

**Artemisinin content of sc-CO₂ derived extracts
from *Artemisia annua***

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B.Sc. (Honours)**

July 2004

**This dissertation is part of the requirements for
Magister Scientiae at the North-West University
(Potchefstroom Campus)**

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ACKNOWLEDGEMENTS

CHAPTER 0 A BIRD'S EYE VIEW OF THE PROJECT

**All religions, arts and sciences are branches of the same tree.
All these inspirations are directed towards ennobling man's life,
lifting it from the sphere of mere existence and leading it
towards freedom.**

Albert Einstein

One of the principal research themes of the supercritical fluid research group within the Centre of Separation Science and Technology (SST) at the North-West University (Potchefstroom Campus) is botanical extraction. The group produces botanical extracts from locally cultivated plants which contain substances (essential oils, natural waxes) relevant to the food, flavour, pharmaceutical, medical and cosmetic industries while utilising the advantages of sc-CO₂ extraction over traditional steam distillation and solvent extraction.¹⁻³

In this study, which represents a contribution to a series of botanical extractions performed within the core programme outlined above, sc-CO₂ extraction of artemisinin from *Artemisia annua* (or wormwood) was investigated. The active component is a potential cure against malaria.⁴ It would have been desirable to extract it with no solvent residues left in the final product to prevent side-effects on taking the medicine. The extraction by clean technology, using environmentally friendly sc-CO₂, could be relevant as it offers an affordable alternative for synthetically prepared medicines in the marketplace.

The manipulation of the conditions (temperature and pressure, or density) for sc-CO₂ extraction could facilitate more selective isolation of the active component and thereby enhance the medicinal value of wormwood. The unique solvent strength and mass transport characteristics of sc-CO₂ offer the possibility of obtaining better results than with other solvent based extraction methods.

The specific goals with the project were

- to extract an active component or ingredient (artemisinin) from the leaves of wormwood with sc-CO₂ on laboratory scale by using an advanced benchtop supercritical fluid extractor and other available laboratory infrastructure;
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- to compare the results of sc-CO₂ extraction with those of classical extraction methods, such as solid-liquid extraction, to ascertain the advantages and disadvantages of different extraction methods;
 - to identify and implement suitable analytical techniques (HPLC and/or GC) with which artemisinin containing extracts can be analysed qualitatively and quantitatively;
 - to identify process parameters and to vary these according to a statistical design using a suitable software programme (Statistica for Windows[®]) to establish optimum conditions for the extraction of the target component;
 - to process the extraction data mathematically and/or graphically in such ways as to reveal the principal features of the extraction process, to facilitate modelling of the process and to enable calculation of mechanism directive activation parameters;
 - to complement comparable studies performed in the research group.

In addition to these specific goals, the project also served the purpose to contribute to a lesser extent to the following relevant issues:

- The essential oils, natural waxes and other chemical components derived from plants are low-volume high-value products and therefore have significant commercial value.⁵
- The importance of clean technology for “green” or sustainable chemistry is increasingly emphasised.⁶ sc-CO₂ is an environmentally friendly solvent with which solvent-free extracts can be derived.
- There are academic interest in and financial support for the development of knowledge about indigenous plants.⁷ The suitability of sc-CO₂ for the isolation of plant components derived by traditional healers for centuries can help to better understand the beneficial effects of plant medicines.
- The relevance of supercritical fluid based processes for daily life creates science awareness and renders the research done in this investigation suitable for the popularisation and promotion of chemistry since the replacement of natural products in ordinary household products (beer, shampoo) captures the attention and imagination of the public.
- Finally, this investigation can help to convince industry to apply the technology despite of the negative perceptions about extreme conditions and the high capital investment needed to put up the required infrastructure.

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5. The price of harpogoside (derived from devil's claw or *Harpagophytum procumbens*) in the marketplace is estimated at \$120 for 10 mL of the pure substance.
6. ICS/UNIDO Workshop on *Cleaner Technologies for Sustainable Chemistry*, Cape Town, 9-11 December 2002.
7. The National Research Foundation (NRF) has identified indigenous knowledge systems as one of its research focus areas and makes substantial funding available to prospective investigators.

CHAPTER 1 ARTEMISIA ANNUA

Science is organized knowledge. Wisdom is organized life.

Immanuel Kant

The plant investigated in this study was selected for the pharmaceutical importance of one of its active components (artemisinin) as an antimalarial drug. This component is mostly extracted by solvent extraction, with the result that the extracted material needs to be purified of solvent residue to obtain a product suitable for human intake. This study focuses on the solvent-free extraction of artemisinin by using non-hazardous sc-CO₂.



Figure 1.1: *Artemisia annua*

1.1 Origin of Plant

The herb *Artemisia annua* L. shown in **Figure 1.1** is a member of the Asteraceae family and has been used by the Chinese in traditional medicine against fever and malaria from as early as 168 B.C.¹ The recipes for the prescriptions found in the Mawangdui Han Dynasty Tombs recommended that it could also be used for haemorrhoids.² Wormwood, as *A. annua* is more commonly known, is an annual herb native to Asia, more specifically China, where it is named *qinghao*.³ Traditionally, *A. annua* was harvested from wild strands in China with artemisinin

concentrations ranging from 0.01 to 0.5% (m/m), with varieties in Sichuan Province giving the largest yield.^{1,4} The plant also grows in Europe, America and Africa.¹

Artemisia, the largest genus of the tribe Anthemideae, is well known for bitter and toxic substances, and a number of species, in addition to *A. annua*, has long been famous as medicinal and culinary herbs. It is used in the crafting of aromatic wreaths, as a flavouring agent for spirits, such as vermouth, and as a source of artemisinin, an important natural antimalarial drug.

1.2 Botanical Description

A. annua is a large shrub⁵, often 2 m in height and single-stemmed with alternative branches. The aromatic leaves are deeply dissected and range from 2.5 to 5 cm in length. The leaves contain both 10-cell biseriate trichomes and 5-cell filamentous trichomes. It is a short-day plant with a photo period of 13½ h. Vegetative shoots are transformed into flowering shoots on receiving an inductive stimulus. The nodding flowers (capitula), only 2 to 3 mm in diameter and enclosed by numerous, imbricated bracts, are greenish-yellow. The florets contain small central flowers, which can be fertile or sterile.

The glandular trichomes, which are abundantly present on the surface of the leaves and the flower organs, sequester artemisinin as well as highly aromatic volatile oils. These components are absent in the foliar tissues of plants of a biotype of *A. annua* lacking glandular trichomes.⁶ The glandless biotype of *A. annua* grows spontaneously among field-cultivated plants, and is being used as a model plant to study the biosynthesis of artemisinin and other isoprenoids. The anatomy of the glandless biotype is virtually indistinguishable from its glanded counterpart, except for the absence of peltate, secretory glands.

1.3 Cultivation, Distribution and Harvesting

Artemisinin is extracted from the leaves of *A. annua*. The substance contains both mono and sesquiterpenes. The yield of artemisinin varies considerably, and does not depend only on plant strain and stage of development but also on environmental and soil conditions.⁷ In previous work⁴ the dry leaf matter of the Yugoslavian cultivar varied between 1.1 and 7.2 g/plant, and between 7.3 and 10.6 g/plant in the Chinese cultivar. The maximum yield of artemisinin from cultivated *A. annua* amounts to 2 % (m/m) of the dry plant material, but the values for plants

growing in the wild are usually only 0.01-0.5 % (m/m). The pH was shown to have little effect on the artemisinin content in the range $5 < \text{pH} < 8$. For commercial purposes, yields of artemisinin need to approach 15 kg/hectare.⁴

Leaves produced in the Democratic Republic of the Congo at an altitude of 1 650 - 2 000 m yielded 0.63 - 0.70 % artemisinin per dry mass. In comparison, the leaf material of plants cultivated and dried by professional methods in Europe was shown to have a content of 0.58 % per dry mass.⁵

The artemisinin content differs at various stages of the development of the plant. The highest content is reached just before or at the time of flowering, when the plant is still a “green herb”. Later in the season, due to loss of leaves, the lower parts of the plant contain only little artemisinin. It is therefore recommended that the plant should be harvested prior to flowering.¹

The leaves of *A. annua* could be machine harvested by leaf stripping or forage harvesting, but problems like the large bulk involved and the necessity to lower the moisture content of the plant to reduce the cost of kiln drying need to be taken into account. Drying of the plant material in the sun can reduce the moisture content by as much as 50 %.⁴

1.4 Constituents

The genus *Artemisia* comprises over 100 species, two of which can be distinguished in terms of the nature of the principal constituents. Several species are characterised by the occurrence of eudesmanolide and guaianolide sesquiterpene lactones. *A. annua* yields an aromatic essential oil rich in monoterpenes.⁶

1.4.1 Artemisinin

Specialised plastids present in the apical and subapical cells of capitate glandular trichomes of *A. annua* have been proposed as the site of artemisinin synthesis.⁶ In 1972, this sesquiterpene lactone was isolated from the plant, and in 1979 its structure, shown in **Figure 1.2**, was determined by combined spectral, chemical and X-ray analysis.^{1,7} This novel compound contains an endoperoxide moiety, which is a rare feature in natural products. Artemisinin is also referred to as arteannuin, quinghaosu and QHS.⁸

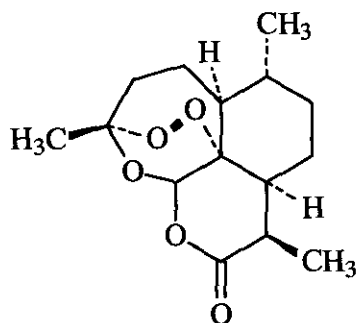


Figure 1.2 Structure of artemisinin¹

Artemisinin has a peroxide bridge to which antimalarial properties are attributed. It has an unusual structure and lacks a nitrogen containing heterocyclic ring found in most antimalarial compounds. Artemisinin is an odourless, colourless compound and forms crystals with a melting point of 156-157 °C. It has an empirical formula $C_{15}H_{22}O_5$ and a molar mass of 282.2 g/mol as determined by high resolution mass spectrometry. The compound is poorly soluble in water and decomposes in other protic solvents, probably via the opening of the lactone ring. Substitutions at the lactone carbonyl group increases potency.⁷ Artemisinin has been identified to be effective against both chloroquine and maffloquine resistant *Plasmodium falciparum* associated with cerebral malaria.⁹ It is particularly useful in the treatment of cerebral malaria in view of a rapid clearance of parasites and fever. Neither cross-resistance with other currently used antimalarial drugs nor serious side-effects in humans have been observed.¹⁰

1.4.2 Other Constituents

The first-generation artemisinin derivatives, including esters and ethers of dihydro-artemisinin, are more potent than artemisinin but have a shorter half-life on circulating in the human body.¹⁰ The common structural entity of these derivatives is shown in **Figure 1.3**. Artensunate, artemether and arteether are more effective than artemisinin, the first-mentioned two being the most widely used. Arteether is utilised in clinical trials.

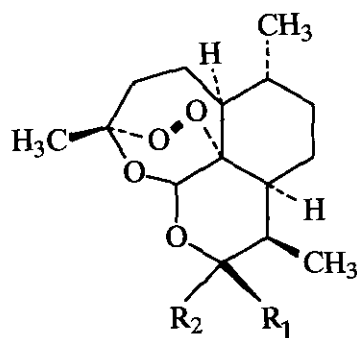


Figure 1.3 Chemical structure of some artemisinin derivatives

Other sesquiterpene lactones from *A. annua* and related to artemisinin include arteannuin B and arteannuic acid shown in **Figure 1.4** and **Figure 1.5** respectively.⁷ The presence of guaianolides and seco-guaianolides has been reported.

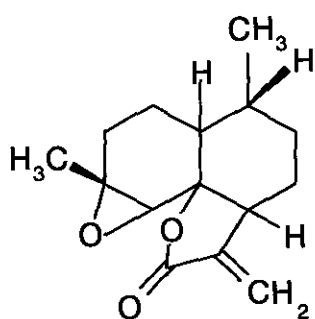


Figure 1.4 Arteannuin B⁷

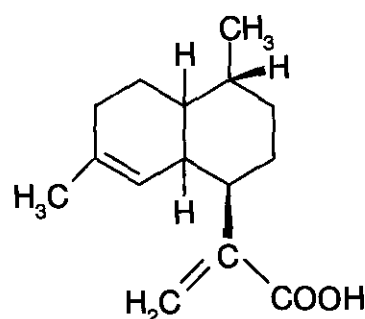


Figure 1.5 Arteannuic acid⁷

The essential oil (3 %) in the leaves contains sesquiterpenes and quite a few monoterpenes. The principal component is artemisia ketone, an irregular type of monoterpene which does not comply with the classical isoprene rule. Other relatively abundant monoterpenes include borneol, 2,8-cineole, α -pinene, thymol, carvacrol and camphor.¹¹ Caryophyllene and its oxide are the most important sesquiterpenes.

