# Extraction of Lutein from Marigold Flower with Supercritical Carbon Dioxide

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*"He is no fool who loses what he cannot keep, to gain what he cannot lose!"*

# Contents

# Summary

The objective of the study was to extract lutein, an active component of marigold flower (*Tagetes erecta*) by means of supercritical carbon dioxide (*sc*-$CO_2$).

Extractions on freeze-dried marigold petals were performed with a laboratory scale supercritical fluid extractor (LECO TFE/M2000) of highly advanced design and performance. Extracts were analysed for lutein by high-performance liquid chromatography (Agilent Technology 1100 Series HPLC System) using a calibration line constructed by virtue of solutions of a commercially available standard (Indofine Chemical Company, Inc.).

The conditions at which a maximum yield of lutein is obtained (52°C, 400 bar, 60 min) were determined by performing runs according to a statistical design and processing the data by computer assisted surface response analysis. A mass balance proved effective recovery of the extracted material from the supercritical extractor.

The density of *sc*-$CO_2$ was found to be the variable which controls the extraction as the fluid becomes capable to chemically dissolve lutein once its density (and thus solvent strength) takes on liquid-like values (between 0.8 and 1.0 g/mL). The large negative value of the volume of activation $\Delta^{\neq}V$ relates to the decrease in volume associated with melting and solvation of lutein while it dissolves in the highly compressed supercritical fluid.

A function combining several process variables into a dimensionless quantity was used successfully to mathematically describe the extraction process.

# Opsomming

Die doelwit met die studie was om luteïen, 'n aktiewe komponent van die afrikanerblom (*Tagetes erecta*), met behulp van superkritieke koolstofdioksied (*sc*-$CO_2$) te ekstraheer.

Ekstraksies is op gevriesdroogde afrikanerblomblare met 'n laboratoriumskaal superkritieke-fluïed-ekstraktor (LECO TFE/M2000) van gevorderde ontwerp en werkverrigting uitgevoer. Ekstrakte is vir luteïen geanaliseer deur gebruik te maak van hoëverrigtingvloeistofchromatografie (Agilent Technology 1100 Series HPLC System) en 'n kalibrasielyn wat gekonstrueer is met behulp van oplossings van 'n kommersieel beskikbare luteïenstandaard (Indofine Chemical Company, Inc.).

Die kondisies waarby 'n maksimum opbrengs luteïen verkry word (52°C, 400 bar, 60 min) is bepaal deur ekstraksielopies volgens 'n statistiese ontwerp uit te voer en die data met rekenaargesteunde oppervlakresponsanalise te verwerk. 'n Massabalans het die doeltreffende herwinning van die geëkstraheerde materiaal vanuit die superkritieke ekstraktor bevestig.

Die digtheid van *sc*-$CO_2$ is bepaal as die veranderlike wat die ekstraksie beheer aangesien die fluïed in staat is om luteïen chemies op te los sodra die digtheid (en dus die oplosmiddelsterkte) van die fluïed vloeistoftipe waardes (tussen 0.8 en 1.0 g/mL) aanneem. Die groot negatiewe waarde van die aktiveringsvolume $\Delta^{\neq}V$ hou verband met die volumeafname tydens die smelting en solvering van luteïen wanneer dit in die hoogs saamgeperste superkritieke fluïed oplos.

'n Funksie wat verskillende prosesveranderlikes tot 'n dimensielose grootheid saamgroepeer, is suksesvol gebruik om die ekstraksieproses wiskundig te beskryf.

# Chapter 0

# Actuality

*As for marigolds, poppies, hollyhocks, and valorous sunflowers, we shall never have a garden without them, both for their own sake, and for the sake of old-fashioned folks, who used to love them.*

*Henry Ward Beecher*

## *0.1 Introduction*

Botanical extraction is one of the principal research topics of the supercritical technology group within Separation Science and Technology (SST) at the North-West University (Potchefstroom Campus).

The aim of this study, which represents a further contribution in a series of botanical extractions[1-8], was to extract lutein, an active component of the petals of *Tagetes erecta* (marigold flower), by means of supercritical carbon dioxide (*sc*-$CO_2$).

Lutein is a dihydroxy carotenoid or xanthophyll that has recently received the attention of many researchers for its anticancer activity and effect on degenerative diseases of the eye.[9, 10] In the past few years, it has been the subject of many studies associated with risk reduction of failing eyesight due to diseases such as age-related macular degeneration (AMD), the leading cause of irreversible blindness.[11]

Although many fruits and vegetables contain lutein, marigold (*Tagetes erecta* L.) is the best commercial source of pure lutein. It is part of the *Compositeae* family, and its flowers are the richest common source of xanthophylls, ranging from 0.6 to 2.5% on a dry basis. Of the total xanthophylls in marigold flower 88–92% are lutein and zeaxanthin, with lutein being the predominant one.[11]

The pharmaceutically relevant properties of lutein emphasise the actuality of investigating alternative methods to extract this substance and more than justifies a contribution by virtue of this project.

