be an empty vessel or a vessel containing a small volume of solvent. During the decompression, volatile and semi-volatile analytes may be lost. This potential analyte loss during collection has been a big problem for the quantitative extraction of essential oils, which comprises volatile and semi-volatile compounds. Research has shown that faulty collection rather than non-quantitative extraction could explain many of the reported low extraction yields of volatile compounds.[69-72] Thus, collection step is very important in the quantitative extraction of essential oils.

In this study, several parameters that influence the collection of extract after SFE by trapping with a small volume of an organic solvent were investigated. This study was done with an aim of eliminating the least important variables so that the important variables could be modeled. Then the resultant empirical model would be used in determining optimal conditions for quantitative extraction of essential oils. The

parameters considered included solvent type (chosen according to polarity and viscosity), solvent volume, decompression flow rate, restrictor positioning, restrictor temperature, cooling position, collection time, and collection temperature.

A design of experiments approach, which entails a collection of mathematical and statistical techniques, was used. This technique assisted in identifying key variables, understanding of the relationship between these variables and the response, and building a mathematical model. The model was subsequently used in determining the optimal conditions for quantitative extraction of essential oils from selected plants. This technique is more efficient than the collection of data by one-factor-at-time experimentation or a series of trial and error tests, which are time-consuming considering the number of runs involved and do not consider the interaction among the variables. Therefore, they do not depict the true representation of the process.

Plackett-Burman (P-B) design, which is a fractional-factorial design was used for screening to establish significant variables, while a response-surface design, Box-Behnken, was used for optimization of the significant parameters established.

Time, flow rate and cooling temperature were found to be the most important factors with a strong effect on analyte recovery. The amount of solvent was found to be significant with a less viscous solvent and insignificant in more viscous solvents. Cooling position and restrictor position was found to be insignificant. Time had a negative effect on the trapping efficiency with isopropanol and dichloromethane, while acetonitrile had a positive effect. Flow rate had a negative effect with all solvents. Thus, higher recoveries were realized at lower flow rates. The interaction between time and flow rate was found to have a positive effect with isopropanol and dichloromethane, while with acetonitrile it

was negative. The time and temperature interaction was found to have a positive effect with acetonitrile and negative effect with dichloromethane. The flow rate and temperature interaction was found to have a negative effect in all the solvents. The optimal condition for total recovery was as follows: isopropanol (25.58 min, 2.07 ^{o}C, and 0.3 L/min), acetonitrile (28.30 min, -8.20 ^{o}C, and 0.3 L/min) and dichloromethane (26.8 min, 3.21 ^{o}C, and 0.3 L/min).

2.2 Introduction

SFE extracts solutes from the sample which are then isolated via the collection step. The faulty collection can lead to an incorrect conclusion on the efficiency of the extraction step or the transfer of the analyte from the extraction vessel to the collection system. To achieve quantitative extraction, the extraction step and subsequent collection step should be considered as integrated.[73, 74] The analytes must be extracted efficiently from the sample matrix and must be trapped, or collected, efficiently.

The extraction step has been widely researched,[23, 35, 48, 54, 75] but there is minimal literature on the collection step. Lately, the importance of the collection step has been emphasized, especially for volatile and semi-volatile compounds, as low recoveries have been attributed to a faulty collection as opposed to non-quantitative extraction.[72, 73, 76] During collection after supercritical carbon dioxide extraction, the decompression of supercritical CO_2 causes a sharp drop in density as it changes from fluid to a gas. The volumetric flow rate increases by the same factor, which makes trapping of volatile and semi-volatile compounds difficult as they are purged from the system.

The primary goal of the work in this chapter is to investigate and determine the collection efficiency following SFE. This will be done by screening for important parameters affecting the collection of essential oils using a Placket-Burman model. The significant parameters will then modeled using Box-Behnken response-surface methodology to establish optimal collection conditions. Analytes were added onto an inert matrix then extracted using supercritical carbon dioxide and collected in varying small volumes of organic solvents. Considering the interaction of the analyte in the inert matrix and the native sample matrix differ, spiking is not always a valid method to

determine extraction efficiency. However, in this work it must be noted that spiked analytes were introduced into the collection system under the same SFE conditions experienced by authentic samples. Therefore, this approach is an effective and valid approach for determining trapping efficiency.[76]

The response-surface methodology was used to draw a statistically appropriate conclusion from the experiments. This approach is a well-establish and proven statistical method and has a versatile application across many disciplines and industries.[46, 50, 77-79] There are a number of designs available under design of experiment (DOE) that range from simple two-level fractional-factorial designs like Placket-Burman design to multi-level designs like Box-Behnken design, central composite design and Taguchi design, among others.[77] These designs are used in the identification of critical factors, identification of the interaction between factors, and facilitation of optimization from surface-area designs.

2.3 Background

2.3.1 Offline Collection Modes

There are four main commonly used trapping modes for offline collection following SFE, solid-phase sorption, cryogenically cooled surface trapping, open-vessel collection, and liquid collection.[73] The choice of collection mode depends on the properties of the analyte of interest and technique to be used for analysis of the analyte.

Among the trapping modes, analysts have been able to achieve over 80% recovery using sorbent trapping.[22] However, this is only applicable to compounds of the same physical and chemical properties. It has been virtually impossible to trap analytes of varying chemical and physical properties using a single trap.[73] Sorbent trapping has more

variables to be considered ranging from the type of sorbent to be used, the solvent for elution, elution temperature, to the solvent used to pre-rinse the trap.[76] Extra hardware is needed, i.e. pump for the elution solvent, as well as heating and cooling capability. Additional challenges include irreversible binding of the analytes to the solid-phase sorbent, solvent used for elution being chromatographically strong for the stationary phase, and, if solvent modifiers are used, they condense in the trap region and may elute the analytes from the sorbent. Cryogenic trapping has also been used in trapping C_{10} hydrocarbons with reasonable efficiency. Cryogenic trapping is mostly used for on-line coupled SFE systems like SFE-SFC, SFE-GC and SFE-SPE-GC.[80] Apart from extra instrumentation needed like cryogenic pumps, too low temperatures may cause restrictor plugging as ice forms with samples containing a significant amounts of water. This plugging disrupts the gas flow, making extraction reproducibility difficult. The open-vessel collection mode is the least commonly used mode. It is used in the collection of higher molecular weight compounds. Depressurizing CO_2 into a small volume of the organic solvent for collection is the most commonly used collection mode.[70] It is relatively simple and inexpensive to perform and the collected extract can be immediately analyzed without further preparation. This method has successful been used in the collection of 66 polycyclic aromatic hydrocarbons with a wide range of polarity and volatility. Loses of 5-20% of the more volatile compounds was reported.[70] The recovery was found to be dependent not only on solubility and volatility of the test analyte, but also to trapping temperature, collection solvent volume and height, and type of solvent used.[70] It was also reported that loss of volatile compounds occurred during concentration

of the extract and was due to purging of compounds from the collection vessel during depressurization of CO_2, especially at high flow rates.[70]

2.3.2 Trapping Process

During solvent trapping, the analyte undergoes four main steps, as depicted in Figure 2.1.[69] The first step involves the analyte exiting the restrictor. For successful exit, the solute must have solubility all the way to the tip and should not adsorb to the inside of restrictor. This is achieved by uniform heating of the restrictor. Step two is diffusion of the analyte through the gas bubble to the gas-liquid interface. This step is a function of the diffusion constant of analyte in the gas phase. Smaller bubbles result in shorter diffusion paths so an analyte reaches the gas-liquid interphase faster. Smaller bubbles can be achieved by lower decompression flow rates and also by using collection solvents with high viscosity. The third step is solvation of the analyte into the solvent. The solubility of the analyte in the collection solvent is an important factor. Solubility, temperature and time of exposure are the most important parameters. Though slightly higher temperatures may improve solubility of some compounds, a lower temperature is preferred for the collection of volatile compounds as it results in a lower vapor pressure of the analyte, reducing the loss of solutes. The last step is maintaining of the trapped analyte in the collecting solvent before it is taken to the analysis step. The most important parameter in this step is temperature. It should be kept low enough to avoid evaporation of the analyte and high enough to avoid restrictor plugging.

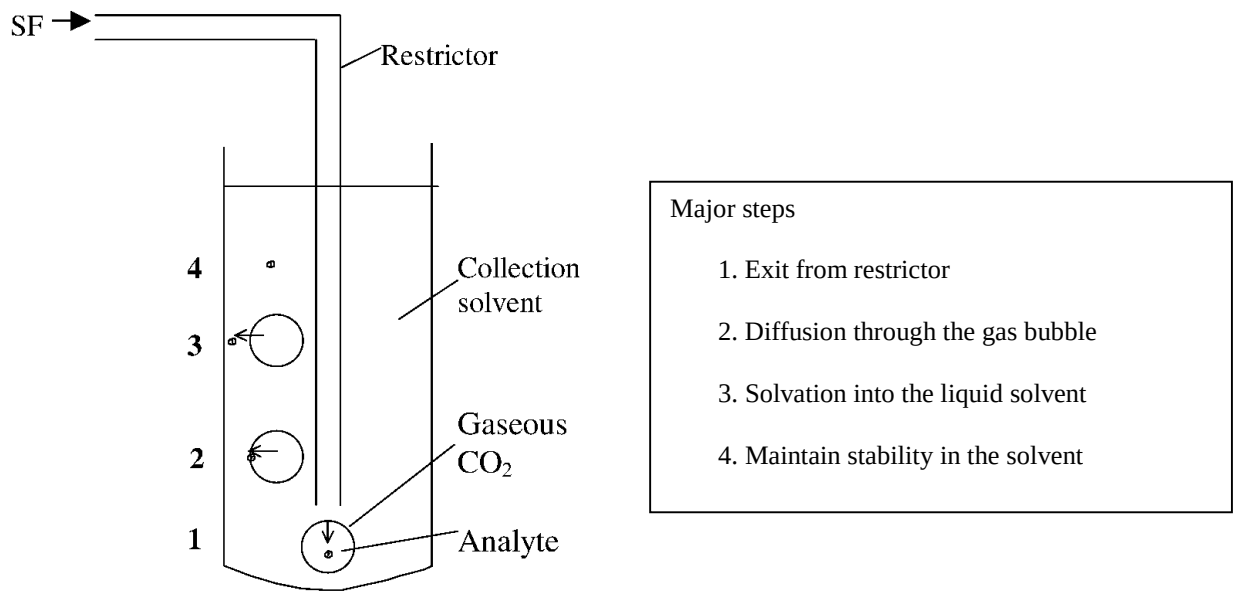

Figure 2.1. Schematic of solvent collection, showing four main steps of the analyte collection procedure: (1) exit from restrictor, (2) diffusion of analyte through the gas bubble, (3) solvation into the solvent, and (4) maintain solubility.[69]

2.3.3 Design of Experiment (DOE)

Development of analytical methods involves monitoring parameters affecting the response in question to determine the optimal conditions. Traditionally, optimization is done by varying one variable at a time while holding the rest of the independent variables constant. This method is time consuming considering the number of runs involved and does not consider the interactions among the variables. Therefore, it does not depict the true representation of the process.

Response-surface methodology (RSM), a collection of mathematical and statistical techniques, is used in designing experiments in which the outcome of the experiment (that is, the response) is influenced by several variables when the true relationship between the variables and the response is unknown.[77] It involves fitting empirically obtained response data to an appropriate polynomial equation that expresses

the behavior of various variables. The outcome of the experiment is usually assumed to depend on experimental conditions. Therefore, the response, or outcome, can be described as a function based on the experimental variables, equation 2.1[77]

$$\boldsymbol{y = f'(x)\beta + \varepsilon} \qquad \textbf{2.1}$$

where x is an independent variable and can be x_1, x_2,....x_k, ε denotes experimental error, and β is regression coefficient.

In most cases, the true relationship of between the variables and the response is not known. The approximation of the relation can be done by a first-order model whereby independent variables are expressed as shown in equation 2.2.[77]

$$\boldsymbol{y = \beta_o + \sum_{i=1}^{k} \beta_{x_i} + \varepsilon} \qquad \textbf{2.2}$$

Equation 2.2 contains only linear terms and describes only the linear relationship between the experimental variables and the response. To describe the interaction between different independent variables, additional terms are added as illustrated by a second-order interaction model, equation 2.3.[77]

$$\boldsymbol{y = \beta_0 + \sum_{i=1}^{k} \beta_i\, x_i + \sum_{i<j} \sum \beta_{ij}\, x_i x_j + \varepsilon} \qquad 2.3$$

The two empirical models, equation 2.2 and equation 2.3, are mainly used for screening and robustness tests.

To determine the optimal (maximum or minimum) conditions, quadratic terms are introduced in the model. Equation 2.4 includes the linear terms, interaction terms, and the quadratic terms.[77, 79]

$$\boldsymbol{y = \beta_0 + \sum_{i=1}^{k} \beta_i\, x_i + \sum_{i<j} \sum \beta_{ij}\, x_i x_j + \sum_{i=1}^{k} \beta_{ii}\, x_i^2 + \varepsilon} \qquad 2.4$$

The purpose of these equations is to establish the interaction between factors and their effect on the response. They also establish, through hypothesis testing, the significance of the factors. Finally, these functions are used to determine the optimal conditions that result in the maximum or minimum response over given region.

2.3.4 Experimental designs

There are several types of designs, and the appropriate choice depends on the objective or goal of the experiment and the number of factors being investigated. Figure 2.2 shows different designs with their respective number of variables.

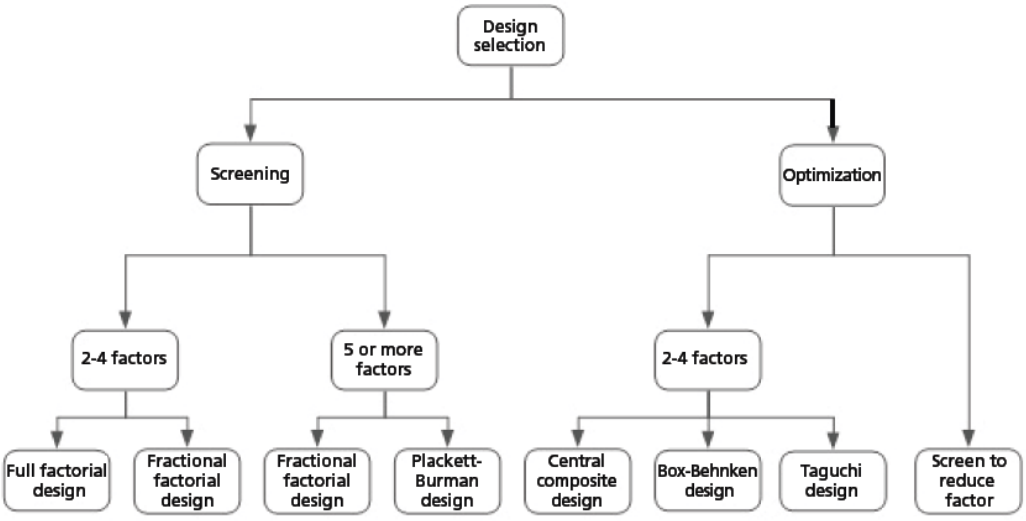

Figure 2.2. Flow chart showing the appropriate screening and optimization designs according to the number of factors.[78]

2.3.4.1 Screening Designs

The level of significance of different factors varies. It is usually practically impossible to consider the effects of all parameters. Therefore, it is necessary to identify the main factors that significantly affect the response. Screening designs usually assume a linear response where only the main effects or main effects plus interaction effects are

considered. Experimental designs popularly used in screening are full and fractional two-level factorial designs, Plackett-Burman, and supersaturated designs.[81]

2.3.4.1.1 Two-Level Full Factorial Designs

When two to four factors are involved, full factorial in two levels is used. It combines a high and low combination of all of the output factors, and the number of runs is 2^k, where k is the number of factors. When more than four factors are involved, 2^k can result in a large number of runs to be made. For example, a full-factorial design with ten factors requires 2^{10}, which is equal to 1024 experimental runs. However, some interactions, especially individual higher-order interactions, have no distinguishable effect on response and can be ignored to reduce the number of experimental runs. As a result, a well-designed two-level fractional factorial can be used to estimate the model parameters with few runs. The advantages of full-factorial design are orthogonality, no aliasing concerns, and all main factors and all interactions can be evaluated.[77, 82] The disadvantage is the cost, time, and resources needed to do all experimental runs required by a full factorial, especially when the number of factors is large.[82]

2.3.4.1.2 Two-Level Fractional-Factorial Designs

Fractional-factorial designs use a fraction of the runs required by full-factorial designs. Considering that some interactions, especially three way and higher do not significantly affect the response, a subset of the experimental treatment is selected. This type of experimental design allows the estimation of all linear effects and desired interactions while requiring fewer runs. It is usually an orthogonal design, and it can separate these effects.

Fractional-factorial designs are usually denoted by roman numerals depending on design resolution or the aliasing of effects involved.[82] If main effects are clear from other effects, but the main effects are confounded with a two-way interaction, it is denoted as Resolution III. Resolution III designs are typically used in screening when a large number of factors are involved. If main effects are estimated clear of any two-way interactions, but two-way interactions are confounded with each other, the design is denoted as Resolution IV.[81, 82] Resolution IV designs are used for building prediction equations when resources are limited and do not permit the use of Resolution V. In Resolution V, the main effects and two-way interaction are estimated clear of any other main effect or two-way interaction, but two-way interactions are confounded with three-way interaction. Resolution V designs are used to build prediction equations that typically do not have serious interaction concerns.

2.3.4.1.3 Plackett-Burman Design

The Plackett-Burman design is a two-level fractional-factorial screening design based on a Hadamard matrix, which has more flexibility.[77, 79] It is excellent for screening as the number of experimental runs required are very few, leading to saving time, chemicals, and manpower. For example, to study nine factors, only twelve runs are needed as compared to thirty-two runs needed in the standard fractional-factorial design. In this design, N variables require N+1 number of experimental runs, which is usually a multiple of four plus the center points. Only main effects are considered. Placket-Burman designs are Resolution III designs.[82] This means that the main effects can be estimated clear of other main effects. This design is suggested for studies involving five or higher number of factors. Although it is useful mostly for fitting first-order models, it can also

be used to provide information on the existence of second-order effects (curvature) by the inclusion of center points.

2.3.4.2 Optimization Designs

Optimization designs are used to examine in more detail the factors selected from screening or experience. Response-surface designs are usually used in optimization. There are two main types of response surface designs, central-composite design (CCD) and Box-Behnken design (BD). Response-surface designs include quadratic terms which describe the curvature in the model. This makes them useful for understanding and mapping a region of a response, finding levels of variables that optimize response and help in selecting operating conditions meeting a specific target.

2.3.4.2.1 Central-composite design

Central-composite design is a five-level fractional-factorial design with center points, augmented by a group of axial points called star points which facilitates the estimation of curvature. It is often possible to build on previous factorial experiments by adding axial and center points. The number of points in CCD contains a factorial run 2^k, axial runs of 2k, and C_o center point runs, as shown in Figure 2.3. The total experimental runs N is given by $2^k+2k+Co$ where k is the number of factors and C_o are the number of center-point runs.[77, 82]

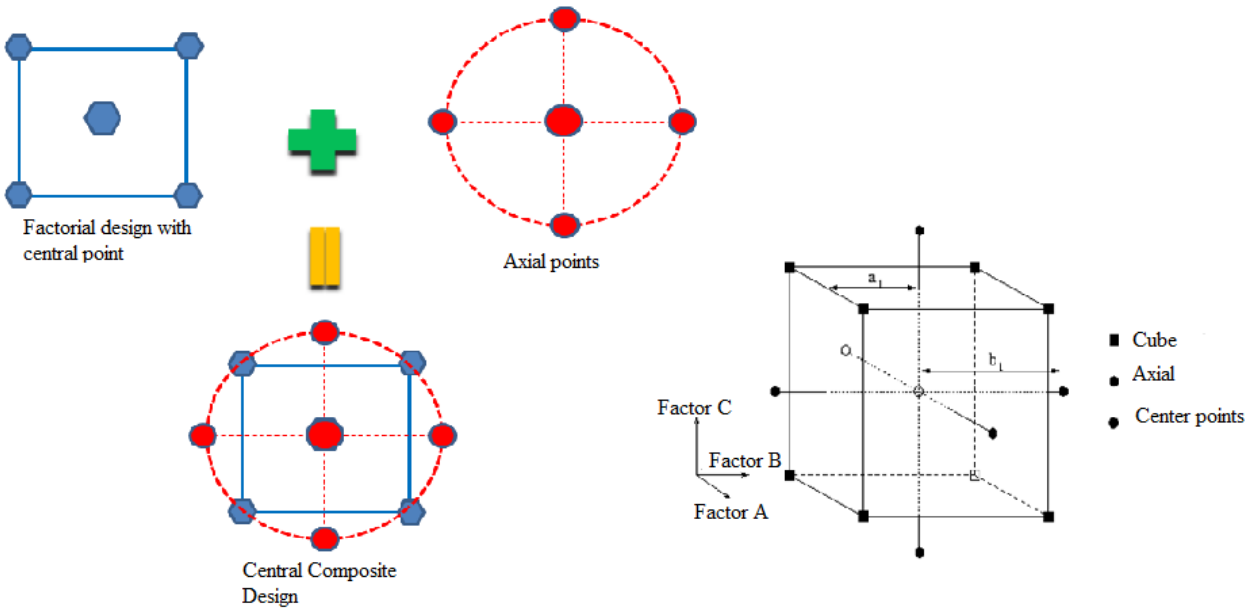

Figure 2.3. Illustration of the points in central-composite design.[82]

CCD is the most effective and efficient second-order modeling design for quantitative factors. It has flexibility in resolution, as the factorial portion of any resolution can be build. It saves resources as the experimental runs can be done sequentially, i.e., factorial and center points can be run first to build a linear model then add the axial points to complete a quadratic model.