A few flavones, such as casticin, chrysoplenetin and cirsilineol, markedly enhance the antimalarial activity of artemisinin. Casticin is the most effective inhibitor of parasite mediated transport systems controlling the influx of L-glutamine and myo-inositol across the host cell membrane in erythrocytes infected with human and murine malaria.¹²

1.5 Mechanism of Biological Activity

Insight into the mechanism of biological activity can assist in the design of new and more effective derivatives. Artemisinin, and its derivatives, combat parasites more rapidly than any other antimalarial agent due to its unique activity and despite its nanomolar concentration in comparison to that of the malarial parasite.¹² Studies on the mechanism of biological activity revealed that artemisinin causes structural changes in the erythrocyte stage of the parasite, which affects the membrane surrounding the food vacuole, the nucleus, the mitochondria, the endoplasmic reticulum and the nucleoplasm.^{2,12} These changes lead to the formation of autophagous vacuoles and the loss of vacuoles, which kills the parasite. The peroxide functionality in artemisinin and related compounds is vital for antimalarial action. It is proposed that the killing of the malarial parasite by artemisinin and its derivatives is mediated by the production of cytotoxic compounds such as free radicals and reactive aldehydes.¹²

Chinese investigators were the first to suggest that the membrane of the parasite is the main site of biological activity of artemisinin.¹ Changes in the ultrastructure of parasite membranes after exposure to the drug have been described. Such changes of the erythrocytic stage appeared within 1-4 hours after exposure *in vitro*. Morphological changes were found in the ribosomes and the endoplasmic reticulum, and accumulation of tritium labelled dihydroartemisinin in the membranes was shown to occur.¹ Artemisinin compounds concentrated in parasitised erythrocytes and structure/activity relations suggest that the endoperoxide bridge is essential for antimalarial activity.⁷

The biochemical action of artemisinin comprises two sequential steps shown in **Figure 1.6**.² In the first step haem or Fe^{2+} catalyses the opening of the peroxide bridge, leading to the formation of free radicals. The intraparasitic haem activated artemisinin irreversibly decomposes during the second step, generating free radicals that alkylate and oxidise proteins and oxidise lipids.⁷ Malaria parasites are known to be sensitive to free radicals. A mechanistic framework for the Fe^{2+} induced cleavage of artemisinin and its derivatives has been proposed to explain the formation of metabolic products and the occurrence of the most important pathways.¹³ Artemisinin compounds act rapidly in terminating parasite development. The artemisinin derivatives are 20-100 times more active *in vitro* than the parent compound, and dihydroartemisinin is about 3 times more active than artemether.⁷

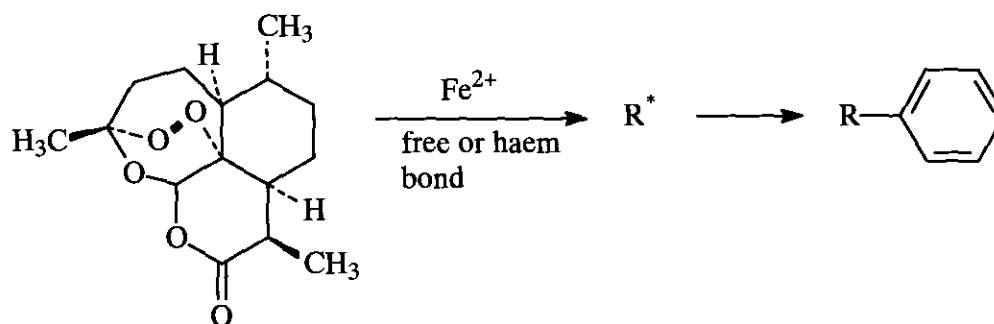


Figure 1.6 Mechanism of biological action of artemisinin¹²

1.6 Therapeutic Function and Value

The efficiency of artemisinin derivatives in the treatment of malaria, including severe, cerebral and multi-resistant malaria, has been proved. Moreover, these compounds are highly regarded in view of an absence of major side-effects and the relatively low cost of manufacture and distribution.¹³

Artemisinin suppositories represent a major advance in the treatment of severe malaria in rural areas where injections cannot be administered. They are particularly valuable for the treatment of children. Artemisinin compounds are active when taken orally, but the optimum dosage still needs to be determined.⁷

It is clear from the available literature that artemisinin and its derivatives received much attention during the last decade. However, to allow a rational and worldwide use of this group of peroxide containing sesquiterpene lactones as antimalarials, a considerable amount of work remains to be done by investigators in various research areas.

Finally artemisinin is known to inhibit seed germination and seedling growth of several mono and dicotyledonous plants, both cultivated and as weeds.¹⁴ Several compounds related to artemisinin, including artemisinic acid, arteannuin B deoxyartemisinin and artesunic acid were also shown to be phytotoxic to different extent.

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CHAPTER 2 EXTRACTION METHODS

It is a tension between creativity and scepticism that has produced the stunning and unexpected findings of science.

Carl Sagan

There are several classical techniques suitable for botanical extraction, among which steam distillation and solvent extraction are the most important. Recently, supercritical fluid extraction (SFE) proved to be a viable alternative, and since it is the technique used in this investigation, it is dealt with in more detail in this chapter.

2.1 Steam Distillation

The most successful method for the production of essential oils, i.e. volatile oils contained in plant material, is steam distillation. Constituents having boiling points between 150 and 250 °C are readily separated from the base material by steam.¹

Steam distillation is a useful technique for the distillation of immiscible mixtures of an organic compound and water (steam). In such mixtures, each component maintains its own vapour pressure independently of the other. When the total vapour pressure, which is the sum of the individual vapour pressures, equals the external pressure, the mixture boils at a temperature lower than the boiling points of the pure liquids.² The reason for this is that the added steam increases the pressure inside the extraction vessel so that pressure equilibrium between inside and outside the vessel is attained at a temperature lower than the normal boiling point of water.

The situation is mathematically described by

$$p_{\text{total}} = X_A p_A^{\circ} + X_B p_B^{\circ} \quad (2.1)$$

where p_{total} represents the total pressure of all components, X the mole fraction of each component and p° the partial pressure of each component.

Water and organic oils do not mix, and are thus independent of each other. Each has a mole fraction of 1, so that equation 2.1 simplifies to

$$p_{\text{total}} = p_A + p_B \quad (2.2)$$

The boiling points should thus be lower for the pressures to add up to atmospheric pressure. This allows successful distillation to be performed at a temperature lower than the normal boiling point of either component in the mixture.

A disadvantage of the technique of distilling natural material is that hydrolytic processes often occur during the heating-up phase of the water and causes undesirable flavours. Also, terpene alcohols remain partially dissolved in water, and are thus lost in the essential oil. The loss of phenylethyl alcohol in rose oil is an example. The essential oil composition in the distillate may be different from that of the herbal raw material. An example is the blue colour of German chamomile steam distillates, which is the consequence of the decomposition of matricine, a sesquiterpene lactone, into blue chamazulene, which is related to the loss of anti-inflammatory efficacy.³

The positive aspects of steam distillation, however, are the simplicity of the method, its universal application, and the inexpensive equipment required. The equipment should be made of stainless steel in order to avoid a colour change of essential oils. In addition, distillation is suitable for fresh plant material.

The equipment required for the extraction of essential oils from plant material by steam distillation consists of three basic parts; viz a still, a condenser, and a separator, as shown in **Figure 2.1**.

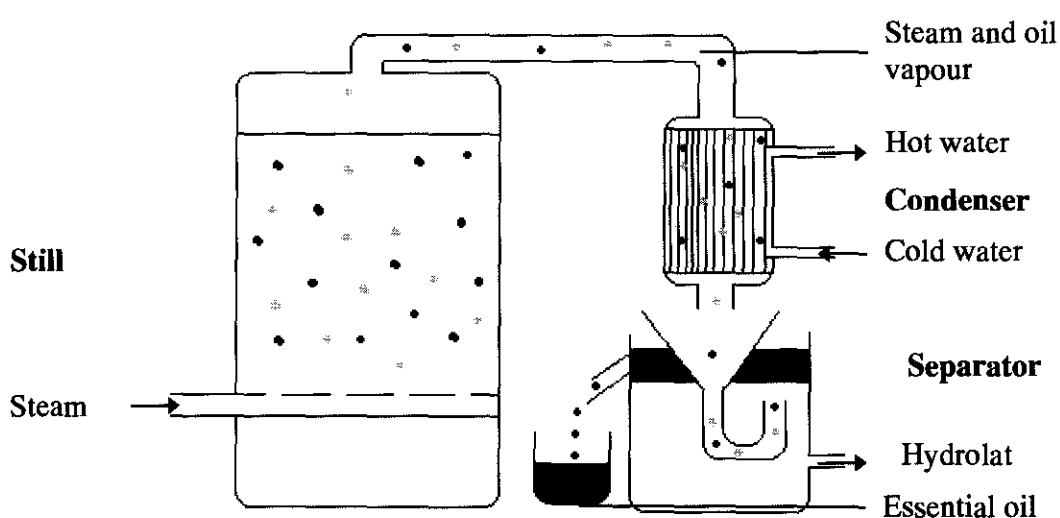


Figure 2.1 Main components of steam distillation equipment for plant extraction

The still contains the plant material with the volatile oil. Steam is let into the still at the base where a perforated grid supports the sample of green herb. The steam passes through the plant material, heating and saturating it with water. The resulting vapour, a mixture of steam and essential oil vapours, leaves the still at the top and is transferred to the condenser.⁴

The vapour from the still passes down a long spiral of tubing or plates cooled by circulated water. The resulting condensate flows by gravity into a separator, where the lighter insoluble oil floating on the surface is slowly drawn off periodically. The water soluble constituents extracted by the steam, known as the hydrolat or floral water, is drawn off continuously from the bottom of the separator.⁴

One of the advantages of steam distillation is that the temperature never exceeds the boiling point of water. This permits the purification of high-boiling substances, which are too heat sensitive to withstand ordinary distillation. However, the heat requirements of the steam distillation process are high, since large amounts of water need to be evaporated.⁵

Even a temperature lower than the boiling point of water (100 °C) may be too high for certain thermolabile natural compounds. In addition, water may induce hydrolytic modification of the natural material. In many distillations, decomposition products like hydrogen sulphide, ammonia, acetaldehyde, acetic acid and others may require subsequent steps for purification.⁶

2.2 Solvent Extraction

Liquid-liquid extraction is a process suitable for separating components in a solution by distribution between two immiscible liquid phases. Such a process can also be simply referred to as liquid or solvent extraction.

Since liquid-liquid extraction involves the transfer of mass from one liquid phase into a second immiscible liquid phase, the process can be carried out in many different ways. The simplest method involves the transfer of one component from a binary mixture into a second immiscible liquid phase.⁶

Solids can also be extracted with organic solvents. Solid-liquid extraction is generally utilised for the removal of natural products from dried tissue originating from plants, fungi, seaweed,

mammals, etc. Volatile natural products (e.g. those occurring in essential oils) such as alcohols, esters and carbonyl compounds of aliphatic and simpler aromatic systems, are removed by steam distillation. The non-volatile compounds may be removed by solvent extraction using a batch or continuous process.

The following properties of a solvent should be considered before using it in liquid-liquid extraction:

1. **Selectivity:** The relative separation or selectivity of a solvent is the ratio of two components in the extraction solvent phase divided by the ratio of the same components in the feed solvent phase.
2. **Recoverability:** The solvent should usually be recoverable from both the extract and the raffinate streams in an extraction.
3. **Distribution coefficient:** The distribution coefficient for a solute should preferably be large, so that a low ratio of solvent to feed can be used.
4. **Toxicity:** Low toxicity is preferred in view of potential solvent vapour inhalation or skin contact during repair of equipment or when connections are broken after a solvent transfer. Often solvent toxicity is low if water solubility is high.

A special case of solid-liquid extraction is soxhlet extraction. The solid to be extracted is packed into a special thimble made of thick filter paper. The thimble is placed in the apparatus shown in **Figure 2.2**, and the entire soxhlet extractor is placed on top of a well-supported round-bottom flask containing the organic solvent. A reflux condenser is placed on top of the soxhlet extractor. The flask is heated using a water or steam bath (for flammable solvents) or some form of electrical heating, so that the solvent boils. Solvent vapour passes up the large diameter outer tube of the apparatus, and condensed solvent drips down through the thimble containing the solid. Material is extracted out of the solid into the hot solvent. When the solution level reaches the top of the siphon tube, it automatically flows through the narrow tube and returns to the flask where the extracted material accumulates.²

The process is efficient, since the same batch of solvent is repeatedly recycled through the solid. If the extraction is run for prolonged periods it is possible to extract materials that are only slightly soluble in organic solvents. The technique is often used for the extraction of natural products from biological materials such as crushed leaves or seeds.