The main objectives of the project were to

1. identify process parameters (temperature, pressure, time, fluid flow rate and more) influencing the amount of extract and the lutein content of an extract;

2. develop an analytical protocol for lutein determination using a commercially available standard and a suitable instrumental analytical technique (HPLC);

3. optimise process conditions by performing extraction runs at selected conditions (time, temperature, pressure, density) according to a statistical design suitable for surface response analysis;

4. compare the physical-chemical properties (colour, composition and stability) of the extract obtained by *sc*-$CO_2$ with those acquired by solvent extraction (hexane);

5. propose a mechanism according to which lutein is extracted by *sc*-$CO_2$ and to utilise the mechanism as a basis for process modelling.

## *0.2 Workplan*

The suggested workplan comprised

1. a comprehensive literature study on aspects of *sc*-$CO_2$ technology relevant to the acquisition of botanical extracts in general and the isolation of lutein from marigold flower with this technology in particular;

2. selection of suitable plant material of consistent quality and reliable origin for extraction by *sc*-$CO_2$;

3. construction of a calibration line for quantitative HPLC analysis of lutein using a commercially available standard;

4. evaluation of the quality and quantity of *sc*-$CO_2$ extracted lutein to make a critical assessment of the advantages and disadvantages of different methods used for the extraction of this substance;

5. optimisation of extraction conditions by performing runs based upon a statistical design which meets all mathematical requirements and relates yield to different process parameters by virtue of a surface response graph;

6. deduction of the most significant process characteristics in order to elucidate the mechanism of lutein extraction;

7. development of a mathematical equation based on a dimensionless grouping of variables by virtue of which extraction data can be fitted as a way of process modelling.

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# Chapter 1

# Tagetes erecta

This chapter covers different aspects of *Tagetes erecta* L. commonly known as marigold. The history, origin and medicinal uses of the plant will be discussed. Lutein, an active compound derived from the marigold flower, and its relevance to the food and pharmaceutical industry, will be emphasised. Even though *Tagetes erecta* is indigenous to Mexico and Latin America, the plant has also been successfully cultivated in South Africa. Marigold flower petals contain up to 2000 ppm of carotenoids and are the richest source of xanthophylls with lutein being the predominant one.

The use of medicinal plants in daily life is an important part of the South African cultural heritage. Plants were once a primary source of all medicines and they continue to provide mankind with new remedies. Natural products and their derivatives represent more than 50% of all drugs in clinical use in the world today.[1]

*Tagetes* is a medicinal plant originating from Latin America. The genus comprises several species of which especially *T. erecta* L. is of predominant commercial relevance.[2]

## *1.1 History and origin*

*Tagetes* (family Compositae) is a diverse genus comprised of strongly scented species. Its natural range extends from south western United States to Argentina, and the area of largest diversity is documented as south-central Mexico. Several species have become well established horticulturally. Records

of their cultivation and extensive use by Indian tribes in Mexico and South America extend back to before the time of the Conquistadores. These plants, from both cultivated and uncultivated sources, have had an unbelievable array of uses ranging from the religious to the ordinary.[3]

## 1.2 *Botanical classification*

**Family:** Compositae / Asteraceae
**Genus:** *Tagetes* L.
**Species:** *Tagetes erecta* L.
**Common name:** African / Aztec / American marigold

**Figure 1.1** Marigold, ***Tagetes erecta* L**

## *1.3 Compositae family characterisation*

The Compositae is also known as the Asteraceae or sunflower family. The plants are herbs or shrubs, with leaves often in basal rosettes. The flower head is subtended by involucres of bracts (phyllaris) containing small, epigynous flowers (florets). These consist of tubular or strap-shaped sympetalous corolla, a reduced calyx (pappus), 5 syngenecious, appendaged anthers, a bicarpellate ovary with a single basal ovule, bifid styles with pollen-collecting hairs and restricted stigmatic surfaces. Achene, with persistent pappus and nonendospermous seeds, is characteristic of this family. Most of the plants have inulins (unusual storage polysaccharides) in the roots and tubers. An anatomical feature is the unitegmic and tenuinucellate ovules.[4]

The genera/species ratio of the Compositae family is 1 160/19 085, making it one of the largest angiosperm families known.[4] The family contains a number of economically important species, such as *Tagetes*, from which valuable plant products have been obtained.[5] Various *Tagetes* species are listed in **Table 1.1**.