2.3.4.2.2 Box-Behnken Design

Box-Behnken is a fractional three-level factorial design. It is built from combining a two-level factorial design with incomplete block design in such a way that the sample size is kept to a value that is sufficient for estimation of second-degree polynomial coefficients. The design does not contain any points at the vertices of the experimental region where factors are at their highest levels. This is an advantage when

points on the corners of the cube represent factor level combination that are prohibitively expensive or impossible to test because of physical process constraints.[77, 81, 82] It is considered as a nearly orthogonal, Resolution V design, allowing the estimation of linear effects, quadratic effects, and all two-way interactions. The total number of runs are based on $N = 2k(k-1) + C_o$, where k is the number of factors and C_o is the central-point numbers, as shown in Figure 2.4.[79]

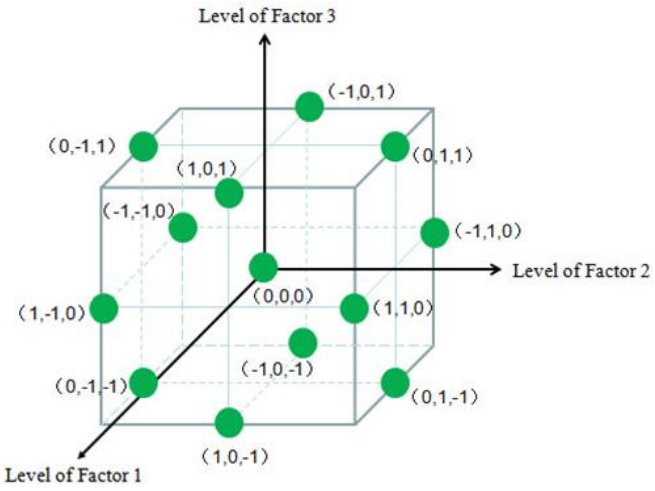

Figure 2.4. Figure showing Box-Behnken design of three factors, it includes experimental points that defines the design.[83]

Comparing BBD with CCD, BBD design requires fewer experimental run for three and four factors, as illustrated by Table 2.1. This advantage disappears for factors greater than four. The primary disadvantage of BBD is that the number of runs is always large enough to estimate all factors, second-order effects and all linear two-way interactions, whether they are wanted or not.[79, 82]

Table 2.1. Number of runs required for BB and CCD design according to number of factors.[82]

Number of Factors	**Central composite**	**Box-Behnken**
2	13 (5 center points runs)	-
3	20 (6 center runs)	15
4	30 (6 center point runs)	27
5	33 (fractional factorial) or 52 (full factorial)	46
6	54 (fractional factorial) or 91 (full factorial)	54

2.4 Experimental

2.4.1 Materials and Reagents

A test mixture consisting of ten fragrance compounds, which included d-limonene, linalool, carvone, citral, cineol, geraniol, caryophyllene, β-pinene, phellandrene, and bisabolol, were used. These compounds were representative of monoterpenes, sesquiterpenes, diterpenes, and their oxygenate derivatives. Methyl hexyl ketone was used as an internal standard. All of the test components were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Ottawa sea sand was from Thermo-Fischer Scientific (Pittsburgh, PA). Carbon dioxide 99.9995% purity SFE-grade CO_2, with helium pressure and dip tube, was obtained from Airgas (Radnor, PA). Five solvents (isopropanol, acetonitrile, methanol, dichloromethane, and cyclohexane) were obtained from Thermo-Fischer Scientific (Pittsburgh, PA).. these solvents were chosen according to viscosity, polarity, and vapor pressure, shown in Table 2.2.

Table 2.2. Solvents investigated for trapping efficiency.

Solvent	Viscosity (cP)	Polarity Index	Vapor pressure (Torr)
Isopropanol	2.4	3.9	8.8
Acetonitrile	0.36	5.8	88.8
Methanol	0.55	5.1	125
Dichloromethane	0.44	3.1	436
Cyclohexane	1.0	0.2	77.5

2.4.2 Methods

2.4.2.1.1 Modeling of Trapping Step

Fractional-factorial Plackett-Burman (P-B) design matrix of Resolution III was used to screen for important variables. Nine factors that included solvent polarity, solvent viscosity, solvent temperature, solvent volume, decompression flow rate, restrictor positioning, restrictor temperature, cooling position and decompression temperature, were investigated. Solvents used were chosen according to their viscosity and polarity index. Remaining factors were considered as controllable factors. The levels of variables were selected based on the preliminary study done by a univariate method. The levels for quantitative variables were coded as high (+1), medium (0), and low (-1). Cooling position and restrictor position were coded as illustrated in Table 2.3.

Table 2.3. Variables with their corresponding actual values and coded values

Variables	Symbols	Experimental value	
		Low (-1)	High (+1)
Time (Min)	A	10	60
Flow Rate (L/Min)	B	0.3	1.2
Solvent Temperature (°C)	C	-10	25
Restrictor Temperature (°C)	D	25	50
Solvent Volume (fraction of vial)	E	0.25	0.75
Restrictor position	F	Headspace (+1), Middle (0), Inside (-1)	
Cooling position	G	Top (+1), Whole (0), Below (-1)	

Solvent volume was defined as the fractional of the vial used for collection. Restrictor positioning during decompression was either in the headspace, the middle of the collecting solvent or at the bottom most of the vial, as illustrated in Figure 2.5.

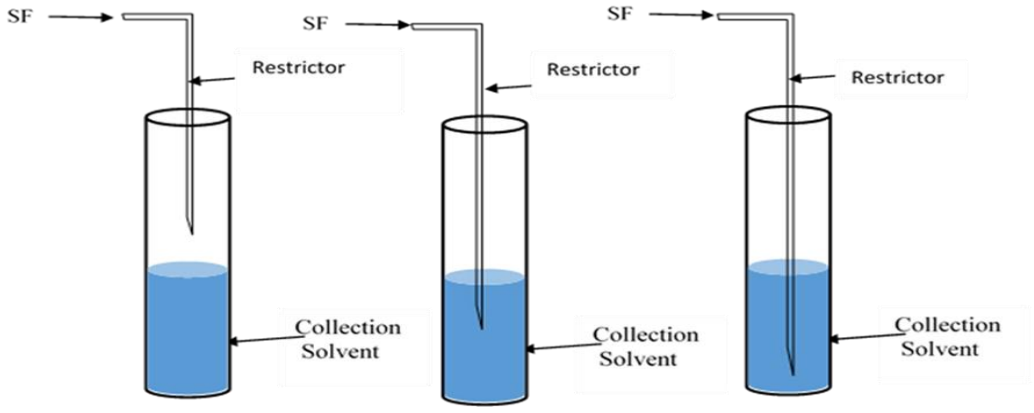

Figure 2.5. Schematic of solvent collection, showing the three restrictor positioning coded as headspace (+1), middle (0), and below (1), respectively, in the experimental design.

The cooling setup was as shown in Figure 2.6 where the collecting vial was cooled from the top, below, or the cooling was done to the whole vial.

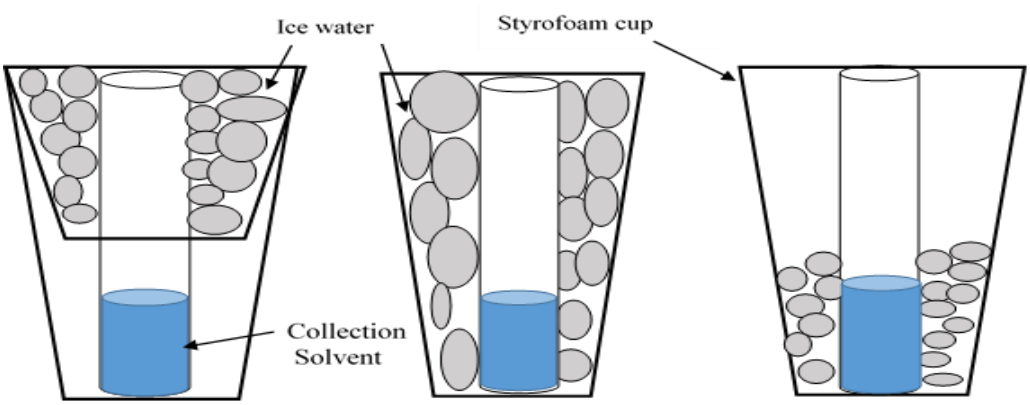

Figure 2.6. Diagram showing the cooling set up during screening, the cooling positions were coded as top (+1), whole (0), and below (-1).

The setup shown in Figure 2.6 was only used during the screening. The cooling system controlled by a chiller, Figure 2.7, was used for optimization studies. This was easy to control and maintain uniform cooling as opposed to the use of ice water.

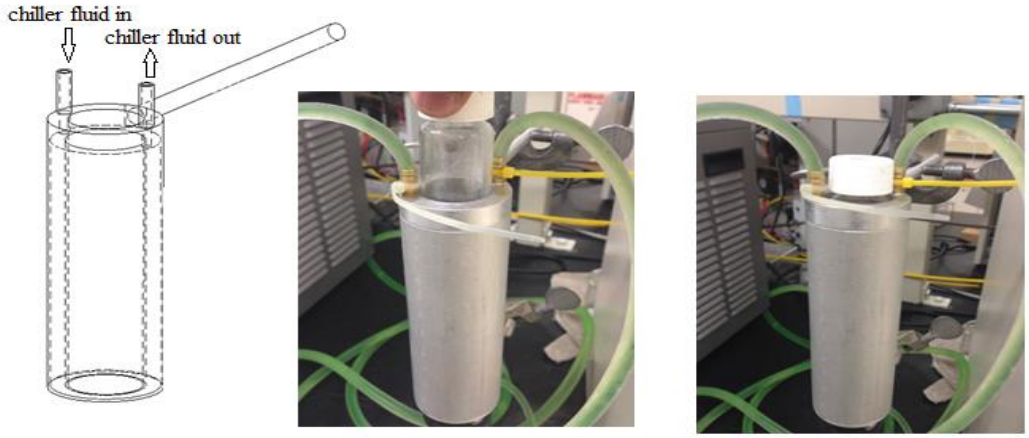

Figure 2.7. Diagram showing the cooling jacket used as cooling system during optimization studies.

The P-B design generated a total of 15 runs consisting of 12 base runs and three center points. The center points were included to provide information on the existence of curvature and to ensure repeatability. All the runs were done in duplicate. Table 2.4 gives

the summary of the P-B design generated, while Tables 2.5 and 2.6 present the variable combination for each run in coded and uncoded form, respectively.

Table 2.4. Plackett-Burman Design Summary.

Factors: 7	Replicates: 2
Base runs: 12	Total runs: 15
Base blocks: 1	Total blocks: 1
Centre points: 3	Risk Level: 0.05

Table 2.5. Placket-Burman screening design work sheet with seven variables with their coded values.

Std Order	Run Order	Time	Flow Rate	Solvent Temp	Restrictor Temp	Solvent Volume	Restrictor Position	Cooling Position
6	1	0	0	0	0	0	0	0
15	2	1	-1	1	1	1	-1	-1
1	3	1	1	-1	-1	-1	1	-1
9	4	1	1	1	-1	-1	-1	1
8	5	-1	1	-1	1	1	-1	1
11	6	-1	-1	-1	1	-1	1	1
2	7	-1	-1	-1	-1	-1	-1	-1
14	8	-1	-1	1	-1	1	1	-1
7	9	-1	1	1	1	-1	-1	-1
4	10	1	-1	-1	-1	1	-1	1
12	11	0	0	0	0	0	0	0
3	12	0	0	0	0	0	0	0
10	13	1	1	-1	1	1	1	-1
13	14	1	-1	1	1	-1	1	1
5	15	-1	1	1	-1	1	1	1

Table 2.6. Placket-Burman screening design with seven variables with their true (uncoded) values.

Std Order	Run Order	Time	Flow Rate	Solvent Temp	Restrictor Temp	Solvent Volume	Restrictor Position	Cooling Position
6	1	35	0.75	7.5	37.5	0.5	Middle	Whole
15	2	60	0.3	25	50	0.75	Inside	Below
1	3	60	1.2	-10	25	0.25	Headspace	Below
9	4	60	1.2	25	25	0.25	Inside	Top
8	5	10	1.2	-10	50	0.75	Inside	Top
11	6	10	0.3	-10	50	0.25	Headspace	Top
2	7	10	0.3	-10	25	0.25	Inside	Below
14	8	10	0.3	25	25	0.75	Headspace	Below
7	9	10	1.2	25	50	0.25	Inside	Below
4	10	60	0.3	-10	25	0.75	Inside	Top
12	11	35	0.75	7.5	37.5	0.5	Middle	Whole
3	12	35	0.75	7.5	37.5	0.5	Middle	Whole
10	13	60	1.2	-10	50	0.75	Headspace	Below
13	14	60	0.3	25	50	0.25	Headspace	Top
5	15	10	1.2	25	25	0.75	Headspace	Top

2.4.2.1.2 Optimization of trapping step

The results from the Placket-Burman screening model, Table 2.19, indicated that in all the collection models, flow rate, cooling temperature, and depressurizing time were the most significant variables. The type of trapping solvent was also found to be a significant as different solvents had different collection capacity. Three of the five solvents (isopropanol, dichloromethane, and acetonitrile) were chosen for the optimization studies based on recovery of over 80% across all the individual compound families. Dichloromethane is used in the liquid-liquid extraction of essential oils. It is

easy to evaporate owing to its high vapor pressure. This is ideal if concentration is needed. Isopropanol is known to dissolve most of the essential oils and aromatic resins.[84] Acetonitrile is good at making a biphasic system in the purification of essential oils.[85]

Solvent volume was found to be a significant variable in less viscous solvents (dichloromethane and acetonitrile). However, compared to the total recovery when the collection was done in higher solvent volume and when was done in lower volume with the restrictor inside, there was no difference in total recovery. Thus, the subsequent collection was done using 20 mL of the collection solvent, and depressurization was done inside the solvent.

Restrictor temperature was not a significant variable. However, real samples usually contain water and to avoid plugging due to water freezing in the restrictor, 50 ^{o}C was chosen for subsequent experiments.

The cooling position was found to be insignificant. Thus, the subsequent experiment was done by cooling the whole vial in a cooling pocket as illustrated in Figure 2.7. The cooling system was connected to a chiller making it easier to control the temperature. Table 2.7 gives a summary of the experimental values and condition for each parameter.

Table 2.7. Variables and their corresponding actual and coded values.

Variables	Symbols	Experimental value	
		Low (-1)	High (+1)
Time (min)	A	10	45
Flow Rate (L/min)	B	0.3	1.2
Solvent Temperature (°C)	C	-10	25

Solvent Volume	20 mL
Restrictor Temperature	50 ^{o}C
Restrictor position	Inside bottom most
Cooling position	Whole vial

2.4.2.1.3 Response-Surface Methodology

Optimization was done using a Box-Behnken design (BBD). BBD is a multiple-regression model utilizing a second-order polynomial equation. Twenty-seven experimental runs that included twelve base runs in duplicate and three center points were generated by statistical software. The resultant variables combination was as illustrated in Table 2.19. The Table contains the actual factor combinations in a random order. This was the guide to experiments performed. To normalize the parameters during modeling, the variables levels were coded as high (+1), medium (0), and low (-1).

Table 2.8. Box-Behnken design experimental runs in their actual variables values generated using a statistical software, generated for the three solvents.

Time (min)	Temperature (°C)	Flow Rate (L/min)
10	7.5	0.3
27.5	25	0.3
27.5	-10	0.3
27.5	25	1.2
10	25	0.75
27.5	7.5	0.75
27.5	25	0.3
45	-10	0.75
10	-10	0.75
27.5	-10	1.2
45	7.5	0.3
27.5	25	1.2
45	-10	0.75
27.5	7.5	0.75
45	7.5	0.3
10	7.5	1.2
45	7.5	1.2
10	25	0.75
27.5	-10	0.3
27.5	-10	1.2
45	25	0.75
45	7.5	1.2
10	7.5	1.2
27.5	7.5	0.75
10	-10	0.75
45	25	0.75
10	7.5	0.3

2.4.2.1.4 Supercritical fluid extraction

Extraction was performed using a Spe-ed SFE Helix Model 7401 (Applied Separations, Allentown, PA, USA). SFC-grade carbon dioxide was used for all extractions. A 24-mL vessel was filled with glass beads or clean sea sand. A test mix, 100 μL of 600 ppm, was spiked onto the center of the vessel containing the glass beads or sea sand. The vessel was sealed immediately to prevent any loss of added components. The vessel was mounted onto a thermostat-controlled oven and CO_2 was introduced into the vessel. Temperature and pressure were set at 45°C and 5000 psi. These values were preliminarily determined using a one variable at a time approach. The pressure was adjusted to 5000 psi after the set temperature was achieved. Extraction was carried out in the dynamic mode, and the extract was collected by decompression of CO_2 into a 60-mL collecting vial containing the solvent. Collection conditions were set according to the working sheet suggested by the design of experiment software, Table 2.8. Solvent volume was maintained by small additions during SFE.

2.4.2.1.5 Gas chromatographic analysis

GC analysis was done with an Agilent 7890 gas chromatograph (Agilent Technologies, Little Falls, DE) coupled to an Agilent Technologies 5975C mass spectrometer and fitted with a 30-m x 0.25-mm, 0.25-μm DB-5 column (Agilent Technologies, Little Falls, DE). A Hewlett-Packard Model 5890 gas chromatograph with flame ionization detection was used in some studies. The oven-temperature program was held at 45 °C for 2 min, and then ramped at 5 °C/min to 240 °C and held for 10 min. The hydrogen carrier flow was kept constant at 1.2 mL/min (equivalent to a pressure of 45.5 kPa at 165 °C). Splitless injection (2 μL) was performed with an HP7673A automatic

sampler with an injection port at 280 °C, The transfer line temperature to the mass spectrometer was kept at 300 °C. The MS temperatures were ion source 230 °C and quadrupole 150 °C. The scan range was 40-550 U (2.91scans/s). The test mixture solution plus the internal standard was added to the same volume of solvent used in trapping and analyzed by GC. This was taken as the 100% recovery. This was used for relative quantification. An internal standard was prepared in each test solvent to ensure that any difference in SFE collection efficiency was not as a result of gas chromatographic analysis difference caused by the solvent. The internal standard was added to the extract after the extraction prior to gas chromatographic analysis.