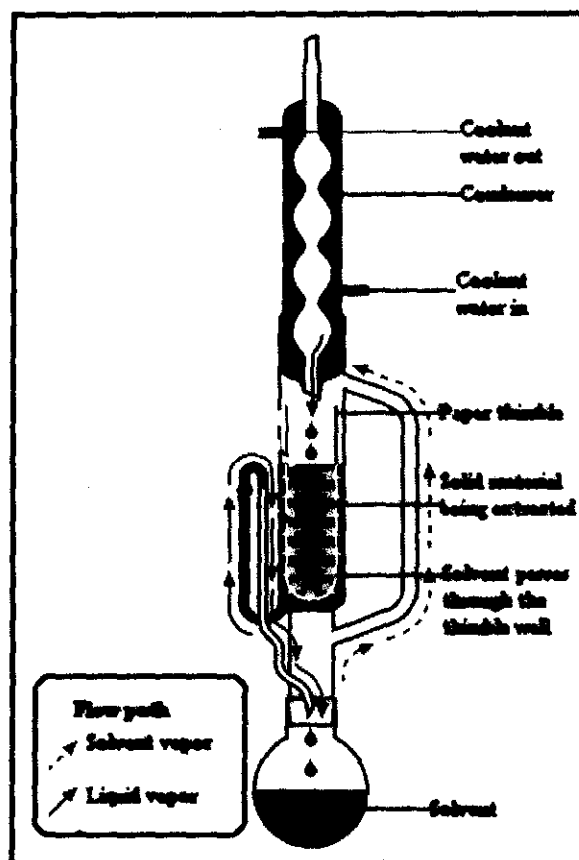


Figure 2.2 Soxhlet extractor

2.3 Enfleurage

Enfleurage⁷ is a historical procedure to recover volatile fragrant substances by absorption in lard. Plates with thin film of lard are positioned on top of strong smelling flowers like rose or jasmine, and the volatile substances are transferred through the air and are absorbed into the lard. One exciting aspect of this procedure for living flowers is the higher yields obtained, since oil production is continued for a certain period, whereas picked flowers provide only the amount present at the time of harvest. The collected fragrances are washed out of the lard in further steps by hydro-alcoholic solvents, and are then removed by distillation in order to obtain the principal smelling agent. This procedure is complex and uneconomical, restricted to

highly volatile floral fragrances, unsuitable for the production of larger amounts, and therefore not industrially viable.

2.4 Supercritical Fluid Extraction (SFE)

Supercritical fluids have received widespread attention during the past few years in view of their potential application in extraction processes relevant to the food, pharmaceutical and petroleum industries.⁸

One of the best known applications of supercritical fluid extraction (SFE) is the decaffeination of coffee beans with supercritical carbon dioxide (sc-CO₂), by which caffeine, but none of the aroma substances, is removed as a result of the high selectivity of the supercritical fluid for this substance.⁸ This was followed by the extraction and refining of edible oils such as coconut, peanut, soya bean and sunflower seed oil. The interest in fats extracted from fish, meat and eggs partly lies in the appreciable amounts of glycerides important for their pharmaceutical use. In the extraction of egg yolk, total lipids (particularly cholesterol) are largely reduced, especially at high pressures and temperatures.⁹ An advantage is the high-quality extract obtained, which can be directly used for human consumption without further refining as no hazardous solvent residues are left.

The production of food colouring agents, flavours and aromas is another potentially fertile field for application of sc-CO₂. The extraction of bitter components from hop is significant to beer brewers. Extracts containing the principal constituents of spices have the advantage of being sterile and usable for extended periods of time. Mainly essential oils and flavour components are being extracted.⁹

A drawback of supercritical fluid extraction is the high initial cost of equipment. Another problem is that SFE fails to achieve the same extracting efficiency for real and synthetic samples.

2.4.1 Supercritical Fluids

A supercritical fluid (SCF) is a substance which, above but close to its critical point, can no longer be classified as either a liquid or a gas but shares the physical properties of both. It exhibits gas-like diffusivity and viscosity, while it retains liquid-like solvating characteristics

and densities (0.1 – 1 g/mL).¹⁰ The high diffusivity allows penetration of solid materials, and the high densities enable analytes to be dissolved from a solid matrix. SCFs are compressible, and small pressure changes produce significant changes in density and in the ability to solubilise compounds. Also, SCFs have no surface tension. They can, therefore, penetrate low porosity materials. In addition, the very low viscosity provides favourable flow characteristics. These properties enable SCFs to provide excellent extraction efficiency and speed. Mass transfer limitations ultimately determine the rate at which an extraction can be performed.

The fluid density and, with it, the solvent strength and selectivity, undergo drastic changes as a result of moderate changes in temperature and/or pressure within the critical domain. The solvent strengths of SCFs approach those of liquid solvents as their density is increased. Since SCFs have diffusivities of an order of magnitude higher (10^{-4} vs. 10^{-5} cm²/s) and viscosities of an order of magnitude lower (10^{-4} vs. 10^{-3} N s/m²) than liquid solvents, they have much better mass transfer characteristics. Quantitative SFE is generally completed in 10-60 min, whereas solvent extraction can run from several hours to days.¹⁰

The substances proposed to be used as SCFs are rather limited: CO₂, ethane, propane and higher alkanes up to hexane, ethylene, nitrous oxide, SF₆, CHClF₂, methanol, ethanol, butanol, acetone, toluene, pyridine, ammonia and water. **Table 2.1** lists the physical-chemical characteristics of a few selected SCFs.

Table 2.1: Critical parameters for selected SCFs						
Critical Values						
SCF	Temperature (K)	Pressure (atm)	Density (g/mL)	Volume (mL)	Solubility (g/mL)	Dipole moment (D)
CO ₂	304.3	72.9	0.472	93.9	10.7	0.0
N ₂ O	309.8	73.4	0.445	97.4	10.7	0.2
NH ₃	405.5	115.0	0.211	72.5	13.2	1.5
CH ₃ OH	513.2	82.0	0.246	118.0	14.4	1.7
C ₂ H ₆	305.4	322.7	0.204	148.3	6.6	0.0
C ₂ H ₄	283.3	51.2	0.201	-	6.6	0.1

2.4.2 SFE Apparatus

SFE requires fairly simple equipment. **Figure 2.3** shows the essential components. The extraction solvent, e.g. CO₂ from a cylinder (1), is supplied by a pump (2) and regulating valve (3) to an extraction vessel (4) placed in an oven (5) to maintain it at a temperature above the critical temperature of the solvent. During SFE, the soluble analytes are partitioned from the bulk sample matrix into the SCF and then separated at a flow restrictor (6), where ambient conditions are restored, into a collection device (7). The gas is vented from the restrictor while the extracted analytes are retained.

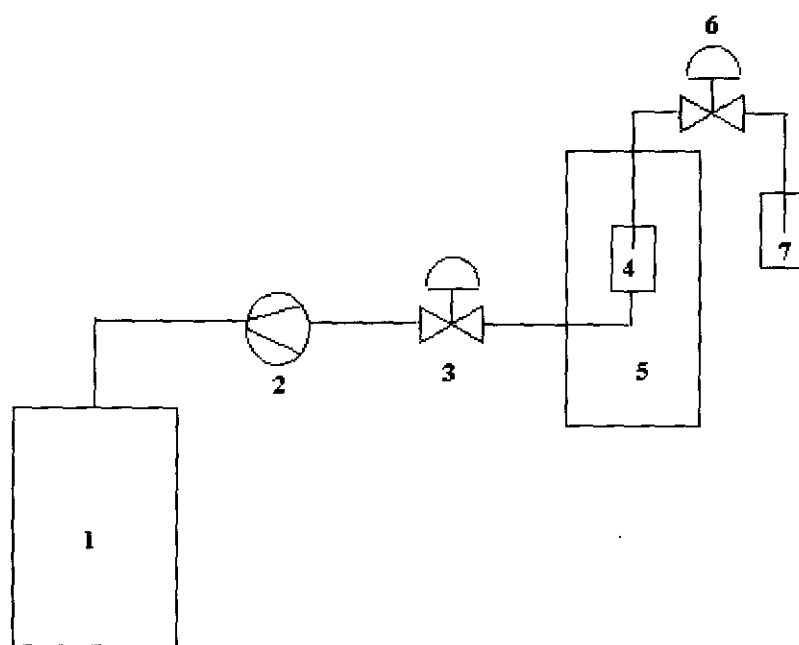


Figure 2.3: Schematics of typical SFE system. 1. Liquid CO₂, 2. Pump, 3. Regulating valve, 4. Extraction vessel, 5. Heated zone, 6. Restrictor, 7. Collection vessel

Separation of the desired product from the SFE exit stream is achieved in different ways. One option is to precipitate the solute by reducing the solvent density through pressure reduction (isothermal cycle), temperature increase (isobaric cycle) or mixing the extract with gases like Ar or N₂. The precipitated product is collected either directly in a collection vessel or adsorbed onto a suitable adsorbent, e.g. glass beads, and removed in a subsequent rinse cycle.¹²

Several experimental variables need to be considered and optimised for SFE to be successful, including choice of SCF, pressure and temperature, extraction time, sample size, method of collection of extracted materials and equipment needed. If the extraction requires the use of a modifier, cosolvent or entrainer to adjust the polarity of the selected SCF, an additional modifier pump introduces small quantities of such solvent from a storage bottle into the flow line where it is mixed with the SCF.

2.4.3 Modifier

The extraction efficiency of polar organic substances can be increased by adding small amounts of a suitable modifier to a non-polar SCF such as CO₂. The mechanism by which a modifier operates, depends on the type of matrix and analyte, and can be

- by increasing the analyte solubility in the SCF through interaction with the solute in the fluid phase;
- by facilitating desorption of the analyte from the matrix through interaction with the bound solute or the matrix active sites;
- by favouring diffusion of the solute within the matrix through swelling of the matrix;
- by hindering diffusion of the solute within the matrix through contraction.

The first two mechanisms occur when a polar modifier is used, whereas the latter two can result from the use of both polar and non-polar modifiers. The more polar substances are, the larger the amounts of modifier needed. The following organic solvents are most frequently used as modifiers for sc-CO₂: methanol, propanol, acetone, formic acid and toluene. Methanol is the most common modifier, and is used at concentrations between 10 and 20 % (v/v) for extracting pharmaceutical compounds.

2.4.4 Mechanism of Extraction from Solid Plant Matrices

The transport of substances within a solid plant matrix may differ from substrate to substrate as the primary structure of the same type of plant is different from one harvesting period to another and varies with conditions and treatment after harvesting, age and treatment prior to extraction. Moreover, the solid material may consist of particles of different size and form, and the size distribution of the particles may differ.¹³

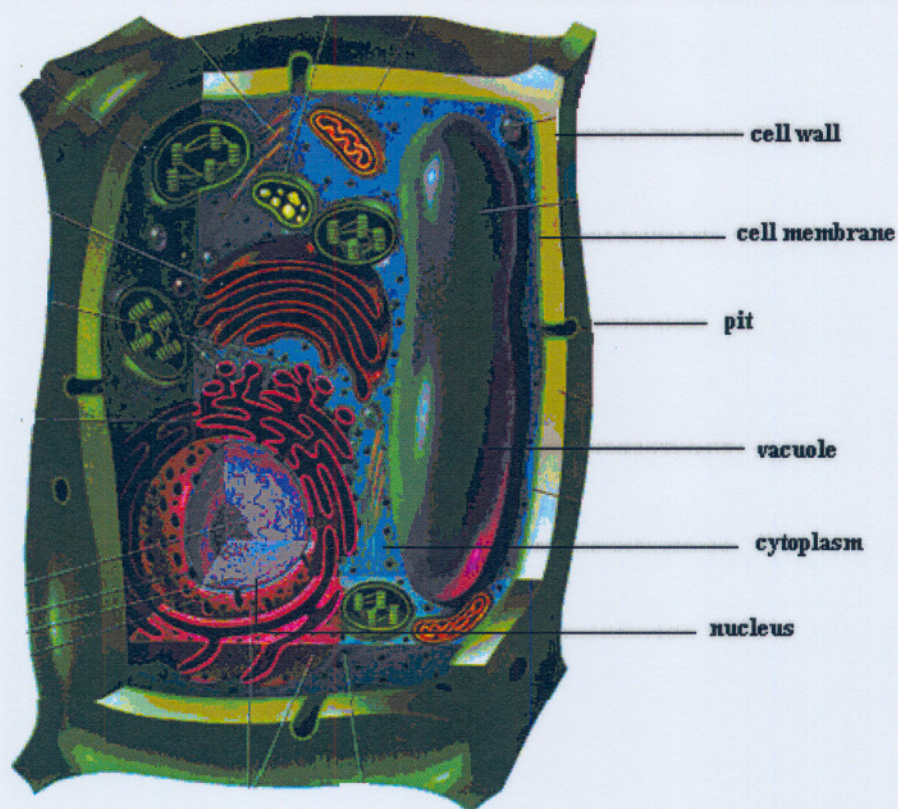


Figure 2.4: Schematic diagram of plant cell

A plant cell, which is shown in **Figure 2.4**, is part of a cell structure which, in turn, is part of a particle and an aggregate of particles in a fixed bed of material placed in an extraction vessel. The substructure of the cell, like the cytoplasm with the vacuoles, the cell wall, the intercellular cavities and the plasma membrane, the tonoplasts and the pit within the cell wall, are determining the mass transfer during extraction if the substances to be extracted are part of the cell.¹⁴

In certain cases the cell structure and the cells remain intact during extraction. An example is the decaffeination of green coffee beans, which are roasted only after the extraction of caffeine. During roasting the aroma substances are formed under pressure. If the cells and cell structures were damaged, the resulting pressure would probably be much lower and thus detrimental to the coffee aroma.