**Table 1.1** Various species of *Tagetes*[3]

| **Specie** | **Common name** |
|---|---|
| Tagetes erecta | African marigold |
| Tagetes patula | French marigold |
| Tagetes tenuifolia | Signet marigold |
| Tagetes lemonii | Tangerine scented marigold |
| Tagetes lucida | Spanish tarragon |
| Tagetes filifolia | Irish lace marigold |

## 1.4 Botanical description

Native to the western hemisphere, the *Tageteae* tribe is a small group of 15 genera. The presence of conspicuous brown-black glands (mainly on phyllaries and leaves) containing abundant amounts of strong-smelling volatile constituents is one of the primary characteristics of the majority of *Tageteae* members.[5]

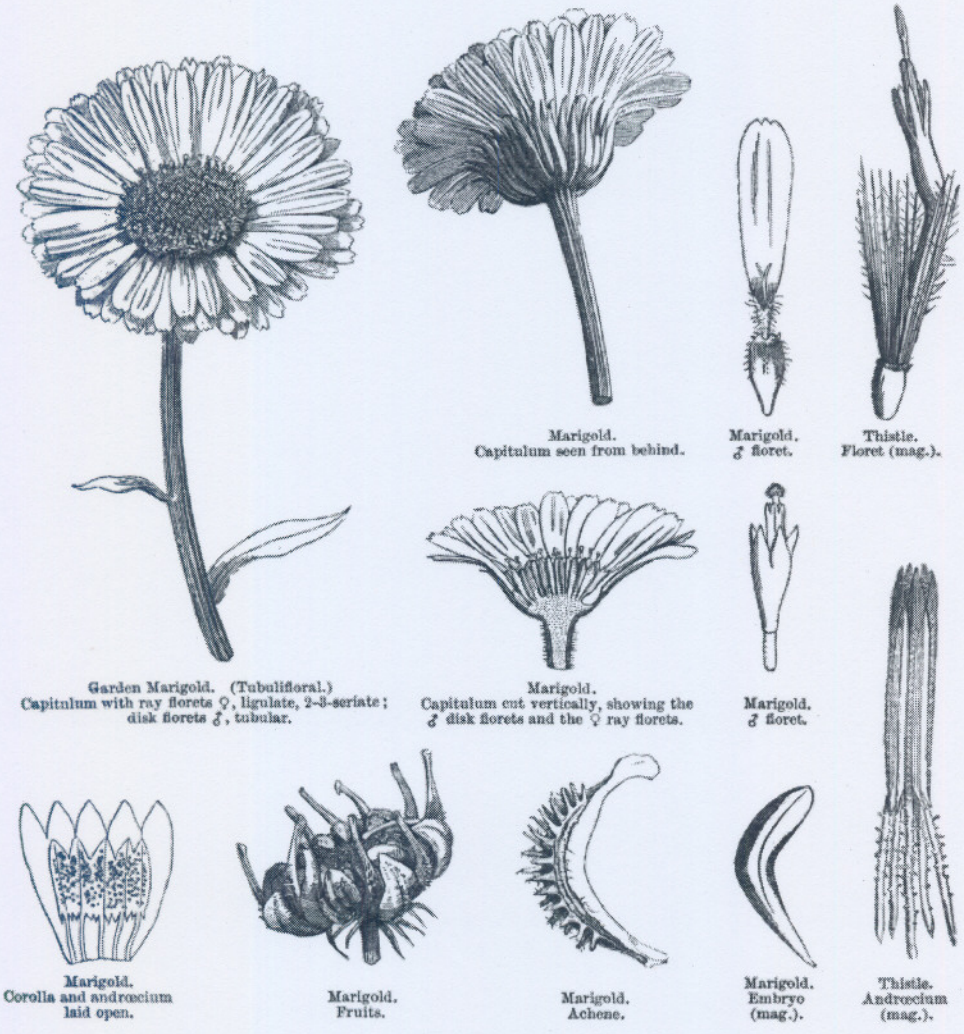



**Figure 1.2** Botanical description

## 1.5 Geographical distribution

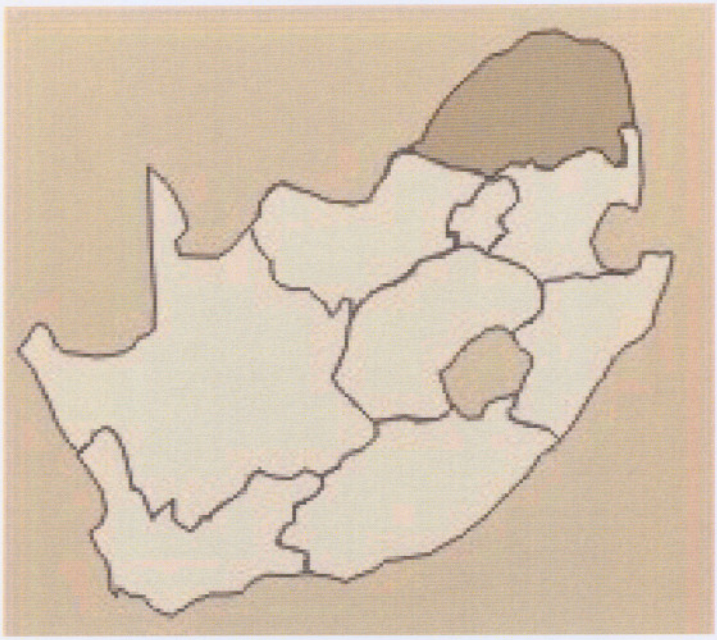


**Figure 1.3** Occurrence of *Tagetes erecta* L used in this study

Marigold (*Tagetes erecta*) is a plant native to Mexico and has been used in traditional Mexican medicine.[6-8] In Central America several varieties of marigold are cultivated for the purpose of carotenoid extraction.[9]

Even though *Tagetes erecta* is indigenous to Mexico and Latin America, the plant has also been successfully cultivated in South Africa. About 50 species, native in America from New Mexico and Arizona to Argentine are commonly cultivated in South Africa.[10]

*Tagetes erecta* plant material used in this study was cultivated locally in the Giyani region, Limpopo Province, South Africa.

## 1.6 Cultivation

Marigold is cultivated commercially and extracts of the flower are used as poultry feed supplements for the colouring of egg yolks.[7]

Due to the short period of time needed for marigold cultivation, it is conveniently grown as part of a multi-crop system, rotated with other agricultural or horticultural crops. It is also grown as a mixed crop on the borders with other plants (e.g. tomatoes) with beneficial effects to the latter.[11]

The plants are usually grown in double rows on 75 or 100 cm beds. An early start and sufficient phosphorus promotes flowering. Additional nitrogen should be applied two or three times during the growing season. The final stands in the row should be 15 to 25 cm. Under optimal germination rates, the seeding level should be 0.37 kg/ha.