2.4.2.1.6 Quantification of essential oils components

The gas chromatographic results were evaluated by relative quantification. A mixture containing all the essential oils to be subjected to SFE was run. The total area, which was the ratio of compound peak area and internal standard peak area, was assumed to be 100% recovery. To ascertain the percentage of the compounds collected, total peak area from the gas chromatographic analysis after performing SFE was compared to the total area obtained before SFE. Percentage amount of the total compounds collected was found by equation 2.5.

$$\%\ \text{relative of total essential oils collected} = \frac{\text{Total area after SFE}}{\text{Total area before SFE}}\ \text{X}100\% \quad \textbf{2.5}$$

To evaluate the percentage amount of each component collected, equation 2.6 was used.

$$\%\text{relative abundance of component} = \frac{\text{component area}}{\text{total area}}\ \text{X}\ 100\% \quad \textbf{2.6}$$

2.4.2.1.7 Model Evaluation

To investigate the fitness of the model and significance of the variables, an analysis of variance (ANOVA) was performed using ReliaSoft DOE++ software. ANOVA compares the variation that is caused by the changing of the combination of variables level and random errors. Fisher-distribution test values (*F*-value) and p-values were used in drawing the conclusion on the significance of the model and variables. To determine if the model was well fitted, the ratio of media of square of the mean (MS) of regression with the MS of residual was compared using the Fisher distribution (*F*-test). If the ratio was higher than the tabulated value of *F*, the model was considered to be statistically significant. Media of square of the mean (MS) is the division of the square of each source of variance by the respective degree of freedom. A critical p-value of 0.05, which means that there is only 5% chance that *F*-value calculated occurred due to noise, was used in determining the significance of variables. In ANOVA, a term is considered to have a statistically significant effect on the response if its corresponding p-value is less than 0.05. Terms with p-values less than 0.05 were chosen for further optimization studies using response-surface methodology.

Only main effects were considered in the Plackett-Burman design. Therefore, the data was fitted to the first-order model to detect linear effects. In optimization experimental design, two-way and higher interactions were considered. Therefore, the data was fit to a second-order polynomial.

Graphical representations including Pareto plots of effects, a normal probability of effects plot, response-surface plot, and interaction plot were used in data interpretation. Pareto charts displayed the absolute value of the effect and a reference line corresponding

to critical *F*-value. Any effect that extended past the reference line was considered important. The normal probability of effects plot was used in determining the extent and direction of effect of each variable had on the response. Response-surface plots were used to establish desirable response values and operating conditions. They are three-dimensional plots of variable conditions and the corresponding response.

2.5 Results and Discussion

2.5.1 Screening Results

The primarily objective of this chapter was to reduce large a number of factors to a manageable subset of important factors that can be used to model response surfaces, which were used in quantitative extraction of essential oils. Analysis of variance (ANOVA) and graphical approaches were used in data analysis and validation. ANOVA compares the variation caused by the changing combinations of the variables and random errors because of response measurements. The source of variation in response is caused by regression, residual, lack of fit, and pure error. Tables 2.9 to 2.13 contain the analysis of variance results. The significant parameters are in red. All the statistical calculations were done using statistical software ReliaSoft DOE++ software.

Table 2.9. Analysis of variance for acetonitrile design model, significant terms are in red.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	P Value
Model	8	635.89	79.49	36.92	0.000151
Main Effects	7	497.57	71.08	33.02	0.000223
Curvature	1	138.32	138.32	64.25	0.000201
Residual	6	12.92	2.15		

Lack of Fit	4	11.46	2.86	3.92	0.213288
Pure Error	2	1.46	0.73		
Total	14	648.81			

S = 1.47, R^2 = 98.01%, R^2 (adj) = 95.35%

Table 2.10. Analysis of variance for methanol design model, significant terms are in red.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	P Value
Model	8	488.55	61.07	32.45	0.000219
Main Effects	7	480.26	68.61	36.46	0.000168
Curvature	1	226.82	226.82	182.32	0.000106
Residual	6	11.29	1.88		
Lack of Fit	4	11.23	2.81	4.34	0.17340
Pure Error	2	0.06	0.03		
Total	14	499.84			

S = 1.37, R^2 = 97.74%, R^2 (adj) = 94.73%

Table 2.11. Analysis of variance for isopropanol design model, significant terms are in red.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	P Value
Model	8	699.57	87.45	141.17	0.000003
Main Effects	7	478.77	68.40	110.42	0.000007
Curvature	1	220.80	220.80	356.45	0.000001
Residual	6	3.72	0.62		
Lack of Fit	4	2.26	0.56	0.77	0.63133
Pure Error	2	1.46	0.73		
Total	14	703.29			

S = 0.79, R^2 = 99.47%, R^2 (adj) = 98.77%

Table 2.12. Analysis of variance for dichloromethane design model, significant terms are in red.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	P Value
Model	8	702.65	87.83	78.46	0.000017
Main Effects	7	485.67	69.38	61.98	0.000036
Curvature	1	216.98	216.98	193.83	0.000009
Residual	6	6.72	1.12		
Lack of Fit	4	5.26	1.31	1.80	0.38749
Pure Error	2	1.46	0.73		
Total	14	709.37			

S = 1.05, R^2 = 99.05%, R^2 (adj) = 94.79%

Table 2.13. Analysis of variance for cyclohexane design model, significant terms are in red.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	P Value
Model	8	835.61	104.45	125.09	0.000004
Main Effects	7	498.60	71.23	85.31	0.000014
Curvature	1	337.01	337.01	403.61	9.876284E-7
Residual	6	5.01	0.84		
Lack of Fit	4	4.48	1.12	4.26	0.199195
Pure Error	2	0.53	0.26		
Total	14	840.62			

S = 0.91, R^2 = 99.40%, R^2 (adj) = 98.61%

The obtained *F* value (Fisher-variation ratio, the ratio of mean square for regression to mean square for residual) was compared with the theoretical value at a confidence level of 95% to test the significance of the regression model. *F*-ratio values

obtained were found to be greater than the theoretical *F* value, with a low probability of $p < 0.001$ for each regression model. Higher *F*-ratios mean that the variation among the group is more than what is expected to be seen by chance or by sampling error. A *p*-value is computed with an assumption that the difference observed is due to sampling error, which is the null hypothesis. A *p*-value measures the strength of evidence for rejecting the null hypothesis. A term is considered to be statistically significant if its corresponding *p*-value is less than the chosen α value, in this case 0.05. This indicates that each regression model was significant with a confidence of 95%.

The Placket-Burman design is a first-order model and it considers only the main effect. The results showed that the main effects in each the model were significant. Though each model had an insignificant lack of fit, this was only for the main terms. The presence of significant curvature indicates that the model did not depict the full relationship the variables have with the response. This is consistent with the purpose of screening design whereby they are only used for screening and not optimization or prediction. This indicates that it is necessary to investigate a better model using higher interactions and quadratic effects. The results were able to provide information on the existence of second-order effects. This is one of the advantages of using Placket-Burman design since it allows the inclusion of center points.

2.5.1.1 Determination of Significant Variables

Pareto plots of factor effects and normal probability of factor effects

Determination of the extent of the effect of each variable have on the response (total recovery) was based on the statistical ANOVA results with a confidence level of 95%. The effect was considered significant provided that its *p*-value is smaller than 0.05.

Tables 2.14 to 2.18 contain the ANOVA results. The Tables contain unstandardized effects where the variable has no the response, coefficient associated with each variable, T-value (the measure of the size of difference relative to the variation in sample data), and *p*-value (the probability of the null hypothesis being true, the null hypothesis was that there is no significant difference). The t-test is done to find evidence of a significant difference between the means. The T-value can either be positive or negative. The closer *p*-value is to 0 the greater the evidence is against the null hypothesis that there is no significant difference. The significant variables are highlighted in red.

Table 2.14. Analysis of variance results for acetonitrile, estimated containing effects and regression coefficient of each term.

Term	Effect	Coefficient	T Value	P Value
Intercept		79.7083	188.1892	1.518896×10^{-12}
A:time	9.7512	4.8750	11.5097	0.000026
B:flow rate	-5.0167	-2.5083	-5.9221	0.001033
C:solvent temperature	-5.2145	-2.6251	-6.1975	0.000813
D:restrictor temperature	1.3215	0.6750	1.5937	0.162122
E:solvent volume	3.9155	1.9753	4.6629	0.003457
F:restrictor position	0.6833	0.3416	0.8066	0.450642
G:cooling position	-0.4166	-0.2083	-0.4918	0.640287
Curvature		7.5917	8.0157	0.000201

Table 2.15. Analysis of variance results for methanol, containing estimated effects and regression coefficient of each term.

Term	Effect	Coefficient	T Value	P Value
Intercept		79.4416	200.6171	1.034950×10^{-12}
A:time	9.6854	4.87511	12.3110	0.000018
B:flow rate	-5.1266	-2.50833	-6.3344	0.000724
C:solvent temperature	-5.2035	-2.6251	-6.6290	0.000568
D:restrictor temperature	2.8166	1.4083	3.5565	0.011977
E:solvent volume	1.9166	0.9583	2.4201	0.051855
F:restrictor position	0.7166	0.3583	0.9049	0.400387
G:cooling position	-0.41667	-0.2083	-0.5261	0.617682
Curvature		1.8583	2.0987	0.080618

Table 2.16. Analysis of variance results for isopropanol, containing estimated effects and regression coefficient of each term.

Term	Effect	Coefficient	T Value	P Value
Intercept		77.7083	342.0244	4.218847×10^{-14}
A:time	9.75	4.8751	21.4567	$6.685837\ 10^{-7}$
B:flow rate	-6.01667	-3.0083	-13.2408	0.000011
C:solvent temperature	-5.25	-2.6253	-11.5536	0.000025
D:restrictor temperature	0.35	0.1754	0.7702	0.470386
E:solvent volume	-0.05	-0.0250	-0.1100	0.91597
F:restrictor position	0.683333	0.3416	1.5038	0.183327
G:cooling position	-0.41667	-0.2083	-0.9170	0.394529
Curvature		9.5917	18.8799	0.000001

Table 2.17. Analysis of variance results for dichloromethane, containing estimated effects and regression coefficient of each term.

Term	Effect	Coefficient	T Value	P Value
Intercept		77.79167	254.6962	2.472467×10^{-13}
A:time	9.916667	4.958333	16.23398	0.000003
B:flow rate	-5.85	-2.925	-9.57669	0.000074
C:solvent temperature	-4.75	-2.375	-7.77594	0.000238
D:restrictor temperature	0.85	0.425	1.391484	0.213478
E:solvent volume	2.45	1.225	4.010749	0.007032
F:restrictor position	0.183333	0.091667	0.300124	0.774209
G:cooling position	0.083333	0.041667	0.13642	0.895951
Curvature		9.508333	13.92223	0.000009

Table 2.18. Analysis of variance results for cyclohexane, containing estimated effects and regression coefficient of each term.

Term	Effect	Coefficient	T Value	P Value
Intercept		74.11667	280.9721	1.371125×10^{-13}
A:time	9.6	4.8	18.19653	0.000002
B:flow rate	-8.2	-4.1	-15.5429	0.000004
C:solvent temperature	-1.4	-0.7	-2.65366	0.037843
D:restrictor temperature	0.5	0.25	0.947736	0.379855
E:solvent volume	2.1	1.05	3.980491	0.007279
F:restrictor position	-0.13333	-0.06667	-0.25273	0.808913
G:cooling position	0.4	0.2	0.758189	0.477052
Curvature		11.85	20.09004	9.876284×10^{-7}

Graphical representation

Pareto plots and the normal probability of factors, Figures 2.8 to 2.12, were used for graphical presentation. Pareto plots display the absolute standardized effects values, which are standardized by dividing each effect by its standard error. It contains a vertical reference line, which indicates the confidence limit. The confidence limit was 0.05 or 95%. This helps in determining the factor and interaction effects that are important. The bars are displayed on the order of the size of the effect. Any effect that extends past the reference line on the chart is considered as important.

The normal probability of factors displays same information as Pareto plots. However, the effect values are not absolute values and are plotted against cumulative probability. It displays negative effects on the left side of the fitted line and positive effects on the right side of the fitted line. The fitted line indicates where the points would fall if the effects were zero. They are used in determining the direction of the effect the variable has on the response.

The graphical analysis provided only visual understanding of the relative importance of each effect but did not provide a quantitative measure of confidence for conclusion. To estimate this confidence, analysis of variance (ANOVA) was used.

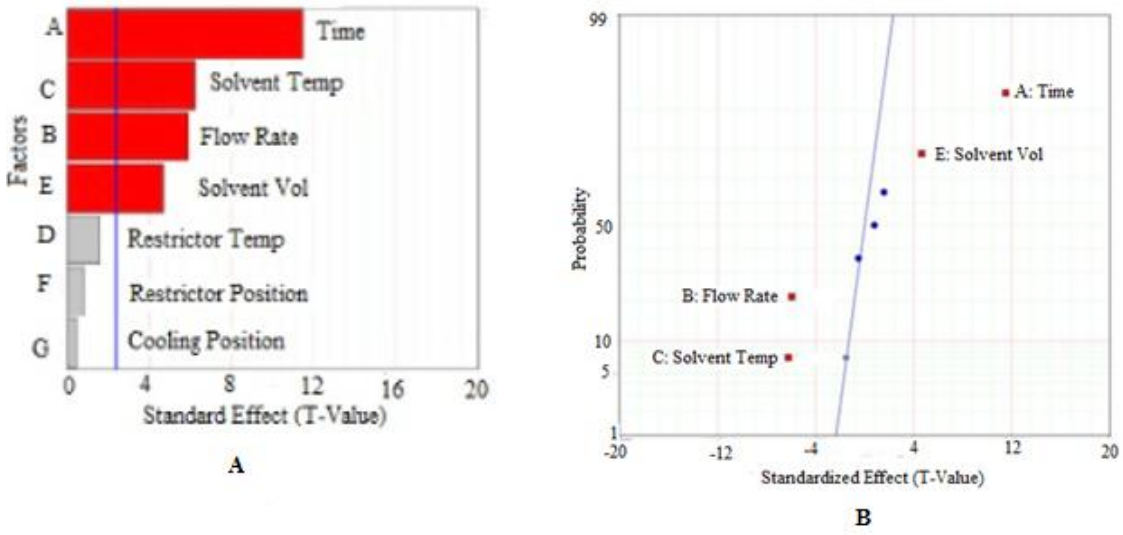

Figure 2.8. Pareto chart (A) and normal probability chart (B) for acetonitrile.

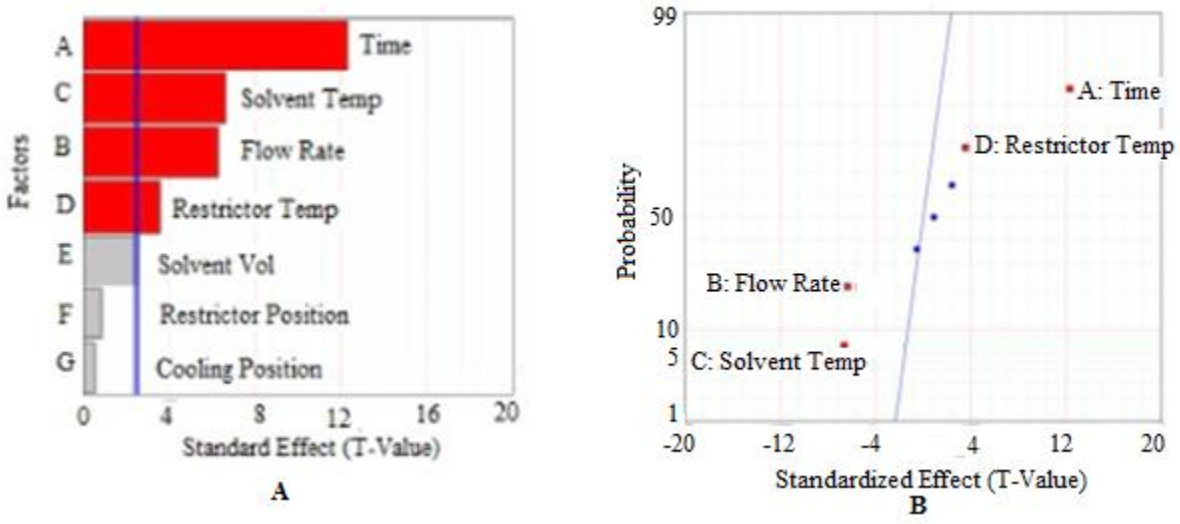

Figure 2.9. Pareto chart (A) and normal probability chart (B) for methanol.

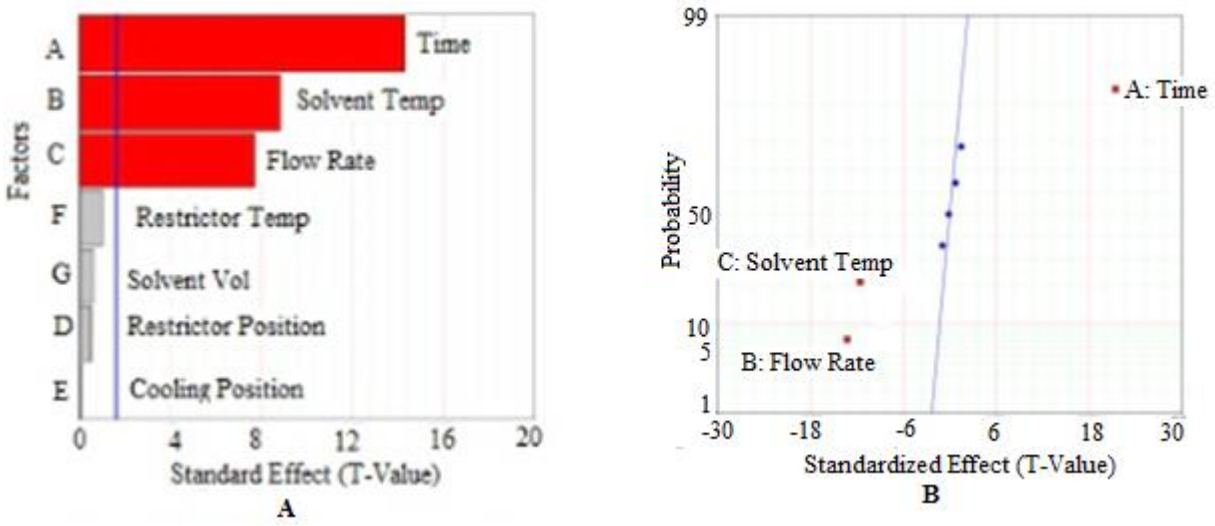

Figure 2.10. Pareto chart (A) and normal probability chart (B) for isopropanol.

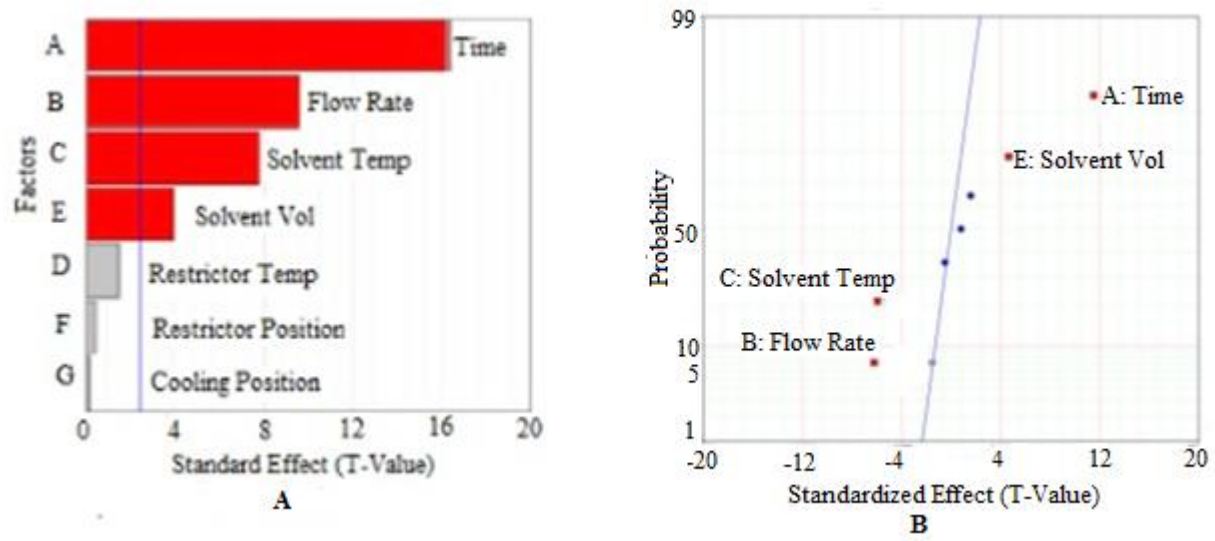

Figure 2.11. Pareto chart (A) and normal probability chart (B) for dichloromethane.