Transport paths are shortened and mass transfer enhanced if the plant material is crushed and the cells and the cell structures are destroyed. The transport resistance across membranes will

then be of minor importance. Other transport mechanisms, like diffusion in the solid, desorption from the solid surface and diffusion in laminar flowing layers of solvent still prevail.

2.4.5 Solubility of Substances in SCFs

In an effort to understand the parameters influencing SFE, researches have studied thermodynamic properties such as the solubility of substances in SCFs.¹⁰

The semi-empirical equation

$$\delta = 0.47 P_c^{1/2} \rho \quad (2.3)$$

relates the Hildebrand solubility parameter (δ) to the density (ρ) of a SCF. It is used to calculate the Hildebrand solubility parameter for a given SCF and even for binary fluids provided that the mixture does not contain a polar component (modifier). Hildebrand solubility parameters predict extraction efficiency fairly well if the sample matrix has no strong adsorption sites. If polar analytes are adsorbed onto a polar sample matrix with relatively strong adsorption sites, small amounts of polar modifier will greatly enhance the desorption rate. Recovery is vastly improved as compared to the pure, unmodified SCF.

The effect of temperature on the solubility of solids is different at pressures in the critical range or when the pressure exceeds the critical value by a factor of two or more. Near the critical pressure the fluid density is so sensitive to temperature that even a decrease in fluid density occurs with a moderate increase in pressure, with a consequent reduction in the solubility of solids.

2.4.6 Advantages of sc-CO₂

The choice of a supercritical solvent depends on the polarity of the target analyte. Another consideration is the temperature and pressure needed to convert the substance into the supercritical state. CO₂ becomes supercritical under rather mild conditions, viz. above 31.1 °C and 7 380 kPa.¹⁰ It eliminates many hazardous chlorinated solvents to which environmental limitations currently apply, and is relatively inert and pure.¹⁴ Using sc-CO₂ results in rapid preparation procedures and often eliminates subsequent sample clean-up steps.¹⁵ CO₂ is readily

available at low cost. It has low toxicity and reactivity, and it provides a clean alternative to conventional extraction techniques. All these make sc-CO₂ the most widely used extraction fluid, especially for non-polar analytes. Although sc-CO₂ is a poor solvent for polar compounds, it can be modified into a more polar solvent by adding small amounts of acetone or methanol, and can thus be used to extract slightly polar compounds.

Other advantages of using sc-CO₂ include the following: The extracted material is collected in a concentrated form since the fluid evaporises at ambient conditions. The solvent strength can be adjusted by changing the density (pressure and temperature) and by adding a modifier. Extraction times are fairly short since sc-CO₂ has superior mass transfer qualities.¹⁰ Mass transfer limitations ultimately determine the rate at which an extraction can be performed. Since CO₂ has a relatively low critical temperature, SFE can be performed at low temperatures to extract thermally unstable compounds.¹⁴

References Chapter 2

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CHAPTER 3 TECHNICAL ASPECTS

Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under your observation in life.

Thomas Carlyle

The technical aspects of the investigation are outlined in this chapter. These include sample preparation, extraction procedures and method of analysis, and also cover the principles underlying the adopted data processing and process description approaches.

3.1 Materials and Reagents

The plant material was donated with a certificate of authenticity provided by the overseas supplier. The dried material was finely crushed. Fresh plant material was also investigated. It was purchased from Lifestyles nursery in Gauteng, and its identity was verified by the in-house horticulturist.

The CO₂ used for extraction was of SFE-grade (99,9999%) and purchased from Air Products. Other gases used for extraction and/or GC-analysis included H₂, N₂, and compressed air, which were all supplied by Afrox. HPLC-grade methanol was used as a cosolvent to slightly modify the polarity of the CO₂, and hexane was selected as a suitable solvent to collect the extract.

An artemisinin standard was purchased from Sigma-Aldrich in Germany. It was 98.6 % pure and was used without any further purification. The standard was stored in a refrigerator at 5 °C pursuant to the manufacturer's instructions. It was used for the identification of artemisinin present in the botanical extracts and for the construction of a calibration line to quantitatively analyse for artemisinin content.

3.2 Extraction Procedures

Extractions were performed by steam distillation, soxhlet and sc-CO₂ in order to mutually compare the techniques and to establish any advantages sc-CO₂ extraction may have over the traditional techniques in isolating artemisinin from plant material. The procedures used with the different techniques are described in the subparagraphs below.

3.2.1 Steam Distillation

The weighed (5 g) plant material was mixed with 100 mL water in a round-bottom distillation flask as shown in **Figure 3.1**. The steam was generated in a separate flask and then passed through the plant material. The heat of the steam opens the tiny pores that hold the extractable material to be released. The added steam causes the heated mixture to boil at a temperature lower than the normal boiling point of water as described earlier. The extraction was performed for an entire day and repeated for 5 days.

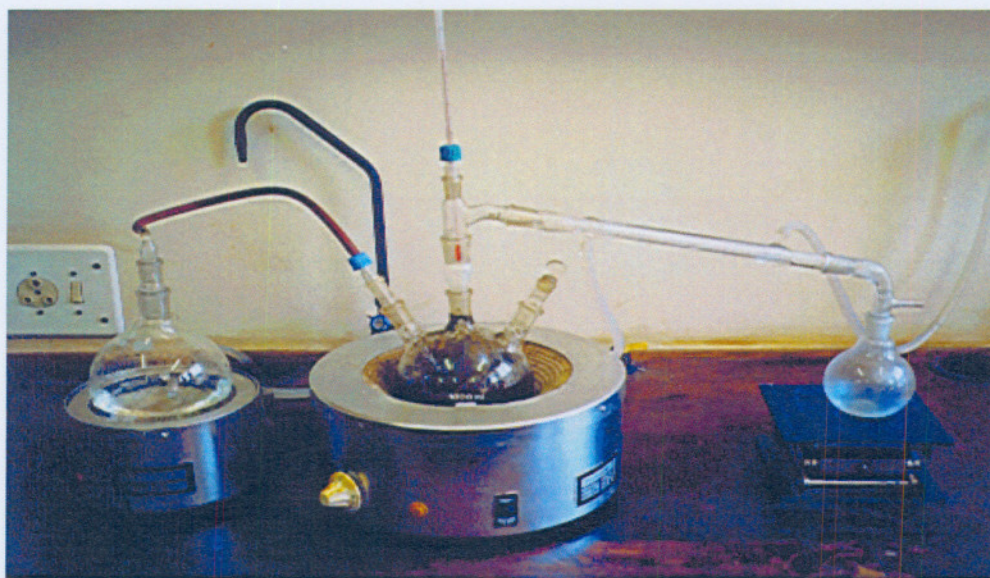


Figure 3.1: Steam distillation apparatus

3.2.2 Soxhlet Extraction

The weighed (5 g) dried plant material was placed in the thimble in the apparatus shown in **Figure 3.2**. A suitable solvent (100 mL n-hexane) was placed in the flat-bottom flask and heated on the hotplate of a magnetic stirrer. The vapour rises and enters the thimble containing the plant material. It subsequently condenses in the reflux condenser, and the extract is collected in the flat-bottom flask. There is thus a continuous circulation of organic solvent by alternating heating and cooling.

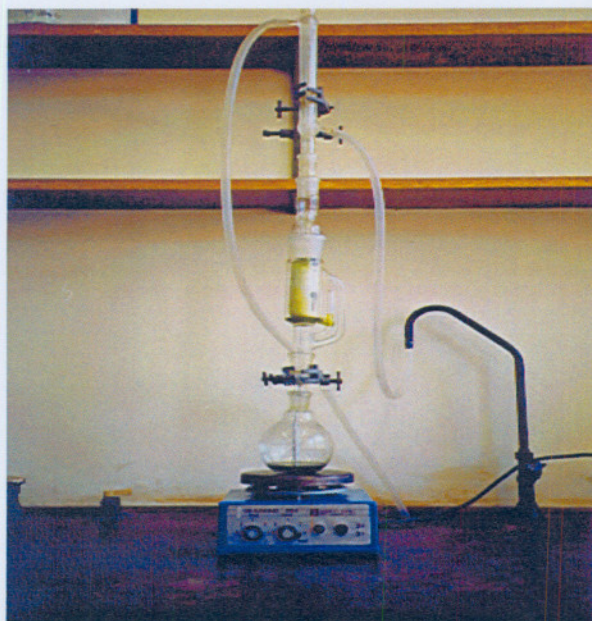


Figure 3.2 Soxhlet apparatus

3.2.3 SFE

In the case of SFE the extraction vessel of the Isco SFX 220 supercritical fluid extractor shown in **Figure 3.3** contained about 1.0 g of finely ground plant material. The initial conditions used are summarized in **Table 3.1**, but these conditions were varied during the investigation in order to optimise the extraction yield of artemisinin and to identify the limiting process parameters. The extract was collected in 5 mL methanol, filtered and transferred into a GC vial from where 1 μ L was injected into a gas chromatograph (**Paragraph 3.3**) for analysis.

Table 3.1 Initial conditions

Pressure	150 atm
Temperature	40°C
Flow rate	2 mL/min
Time	15 min
Modifier	none

The operation of the supercritical fluid extractor in **Figure 3.3** can be described as follows: CO₂ is introduced from a cylinder by virtue of a single piston pump. The compressed gas enters the extraction vessel, which is manufactured of a hard-wearing polymer material and which contains the finely crushed plant material and a small amount of modifier (if applicable).

The vessel is screwed into the extraction chamber and heated to a temperature which ensures supercritical conditions at the selected pressure. The extracted material is subsequently released in a stainless steel capillary restrictor by restoring ambient conditions and allowing the fluid to escape into the atmosphere as a gas. The extract was collected in a small glass tube containing methanol.



Figure 3.3 SFE apparatus

3.3 Analysis

Gas chromatography (GC) was used for the analysis of the acquired extracts. An HP 6890 gas chromatograph with HP-5 column (30 m x 320 μm x 0.25 μm ; 5 % phenyl methyl siloxane) shown in **Figure 3.4** was used. The oven temperature was set at 150 $^{\circ}\text{C}$ and controlled to a few tenths of a degree. The elution time was 10 min. An auto-sampling system with a microsyringe was used to inject the liquid sample through a septum into a flash vaporiser port located at the head of the column. Elution was effected by an inert gaseous mobile phase (mixture of H_2 and N_2) which does not interact with the analyte. A flame ionization detector was used. The extracts were diluted to a fixed volume with methanol prior to injection into the gas chromatograph.



Figure 3.4 Gas chromatograph

3.4 Data Processing

3.4.1 Surface Response Analysis

Response surface methodology (RSM)¹ comprises a set of techniques designed to find a “best” value of a response. The measured response is governed by laws approximated by a deterministic relationship between the response and a set of experimental factors. It should thus be possible to determine the best conditions (levels) of the factors to optimise a desired output. The data collected from an experiment are used to empirically quantify, through some form of mathematical model, the relationship between the response variable and its influencing factors. The response variable is called the dependent variable and the influencing factors are referred to as explanatory variables.