The marigold flowers are harvested by hand when the plants have, on average, two or three fully developed flowers (about 90 days after planting). Depending on plant vigour, subsequent harvests (up to two) can be made at intervals of 3 to 5 weeks. Mechanical harvesters are also used; however, they generally limit the number of harvests to one as a result of plant damage.[12]

## 1.7 Applications

The xanthophyll lutein is primarily responsible for the intensive yellow-orange colour of the crown petals.[2] The petals are commercially valuable as a natural source of lutein pigments. The poultry industry uses these primarily as feed additives to colour egg yolks (orange) and poultry skin (yellow) and to augment the xanthophyll present in corn and alfalfa feed to standardise the feed's

xanthophyll content.[6-7, 9, 11, 13-17] Lutein is a food colourant allowed by EU and referred to as E 161 b.[15, 18]

Apart from colouring attributes, marigold extracts presently gain a great deal of attention in so-called "functional foods". Lutein has been reported to possess pharmacological activity (radical scavenging properties) and is associated with risk reduction for failing eyesight due to diseases such as age-related macular degeneration (AMD), the leading cause of irreversible blindness amongst senior populations.[2, 15, 19, 20] Lutein is also a potent antioxidant and is found to enhance immune function as well.[19] The pharmaceutical properties of the substance are fully discussed in **Paragraph 1.10**.

Bioactive extracts of *Tagetes* partly exhibit nematocidal, fungicidal and insectical activity.[11] It has been reported that nematode populations are greatly reduced when *Tagetes erecta* is intercropped with other plants.[12-13, 21]

## *1.8 Medicinal uses*

The reported medicinal uses are divided into the following categories: analgesics, antiseptics, carminatives, diuretics, expellants, stimulants and vermifuges. High concentrations of some of the components contained in *Tagetes* plants do affect human physiological homeostasis, and the flowers of *T. erecta* are officially listed in the Mexican Pharmacopoeia.[3] **Table 1.2** lists reported uses of *Tagetes erecta* along with the portion of the plant utilised.