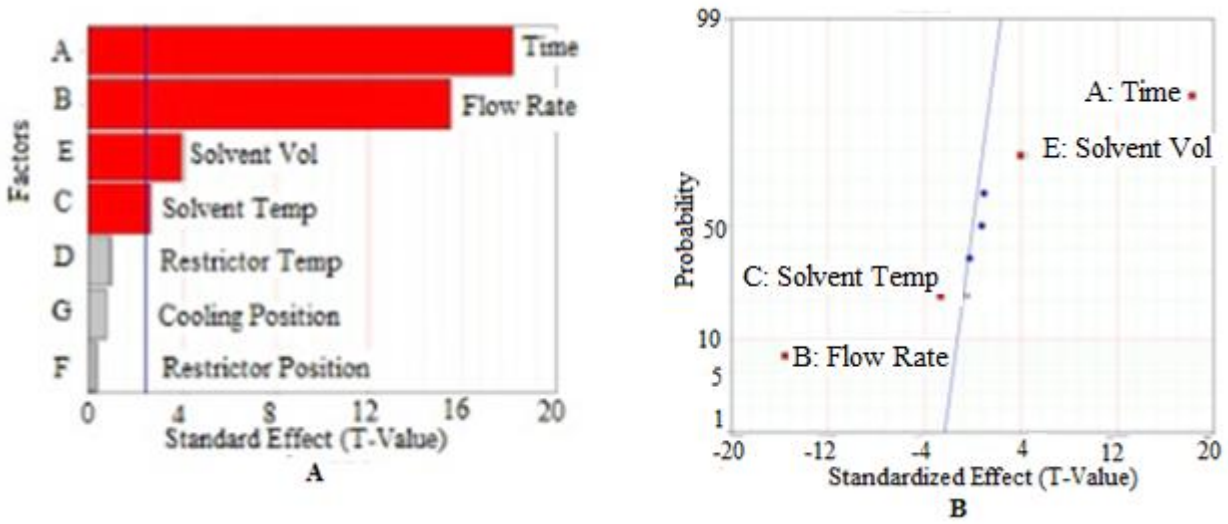

Figure 2.12. Pareto chart (A) and normal probability chart (B) for cyclohexane.

The summary of the significant and insignificant variables for each model is presented in Table 2.19. The Table summarizes ANOVA results for each regression model. T-values with a *p*-value less than or equal to 0.05 was considered as significant and those with values greater than 0.05 were considered insignificant.

Table 2.19. The summary of each model significant and non-significant factors.

Solvents	**Significant Factors**	**Non-significant Factors**
Acetonitrile	Time, solvent temperature, flow rate, solvent volume	Restrictor temperature, restrictor position, cooling position
Isopropanol	Time, solvent temperature, flow rate	Restrictor temperature, cooling position, restrictor position
Methanol	Time, solvent temperature, flow rate, restrictor temperature	Solvent volume, restrictor position, cooling position

Dichloromethane	Time, flow rate, solvent temperature, solvent volume	Restrictor temperature, restrictor position, cooling position
Cyclohexane	Time, flow rate, solvent volume, solvent temperature	Restrictor temperature, cooling position restrictor position

In each model, time, solvent temperature, and flow rate were found to be significant. Temperature and flow rate were found to have a negative effect, while time was found to have a positive effect. A conclusion to what extent this trend is viable cannot be drawn using screening models since the presence of higher polynomial coefficient terms might reverse the trend. For instance, higher decompression times increases the duration of contact the extract has with trapping solvent while increasing the time for the analytes to be purged.

Comparing isopropanol (viscosity, 2.4 cP) with acetonitrile (viscosity, 0.36 cP), solvent volume had a greater effect with acetonitrile and was significant, but with isopropanol the effect was minimal and was insignificant. Viscosity has an effect on the rate at which the bubble rises. It can be implied that volume of the solvent was important in a less viscous solvent because the resistance was less and higher volumes of solvent increased the contact time the bubble, which contained the extract, had with the trapping solvent.

The bubbles formed during decompression have an effect on the diffusion of the solute through the expanding fluid. Higher flows rates make larger bubbles and, hence, longer diffusion pathways. Viscosity also affects the bubble size. Viscous solvents makes

smaller bubbles as compared to less viscous solvents. Higher total recoveries were achieved using isopropanol. Different solvents had different recoveries for individual essential oil compounds. Those results are discussed in the optimization section.

The cooling position was insignificant in all the models. The supercritical fluid expands during decompression with a decrease in temperature, due to the Joule-Thomson effect. Therefore, there is limited control of where the vial is cooled. Cooling the whole vial was considered for subsequent studies.

2.5.2 Optimization Results

2.5.2.1 Polynomial Model Equations and Response Surface

The polynomial functions describing how the experimental variables and their interactions influenced the response (total percent recovery) for each model was as illustrated in Equations 2.7 to 2.9. The coefficient for each term describes the estimate of the effect each respective variable had on the total recovery. The respective resultant surface plots were as illustrated by Figures 2.13 to 2.15. The plots display the three-dimensional relationship. The predictor variables are displayed on x- and y-scales, and the response (z) variable is represented by a smooth surface. The plot assisted in visualizing the relationship between different variables, and also in the approximation of desired factor-level combinations that gave the maximum, or target, response depending on the objective of the study.

In this work, the plots were used to predict the maximum total recovery and maximum recovery conditions for individual test compounds.

The second polynomial equation for acetonitrile collection model is Equation 2.7

$$Y = 77.4257 + 1.1258A{:}Time - 13.0467B{:}Flow\ Rate - 0.1391C{:}Temp - 0.0039A.B + 0.0009A.C - 0.0354B.C - 0.0196A.A + 5.2253B.B - 0.0074C.C \quad \textbf{2.7}$$

Figure 2.13 illustrates the three dimension response-surface plots for percent recovery using acetonitrile.

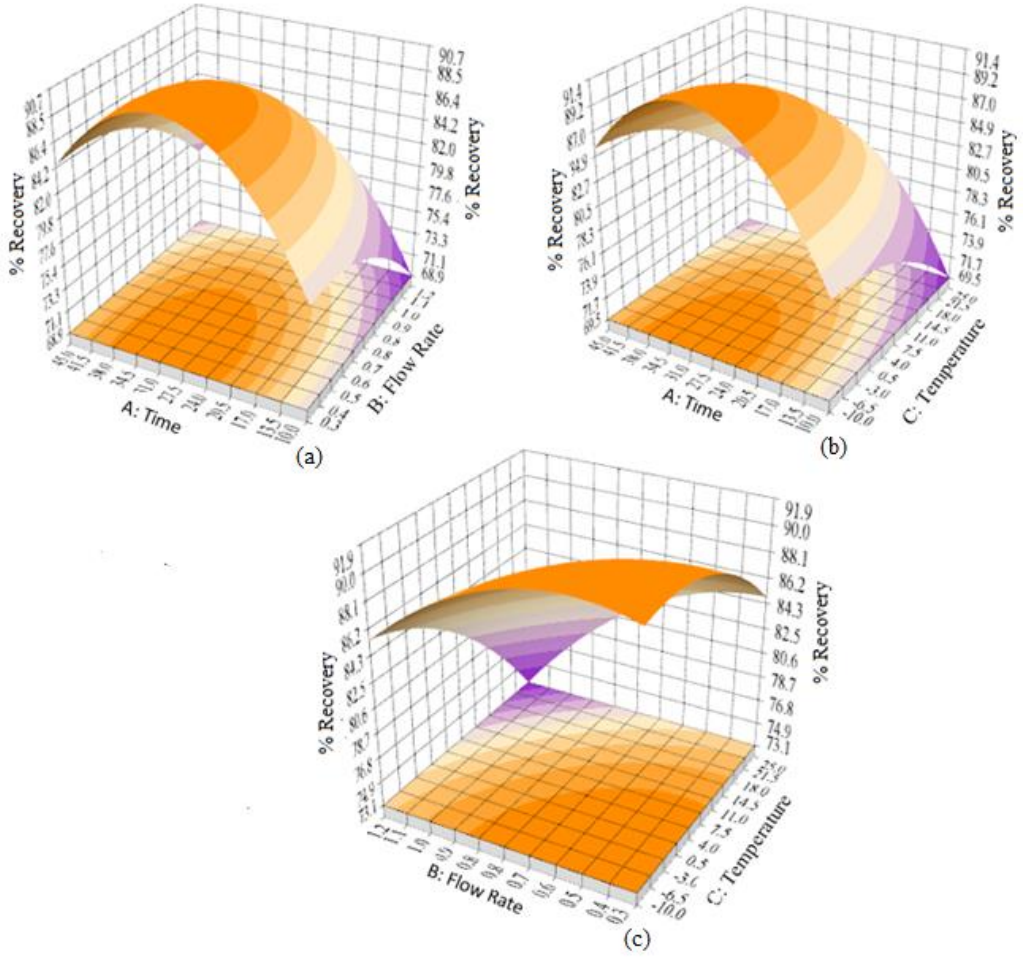

Figure 2.13. Response surface of acetonitrile % recovery versus (a) time and flow rate, (b) time and temperature, and (c) flow rate and temperature. The parameters range was temperature (-10-25˚C), time (10-45 min, flow rate (0.3-1.2 L/min).

Equation 2.8 is the second polynomial equation for the dichloromethane collection model.

$$Y = 69.5902 + 1.7347A{:}Time + 0.3716B{:}Temp - 4.0852C{:}Flow\ Rate - 0.0062A.B + 0.0038A.C - 0.0682B.C - 0.0318A.A - 0.0286B.B - 4.6630C.C \quad 2.8$$

Figure 2.14 illustrates the three-dimensional response-surface plots for percent recovery using dichloromethane.

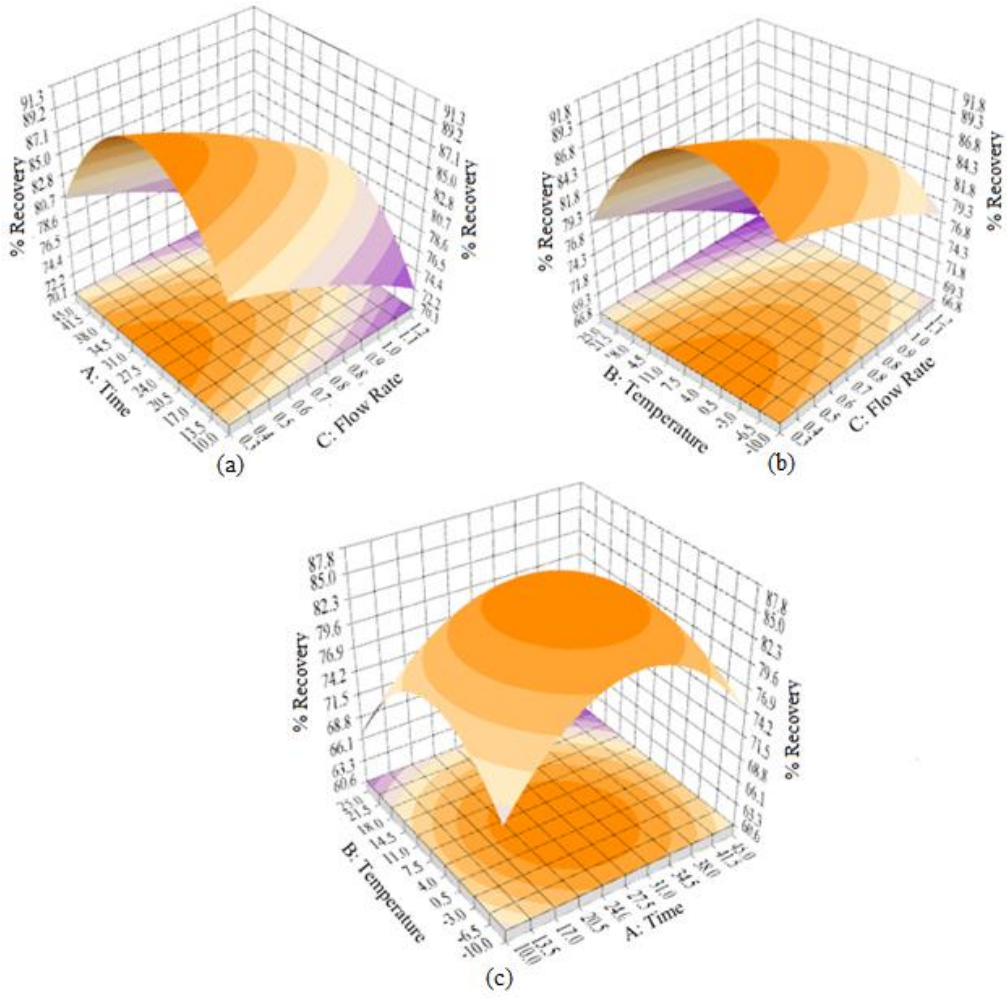

Figure 2.14. Response surface of dichloromethane % recovery versus (a) time and flow rate, (b) flow rate and temperature, and (c) time and temperature. The parameters range was temperature (-10-25˚C), time (10-45 min), flow rate (0.3-1.2 L/min).

Equation 2.9 is the second polynomial equation for isopropanol collection model.

$$Y = 75.8619 + 1.5085A{:}Time + 0.150B{:}Temp - 7.1607C{:}Flow\ Rate + 0.0007A.B + 0.0174A.C - 0.0193B.C - 0.0296A.A - 0.0266B.B - 1.0740C.C \qquad 2.9$$

Figure 2.15 illustrates the three-dimensional response-surface plots for percent recovery using isopropanol.

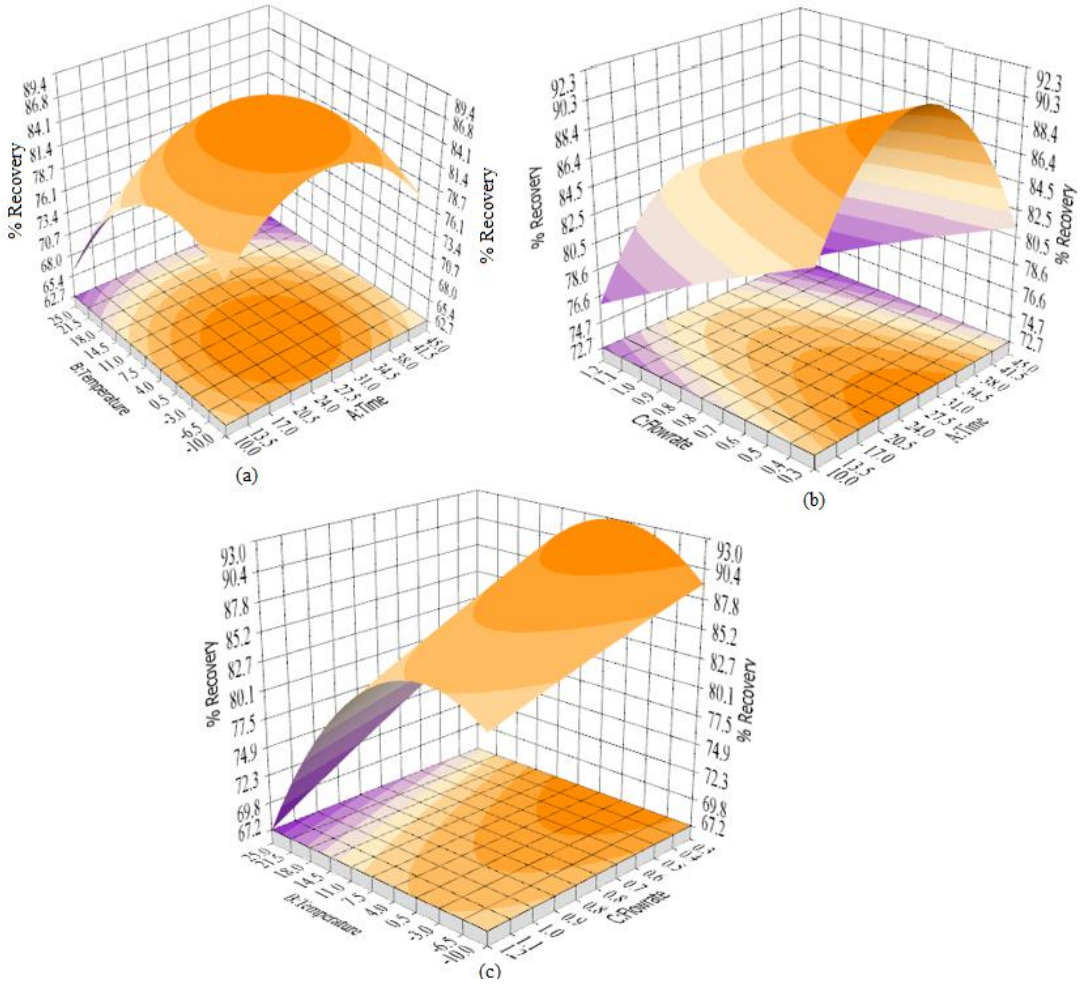

Figure 2.15. Response surface of isopropanol % recovery versus (a) temperature and time, (b) flow rate and time, and (c) temperature and flow rate. The parameters range was temperature (-10-25˚C), time (10-45 min), flow rate (0.3-1.2 L/min).

Model evaluation

The effect of each variable was indicated by its respective coefficient in the polynomial function. Table 2.20 contains the summary of each coefficient value with their respective t-test values. The t-test was used to establish the significance of each term. This was the done by evaluating the *p*-values at a confidence level of 95 percent. The larger the t-value and smaller the *p*-value, the more significant the corresponding coefficient is.

Table 2.20. The t-value, p-values for the three models estimated using DoE^{+++}software. The results tabulate the significance of the variable and their interactions. Significant F-values are highlighted in red.

Term	DCM			Isopropanol			Acetonitrile		
	Coefficient	T-value	p-value	Coefficient	T-value	p-value	Coefficient	T-value	p-value
A: Time	1.734	-2.68	0.016	1.508	-3.50	0.003	1.120	7.50	<0.001
B: Flow Rate	-4.085	-12.51	<0.001	-7.161	-3.50	<0.001	-13.040	-23.87	<0.001
C: Temperature	0.372	-11.86	<0.001	0.015	-12.88	<0.001	-0.140	-41.67	<0.001
A . B	0.004	0.05	0.960	0.017	0.19	0.851	-0.004	-0.21	0.836
A . C	-0.006	-3.26	0.004	0.001	0.31	0.764	0.001	2.00	0.061
B . C	-0.068	-0.92	0.370	-0.019	-2.11	0.049	-0.035	-1.87	0.078
A . A	-0.031	-14.04	<0.001	-0.030	-10.58	<0.001	-0.019	-33.95	<0.001
B . B	-4.663	-1.36	0.019	-1.074	-0.253	0.802	+5.225	5.97	<0.001
C . C	-0.029	-12.60	<0.001	-0.027	-9.51	<0.001	-0.007	-12.88	<0.001

The model shows that flow rate had the greatest effect on total recovery, followed by time, and lastly temperature. This trend was similar in all the models. In all the models, flow rate had a negative effect on recovery. Higher recovery was realized at lower decompression flow rates. Higher flow rates result in large bubble size that increases the diffusion path the analyte have to cover to diffuse into the trapping solvent. Also, higher flow rates increase the rate of purging of the volatile analytes.

The effect of flow rate was found to be higher in the acetonitrile model, which is the least viscous solvent. The viscosity of the solvent has an effect on the rate of the carbon dioxide bubble containing the analyte. Less resistance is experienced in a less viscous solvent. The rate is further increased by higher flow rates. This reduces the contact duration the analyte has with the trapping solvent, thus reducing the chance of the analyte to be trapped.

The total recovery increased as the temperature decreased to around 5^{o}C, and then it started decreasing. These results are illustrated by response-surface plots, Figure 2.13 to 2.15. Higher temperature may affect the solubility of some compounds, improving the trapping, and also can cause the evaporation of the volatile compounds. The temperature had an overall negative effect and higher recoveries were realized at lower temperatures.

The total percent recovery increased with time to around 30 minutes and then started to decrease. The increase can be attributed to increasing contact the analytes had with the collection solvent. However, as the duration of decompression increases, more of analytes are purged, especially at higher flow rate. Higher flow rates coupled with

long extraction times resulted in lower recoveries. This is as a result of volatile compounds being purged. Time had an overall positive effect.