Surface response techniques were employed for this investigation. It was necessary to first design the required experiments, i.e. to select the explanatory variables, viz. temperature and pressure, and to designate the values to be used for these two controllable factors during actual experimentation. Statistica for Windows[®] was used for the experimental design and for the presentation of the yield of extracted artemisinin as a function of these two variables.²

Other authors^{1,2} have also noted the decomposition of artemisinin in protic solvents and at high GC injection temperatures and investigated alternative methods (HPLC, SCF chromatography) to avoid the problem. The peaks are rather wide and asymmetrical, indicating a less efficient separation and a need to derivatise the compound prior to analysis. This was, however, not considered desirable, as post-column derivatisation could change the structure of artemisinin.

4.2 Calibration Graph

The artemisinin standard was used to construct the calibration graph in **Figure 4.2**. A weighed amount of artemisinin was dissolved in 1 mL of HPLC grade methanol to prepare different test solutions for injection into the GC. The concentration range was selected on the basis of the expected content of artemisinin in the plant itself.

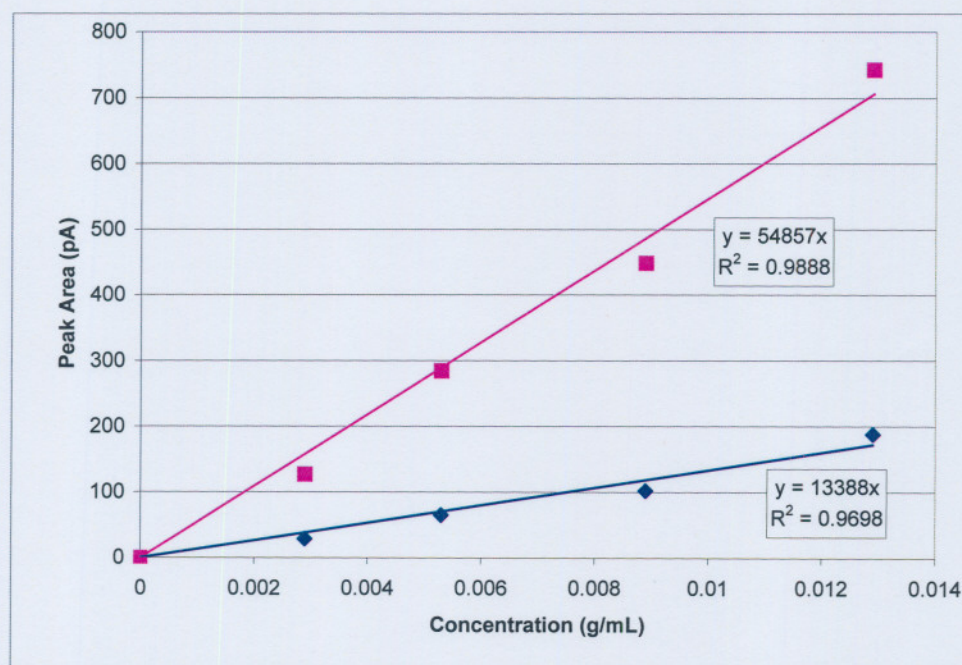


Figure 4.2 Calibration line of artemisinin

The calibration lines were used to read off the concentration of artemisinin from the measured peak areas obtained for extracts of the plant material. The two peaks at the selected gas chromatographic conditions (**Paragraph 3.3**) both exhibit a linear relationship between peak area and concentration of artemisinin at the respective retention times. For the artemisinin standard no other peaks were detected beyond the retention times mentioned.

An experimental design is a set of combinations of the levels of k coded variables which specifies the settings for the runs to be performed during experimentation. With $k = 2$, i.e. for two different controllable variables x_1 and x_2 , the orthogonal 2×2 design array or experimental layout in **Table 3.2** is applicable.

Table 3.2 Orthogonal 2×2 design		
Runs	Controllable variable x_1	Controllable variable x_2
1	1	1
2	1	-1
3	-1	1
4	-1	-1

In the first two runs, both variables x_1 and x_2 are set at their high levels (+1). In the last two runs, both are set at their low levels (-1). The two columns in a design matrix are orthogonal if the sum of the products of their elements within each row is equal to zero. Generally, the more orthogonal the columns the better the design. Thus, one consideration for choosing standard composite designs is to find designs that are orthogonal or nearly orthogonal.

For a simple 4-run orthogonal experiment the information remains constant around a circle with the origin as the midpoint. Thus any kind of rotation of the original design will generate the same information. The 2×2 orthogonal design in **Table 3.2** is therefore said to be rotatable.

In order to determine the second-order, quadratic, or non-linear component of the relationship between a factor and the dependent variable, at least three levels are required for the respective factors. The information function is then non-constant on circles around the origin, and the design is non-rotatable. This means that different rotations of the design points will extract different information from the experimental data. By adding so-called star-points to the simple 2×2 factorial design points, it is possible to obtain rotatable, and often orthogonal or near orthogonal designs, like that shown in **Table 3.3**. The first four runs in this design are the previous 2×2 orthogonal design points, runs 5 through 8 are the so-called star or axial points, and runs 9 and 10 are centre points. The information function for this second-order (quadratic) design is rotatable, i.e. constant on a circle around the origin.³

Table 3.3 Orthogonal design		
Runs	Independent variable 1	Independent Variable 2
1	1	1
2	1	-1
3	-1	1
4	-1	-1
5	-1.414	0
6	1.414	0
7	0	-1.414
8	0	1.414
9	0	0
10	0	0

It will be shown later (**Paragraph 4.5**) that, based on the orthogonal design in **Table 3.3**, 10 runs were performed with the temperature and pressure selected within the ranges $40 < T < 100$ °C and $135 < p < 450$ atm to establish optimum extraction conditions.

3.4.2 Modelling of Extraction

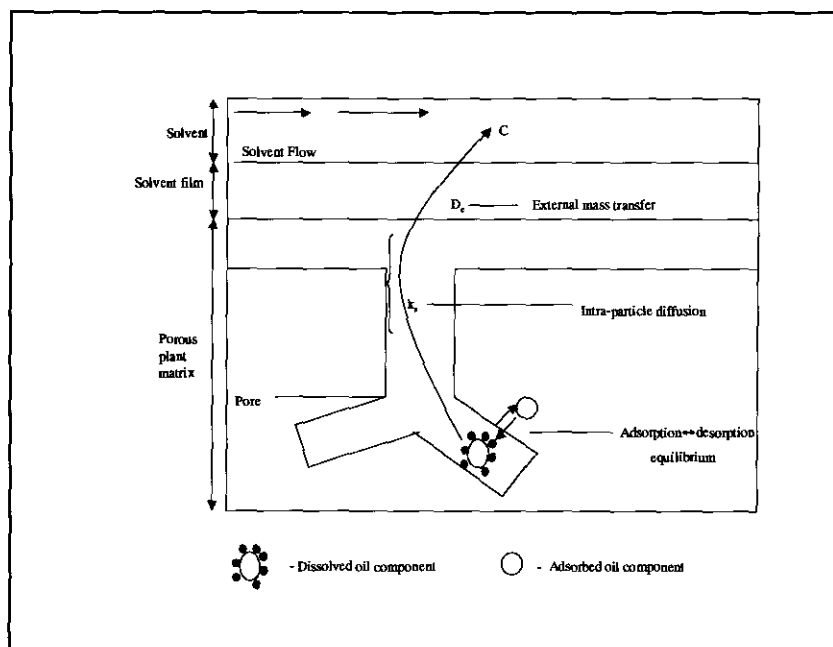


Figure 3.5 Schematic representation of extraction process⁴

Essential oils are found within porous plant matrices. The distribution of essential oils and other active ingredients in such matrices depends on various factors including botanical species, crop time and method of harvesting. In the case of *A. annua* the glandular trichomes sequester artemisinin as well as aromatic volatile oils (essential oils). Artemisinin accumulates in the sub-cuticular space of capitate glandular trichomes abundantly present on the surface of the leaves and flower organs.

A dynamic (i.e. continuous flow) extraction as illustrated in Figure 3.5 is described mathematically⁴ by equations (1) – (3):

$$\alpha \frac{\partial C}{\partial t} + u \frac{\partial C}{\partial z} = D_L \frac{\partial^2 C}{\partial z^2} - (1 - \alpha) a_p k_f (C - C_p) \quad (1)$$

$$\beta \frac{\partial C_p}{\partial t} = D_e \frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial C_p}{\partial r} \right) - \mathfrak{R} \quad (2)$$

$$\frac{\partial C_s}{\partial t} = \mathfrak{R} \quad (3)$$

where

C_s = concentration extractable in matrix [kg/m³]

C_p = concentration in pore volume of matrix [kg/m³]

C = concentration in empty pore volume of matrix [kg/m³]

α = fraction in empty pore volume of matrix

t = time [s]

u = surface rate [m/s]

z = axial coordinate [m]

D_L = axial dispersion coefficient [m²/s]

a_p = specific surface area [1/m]

r = radial coordinate [m]

β = particle porosity [m]

D_e = effective intraparticle diffusivity [m²/s]

$\mathfrak{R}$ = extraction rate [kg/m³ s]

k_f = mass transfer coefficient [m/s]

For the situation where no substance is extracted equations (4) – (10) apply:

$$C(t = 0) = C_0 \quad (4)$$

$$C_p(t = 0) = C_{p,0} \quad (5)$$

$$C_s(t = 0) = C_{s,0} \quad (6)$$

$$\left(\frac{\partial C}{\partial z} \right)_{z=L} = 0 \quad (7)$$

$$D_L \left(\frac{\partial C}{\partial z} \right)_{z=0} = u(C|_{z=0} - C_0) \quad (8)$$

$$D_e \left(\frac{\partial C_p}{\partial r} \right)_{r=r_0} = k_f (C - C_p|_{r=r_0}) \quad (9)$$

$$\left(\frac{\partial C_p}{\partial r} \right)_{r=r_0} = \left(\frac{\partial C_s}{\partial r} \right)_{r=0} = 0 \quad (10)$$

where r_0 = particle radius [m]. The two possible mechanisms of extraction is (1) desorption of the extractable components from the solid matrix, or (2) the dissolution of the components in the supercritical solvent.⁵ The extraction process is described in terms of adsorption/desorption when interaction between the components and the solid matrix is the determining factor. When the solubility of the components in the solvent determines/limits the extraction, the process is best described as dissolution, which comprises melting (if solids are present) and solvation by the solvent/fluid.

In the case of an adsorption/desorption mechanism, the rate of extraction can be described by the equation

$$\mathfrak{R} = \left(1 - \frac{C_s}{C_m} \right) C_p - k_d C_s \quad (11)$$

which, on applying boundary conditions at equilibrium, simplifies to a Langmuir type adsorption isotherm

$$C_s = \frac{KC_m C_p}{C_m + KC_p} \quad (12)$$

where

$K = k_a/k_d$ = adsorption equilibrium constant

k_d = desorption rate constant [1/s]

C_m = adsorption capacity [kg/m³]

Extraction data can be fitted by non-linear regression based on equation (12), or its inverse

$$\frac{1}{C_p} = \frac{K}{C_s} - \frac{K}{C_m} \quad (13)$$

can be represented graphically as a straight line by plotting $1/C_p$ against $1/C_s$. The equilibrium constant K of the adsorption process can be determined from the gradient and the adsorption capacity C_m from the intercept. The magnitude of K indicates the direction of the adsorption/desorption equilibrium and reflects the relative ease with which these reaction steps occur.

If $C_m \gg C_s$, equation (13) simplifies to

$$C_p = C_s/K \quad (14)$$

which yields a straight line by plotting C_p against C_s . The inverse of the gradient yields a duplicate value of K .

If dissolution is the determining factor in extracting material from the pores, the solubility of the material in the solvent/fluid is the important parameter. The situation can then be thought of as a shrinking nucleus of unextracted material and an outer layer of solute that diffuses out. The average concentration $\bar{C}_s$ in the solid matrix relates mathematically to the nucleus radius r_c according to

$$\frac{\bar{C}_s}{C_{s,0}} = \left(\frac{r_c}{r_0} \right)^3 \quad (15)$$

The concentration within the matrix, excluding the region of the nucleus, is independent of time, so that

$$\left(\frac{\partial C_s}{\partial t} \right)_{r \neq r_c} = 0 \quad (16)$$

while the concentration in the pores in the region of the nucleus is saturated so that

$$C_p(r = r_c) = C^* \quad (17)$$

where $C^* = \text{solubility [kg/m}^3\text{]}$

A mass balance in the region of the nucleus gives

$$\frac{C_{p,0}}{D_e} \frac{dr_c}{dt} = \left(\frac{\partial C_p}{\partial r} \right)_{r=r_c} \quad (18)$$

which relates the change in concentration in the pores to the decrease of the radius of the contracting nucleus over time.