**Table 1.2** Reported uses of *Tagetes erecta* L[3]

| Specie | Use | Portion of plant utilised |
|---|---|---|
| *T. erecta* L. | Aphrodisiac | Juice and ground leaves |
| | Appetiser | Juice and leaves |
| | Blood purifier | Juice of flowers |
| | Boils and carbuncles | Leaf |
| | Bronchitis | Entire plant |
| | Carminative (relieve colic and intestinal gas) | Flowers and leaves |
| | Colds and respiratory diseases | Entire plant |
| | Diaphoretic (to increase perspiration) | Juice and leaves |
| | Diuretic | Flowers and leaves |
| | Dropsy (edema) | Juice and leaves |
| | Dye for clothes, butter and cheese | Flowers |
| | Earache | Juice |
| | Emetic, to induce vomiting | Leaves |
| | Eye infections | Juice |
| | Fever reducer | Juice and leaves |
| | Horticultural ornamentals | Entire plant |
| | Laxative | Roots |
| | Liver remedy | Juice and leaves |
| | Malarial treatment | Juice and leaves |
| | Menstrual flow regulator and inducer | Juice and leaves |
| | Muscle relaxer | Entire plant |
| | Ornamentals | Flowers and entire plant |
| | Piles | Juice of flowers |
| | Religious rituals and celebrations | Flowers and entire plants |
| | Rheumatic pain | Entire plant |
| | Stimulant | Entire plant |
| | Vermifuge | Leaves |

## 1.9 Components

The species *Tagetes erecta* L. is recognised as a source of natural carotenoids and other biologically active components such as essential oils and thiophenes.[15]

### 1.9.1 Thiophenes

Most species accumulate a range of thiophenes in their roots.[22] Oil extracted from various parts of *Tagetes minuta* L. (Mexican marigold) (Asteraceae) are used in the tropics as a dressing for livestock to control blowfly. The (terthienyl (2,2':5',2''-terthiophene) present in the oil has been identified as the active phototoxic compound against mosquitoes. Its high level of activity facilitates its commercialisation as a mosquito larvicide.[23]

### 1.9.2 Fatty acids

The following fatty acids are found in *Tagetes erecta*:

- lauric acid
- myristic acid
- palmitic acid
- oleic acid[5]

## 1.9.3 Volatile constituents

Monoterpenes isolated from *Tagetes erecta* are:

*Acylic*

- tagetone
- linalool
- ocimene

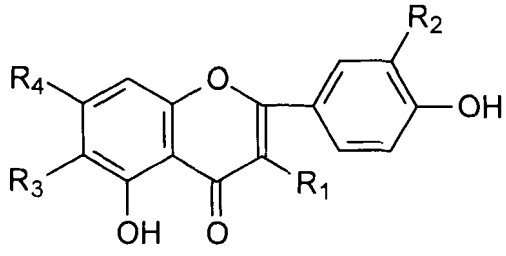
*Monocylic*

- limonene[5]

## 1.9.4 Flavonoids

The flavonoids isolated from *Tagetes erecta* include:

- quercetagetin
- quercetagetin 7-*O*-glucoside (quercetagitrin)
- quercetagetin 3-*O*-glucoside (tagetiin)
- quercetagetin 3-*O*-dirhamnoside




| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| quercetagetin | OH | OH | OH | OH |
| quercetagitrin | OH | OH | OH | OGlc |
| tagetiin | OGlc | OH | OH | OH |
| quercetagetin 3-*O*-dirhamnoside | ORha ORha | OH | OH | OH |

**Figure 1.4** Flavonoids from *Tagetes erecta*[5]

## *1.9.5 Carotenoids*

Of the various classes of natural pigments in nature the carotenoids are among the most widespread and important ones, especially due to their wide distribution, structural diversity and numerous functions.[24]

The carotenoids are essential for photosynthesis. In addition to the provitamin A activity of some carotenoids, these pigments have recently been implemented for prevention of or protection against serious health disorders[2, 3] such as cardiovascular disease, cancer, age-related macular degeneration and cataracts. Carotenoids have also been used for many years in the treatment of individuals suffering from photosensitivity disease, such as erithropoietic protoporphyria.[25]

Petals of *Tagetes erecta* are considered to be one of the richest natural sources of carotenoids, containing up to 2000 ppm.[16, 20] Xanthophylls (dihydroxy carotenoids) are the major type of carotenoid in these flowers. In 1972 a total of 17 carotenoids from dried flowers of *Tagetes erecta* were isolated. Lutein (**Figure 1.5**) and zeaxanthin constituted 88-92% of the total carotenoids, with lutein being the predominant xanthophyll.[5, 19]

**Table 1.3** lists the relative percentage distribution of carotenoids in *Tagetes erecta* in both red and yellow petal types, respectively.