Table 2.21 tabulates the optimum values that resulted in a maximum total recovery for each trapping model. The model was used to predict the desired combination of variables during the quantitative extraction of specific essential oils components.

Table 2.21. Optimization results obtained from differentiation of the each quadratic model equation with respect to individual factor.

Trapping Solvent	Time (min)	Temperature (°C)	Flow Rate (L/min)	Total Recovery (%)
Isopropanol	25.58	2.07	0.3	94.07
Dichloromethane	26.89	3.21	0.3	91.86
Acetonitrile	28.30	-8.20	0.3	90.44

2.5.2.1.1 Evaluation of Model Significance

To establish if the observed variation in response was due to noise or due to variation of the effect of the combination of independent factors, analysis of variance was carried out. The F-test at a confidence level of 95 percent was used in establishing the significance of the model. Table 2.22 tabulates the ANOVA results obtained for each model.

Table 2.22. F-values and p-values for the three model obtained using DoE^{+++} statistical software. The results tabulate the significance of each term. Significant F-values are highlighted in red.

Factors	DCM		Isopropanol		Acetonitrile	
	F-value	p-value	*F*-value	p-value	*F*-Value	p-value
Model	68.55	<0.0001	48.36	<0.0001	427.82	<0.0001
Main effects	101.41	<0.0001	84.12	<0.0001	787.41	<0.0001
2-way interactions	3.82	0.0291	1.53	0.241	2.53	0.0919
Quadratic effects	100.43	<0.0001	59.44	<0.0001	493.53	<0.0001
Lack of fit	3.31	0.0715	3.021	0.0651	0.31	0.8211
R^2	0.9732		0.9624		0.9956	
Adjusted R^2	0.9590		0.9425		0.9933	

F-value is the ratio of the mean square of variance due to the variation of variables to mean square due to the variation of residual (error). Significant models are characterized by higher F-ratio. In this work, F-values are reported at a confidence level of 95%. The model was considered to be statistically significant if the *p*-value of the F-ratio was less than or equal to 0.05. This implies that there is only 5% chance that the F-value as higher as the one obtained is as a result of noise. All three models were found to be statistically significant with higher F-ratio and *p*-value of less than 0.0001, as indicated in Table 2.22 by the model term.

2.5.2.1.2 Evaluation of Model Fitness

To check how well the empirical data fit the polynomial equation (model), the lack of fit of the F-value was evaluated. The p-value of greater than 0.05 for the lack of fit implies that model error (residual excluding replicate variation) is not significantly greater than the replicate error.

The lack of fit for all models was found to be greater than 0.05. Thus, the models were sufficient to describe the process adequately. This was further confirmed by comparing the predicted values of percent total recovery versus the actual values obtained experimentally. The results showed high values for both regression coefficients (R^2 and adjusted R^2), which were closer to unity. The adjusted R^2 was considered as a more accurate indicator as it does not increase with the additional of variables, but it increases or decreases depending on whether the additional variable adds or detracts the response. While the R^2 increases with the increase of variables, a larger value of adjusted R^2 suggests that a significant relationship is captured by the model. Figures 2.16 shows the

linear regression of the predicted total recovery versus the actual experimental total recovery at different variable combinations.

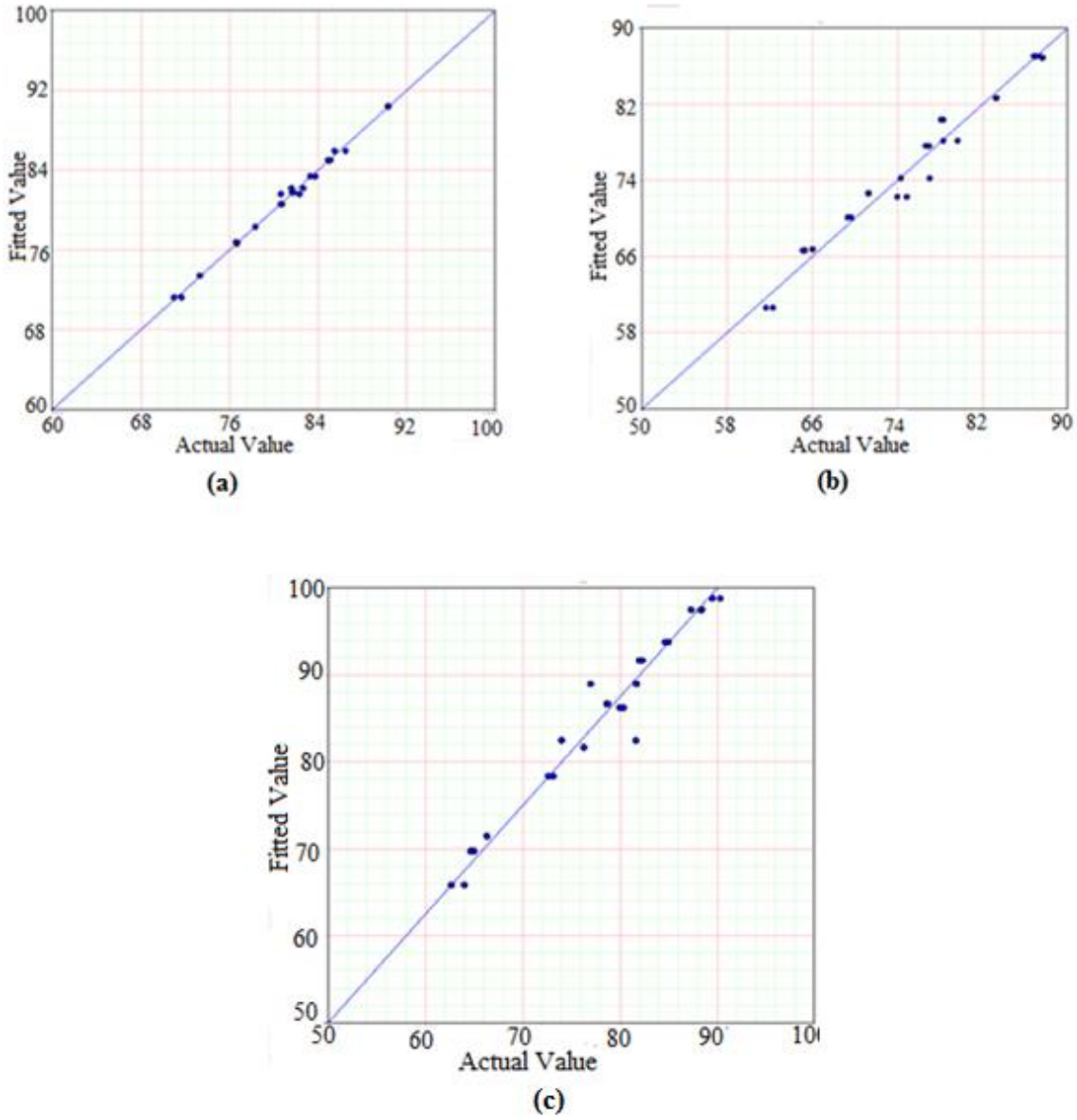

Figure 2.16. Predicted total percent recovery versus experimental total percent recovery for (a) acetonitrile trapping BB design, (b) dichloromethane BB design, and (c) isopropanol BB design.

To further validate the model, confirmation of the maximum percent recovery was done by running experimental runs at optimal conditions. This was done in triplicate.

2.6 Conclusions

The usefulness of the design of experiments approach in the modelling the collection step in supercritical carbon dioxide extraction has been demonstrated. Plackett-Burman screening design was used for screening for important parameters that affect the trapping of essential oils components following SFE. Time, flow rate and collection temperature were found to be the most significant parameters. Box-Benken response-surface methodology design was used in modelling the collection step. The BBD design was found to be an important tool to investigate the interaction of variables, the effect on the recovery and the optimum conditions for collection of essential oils. This was done at a reduced number of experimental trials compared to one-variable at a time method. Main effects and the quadratic effects were found to be significant.

Time had a negative effect on trapping efficiency with isopropanol and dichloromethane, while with acetonitrile it had a positive effect. Flow rate had a negative effect on all the solvents. Thus, higher recoveries were realized at lower flow rates. The temperature had a negative effect in all the models. Higher recoveries were realized at lower temperature (< 5 ºC). The time and flow rate interaction was found to have a positive effect with isopropanol and dichloromethane, while in acetonitrile the interaction was negative. Time and temperature interaction was found to have a positive effect with acetonitrile and a negative effect with dichloromethane. The flow rate and temperature interaction was found to have a negative effect in all the solvents. The optimal condition for total recovery was as follows, isopropanol (25.58 min, 2.07 ºC, and 0.3 L/min),

acetonitrile (28.30, -8.20 ºC, and 0.3 L/min) and dichloromethane (26.8 min, 3.21 ºC, and 0.3 L/min).

The model was adopted to predict the best collection conditions for individual essential oil components within the experimental range. These conditions were used in the collection of essential oils after SFE extraction of essential oil from chamomile, rabbit brush, and skunk brush.

3 CHAPTER 3: Extraction of Essential Oils from *Chrysothamnus nauseosus* (rabbit brush), *Rhus aromatic* (skunk brush), and *Matricaria chamomilla* L (chamomile)

3.1 Abstract

Green extraction is based on the design and discovery of extraction processes that reduce energy consumption, allow the use of alternative solvents and renewable natural products, and ensure a safe and high-quality extract. There is significant interest in obtaining extracts with particular biological activities from renewable feedstocks using environmentally benign processes. In this work supercritical carbon dioxide was used in the extraction of essential oils from three plants, *Chrysothamnus nauseosus* (rabbit brush), *Rhus aromatic* (skunk brush), and *Matricaria chamomilla* L (chamomile).

Carbon dioxide is cheap, readily available in high purity, chemically inert, and supercritical at modest pressure (73 atm) and temperature (31^{o}C). Supercritical fluids have lower viscosities and higher solute diffusivities than liquid solvents. This improves mass transfer and reduces the extraction time needed. The solvent strength and selectivity can be simply controlled by changing the pressure or temperature.

The extraction step was modeled using response-surface methodology (RSM). The collection of the extract was done using the optimized conditions established by RSM. Pressure was found to be the most significant parameter affecting the total yield. The yield increased with pressure while temperature had an inverse effect on the solubility of essential oil components. The extraction of sesquiterpenes and oxygenated compounds are more difficult due to their molecular weight and polarity, respectively, as compared to monoterpenes.

In *Chrysothamnus nauseous* (rabbit brush), the major compounds identified were d-limonene (35.77%), trans-β-ocimene (27.41%), camphor (11.57%), β-phellandrene (4.64%), β-pinene (4.13%), eucalyptol (2.198%), β-cis-ocimene (2.66%), camphene (1.96%), and artemiseole (1.61%). In *Rhus aromatic* (skunk brush) the main compounds identified were d-limonene (20.48%), linalool (37.31%), caryophyllene (12.5%), eucalyptol (9.14%), α-phellandrene (5.5%), and geraniol (1.2%). The major compounds in chamomile samples from three different regions in Kenya were α-bisabolol, α-bisabolol oxide B, α-bisabolol oxide B, matricine, dicycloether, and β-cis-farnesene.

The optimal conditions (temperature, pressure, and time) for total extraction was 35^{o}C, 3570psi, 40 min for skunk brush; 47 ^{o}C, 6620psi, 45min for chamomile oil; and 37 ^{o}C, 1720psi, 43 min for rabbit brush. α-Bisabolol concentrations in Kangari, Kibwezi, and Njabini chamomile plant samples were 1.03±0.006 mg/g, 0.759±0.092 mg/g, 0.900±0.011mg/g respectively. Limonene and camphor concentrations in rabbit brush were 2.052±0.020 mg/g and 0.652±0.010 mg/g respectively. Limonene, linalool, and caryophyllene concentrations in skunk brush were 1.448±0.027 mg/g, 2.28±0.014 mg/g, and 0.956±0.018 mg/g.

3.2 Introduction

In advancement of global green technology based on bioproducts and bioprocesses, there has been an increased focus on the design of green and sustainable extraction methods of natural products.[10, 26, 34, 59] A recent trend in extraction techniques has mainly focused on finding processes that minimize the use of traditional solvents. This should be done while enabling process intensification with the production of high-quality extracts in a cost-effective way. The general definition of green chemistry is the invention, design, and application of chemical products and processes that reduce or eliminates the use and generation of hazardous substances. Based on this definition, green extraction can be defined as the invention and design of extraction processes that reduce energy consumption, allow the use of alternative solvents and renewable natural products, and ensure a safe and quality extract. Six principles of green extraction have been listed as: [4]

Principle 1: Innovation by selection of varieties and use of renewable plant resources.

Principle 2: Use of alternative solvents and preferably water or agricultural-derived solvents.

Principle 3: Reduce energy consumption by recovery and using innovative technologies.

Principle 4: Production of co-products instead of waste to include the bio- and agro-refining industry.

Principle 5: Reduce unit operation and favor safe, robust and controlled processes.

Principle 6: Aim for non-denatured and biodegradable extract without contamination.

The use of supercritical carbon dioxide fluid in the extraction of essential oils, which are a natural source of bioactive agents, is in line with the six principles of green

extraction. Use of supercritical carbon dioxide as extracting solvent offers an alternative to traditional extraction techniques like Soxhlet and steam distillation. The general advantages of SFE include the flexibility of the process due to the possibility of controlling of solvent power or selectivity, elimination of polluting organic solvents, and elimination of expensive post-processing of the extract. Carbon dioxide is safe, cheap, readily available at high purity, nonflammable, nontoxic, and its critical pressure and temperature are convenient.

There are several parameters that affect the extraction efficiency of supercritical fluids. The solvent power and mass transfer are crucial to extraction. These thermodynamic and kinetic parameters are affected by pressure, temperature, sample structure, and time, among other properties.[36, 57] Modeling of extraction step is necessary to determine the interactions between these factors and the effect they have on extraction. Modeling helps to improve the SFE selectivity by determining optimized conditions for extraction of the individual component of interest. It also provides the optimal extraction conditions for total extraction. The model can also be used in the prediction of extraction conditions for desired extract yield within the range considered.[34, 50, 79]

In recent years, the demand for fewer synthetic products has grown tremendously. This is as a result of society embracing ‘green’ consumerism. The demand for products that have a smaller impact on the environment has been preferred. In line with this, the demand for essential oils has increased in the food, pharmaceutical, and cosmetic industries. This has led to sustainability problems.[64] It often takes hundreds of pounds of plant material to produce one pound of essential oil. This has created a biodiversity problem as many plants species have been lost and some are in danger of extinction. To

find a solution to the biodiversity problem, a large number of research projects have been aimed at finding an alternative to the use of threatened species.[64, 68, 86] There has been a significant effort in the natural selection of varieties with much higher concentration of bioactive components. Therefore, analytical methods that can be used to quantitate the amount of these bioactive components are needed.

Although the study for the extraction of essential oils is widespread, there are a limited number of studies that concentrate on quantitative extraction and the study of parameters governing the process. This is due to essential oils being a complex mixture containing volatile and semi-volatile compounds.

In this work, response-surface methodology (RSM) was used to model the extraction of essential oils from selected plants with the ultimate goal to quantitate the major essential oils compounds present in those plants. RSM give the relationship between the measured response and the independent factors. The technique reduces the number of experimental trials and investigates the correlations between factors that can be used for process optimization. The effect of the main process parameters including pressure, temperature, and extraction time on the essential oil yield was investigated. The resultant polynomial empirical model was used in determining the optimal conditions for selective extraction and optimal conditions for total extraction.

3.3 Literature Review

3.3.1 Chamomile (*Matricaria chamomilla* L)

Chamomile (*Matricaria chamomilla* L) is a well-recognized medicinal plant in western culture. It is native to southern and eastern Europe. The plant is found in north and eastern Africa, Asia, North and South America, Australia, and New Zealand. Its

therapeutic use dates back to ancient civilization. The ancient Egyptians used it to alleviate fever and sunstroke. In the sixth century, it was used to treat back pain, rheumatism, insomnia, neuralgia, skin conditions, headaches, indigestion, and gout.[87] Nowadays its extract is widely used in the pharmaceutical, perfumery, food, and cosmetic industries.

There are numerous varieties of chamomile, but the two most popular in traditional herbalism are German chamomile (*Matricaria chamomilla*) and Roman (*Chamaemelum nobile*), both belong to the Asteraceae or Compositae family. They are similar in physical appearance, chemical properties, and general applications.[88] German chamomile is more widely cultivated compared to Roman chamomile. German chamomile has a pleasant apple-pineapple scent. It annually grows two to three feet tall. Its flower head is one inch in diameter and has a hollow conical center covered with tiny yellow florets surrounded by silver-white to cream-colored florets. It has erected branching with finely divided leaves. Roman chamomile, on the other hand, is an aromatic creeping perennial, which grows only one foot in height. Its flower heads are one inch in diameter, with a broad conical disk that is covered in yellow florets surrounded by white florets. It has many freely branching hairy stems and finely divided leaves.[67] Figure 3.1 shows an example of chamomile plant.

In Kenya, chamomile is grown in, among other areas, the Aberdares region, Naivasha, and Kibwezi. It is grown for sale to herbal shops that either blend it with tea to sell as chamomile tea or sell the flowers for further blending by other traders. In the USA, chamomile is found growing freely in cornfields, roadsides, and other sunny, well-drained areas. It is widely used as an ingredient in tea and numerous cosmetics.

Figure 3.1. A photo of chamomile plant.[75]

3.3.1.1 Uses of Chamomile

Chamomile is used mainly as an anti-inflammatory, antiseptic, carminative, sedative, and antispasmodic.[89] It is used internally primarily as an herbal tea for disturbance of the stomach associated with pain, sluggish digestion, diarrhea, and nausea. Externally, the drug in powder form may be applied to wounds slow to heal, for skin eruptions, and infections such as shingles and boils, hemorrhoids, and other inflammations.[90] In addition to medicinal use, chamomile is used as a refreshing beverage tea.

3.3.1.2 Essential Oil Constituents in Chamomile

All organs of the chamomile plant contain essential oils, with the flowers and flower head having the highest quantities, and roots having the least. The composition of the oil differs depending on the source of the flower, growth conditions, and other factors.

The extract contains a large group of therapeutically active compound classes. The most important constituents include sesquiterpenes, flavonoids, coumarins, and polyacetylenes. The oil contains seventy five to ninety percent sesquiterpene derivatives with only traces of monoterpenes, and up to twenty percent polyenes. The main sesuquiterpenes are chamazulene (2.3–10.9%), α-bisabolol-oxides A (25.5–28.7%), α-bisabolol oxides B (12.2–30.9%), and β-farnesene (4.9–8.1%). Other components found in lower concentrations are α- and β-caryophyllene, caryophyllene oxide, spathulenol, and monoterpenes like β-phellandrene (0.8%), limonene (0.8%), β-ocymene (0.4%), and γ-terpinen (0.2%).[91] Pharmacological effects have been connected to essential oil component present.

3.3.1.3 Biological Activities of Main Components in Chamomile

The biological activity of chamomile is mainly due phenolic compounds and essential oils constituents such as α-bisabolol and its oxides and azulenes. The chamomile oil has shown to have antibacterial, antifungal, antiviral, anti-ulcer, sedative, anti-inflammatory, antiseptic, and anti-spasmolytic properties. Among the major constituents, α-bisabolol and chamazulene have been reported to be the most effective than others.[92] Chamazulene comprises about five percent of the essential oil. It has anti-inflammatory, antibacterial, and antispasmodic properties. Bisabolol comprises of fifty percent of the essential oil.[93] It also has anti-inflammatory, antibacterial, antipyretic, ulcer-protective, and antifungal properties.[93, 94]

3.3.2 *Chrysothamnus nauseosus* (rabbit brush)

*Chrysothamnus nauseosus (*rabbit brush) is a perennial shrub that belongs to the Aster family (Asteraceae). It is widely found in deep sandy soils of the desert grassland

and the Great Plains.[95] It typically grows one to seven feet tall and may have several stems from the base that branch to give a rounded appearance. It has narrow yellow-green leaves and flexible twigs, as seen Figure 3.2. It is widely found in the western United States.