If dried material is crushed, the cellular structure is partially destroyed and allows for easier transport of soluble substances without the need for the fluid to penetrate the cell wall and membrane. In extreme cases, where there are only soluble substances on the outside of the partially destroyed cells, only these are extracted. In such circumstances one does not distinguish between C_s and C_p and the last few equations need to be modified accordingly.

3.4.3 Energy and Volume of Activation

The energy of activation for extraction can be determined by studying the temperature dependence of the rate of extraction and plotting $\ln(\text{yield})$ vs $1/T$ according to the Arrhenius

equation cited in physical chemistry textbooks.⁶ The substitution of the rate constant by the yield of extraction does not cause a problem since, although the intercept of the straight line is different from the one obtained by plotting $\ln k$ vs. $1/T$, the slope from which the activation energy is calculated remains unchanged. The activation energy E_a is a mechanistic indicator inasmuch its magnitude indicates whether the extraction takes place by physical or chemical means, i.e. by desorption or by chemical dissolution.

The equation $\ln k = -(\Delta V^\ddagger/RT)p + \text{constant}$ can be used to determine the volume of activation $\Delta V^\ddagger$ by plotting $\ln k$ versus p .⁷ The yield of artemisinin extracted can be used as an alternative for the rate constant without changing the slope of the resulting straight line. The volume of activation is also a mechanistic indicator as it relates to the absolute changes in volume in the transition state. Its sign (positive or negative) specifically indicates whether bonds are ruptured or formed and/or solvational changes occur during the extraction process.

3.4.4 Multivariable Analysis

In process modelling a mathematical equation may be derived which relates an outcome quantity, such as the yield of extraction, to the variables that contribute to that quantity. In the case of supercritical fluid extraction several variables are playing a role. It is important to include as many process variables as possible in the model, as a variation in one single variable may change the outcome of the extraction.

The variables which may play a role in supercritical fluid extraction include the pressure and temperature at which the process is performed, the flow rate of the fluid, the time of exposure of the plant material to treatment with the fluid, the density of the fluid, the amount of extractable artemisinin in the plant material, the amount of modifier used, the moisture content of the plant material and the waxes and oils present in the plant matrix.

Suppose a simple function $y = f(x)$ with one independent variable requires 4 data points to represent y . If a second variable is added, an additional 4 values of z are required for each value of x in the function $y = f(x,z)$ and the total number of experiments needed to determine a good value for y increases to 16. The total number of experiments thus needed for n variables is 4^n , and 3 variables would thus require a total of 64 experiments. A procedure which minimises the number of arguments in a function will decrease the number of experiments

needed. Dimensionless analysis is such a procedure⁸, as the number of experiments required is determined by the number of arguments in the function and not by the number of independent variables selected. In the case of the function $y = f(x,z)$, x itself may consist of a number of variables, say $x = uv/w$ with u , v and w each an independent variable, put together as a dimensionless group of variables (in Afrikaans sometimes referred to as a “bondeltjie” of variables).

Supercritical fluid extraction can grossly be represented by a function based on the following variables: f : flow rate of fluid; p : extraction pressure; T : extraction temperature; t : time of extraction. There may be other factors as well, such as r : amount of artemisinin in plant material; e : amount of modifier; w : moisture content of plant material; σ : amount of oils and waxes in plant material, but these are excluded here to somewhat simplify matters. It may be argued that they contribute to the overall yield to a lesser degree than the first-mentioned variables.

The variables which are likely to play a significant role in the extraction and to contribute to the overall yield H are listed below. The dimensions of these variables are such that they can be expressed in terms of mass M in kg, length L in m and temperature T in K as shown below.

Variable	f	p	T	t	H
Dimension	$\frac{M}{T}$	$\frac{M}{LT^2}$	$\frac{ML^2}{T^2}$	T	$\frac{M}{L^3}$

A product of the variables

$$\left(\frac{M}{T}\right)^a \left(\frac{M}{LT^2}\right)^b \left(\frac{ML^2}{T^2}\right)^c (T)^d \left(\frac{M}{L^3}\right)^e$$

will be dimensionless if

$$M: a + b + c + e = 0$$

$$L: -b + 2c - 3e = 0$$

$$T: -a - b - 2c + d = 0$$

For a first product $c = 1$ and $e = 0$ are selected. A solution for these equations then is $a = -3$; $b = 2$; $c = 1$; $d = 1$ and $e = 0$. Thus

$$\Pi_1 = \frac{p^2 T t}{f^3}$$

For a second product $c = 0$ and $e = 1$ are chosen. The solution then is $a = 2$, $b = -3$, $c = 0$, $d = -1$ and $e = 1$, so that

$$\Pi_2 = \frac{f^2 H}{p^3 t}$$

According to the theorem of Buckingham an equation is dimensional homogeneous if and only if it can be written in the form

$$f(\Gamma_1, \Gamma_2, \dots, \Gamma_n) = 0$$

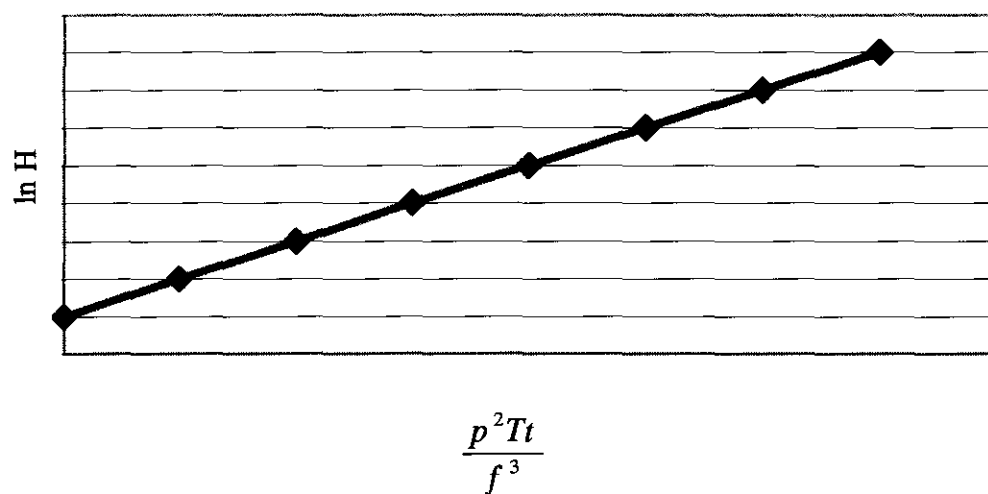
where f is a certain function with a number of arguments and $(\Gamma_1, \Gamma_2, \dots, \Gamma_n)$ represents a complete set of dimensionless products.⁸ Thus

$$H = \frac{p^3 t}{f^2} g(\Pi_1) = \frac{p^3 t}{f^2} e^{\frac{p^2 T t}{f^3}}$$

or

$$\ln H = m_1 + m_2 \Gamma_1 = m_1 + m_2 \frac{p^2 T t}{f^3}$$

with m_1 and m_2 dimensionless constants. This equation can be presented graphically as shown in **Figure 3.6** by plotting $\ln H$ (H = yield of artemisinin) versus the dimensionless grouping of variables. If a straight line is obtained as predicted by the equation, it can be accepted that all contributing process variables have been duly taken into account by the model.



Figuur 3.6 Plot of $\ln H$ versus dimensionless grouping of variables

From such a plot the expected yield of artemisinin can be readily read off once the validity of the model is verified by fitting experimental extraction data to it.

References Chapter 3

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CHAPTER 4 RESULTS AND DISCUSSION

The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.

Albert Einstein

The results obtained from the experimental work are described, processed and discussed in this chapter. The aspects important to the realisation of the project objectives are specifically dealt with. These include an analytical protocol to determine the concentration of artemisinin extracted by virtue of a calibration graph based upon a commercial standard, the optimum conditions for artemisinin extraction with sc-CO₂ using a software-based statistical design and a theoretical model of the extraction to which the experimental data can be fitted to validate the mathematical process description. Finally the results obtained with traditional extraction methods are very briefly discussed.

4.1 GC of Artemisinin Standard

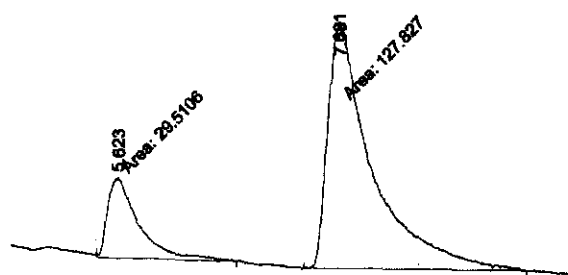


Figure 4.1: Gas chromatogram of artemisinin standard with retention times at $t = 5.6$ min and $t = 7.6$ min

The chromatogram of pure artemisinin exhibits two peaks at two different retention times as shown in **Figure 4.1**. The two peaks probably result from equal concentrations of the products of decomposition of artemisinin in the selected solvent (methanol) and at the temperature of injection concerned, since both peaks were proven to be quantitative indicators of artemisinin.

Other authors^{1,2} have also noted the decomposition of artemisinin in protic solvents and at high GC injection temperatures and investigated alternative methods (HPLC, SCF chromatography) to avoid the problem. The peaks are rather wide and asymmetrical, indicating a less efficient separation and a need to derivatise the compound prior to analysis. This was, however, not considered desirable, as post-column derivatisation could change the structure of artemisinin.

4.2 Calibration Graph

The artemisinin standard was used to construct the calibration graph in **Figure 4.2**. A weighed amount of artemisinin was dissolved in 1 mL of HPLC grade methanol to prepare different test solutions for injection into the GC. The concentration range was selected on the basis of the expected content of artemisinin in the plant itself.

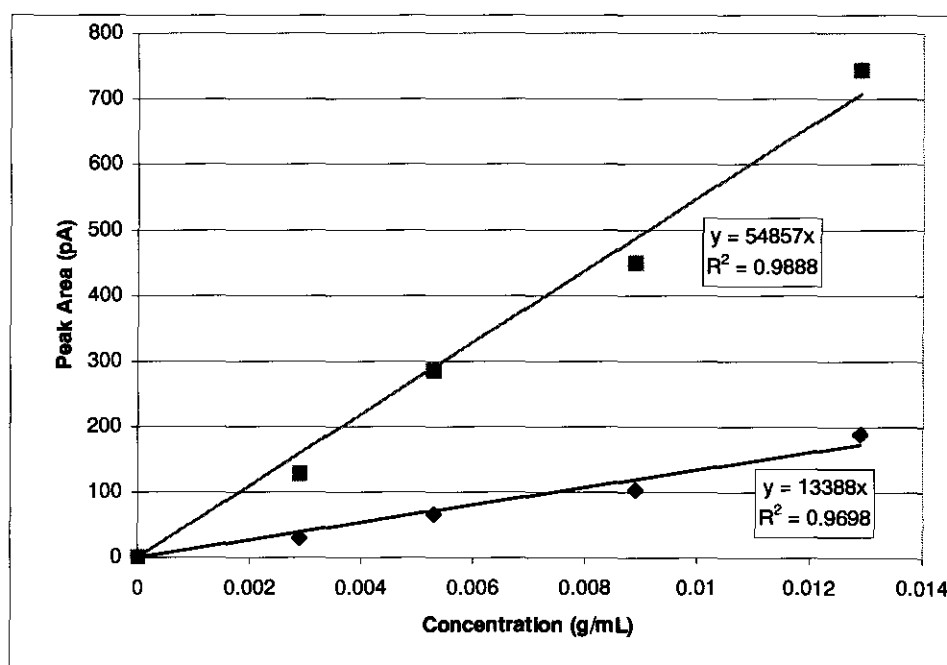


Figure 4.2 Calibration line of artemisinin

The calibration lines were used to read off the concentration of artemisinin from the measured peak areas obtained for extracts of the plant material. The two peaks at the selected gas chromatographic conditions (**Paragraph 3.3**) both exhibit a linear relationship between peak area and concentration of artemisinin at the respective retention times. For the artemisinin standard no other peaks were detected beyond the retention times mentioned.

The concentration of artemisinin in a given extract was obtained from the calibration lines by using **Equation 4.1**.

$$C = \frac{1}{m_i} \times SA_i \quad (4.1)$$

where

C = concentration of artemisinin (g/mL)

m = slope of calibration line

SA = surface area of chromatographic peak

i = peak A or B

The final value of C for each injected sample was taken as an average of the two (practically identical) estimates from the two peaks.