**Table 1.3** Relative percentage distribution in *T. erecta* L. petal types[5]

| Carotenoids | Petal types | |
|---|---|---|
| | Red | Yellow |
| Phytoene | 1.9 | 2.4 |
| Phytofluene | 2.3 | 2.6 |
| α-Carotene | 0.2 | 0.1 |
| β-Carotene | 0.3 | 0.5 |
| β-Zeacarotene | 0.4 | 0.5 |
| δ-Carotene | 0.1 | - |
| α-Cryptoxanthin | 0.7 | 0.8 |
| Zeinoxanthin | - | - |
| Isocryptoxanthin | - | - |
| β-Cryptoxanthin | 0.4 | 0.5 |
| **Lutein** | **87.3** | **72.3** |
| Antheraxanthin | 0.2 | 0.1 |
| **Zeaxanthin** | **4.0** | **16.4** |
| Neoxanthin | 0.2 | 0.8 |
| Chrysanthemaxanthin | 0.3 | 0.8 |
| Flavoxanthin | 0.4 | 1.3 |
| Auroxanthin | 0.1 | 0.1 |

## *1.10 Lutein*

Lutein is the principal colouring component of marigold in the *all-trans*-lutein [(3R,3'R,6'R)-β,ε-carotene-3,3'-diol] form, an asymmetric dihydroxy carotenoid (xanthophyll).[9, 26]

Lutein dipalmitate, the main component of petal xanthophylls, has been reported to possess pharmacological activity and is used as an ophthalmological agent. The larger solubility of lutein esters in vegetable oils compared to that of

synthetic carotenoids is a favourable factor for the use of these compounds as food colourants.[15]

### *1.10.1 Structure*

Structurally, the carotenoids are polyisoprenoid compounds, synthesised by tail-to-tail linkage of two $C_{20}$ geranylgeranyl molecules. All the carotenoids are produced by variations of the parent $C_{40}$ skeleton. One distinguishes between the hydrocarotenoids (carotenes) consisting of C and H only, and the oxidised carotenoids (xanthophylls or oxycarotenoids) having some O-substituent groups such as hydroxy, keto and epoxy groups.[25]

**Figure 1.5** and **Figure 1.6** show the chemical structures of lutein [(3R,3'R,6'R)-β,ε-carotene-3,3'-diol] and its stereo isomer, zeaxanthin, respectively. The spectrophotometric features of the carotenoids are produced by the conjugated double bond system. At the opposite ends of the molecule the carotenoids either have linear or cyclic groups.[25] The conjugated double bond system is the single most important factor in energy transfer reactions, such as those found in photosynthesis. It is this feature of the molecule that also permits the quenching of $^1O_2$ (singlet oxygen).[27]

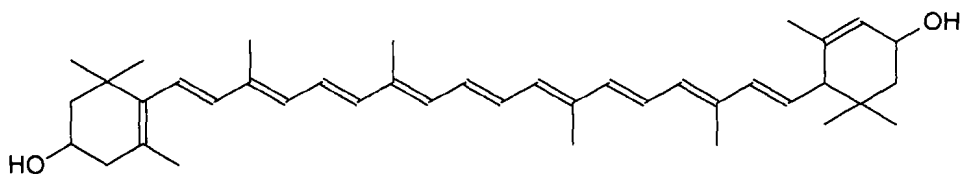



**Figure 1.5** Lutein ($C_{40}H_{56}O_2$; M = 568.88 g/mol)

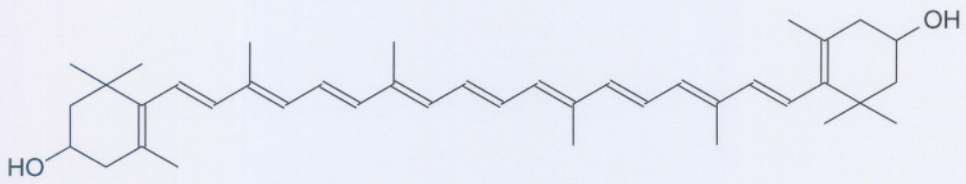



**Figure 1.6** Zeaxanthin ($C_{40}H_{56}O_2$; M = 568.88 g/mol)

Xanthophylls are distinguished from other carotenoids in nature by the presence of characteristic hydroxyl groups. Lutein and zeaxanthin possess two hydroxyl groups, one on each side of the molecule, as shown in **Figure 1.5 and Figure 1.6**. These are believed to play a critical role in their biological function.[28]

## *1.10.2 Occurrence*

Lutein cannot be synthesised by humans and must be obtained through diet and dietary supplements.[29-31] Foods that are rich in lutein include egg yolk, maize, orange juice, honeydew melon, orange pepper, and dark green leafy vegetables such as kale, spinach, collards, turnip greens, and broccoli.[28-29] One of the main sources of lutein is the intense orange coloured flower petals of marigold (*Tagetes erecta*), (**Figure 1.7**).[9, 20]