Figure 3.2. Photo of rabbit brush plant.[96]

3.3.2.1 Uses of Rabbit Brush

Rabbit brush has a history of ethnobotanical uses. Native Americans reportedly used rabbit brush extract as a yellow dye and to make a medicinal tea. The tea was believed to treat coughs and chest pains. They also used the plant as chewing gum.[97] Rabbit brush was used as a source of high-quality rubber during World War II.[98] Recently, it is used in the production of rubber, resins, and other chemicals.[99] Compounds in rabbit brush are being evaluated as nematocides, for anti-malarial properties and as insect repellents. It has also been identified as a potential source of biomass and bio-crude fuels.[100] Essential oil from the plant is used as analgesic, antifungal, antispasmodic, antirheumatic, carminative, and anti-anxiety agents.[101]

3.3.2.2 Essential Oil Constituents in Chamomile

Steam-distilled oil has been previously analyzed and found to constitute 60.7% monoterpenes, 15.9% oxygenated monoterpenes, and 12.2% oxygenated sequiterpenes.[98] The major essential oil components identified were β-phellandrene (14.9-22.8%), β-pinene (8.8-19%), β-caryophyllene (3.3-5%), and β-ocimene (3-6.4%).[95, 101]

3.3.2.3 Biological Activities of Main Components in Chamomile

The *Chrysothamnus nauseosus* essential oil is shown to have antimicrobial, antifungal, and antimalarial activity.[101] Biological activity of individual components has been investigated. Compounds found to be the major contributors to the observed biological activity through synergism were reported.[101]

3.3.3 *Rhus aromatic* (skunk brush)

Rhus aromatic belongs to genus *Rhus* (sumac) and Anacardiaceae family. It is an aromatic, deciduous, small bushy shrub with yellow catkin-like flowers proceeding dark-red, shown in Figure 3.3. The shrub grows six to twelve feet tall. The shrub is native to southeastern Canada to the southern and eastern United States. It grows in many ecological regions, from the Great Plains grassland to mountain shrub land, chaparral, and forest areas. The plant is widely distributed from west to eastern South Dakota, central Nebraska, Kansas, Oklahoma, and Texas.

Figure 3.3. Photo of a skunk brush plant, taken by Neil Reese.[79]

3.3.3.1 Uses of Skunk Brush

Skunk brush has historically been used by Native Americans as food and medicine. The ripe fruits were eaten raw or used as berry tea. The bark and root were chewed or brewed into a drink to treat various ailment including diarrhea, stomachache, toothache, sore throat, skin disease, and eczema.[102] The extract from the bark and leaves has been used in leather tanning. The extract contains a high tannin content. Currently, it is used for treating urinary incontinence, overactive bladder, cystitis, functional bladder problem, and certain types of uterine hemorrhages.[86] It is also being investigated to provide an alternative source of antimicrobial agent to control swine diarrhea, which is a significant problem experienced by swine farmers in South Dakota.[68]

3.3.3.2 Essential Oil Constituents in Skunk Brush

Limited information is available as to the composition of *Rhus aromatica*. Analyses of an alcohol extract showed the presence of around eight percent tannins,

gallic acid, and phenolic compounds. The essential oil content was about 0.01-0.07% with the major components being geranyl acetate, α-ambrinol, dihydro-γ-ionone, farnesyl acetone, and dinorlabdenons.[86]

3.3.3.3 Biological Activities of Main Components in Skunk Brush

The bark alcohol extract has exhibited anti-inflammatory and anti-microbial effects.[103] An aqueous extract has exhibited antiviral against herpes simplex viruses.[86] Antibiotic activity has been reported against mastitis pathogens *E. coli* and *S. aureus.* [104] Antimenatodal activity has also been demonstrated, and it the extract is commercially available in the bionematicide mixture Sincocin.[105]

3.4 Experimental

3.4.1 Materials and Reagents

Chamomile flowers were collected from Kenya. The sampling sites were located at Kibwezi, Kangari, and Njabini. Kibwezi is located in a hot and dry region of Kenya, Njabini is located in the cold part of Kenya, west of the Aberdares range. Kangari is in the east of the Aberdares range. The region is wet and cold. Chamomile flowers from Njabini and Kangari were bought from an organic shop, while those from Kibwezi were obtained from the University of Nairobi farm in Kibwezi. Dry flowers were crushed and sieved to get rid of stalks and petals. The sieved flowers were stored in airtight polythene bags and stored at temperatures below zero.

Rhus aromatic and *Chrysothamnus nauseosus* were collected from Sica Hollow State Park and Oak Lake Field Station, South Dakota. They were all prepared in the field or taken to the laboratory within 2-4 hours. They were cleaned with tap water and stored

at -80°C. d-Limonene, linalool, carvone, citral, cineol, geraniol, caryophyllene, α–pinene, phellandrene, and α–bisabolol, and methyl hexyl ketone were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Commercial hydrodistilled rabbit brush essential oil was from Stillpoint Aromatics (Sedona, AZ). Ottawa sea sand was from Thermo-Fischer Scientific (Pittsburgh, PA). Isopropanol, acetonitrile, and dichloromethane were from Thermo-Fischer Scientific (Pittsburgh, PA). SFE-grade CO_2, 99.9995% purity with helium-pressure dip tube was supplied by Airgas (Radnor, PA).

3.4.2 Methods

3.4.2.1 Experimental Design

Central-composite face-centered response-surface methodology was used to model the extraction step. Three independent factors (temperature, pressure, and time) were investigated. The design needed 20 experiments with eight (2^3) factorial points and six star points (2k) to form a central-composite design and six replications of the center point. The experiments were run in random order to minimize the effect of unexpected variability due to extraneous factors. The design points except the center points were carried out in duplicate. The experimental range for each factor was based on the results of preliminary trials. Table 3.1 lists the independent variables, their symbol, and the coded factor level. All the experimental design, data analysis, and response-surface modeling were conducted using ReliaSoft DOE++ software.

Table 3.1. Independent variables and their actual and coded levels.

Independent Variables	Independent Variable Levels		
	-1	0	+1
Temperature (°C)	35	42.5	50
Pressure (psi)	1500	5750	7000
Time (min)	10	35	60

3.4.2.2 Extraction of Essential Oils using Supercritical Carbon Dioxide

Extraction was performed using a Spe-ed SFE Prime Model 9935 (Applied Separations, Allentown, PA) equipped with a 24-mL stainless-steel vessel that could withstand pressures up to 10,000 psi. Five grams of sample was weighed and packed into the extraction vessel. Carbon dioxide was pumped through the extraction vessel and the extraction chamber was heated and then pressurized to desired value. The pressure range was 1500 to 7000 psi and temperature range was 30-50 ^{o}C. The extraction was done in both dynamic (continual flow) and static modes. The vessel was allowed to stand for ten minutes (static extraction period) for all the runs, and then dynamic extraction of 10-60 minutes was carried out. The extract was collected into 20-mL organic solvent at the optimal conditions determined in the collection studies. Figure 3.4 illustrates the SFE set up.

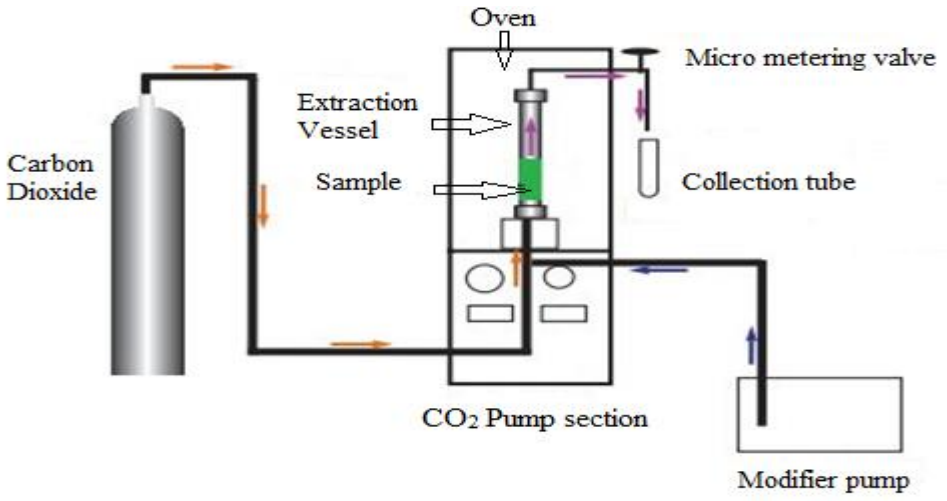

Figure 3.4. Schematic diagram showing the SFE set up and carbon dioxide flow.[44]

3.4.2.3 Soxhlet Extraction

Five-gram samples were weighed and transferred into the extraction thimble and inserted into a 250-mL reflux flask. Using 150 mL of hexane, extraction was done for 12 hours. After the Soxhlet extraction, the extract was concentrated using rotary evaporation at 50 °C.

3.4.2.4 GC-MS Analysis

GC-MS analyses were carried out using with an Agilent 7890 gas chromatograph (Agilent Technologies, Little Falls, DE) coupled to an Agilent 5975C mass spectrometer and fitted with a DB-5 fused-silica capillary column (30 m x 0.25 mm, 0.25-µm film; Agilent Technologies, Little Falls, DE). The MS was operated in the electron impact mode (75 eV) with transfer line and ion source maintained at 250 ^{o}C. The GC operating conditions were 250 °C injector temperature, and the column temperature programmed between 45 to 240 ^{o}C at a rate of 6 ^{o}C/min with an initial isothermal period of two minutes and a final isothermal period of five minutes. The samples were introduced using

splitless injection. The peaks were identified by comparison of their mass spectra with the U.S. National Institute of Standards and Technology (NIST) library. The percent composition of individual components was computed from the chromatographic peak areas. Calibration with six concentration levels (10-600 ppm) were prepared for the major compounds identified. Methyl hexyl ketone, 100 ppm, was used as internal standard. The precision of the gas chromatographic method was confirmed by injecting each sample in triplicate and a standard deviation less than 5% was achieved.

3.5 Results and Discussion

3.5.1 Essential Oils GC-MS Compositional Analysis

3.5.1.1 Chamomile Flower Essential Oils from Three Different Kenyan Regions.

Table 3.2 and Figure 3.5 contain chromatographic results for chamomile essential oil extracted using supercritical carbon dioxide. The samples were from three different regions in Kenya. The extract was collected in acetonitrile at the optimal collection parameters (30 mins, -3.5 ^{o}C, and 0.3 L/min) established using RSM for sesquiterpenes.

The chamomile essential oil was mainly composed of sesquiterpenes. The major compounds were β-farnesene, α-bisabolol oxide B, α-bisabolone oxide A, α-bisabolol, matricine (chamazulene), spathlenol, and dicyloether. Bisabolol, bisabolol oxide, matricine, and dicycloether are known to be the most characteristic and pharmacologically relevant chamomile compounds.[106] α-Bisabolol content was highest in the Kangari sample (36.453%). Kibwezi and Njabini samples had 27.045% and 31.482% respectively. The actual concentration from quantitative analysis was 1.03±0.006 mg/g, 0.759±0.092 mg/g, and 0.90±0.011 mg/g.

Table 3.2. Chamomile essential oil component determined by GC-MS.

SN	RT (min)	% Area[a]			[b]Compound
		NJA	KA	KIBW	
1	8.659	-	0.263	0.23	Unknown
2	8.734	0.109	0.114	0.410	Eucalyptol
3	9.071	0.112	0.105	0.142	β-cis-Ocimene
4	9.226	0.099	0.097	0.131	Carene
5	10.536	-	0.035	-	Unknown
6	11.097	0.468	0.455	0.601	β-Linalool
7	11.371	-	-	0.021	Unknown
8	12.702	-	0.045	-	Grandrule
9	12.768	0.029	0.014	-	cis-Sabine hydrate
10	12.916	0.218	0.050	0.151	Isoborneol
11	13.345	0.025	0.026	0.045	α-Terpineol
12	14.358	0.442	0.279	0.607	Pseudolimonene
13	15.062	0.086	0.031	0.103	Methylverbenol
14	15.709	-	-	0.025	γ-Elemene
15	17.997	0.186	0.120	0.421	Patchoulane
16	18.135	0.263	0.206	0.231	Farnesol
17	18.249	0.556	0.477	0.659	Caryophyllene
18	18.352	4.973	4.264	7.571	β-cis-Farnesene
19	18.707	1.263	0.060	1.005	β-Longipinene
20	19.165	0.019	0.020	0.037	Alloamandrene
21	21.471	1.694	2.30	3.882	Spathulenol
22	22.821	13.929	14.160	22.871	α-Bisabolol oxide B
23	23.445	11.991	13.931	18.442	α-Bisabolone oxide A
24	24.252	3.729	3.75	6.002	Chamazulene/matricine
25	24.749	40.612	41.301	27.045	α-Bisabolol
26	25.327	0.291	0.359	0.420	Herniarin
28	27.347	15.208	15.410	8.435	Cis-ene-yne-Dicyloether
29	27.805	3.154	2.104	0.351	[Z]-ene-yne-Dicycloether

NJA-Njabini, **KIB**-Kibwezi, **KA**-Kangari, -Not detected, [a]GC peak area percentage. Each value is the mean of triplicate analyses, [b]Tentative identification based on MS. Compounds highlighted in yellow were in concentration >3%.

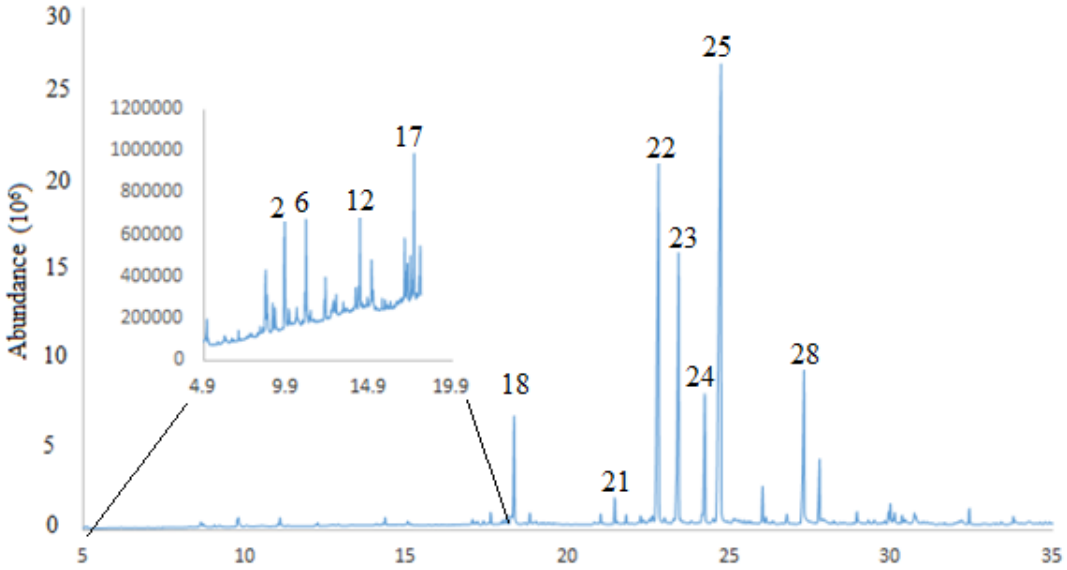

Figure 3.5. GC-MS chromatogram of SFE extracts of Kibwezi sample. Individual peaks are identified in Table 3.2.

Compared to other studies on chamomile samples cultivated in other parts of the world, the main components of essential oils of chamomile cultivated in Estonia was reported as bisabolol oxide A (20–33%) and B (8– 12%), bisabolone oxide A (7–14%), (E)-farnescene (4–13%), α-bisabolol (8–14%), chamazulene (5–7%), and en-yn-dicycloether (17–22%).[75] An Iranian study of chamomile essential oil extracted by hydro distillation reported α-bisabolol oxide A (25.01%) and α-bisabolol oxide B (9.43%) as the major constituents of the oil.[107] This more closely compares with the Kibwezi cultivated sample. Kibwezi is located in a hot and dry region of Kenya and this climate is similar to the Iranian climate. Another study of chamomile samples cultivated in different parts of Romania reported the main components as chamazulene (19.9%), α-bisabolol (20.9%), A and B bisabolol-oxides (21.6% and 1.2% respectively), and β-farnesene (3.1%). Compounds in lower concentrations were identified as α- and β-caryophyllene,

caryophyllene-oxide and spathulenol, and the monoterpenes β-phellandrene (0.8%), limonene (0.8%), β-ocymene (0.4%) and γ-terpinene (0.2%).[108] This compares well with the components found in the extracts from the Kenyan samples.

The composition and amount of herbal extract depend on several factors. It has been demonstrated that climatic conditions, type of soil, and growth stage widely affect the accumulation and composition of essential oil.[109] Kangari and Njabini are cold regions and receive higher rainfall. This tentatively explains the similarity of components in the extract from these regions. Kibwezi is a found in a dry region with minimal rainfall. The manner of which the sample is dried and stored is also a factor. Therefore, the difference in yield and composition could be as a result of one or a combination of various factors. Research has indicated that the pharmacological effect of chamomile is mainly connected with its main components α-bisabolol, bisabolol oxide, chamazulene, and en-yn-dicycloether.[110] Therefore, the quality of the extract can be evaluated by the amount of these compounds. The Kangari sample had the highest amount (90.66%), followed by Njabini sample (88.623%), and the Kibwezi sample had the smallest amount (83.15%) of these compounds. Therefore, Kibwezi extract was of lowest quality compared to that of Njabini and Kangari extract. In all three extracts, α-bisabolol was the dominant compound and, therefore, can be classified as a chemotype C extract. Chemotype classification is used to show the therapeutic values of particular extracts, which depends on the dominant essential oil component. Extracts dominant with bisabolol oxide A, bisabolol oxide B, and 1:1 ratio of bisabolol and bisabolol oxide A and B are classified as chemotype A, B, and D respectively.[111]

3.5.1.2 Comparison of SFE and Traditional Methods

The SFE extract was yellow indicating that no thermal degradation of naturally occurring matricine to chamazulene had occurred. Figure 3.6 shows Soxhlet and SFE extracts. The Soxhlet extract had dark blue color.

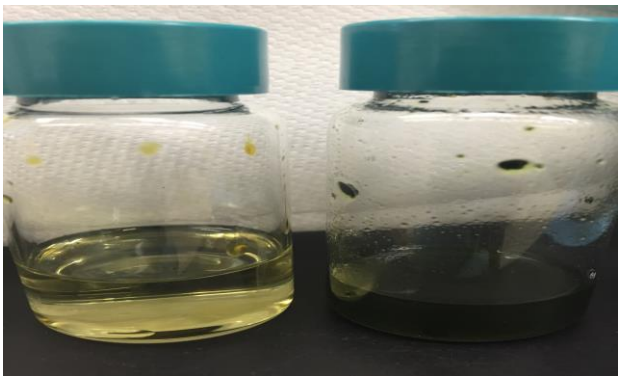

Figure 3.6. SFE extract (left) and solvent extract (right) of chamomile.