4.3 GC of SFE Extract

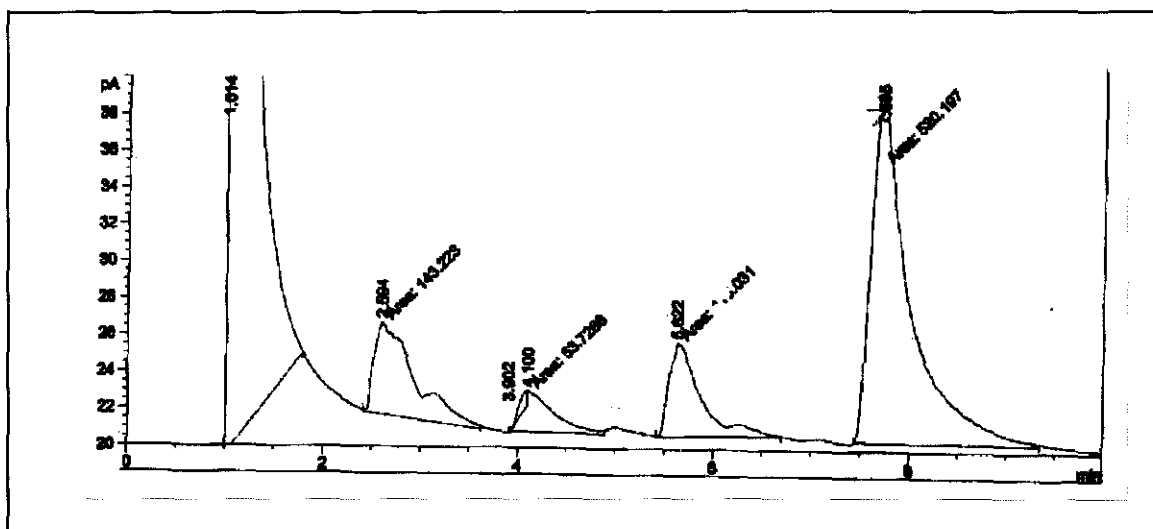


Figure 4.3 Typical gas chromatogram of SFE extract from *Artemisia annua*

Figure 4.3 illustrates that a typical extract consists of more than one component but that artemisinin is one of the major ingredients. The identification of components other than artemisinin was not considered in this investigation as the emphasis was on the extraction and quantification of artemisinin and optimisation of the yield.

4.4 Optimum Extraction Time

A few trial extractions with sc-CO₂ allowed the selection of a set of conditions with which the required time for extraction of a maximum amount of artemisinin could be estimated by virtue of a kinetic (concentration vs. time) curve. The selected conditions are listed in **Table 4.1**.

Table 4.1 Extraction conditions to determine optimum extraction time	
Temperature	100°C
Pressure	450 atm
Flow Rate	2 mL/min
Modifier (methanol)	3 %
Mass of plant material	1 g

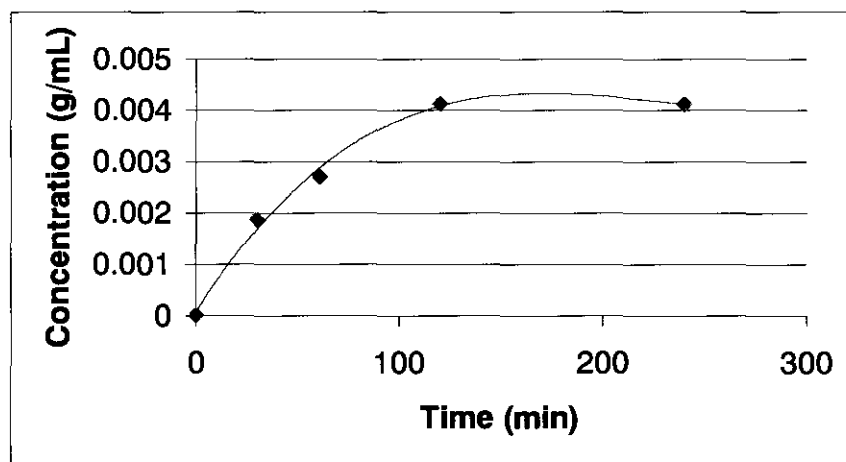


Figure 4.4: Kinetic (concentration vs. time) curve for artemisinin extraction

From the graph in **Figure 4.4** it is noted that the concentration of artemisinin reaches a maximum after extraction of about 120 min at the prevailing conditions. This duration was taken as fixed in all subsequent extraction runs performed to optimise the conditions for sc-CO₂ extraction by statistical surface response analysis as described in the next paragraph.

4.5 Optimisation of Extraction Conditions

The response surface method (RSM) described in **Chapter 3** was used to determine the optimum conditions (temperature and pressure) required for the extraction of artemisinin from

the plant material. The analysis was performed using the software package Statistica 6.0® for Windows.

Table 4.2 Statistical design (10 runs) for determination of optimal temperature and pressure

Run	Pressure (atm)	Temperature (°C)	Density (g/mL)	Yield (mg/g)
1	135	40	0.765	1.29
2	450	40	0.983	1.88
3	135	100	0.291	2.81
4	450	100	0.793	3.91
5	293	40	0.917	2.06
6	293	100	0.658	3.40
7	135	70	0.437	1.68
8	450	70	0.887	2.58
9	293	70	0.787	2.79
10	293	70	0.787	2.79

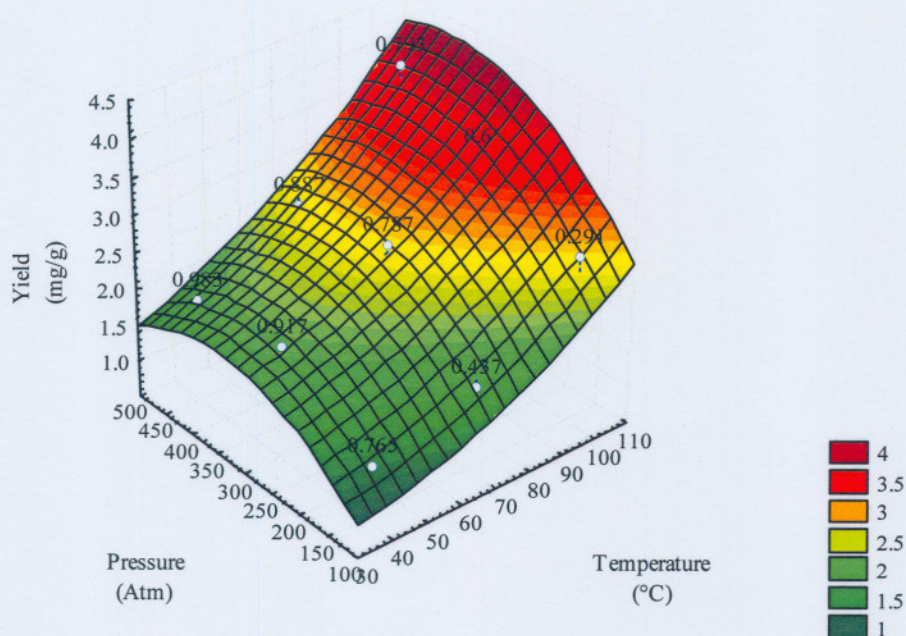


Figure 4.5: Statistical surface response graph relating yield to temperature and pressure

Figure 4.5 presents a 3-dimensional surface response graph relating yield to temperature and pressure (or density). From this relation conclusions regarding the optimum conditions and mechanism of extraction can be drawn.

An approximate amount of 3.5 mg/g (m/m) of artemisinin could be extracted at 450 atm and 100°C. This pressure is close to the maximum capability of the instrument, and the temperature is the highest before significant decomposition of artemisinin occurs.

Along the diagonal from the lowest to the highest yield (1.29 - 3.91 mg/g) the density does not change significantly (0.765 → 0.787 → 0.793 g/mL). This implies that the variation in solvent strength of sc-CO₂ on changing from gas-like (low) to liquid-like (high) densities does not play any significant role and that extraction therefore occurs as a result of physical desorption rather than of chemical dissolution.

4.6 Activation Parameters

The beneficial effect of temperature and pressure according to **Figure 4.5** is compatible with a desorption model. High temperature favours desorption by lowering the activation energy barrier posed by Van der Waals and other adhesion forces³, whereas high pressure facilitates rapid removal of detached material. The value $E_a = (10.8 \pm 2.2)$ kJ/mol obtained by plotting $\ln(\text{yield})$ versus $1/T$ for all three pressures involved (135, 293 and 450 atm) as required by the Arrhenius equation supports the conclusion that the mechanism of extraction has a physical rather than a chemical nature. For a chemical event (bond rupture or formation, dissolution with collapse of crystalline structure) values of $E_a \sim 50$ kJ/mol or more are expected. A value of $E_a \sim 10$ kJ/mol signifies a diffusion controlled process. Such a process features entrance/exit of sc-CO₂ into/from pores within the sample of plant material by film and pore diffusion as shown in **Figure 3.5**.

The average value of the volume of activation calculated at three different temperatures (40, 70 and 100 °C) was calculated as $\Delta V^\ddagger = (-34 \pm 4)$ mL/mol. The relatively large negative value is consistent with the expected significant volume collapse when artemisinin is desorbed from the plant material and taken up within the highly compressed supercritical fluid.

4.7 Model Data Fit

According to values in the literature cited in **Chapter 1** 1 g of plant material (*Artemisia annua*) contains a minimum of 0.01 % artemisinin, but that the content can be as high as 0.5 %. If one optimistically assumes that 1 g of plant material contains 0.5 % artemisinin, then 5.0×10^{-3} g of the substance should be present in a sample of 1 g. In 1.24 g of plant material (actual mass used) there should then be 6.2×10^{-3} g of extractable artemisinin. On dissolution of the extract in 3 mL of methanol for injection into the GC, an amount of $2.07 \times 10^{-3}/282 = 7.33 \times 10^{-3}$ mol/L of artemisinin should prevail, which is the C_s value. The corresponding actual amount extracted at optimal conditions ($T = 100^\circ\text{C}$, $p = 450$ atm and $t = 120$ min) in this investigation turned out to be 5.49×10^{-2} mol/L, which is the C_p value. These two values are listed as the third entry in **Table 4.3** below.

The values for C_s and C_p for different amounts of plant material subjected to sc-CO₂ extraction in **Table 4.3** allow a data fit to the equation

$$\frac{1}{C_p} = \frac{K}{C_s} - \frac{K}{C_m}$$

derived from the model of an extraction process presented in **Paragraph 3.4.2**.

Table 4.3 Extraction data for process modelling		
Mass/g	$10^3 C_s/\text{mol L}^{-1}$	$10^2 C_p/\text{mol L}^{-1}$
0.62	3.66	3.02
0.93	5.50	4.12
1.24	7.33	5.49
1.55	9.16	6.87
1.80	10.64	8.05

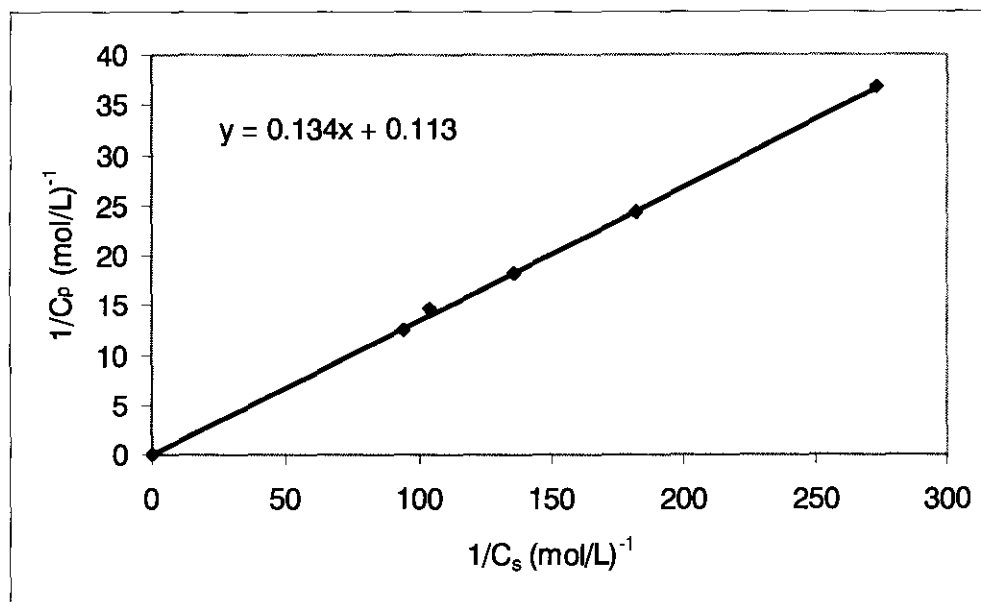


Figure 4.6 Graph of $1/C_p$ against $1/C_s$

The graph of $1/C_p$ against $1/C_s$ shown in **Figure 4.6** represents a straight line with a good correlation coefficient and confirms that desorption plays an essential role in the extraction of artemisinin from the plant material.