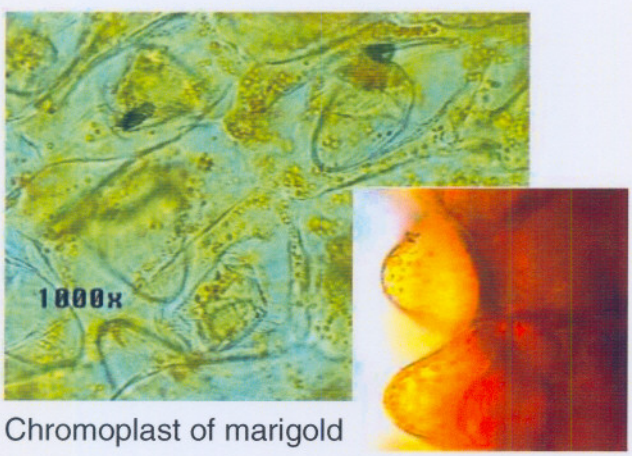



**Figure 1.7** Chromoplast of marigold

Lutein in plants functions as an antioxidant and protects these from photo-induced free-radical damage by screening out blue light (absorbance maximum occurs at λ = 445 nm according to **Figure 1.8**), while allowing through other wavelengths of light critical for photosynthesis. Although not overtly evident in green leafy vegetables due to masking by chlorophyll, purified crystalline lutein actually has a yellow-orange colour (**Figure 1.9**).[28]

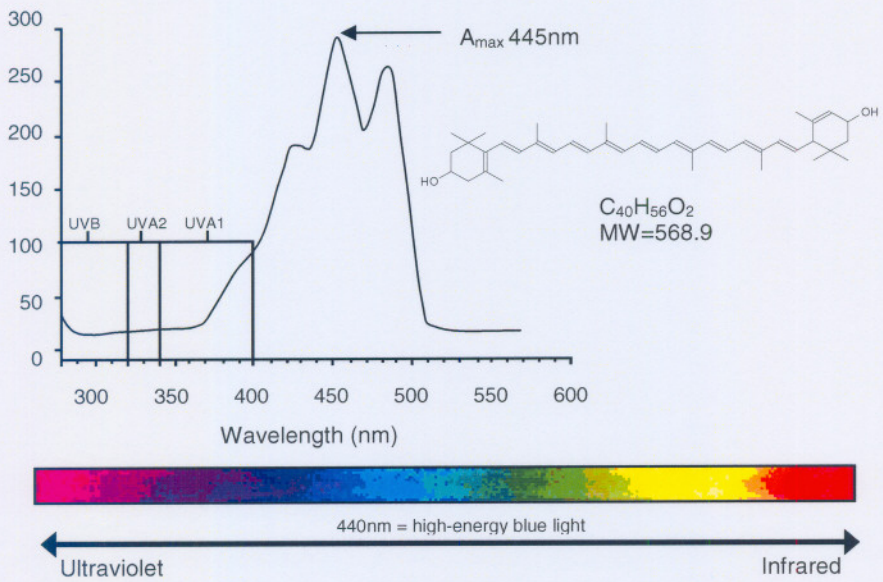



**Figure 1.8** Absorption spectrum of lutein[28]

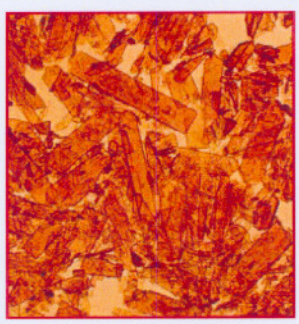


**Figure 1.9** Purified lutein crystals isolated from marigold flower extract[28]

## *1.11 Eye health relevance*

Lutein and zeaxanthin play a similar role in humans as in plants, namely to function as potent antioxidants and as effective screeners of high energy blue light causing free-radical damage to the eye.[32] Their presence in specific eye tissue makes these compounds unique relative to other carotenoids in humans.[28] The two ocular tissues that play a critical role in vision are the macula and the lens. Of all the carotenoids present in the human diet and in serum, only two, lutein and zeaxanthin, are present in these vital two tissues (**Figure 1.10**).[33, 34]