The dark blue color of the chamomile essential oil extract is due to the presence of chamazulene. This compound is formed from matricine during the extraction in a reaction process catalyzed by temperature.[112] Figure 3.7 shows the schematic diagram of degradation of matricine to chamazulene carboxylic acid and further decarboxylation to chamazulene.[113]

The SFE chamomile extracts had matricine instead of chamazulene. However, due to the heating of the samples in the GC-MS system matricine was quantified in the form of chamazulene.[113]

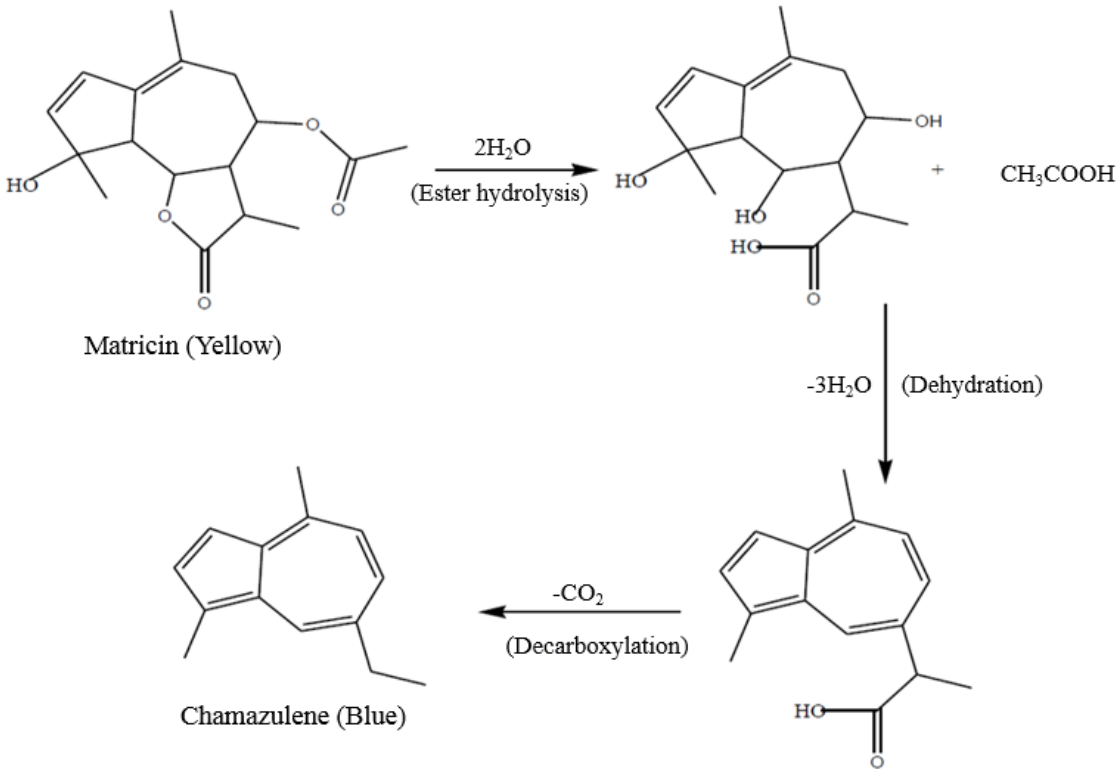

Figure 3.7. Schematic diagram showing degradation of matricine to chamazulene.[113]

Chamomile essential oil containing matricine has been demonstrated to have higher bioactivity compared to essential oils containing chamazulene.[114] Therefore the extract from SFE is considered to be of higher quality, as it can exhibit more valuable pharmacological properties compared to that extracted using traditional methods. In addition, the extract contained higher amounts of terpene compounds (β-farnesene, α-bisabolol oxide B, α-bisabolone oxide A, bisabolol oxide) and had the enriched active components chamazulene and dicycloether. It has been demonstrated that essential oil containing dicycloethers contributes to pharmacological properties mainly exhibiting anti-inflamatory and spasmolyic activity.[115] Therefore, the enrichment of dicycloethers improves the quality of the SFE extract. Comparison of the GC chromatogram of SFE extract and that of steam distillation done by Archana Gawde et al.[116] is illustrated in

Figure 3.8. The composition profile is similar and both had the same major compounds at different ratios. The steam-distilled extract had a dark blue color, indicating thermal degradation of matricine to chamazulene.

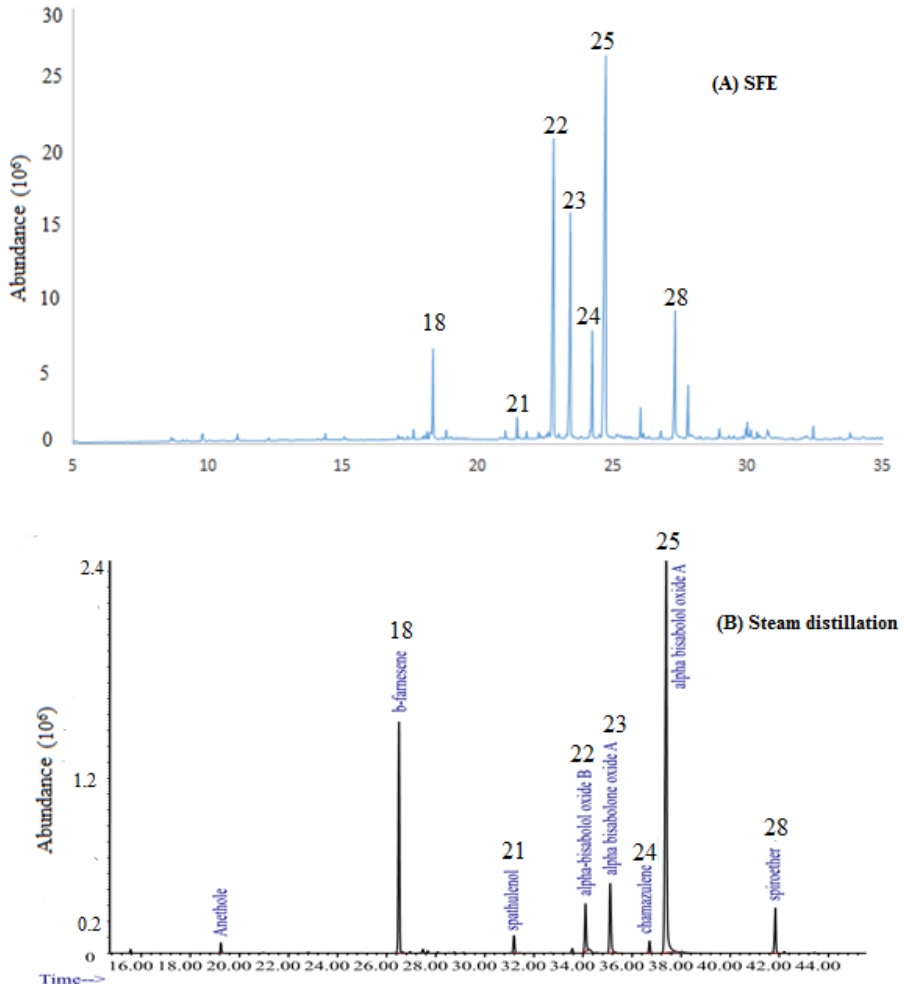

Figure 3.8. Comparison of SFE (A) and steam distillation (B) chromatograms.[116]

3.5.1.3 *Chrysothamnus nauseosus* (Rabbit Brush) Essential Oil Composition

Table 3.3 contains the GC-MS results for SFE and HD rabbit brush essential oils, while Figures 3.9 and 3.10 show the chromatograms for SFE and hydro distilled (HD), commercially acquired extract. Thirty-seven compounds representing 95.63% of the oil

composition were identified in the SFE extract. Among the identified compounds were twenty-nine monoterpene hydrocarbons representing 82.81%, ten oxygenated monoterpenes representing 12.36%, and sixteen sesquiterpenes representing 0.46%. The major compounds identified were d-limonene (35.77%), trans-β-ocimene (27.41), camphor (11.57%), β-phellandrene (4.64%), β-pinene (4.13%), eucalyptol (2.20%), β-cis-ocimene (2.66%), camphene (1.96%), and artemiseole (1.61%).

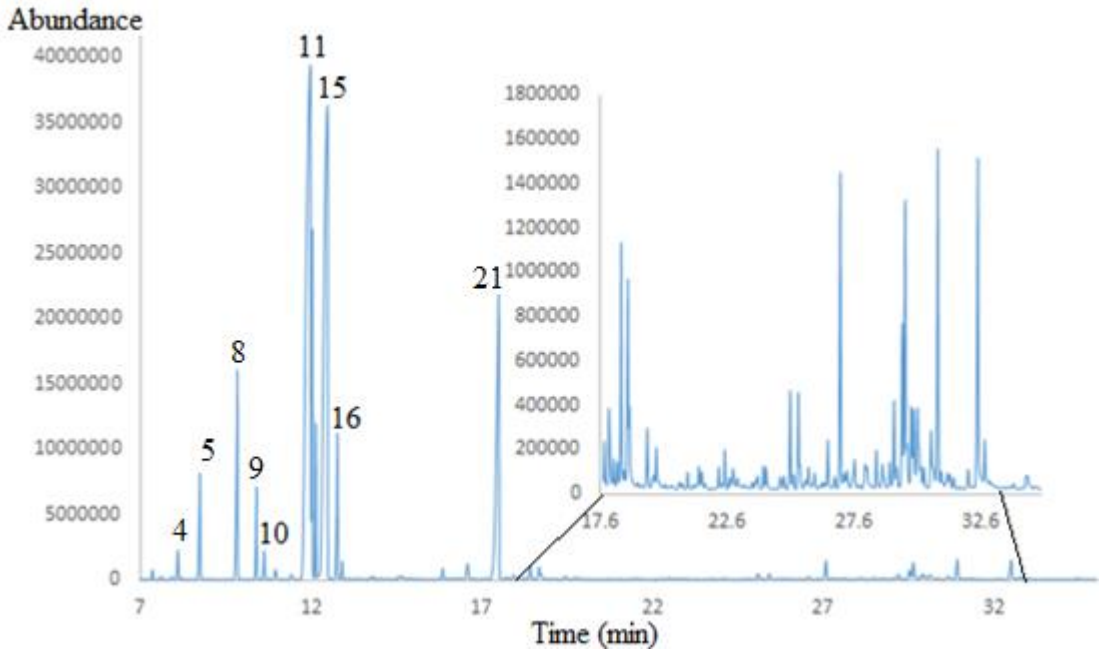

Figure 3.9. GC-MS chromatogram for rabbit brush essential oil extracted with SFE. The upper trace shows a scaled chromatogram for low abundance components. Individual components are identified in Table 3.3.

Table 3.2. Chemical composition of rabbit brush SFE and HD essential oils extract.

[a] SN	Compound[b]	% Area[c]	
		[d]CN SFE	[e]CN HD
1	Santolina triene	0.193	-
2	Tricyclene	0.062	-
3	3-Thujene	0.058	-
4	α-Phellandrene	0.252	0.067
5	α-Pinene	0.566	0.443
6	Camphene	1.958	-
7	Artemiseole	1.609	-
8	β-Pinene	4.133	2.972
9	L-β-Pinene	0.544	0.772
10	Terpinolene	0.163	0.136
11	D-Limonene	35.773	45.956
12	Sabinene	-	2.491
13	β-Phellandrene	4.637	-
14	Eucalyptol	2.198	-
15	Trans-β-Ocimene	27.407	34.111
16	β-cis-Ocimene	2.664	4.667
17	ϒ-Terpinene	0.287	0.278
18	Terpinolene	0.072	-
19	Cosmene	-	0.062
20	Allocimene	0.231	0.368
21	Camphor	11.568	-
22	Myrtenol	0.129	-
23	Terpinen-4-ol	0.310	0.151
24	Linderol	0.289	-
25	α-Terpineol	-	0.044
26	Citronellol	-	0.016
27	Perillal	0.032	0.089
28	L-Perillaladehyde	-	0.010
29	α-Gualene	-	0.032
30	Aromandendrene	0.069	0.015
31	Cis-α-bisabolene	0.064	-
32	α-Copaene	0.026	0.054
33	Modephene	-	0.036
34	Trans-Carane	-	0.648
35	β-Curcumene	0.033	0.029
36	β-Elemene	0.022	0.027
37	β-Isocomene	-	0.020
38	Caryophyllene	0.481	0.123
39	Valencene	-	0.077
40	Alloaromadendrene	-	0.060
41	δ-Cadinene	0.041	0.049
42	ϒ-Muurolene	0.131	0.113
43	Longipinene	0.243	0.305
44	β-Copaene	0.026	0.233
45	α-Ylangene	0.020	0.390
Total Area		**95.627**	**94.944**

[a]Peak numbers refer to the chromatogram in Fig 3.8,3.9, [b]Tentative identification based on MS, [c]GC peak area percentage, each value is the mean of triplicate, [d]SFE extract, [e] hydrodistilled commercial extract. Compounds highlighted in yellow are > 1%.

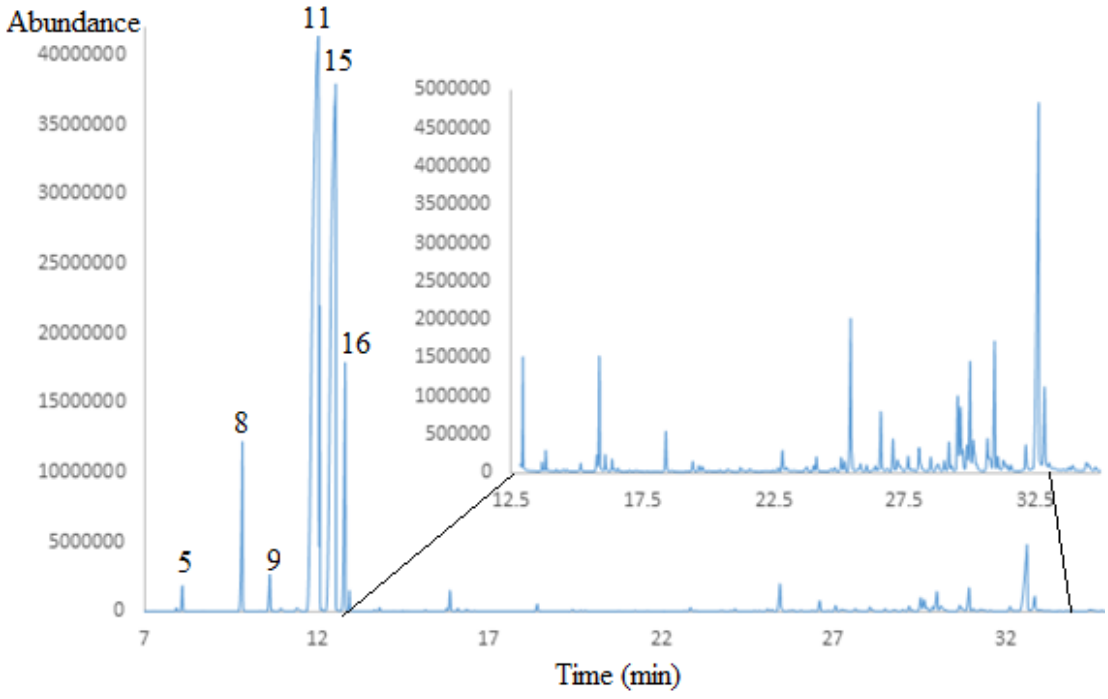

Figure 3.10. GC-MS chromatogram for rabbit brush essential oil prepared by hydro distillation. The upper trace shows a scaled chromatogram for low abundance components. Individual components are identified in Table 3.3.

The composition profile is in agreement with the previous work of Nurhayat et al. that reported monoterpenes hydrocarbons (60.7%), oxygenated monoterpenes (15%), and sesquiterpenes (0.12%).[101] The identified compounds reported here are consistent with those reported for the oils from the *Chrysothamnus* genus. When compared to *Chrysothamnus pulchellus*, over 95% of compounds identified in rabbit brush (*Chrysothamnus nauseosus*) was also present in *Chrysothamnus pulchellus* in different percentages.[95] The results also compare to the results from the analysis of the hydrodistilled essential oils from three *Chrysothamnus nauseous* varieties done by Sue et al.[1] The major constituents in *C. nauseous* var. *albicaulis* were β-pinene (16.8%),

limonene (18.6%), and β-phellandrene (26%). In *C.nauseous* var. *consimilis* were limonene (33.2%), β-phellandrene (18%) and β-ocimene (14.6%). In *C. nauseousus* var. *glabratus* were β-pinene (30%), myrcene (10.5%), limonene (16.5%), and β-phellandrene (10.9%). Compared to the commercially acquired *Chrysothamnus nauseosus* essential oil in Table 3.3, the oil composition was similar with major components being monoterpene hydrocarbons and their derivatives. The SFE extract had more monoterpene hydrocarbons identified than the commercially acquired oil, but the percentage amount of the major components d-limonene and trans-β-ocimene were high in the commercial extract. The exact composition, quantity and quality can vary according to climate, soil composition, plant organ, age, and vegetative cycle stage. Also, the method used for extraction can cause the variation. Therefore, to obtain essential oils of constant composition, the sample should be from the same plant organ, which has been growing in the same climate and has been picked in the same season, and should be extracted under same conditions by same method.

Samples from SFE, Soxhlet and hydro distilled extracts are compared in Table 3.4. Most of compounds in the SFE extract, especially monoterpene hydrocarbons, were absent is soxhlet extract. Soxhlet extraction produces high volumes of dilute solution which needs to be concentrated, leading to loss of volatile compounds. The choice of solvent in Soxhlet extraction controls the selectivity of the analytes extracted.

From the quantification results for selected major compounds (limonene and camphor) in the rabbit brush essential oil, limonene content was found to be 2.052±0.020 mg/g and camphor concentration was 0.652±0.01mg/g in the SFE extract.

Table 3.3. Comparison of composition profile of SFE, hydro distilled, and Soxhlet extract of rabbit brush.

Essential Oil Compounds	SFE % Area	Hydro distilled	Soxhlet
Santolina	0.193	X	X
Tricyclene	0.062	X	X
3-Thujene	0.058	X	X
α-Phellandrene	0.252	√	√
α-Pinene	0.566	√	√
Camphene	1.958	X	√
Artemiseole	1.609	X	X
β-Pinene	4.133	√	√
L-β-Pinene	0.544	√	X
Terpinolene	0.163	√	X
D-Limonene	35.773	√	√
β-Phellandrene	4.637	X	X
Eucalyptol	2.198	X	X
Trans-β-Ocimene	27.407	√	√
β-cis-Ocimene	2.664	√	X
ϒ-Terpinene	0.287	√	X
Terpinolene	0.072	X	X
Allocimmene	0.231	√	X
Myrtenol	0.129	X	X
Camphor	11.568	X	X
Terpinen-4-ol	0.310	√	X
Aromandrene	0.069	√	√
Cis-α-bisabolene	0.064	X	X
β-Curcumene	0.022	√	√
Caryophyllene	0.481	√	X
δ-Cadinene	0.041	√	X
ϒ-Muurolene	0.131	√	X
Longipinene	0.243	√	X
β-Copaene	0.026	√	X
α-Ylangene	0.020	√	X
Other compounds			
Cosmene	X	√	X
Linderol	X	√	X
Citronellol	X	√	X
L-Perillaladehyde	X	√	X
α-Gualene	X	√	X
Modephene	X	√	X
Trans-Carane	X	√	X
Valencene	X	√	X
Alloaromadendrene	X	√	X
Squalene	X	X	√
Phthalic acid	X	X	√
Lachnophyllum ester	X	X	√

X- Compound not present, √-Compound present

3.5.1.4 *Rhus aromatic* (Skunk Brush) Essential Oil Composition

The retention time and chemical composition of the essential oil of skunk brush are presented in Figure 3.11 and Table 3.5. Thirty three compounds, representing 90.7% of the total composition, were identified. Monoterpenes were found to be the major group of compounds (77.1%). Monoterpene hydrocarbons were 40.4% of the total oil composition, while oxygenated monoterpenes were 36.7%. Sesquiterpene compounds represented 13.7% of the total oil. The main compounds identified were limonene (20.48%), linalool (37.31%), caryophyllene (12.5%), eucalyptol (9.14%), α-phellandrene (5.5%), and geraniol (1.2%). The rest of the compounds were present as less than one percent of the total oil. The actual concentration for limonene, linalool, and caryophyllene concentrations were 1.448±0.027 mg/g, 2.28±0.014 mg/g, and 0.956±0.018 mg/g.