The adsorption equilibrium constant is determined from the slope as $K = 0.134$, which shows that desorption is favoured relative to adsorption, explaining the successful removal of artemisinin by sc- CO_2 from the plant matrix. The adsorption capacity is derived from the intercept as $C_m = 0.113 \text{ mol L}^{-1}$, which indicates that more artemisinin was available in the plant material than the amount extracted.

From the extraction data in **Table 4.3** it follows that $C_s \ll C_m$, which means that the mathematical model can be tested alternatively by plotting C_p against C_s as indicated in **Paragraph 3.4.2** and shown in **Figure 4.7**. The value of the adsorption equilibrium constant can be calculated from the gradient $1/K = 7.608$ or $K = 0.131$, which is in good agreement with the value calculated from **Figure 4.6** above.

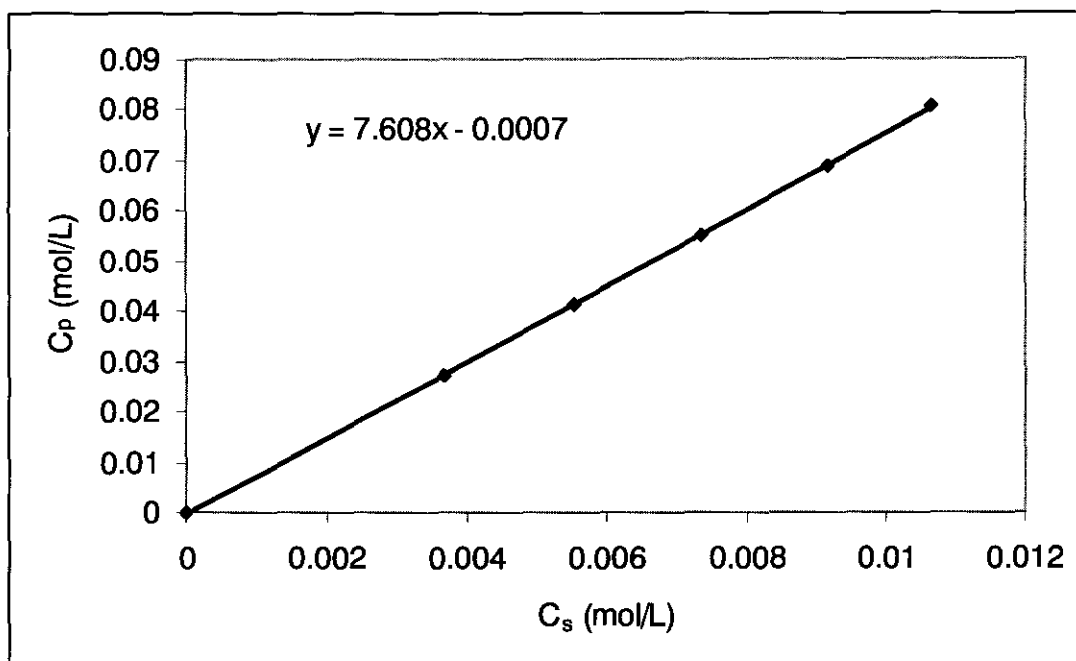


Figure 4.7 Graph of C_p as a function of C_s

In a previous paragraph it was stated that the extraction process is favoured by temperature as well as pressure. This can be explained in terms of the desorption mechanism which underlies the extraction process. High temperatures decrease the energy requirements for desorption so that artemisinin can be removed from the plant material according to the value of the adsorption equilibrium constant ($K < 1$). High pressure facilitates efficient transport of the detached material by the compressed fluid. The high temperature and pressure jointly cancel their opposite contributions towards density, so that density itself has almost no effect as shown by the statistical surface response graph in **Figure 4.5**.

4.8 Multivariable Analysis

An attempt was made to mathematically describe the extraction of artemisinin by fitting the extraction data in **Table 4.2** to the model proposed in **Paragraph 3.4.4**. The model is based on a dimensionless grouping of variables controlling the extraction process. In **Figure 4.8** $\ln H$, where H represents the yield of artemisinin extracted at different conditions, is plotted against the dimensionless grouping of variables $\frac{p^2 T t}{f^3}$ with t and f having fixed values for all data reported in **Table 4.2**.

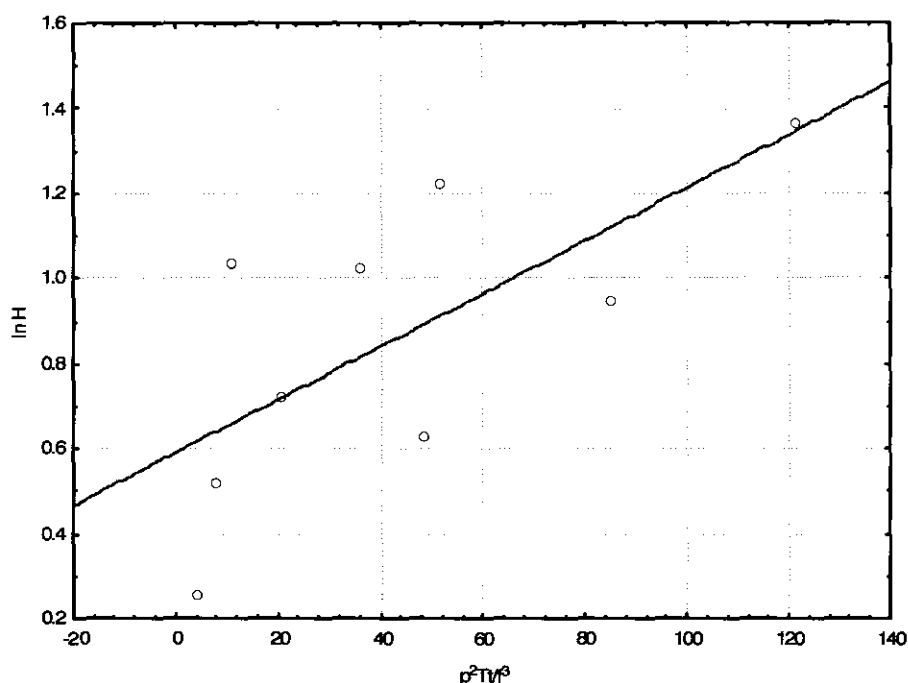


Figure 4.8 Plot of $\ln H$ versus dimensionless grouping of variables

The straight line drawn through the scattered data points indicates that the data only roughly fit the proposed model. The lack of reproducibility of the data is attributed mainly to difficulties encountered with the collection of the artemisinin extract. A further shortcoming of the model is the omission of other variables which probably play a more pronounced role in the extraction than anticipated.

4.9 Alternative Extraction Methods

Steam distillation was performed as a comparative method for artemisinin extraction using the apparatus in **Figure 3.1**. The extracted material was injected into the GC (**Figure 3.4**) for analysis, but no characteristic peaks were noted at the respective retention times. The primary reason for this is that artemisinin is insoluble in water, so that steam distillation is not a viable option for the extraction of artemisinin.

Soxhlet extraction was performed using n-hexane as extracting agent in the apparatus in **Figure 3.2**. Extraction over 5 days resulted in a yield of 0.003 mg/g calculated from **Equation 4.1**. This yield was much lower in comparison to that obtained by sc-CO₂ extraction and did not allow a chromatogram to be recorded for comparison to that of the sc-CO₂ extract.

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CHAPTER 5 EVALUATION AND FUTURE STUDIES

I do not know what I may appear to the world, but to myself I seem to have been only a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

Isaac Newton

The research project is evaluated in this chapter by considering the successes and shortcomings in comparison to the initial objectives. A few perspectives for future research in this field are presented thereafter.

5.1 Successes

A commercially available artemisinin standard was successfully used to construct a calibration line for GC analysis. The optimum extraction time for artemisinin (120 min) could be established by a yield-time-curve, while optimum process conditions (100 °C, 450 atm) could be determined by surface response analysis using a standard software package. The extraction data could be fitted to a mathematical model based on desorption as the underlying mechanism of the extraction. A mathematical description of the process using a dimensionless grouping of variables was also developed. A comparison of different extraction processes proved sc-CO₂ extraction to be the most successful in terms of overall yield from the available plant material and absence of solvent residues in the extract.

5.2 Failures

There are a few failures which call for improvement in continued study on this topic, and these are briefly referred to in the next paragraphs.

After evaluating a few analytical methods, GC was the best choice. HPLC was tried, but found unsuitable as it required post-column derivitisation which could have changed the structure of artemisinin.¹ The extract lacks chromophores as required by the Beer-Lambert law of light adsorption and, therefore, uv-vis spectroscopy was impossible.

More extraction data is needed for more reliable data fits, especially in the case of the analysis based on dimensionless grouping of variables. The reproducibility of the acquired data needs to be improved upon by repeating runs and by taking more care in retrieving the entire bulk of extracted material from the flow line and restrictor of the extractor.

No results could be obtained by steam distillation, and with soxhlet extraction the yield was so low that a workable amount was only obtained after 5 days. This, however, highlights sc-CO₂ as a preferred method to extract artemisinin from dried plant material.

5.3 Future Study

With regard to artemisinin, extraction could be expanded to other species of the plant. One could compare, for example, the amount of artemisinin found in *A. abysinthium* and *A. affra* (wilde-als) and determine which one would give the highest yield.² There are also other issues to be investigated, such as the role played by different climatic conditions, the time of harvesting and distribution of the active component within the plant.³

There are already new technology which could be utilised in future. There is a new generation of supercritical fluid extractors, such as the TFE 2000 marketed by Leco Africa, which offers increased flow-rates (L/min instead of mL/min) and flow-lines for up to three simultaneous extraction runs. Another possibility is microwave-assisted extraction (MAE), which combines microwave and traditional solvent extraction techniques.⁴ It has many advantages, such as shorter extraction times, less solvent, higher extraction rates and better products at lower cost. One could also consider extractions using superheated water.⁵ In many cases extractions with superheated water is cleaner, faster and cheaper than with conventional methods. Utilisation of these technologies, however, requires expensive equipment which was not available when this investigation was undertaken.

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ABSTRACT

The feasibility of supercritical fluid extraction (with sc-CO₂) of artemisinin from *Artemisia annua* was investigated.

The extraction was performed on a laboratory scale supercritical fluid extractor. The extract was analysed quantitatively for artemisinin by gas chromatography (GC) using a calibration line based on a commercially available artemisinin standard.

The optimum time of extraction at a typical set of conditions was determined and a statistical approach used to derive optimum conditions (most suitable temperature and pressure at a fixed flow rate and modifier concentration) for the extraction of artemisinin from ground dried plant material. A yield of 3.5 mg/g (0.35 % m/m) was obtained under optimum conditions, which fits into the range 0.01-0.5 % (m/m) obtained by other methods. A surface response analysis showed that the density of the fluid and thus the solubility of artemisinin in the fluid is not the limiting factor and that extraction is favoured by a combination of high temperatures and pressures.

The experimental data for the extraction of artemisinin from *Artemisia annua* was successfully fitted mathematically to a Langmuir model based on the assumption that desorption of artemisinin from the plant matrix is the limiting step in the extraction process. The compatibility of the observed process characteristics and calculated activation parameters with a desorption mechanism could be proven.

An attempt was made to mathematically describe the extraction by virtue of a dimensionless grouping of process variables. The acquired extraction data was not sufficient to convincingly prove the validity of the model, but process modelling of this nature is a step forward in the description of botanical extractions.

The results obtained in this investigation proved sc-CO₂ to be superior for artemisinin extraction than classical methods of extraction (soxhlet and steam distillation) in terms of total yield and solvent-free extracts.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my parents for their support and encouragement throughout this time, and my brother Yunesh for all the jokes that eased the stressed. My uncle and aunt Champa and Agsie always helped and were willing to listen.

Without Prof. Breet this study was not possible as he was always patient and caring. Not only was he a good supervisor, but also a wonderful person to work with and there was so much that I could learn from him throughout the time that I was his student.

A big thanks to Prof. Vosloo for all the advice and time spent on the analysis. Also Prof Breytenbach deserves thanks for his help.

NRF and Eskom are thanked for financial support. Without them this study was not possible.

Thanks to all my many friends in the School of Chemistry and Biochemistry and to all others that I am not able to mention.