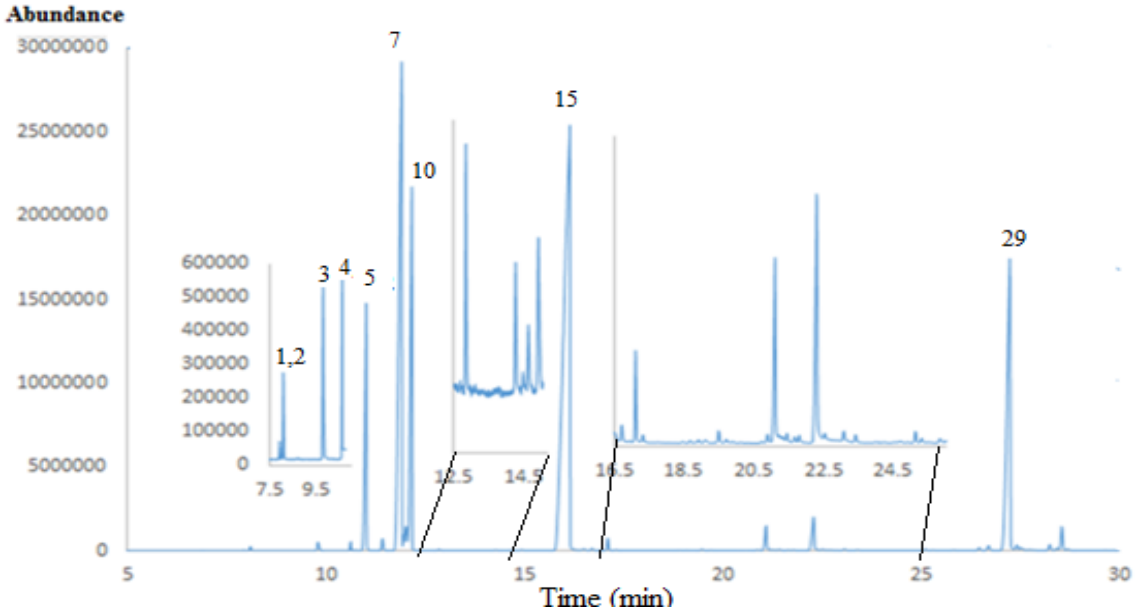

Figure 3.11. GC chromatogram of *Rhus aromatic.* The upper traces show scaled chromatograms for low abundance components. Individual components are identified in Table 3.4.

Table 3.4. Chemical composition of skunk brush SFE essential oil extract.

[a]SN	RT (min)	% Area[c]	[b]Compound
1	7.939	0.020	L-α-Pinene
2	8.094	0.095	α-Pinene
3	9.799	0.234	β-Pinene
4	10.628	0.218	L-β-Pinene
5	11.017	5.585	α-Phellandrene
6	11.424	0.296	Terpinolene
7	11.916	20.481	D-Limonene
8	11.956	0.216	γ-Terpinene
9	12.019	0.773	o-Cymene
10	12.168	9.140	Eucalyptol
11	12.854	0.036	3-Carene
12	14.279	0.023	Cis-Linalool oxide
13	14.651	0.013	Unknown
14	14.937	0.034	Linalool oxide
15	16.156	37.305	Linalool
16	16.511	0.035	Cis-Limonene oxide
17	16.711	0.058	Unknown
18	17.111	0.295	Dihydrolinalool
19	17.312	0.029	Cis-ρ-mentha-2,8-dien-1-ol
20	19.486	0.052	α-Terpineol
21	21.105	0.766	cis-Geraniol
22	21.449	0.025	cis-Carveol
23	21.665	0.006	Carvone
24	21.798	0.027	cis-Verbenol
25	22.301	1.233	Geraniol
26	25.305	0.017	D-Verbenone
27	26.478	0.092	Aromandendrene
28	26.713	0.169	α-Selinene
29	27.251	12.475	Caryophyllene
30	27.417	0.116	β-Ylangene
31	27.520	0.053	β-Longipinene
32	28.258	0.179	Unknown
33	28.561	0.711	Humulene
Total Area		**90.74**	

[a]Peak numbers refer to the chromatogram in Fig 3.10, [b]Tentative identification based on MS, [c]GC peak area percentage, each value is the mean of triplicate analyses. Yellow highlighted compounds are in concentrations >9%.

3.5.2 Extraction Model Results

The effect of extraction parameters (pressure, temperature, and time) on yield was analyzed by considering the total area of all the identified essential oils components. The effect on the yield of individual groups (monoterpenes, oxygenated monoterpenes, and

sesquiterpenes) was analyzed using main components identified in the oils. The selected components were classified into three groups to represent monoterpenes, oxygenated monoterpenes, and sesquiterpenes. The compounds considered were pseudolimonene, β-cis-farnesene, spathulenol, linalool, and α-bisabolol in the chamomile sample; limonene, α-pinene, camphene, eucalyptol, and caryophyllene in rabbit brush; and limonene, linalool and caryphyllene in skunk brush.

3.5.2.1 Model Fitting and Significance of Coefficients

The experimental yield was analyzed using ReliaSoft DOE++ statistical software to get a regression model. The predicted yields were calculated using the regression model and compared with the experimental values. In all the three models, the analysis showed that they were statistically significant at 95% confidence level. The coefficient of regression (R^2) was greater than 0.90 and lack of fit was found to be insignificant. This indicated that the models adequately represented the experimental results. Table 3.6 contains the *p*-values for model significance, the lack of fit, and the coefficients of regression. The regression coefficients for second-order polynomial fit are listed in the Table. They represent the linear, quadratic, and two-way interaction of extraction pressure, temperature, and time. The significant parameters are indicated with an asterisk.

Table 3.5. Regression coefficients for the three RSM model and analysis of variance results.

Regression Term	Regression Coefficient		
	Chamomile	Rabbit Brush	Skunk Brush
A: Pressure	20.70*	9.683*	0.211*
B: Temperature	12.62*	-0.025	-0.07*
C: Time	7.80*	1.448	0.038
A·B	18.81*	1.143*	0.04*
A·C	11.04*	0.794	0.007
B·C	6.09	-0.358	0.02
A^2	-14.02*	-5.555*	-0.117*
B^2	-20.39*	-3.085*	-0.095*
C^2	-25.18*	0.69	-0.072
Model (p-value)	0.00231*	<0.001*	0.00396*
Lack of fit (p-value)	0.27335	0.0563	0.1422
R^2	0.9118	0.9758	0.9648

*Significant ($p<0.05$)

3.5.2.2 Effect of Pressure, Temperature, and Time

Pressure had a significantly positive linear effect on the oil yield, as indicated in Table 3.6 and Figures 3.12-3.14. The yield increased with pressure, most likely due to the improvement of the solvent power resulting from the increased solvent density which enhances the solubility of solutes into the fluid.[23] Though the total yield increased with pressure, pressures greater than 5000 psi resulted in a higher amount of coextracted material. This is consistent with the work of Reverchon et al.[115] on the extraction of rose

flower essential oil at different pressures and temperatures. Pressure greater than 4300 psi resulted in higher quantities of paraffins and steroptens. The interactive effect of pressure with temperature was found to be significant in all the models. Temperature showed a negative quadratic significant effect while the interaction of pressure and temperature had a positive effect on the yield. At constant pressure, the density of CO_2 decreases when temperature is increased.[23] Temperature elevation also affects the vapor pressure of solutes. This inverse transition point is referred to as the crossover point and depends on the nature of the sample. Due to this phenomenon, the effect of temperature elevation is difficult to predict. The linear effect of temperature on chamomile oil yield was positive, while the effect on rabbit brush and skunk brush was negative. Chamomile oil contained a higher percentage of oxygenated sesquiterpenes, while rabbit brush and skunk brush contained higher percentages of hydrocarbon monoterpenes. The effect of temperature on the extraction of volatile compounds is a competition between their solubility in CO_2 (which decreases as the temperature increases) and its volatility (which increases with increasing temperature).[23]

The effect of time on the extraction of monoterpenes was found to be significant, suggesting that these compounds are located on the surface (exogenous sites) and are easily extracted according to free diffusion from the plant surface. In contrast, for the oxygenated monoterpenes and sesquiterpenes, the pressure and time were significant. This indicates that the two compounds are located in both endogenous and exogenous storage sites. The highest yield of monoterpenes hydrocarbons was realized at pressures between 1500-2100 psi, temperature 35-40 ^{o}C, and a dynamic time of twenty five minutes. Longer dynamic times, between 30-45 mins, and pressures above 4000 psi were

needed for extraction of sesquiterpenes. Pressures greater than 5000 psi are not recommended as higher levels of co-extraction occurred. Figure 3.12-3.14 illustrates three-dimensional response surface plots of chamomile, rabbit brush, and skunk brush essential oils. The surfaces illustrate three-dimensional plot of yield, calculated from total peak area, as a function of two variables. The effect extent of pressure, temperature, and time on the total yield were tested by analysis of variance (ANOVA). Table 3.6 contains the regression coefficients of the second polynomial equation fit the three models.

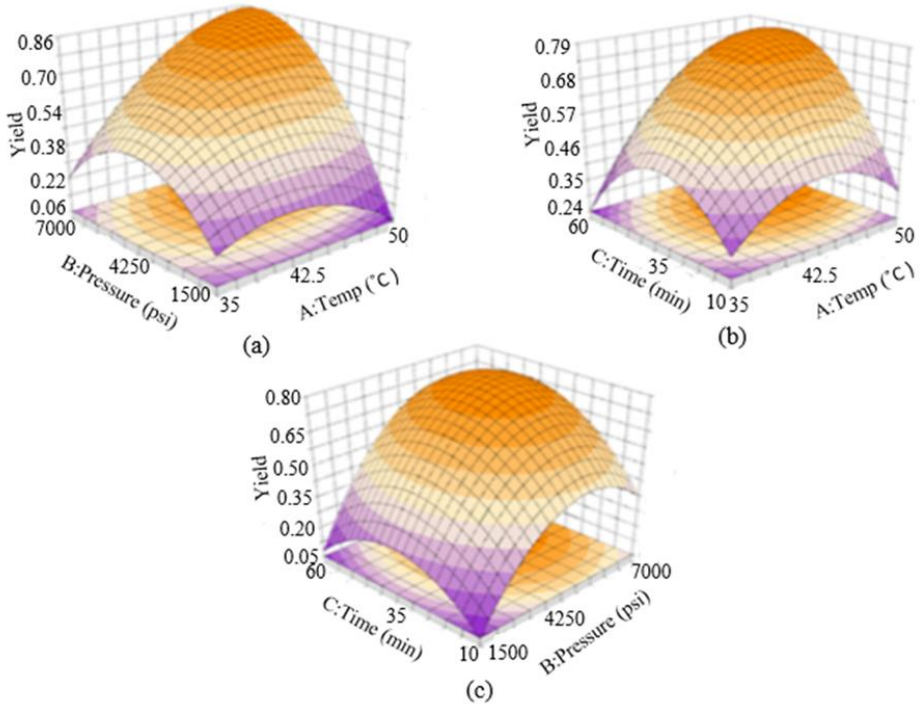

Figure 3.12. Response surface for chamomile total yield recovery versus (a) pressure and temperature, (b) time and temperature, and (c) time and pressure.

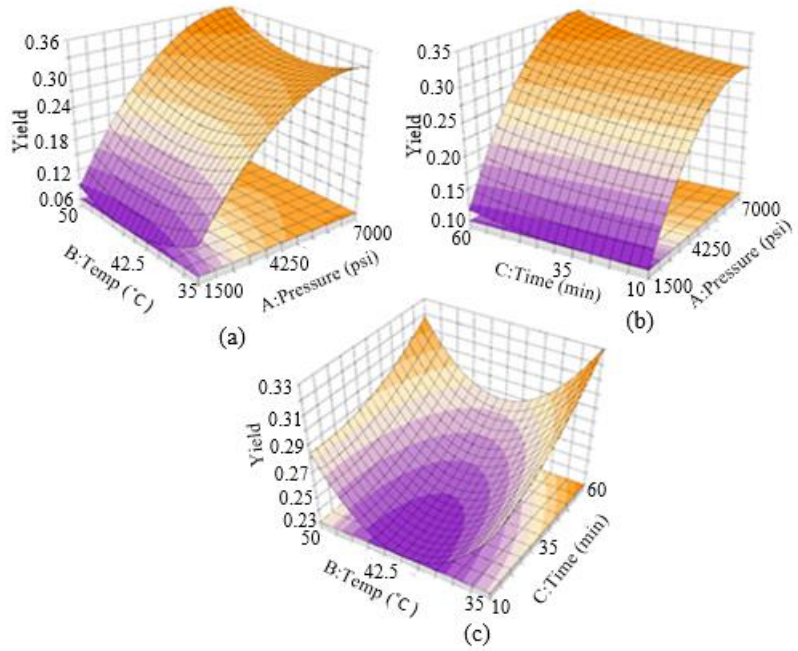

Figure 3.13. Rabbit brush response surface for chamomile total yield recovery versus (a) temperature and pressure, (b) time and pressure, and (c) temp and pressure.

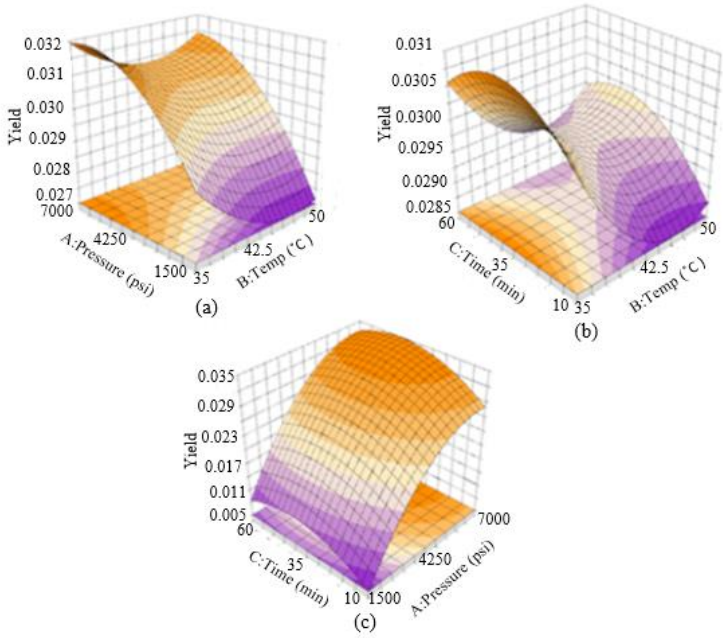

Figure 3.14. Skunk brush response surface for total yield recovery versus (a) pressure and temperature, (b) time and temperature, and (c) time and pressure.

3.6 CONCLUSION

The viability of supercritical extraction of volatile essential oils with minimal loss at collection has been demonstrated. SFE offered considerable advantages over traditional methods. Extraction was performed in a shorter time under milder conditions, thus minimizing degradation of heat-sensitive compounds like matricine. The extract from SFE was of high quality considering the enriched bioactive components identified. Extracts from different plant samples contained different essential oils components. Chamomile extract was composed of mainly oxygenated sesquiterpenes. The major compounds identified were α-bisabolol, α-bisabolol oxide B, α-bisabolol oxide B, matricine, dicycloether, and β-cis-farnesene. Chamomile samples from different regions had different amounts. Chamomile extracts from the three samples from Kenya can be classified as chemotype C since the major compound in each of them was α-bisabolol. Rabbit brush extract was mainly composed of hydrocarbon monoterpenes. The major compounds were limonene (35.77%), trans-β-ocimene (27.41), camphor (11.57%), β-phellandrene (4.64%), β-pinene (4.13%), eucalyptol (2.20%), β-cis-ocimene (2.66%), camphene (1.96%), and artemiseole (1.61%). Skunk brush extract contained monoterpene hydrocarbons, oxygenated monoterpenes, and sesquiterpenes. The main compounds identified were limonene (20.48%), linalool (12.46%), caryophyllene (12.5%), eucalyptol (9.14%), α-phellandrene (5.5%), and geraniol (1.2%).

The results show that the second-polynomial model was sufficient to describe and predict the yield within the experimental range considered. Based on the proposed model, the optimal conditions for total extraction was 35 ^{o}C, 3570 psi, and 40 min for skunk brush, 47 ^{o}C, 6620 psi, and 45 min for chamomile oil, and 37 ^{o}C, 1720 psi, and 43 min for

rabbit brush. Under these optimal conditions, the experimental values were in agreement with the predicted values. Thus, response-surface methodology can provide a basis to examine the effect of the different independent variables on yield. The independent variable affected the yield individually and also interactively. The linear effect of pressure and interactive effect of pressure and temperature had the greatest impact on the extraction yield. It can also be determined from this study that selectivity can be achieved by appropriate altering of SC-CO_2 operating parameters of pressure, temperature, and dynamic time. Therefore, understanding the interaction effect could help in the successful selective extraction of essential oils. The interactive effect of pressure and temperature was that the extraction yield increased with pressure at higher temperatures. The increase can be attributed to the solute vapor pressure increase dominating as compared to the contradicting effect of reduction of solvent density by higher temperature. At lower pressure, the increase of temperature resulted in reduction of extraction yield indicating that solvent density is major factor enhancing the quality of total extract.

4 CHAPTER 4: CONCLUSIONS, BROADER IMPACTS, AND FUTURE WORK

4.1 General Conclusions

The applicability of the design of experiments approach in analytical SFE of essential oils from plant samples has been demonstrated. The SFE process has been described with an emphasis on efficiency of extraction and collection steps. Comparing SFE with traditional extraction methods, SFE offered considerable advantages. The extraction time has been substantially reduced with comparable extract compositions to those achieved with longer extraction times. The SFE extract quality is superior to that of steam distillation and soxhlet extraction with enriched bioactive components.

The collection step has been established as a critical step in achieving quantitative extraction of volatile compounds. The collection conditions have to be carefully adjusted to avoid substantial losses due to the incomplete trapping. Excessive decompression flow rates and long dynamic extraction times should be avoided to minimize the loss of volatile and semi-volatile essential oil compounds. Proper choice of collection solvent and temperature is important for obtaining good collection efficiencies. Viscosity and polarity of the solvent were the most influential properties to be considered for a proper choice of trapping solvent. Solvent volume and height had minimal effect. Large solvent volumes should be avoided due to dilution of the extract, less than 20-mL volume with a 60-mL collection vial is recommended. Narrow collection vials are recommended to enhance the solvent height.

In the extraction step, linear effect of pressure and interactive effect of pressure and temperature were found to have the most impact on extraction yield. The yield increased with pressure at higher temperature. At lower pressure, the increase of temperature resulted to reduction of yield. The solubility of the analytes to in carbon dioxide was found to be the major factor in the extraction of essential oils. The solubility was enhanced by changing pressure and temperature. Polar solvent modifiers are recommended for further enhancement of solubility of polar compounds.

A design of experimental approach was able to explain in depth the supercritical extraction step and collection by solvent trapping. The linear and higher interaction of variables were established. This gives a better representation of the SFE process and yields more accurate results and conclusions in fewer experimental runs compared to the one-variable-at-a-time technique. One-variable-at-a-time optimization overlooks the interaction between different factors, leading to misinterpretation of the results. It is time consuming and expensive, and it cannot be used for prediction as each optimized parameter is at a constant value of other parameters.

The essential oils from different plants differed in total composition, but some compounds were similar. The composition of extract from the same plant from different regions was significantly similar in major compounds present, but in different ratios. From the results of chamomile essential oil extracts from different parts of Kenya, it can be inferred that the composition and the amount of essential oil can be influenced by climatic conditions and type of the soil. Other factors which affect the amount and composition are the growth stage of the harvest, the drying and storage method, and the extraction method used, among others. Therefore to obtain essential oils of constant

composition, the sample should be from the same plant organ, which has been growing in the same climate and has been picked in the same season, and should be extracted under same conditions by same method.

4.2 Broader impacts

The use of carbon dioxide as a solvent in extraction will reduce the amount of pollution and energy consumed by reduction of the use of organic solvents and time. The use of conventional methods like steam distillation and Soxhlet extraction which requires longer heating durations of over twelve hours compared to thirty minutes required by SFE to achieve similar results. Carbon dioxide is nontoxic and nonflammable. Hence use of SFE in the laboratory environment can eliminate the cost associated with solvent disposal, reduce long-term exposure of personnel to potential toxic vapors, and also improve the safety in laboratory by reduction of flammable solvents. The applicability of essential oils in various industries like pharmaceuticals will be enhanced by the use of extracts from SFE. This is due to the enriched composition of SFE extract with higher amount of bioactive components in their natural state. Smaller quantities will be needed as compared to traditional methods.

Although most of industries recognize the importance of design of experiment methodology, they are slow to implement it due to the misperception that statistically designed experiment are costly, time-consuming, and the failure of statisticians to teach these techniques in an easy to understand fashion. With the demonstration of the specific applicability of DOE in the extraction of essential oils, such misconceptions can be lifted. This work can be used in the demonstration of the importance of DOE and in teaching of different DOE techniques in academia as well as industry.

4.3 Future work

The quality of extracts can be further evaluated by correlating recovery at different conditions with the bioactivities. This will give a better representation of the quality of the extract. To achieve a more enriched extract with more polar compounds, modifiers can be introduced in extraction step. Studies on the factors affecting the composition of extract should be extended to season when the sample was harvested, harvesting method, and storage method. The proposed optimal conditions for the extraction of different essential oils groups should be applied to other plant samples with similar major compounds to the samples investigated.

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