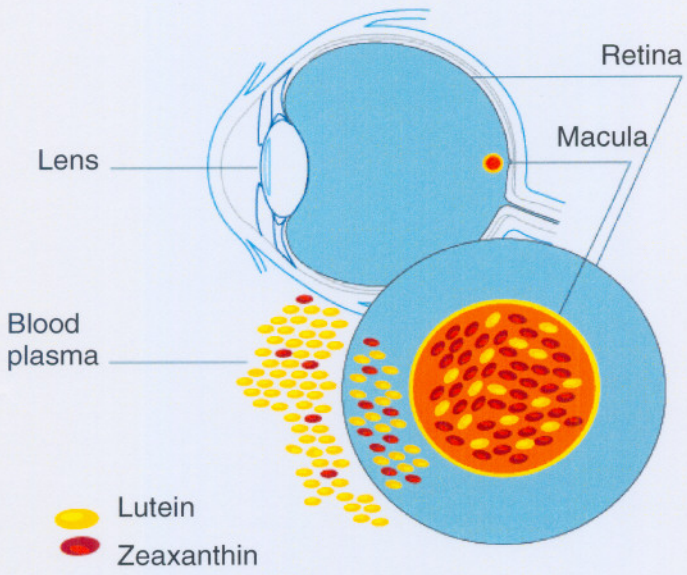



**Figure 1.10** Lutein and Zeaxanthin concentrated in die macula

The *macula lutea* (or "yellow spot", for its characteristic yellow colour) is located in the central and posterior portion of the retina and possesses the highest concentration of photoreceptors responsible for central vision and high resolution visual acuity. It is a circular area 5-6 mm in diameter and possesses a

characteristic yellow pigment, the macular pigment, which is made up entirely of lutein and zeaxanthin.[28, 33]

Maintaining the health of the macula is critical for sustaining normal visual function. Light-induced retinal damage depends largely on the wavelength, exposure time, and power level, with blue light (440 nm) requiring 100 times less energy to cause damage than orange light (590 nm). As in the case of plants, carotenoids protect the macula by absorption of blue light.[28]

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among the elderly.[35-38] AMD is a degenerative disease that affects the outer neural retina, retinal pigment epithelium (RPE), Bruch's membrane, and the choroid.[35] Available evidence supports the involvement of oxidative stress in the development of apoptosis in RPE cells. Exposure of RPE to $H_2O_2$ leads to damage and death of these cells.[37] There are several factors that may increase the risk of developing AMD, *viz.* age, poor diet, excess sunlight, smoking, heredity, gender, eye colour, alcohol usage and heart disease.

"Dry" AMD occurs in 90% of the reported cases and is characterised by small yellow dots called drusen, accumulating behind the macula. It is typically not associated with blindness but with the loss of visual acuity. However, if left untreated, it could progress into "wet" AMD. Its name is derived from the tiny, abnormal vessels that grow behind the retina towards the macula that trickle fluid into the tissue. As a result, the macula is damaged, leading to irreversible blindness. The effect of AMD is shown in **Figure 1.11**.

Normal vision Vision with AMD

**Figure 1.11** The effect of AMD

Lutein plays a protective role against AMD. Protection is provided by the pigment in two ways: through its ability to quench free radicals and singlet oxygen and by absorbing blue light before it reaches the sensitisers which initiate photochemical damage.[39-40]

## *1.12 Human health relevance*

Lutein is used as human nutritional supplement based on its biological capability in cancer prevention, inhibition of auto-oxidation of cellular lipids, protection against oxidant-induced cell damage, and prevention of age-related macular degeneration.[20]

A current trend towards natural food colours is strengthened since a possible correlation between consumption of certain carotenoids (e.g. lutein and age-related macular degeneration) was reported.[18, 33]

Both certified and uncertified colours of the xanthophylls are strictly controlled in the United States by the Food and Drug Administration (FDA) and the Food, Drug and Cosmetic Act (FD and C). In Mexico they are regulated by the General Secretary of Health.[8]

Products currently on the market containing lutein as supplements are shown in **Figure 1.12**).

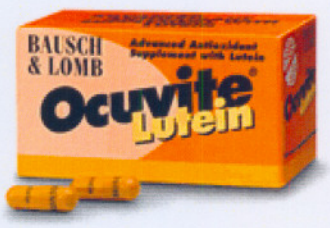




**Figure 1.2** Marigold -Lutein® Eyes for life Bausch & Lomb® Ocuvite Lutein

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# Chapter 2

# Supercritical Technology

There has been an increasing demand for faster, safer and more environmentally friendly extraction methods in analytical chemistry. The use of large amounts of hazardous organic solvents over an extended period can create health problems. Supercritical fluid extraction (SFE) is a viable alternative for the extraction of substances from a variety of matrices. This chapter aims to show several applications of SFE and to indicate some of the avenues which still need to be explored.

## *2.1 Introduction*

In the past few years new extraction techniques have been established in order to reduce the volume of solvents required for extraction, to improve the precision of analyte recovery, and to reduce extraction times and sample preparing costs. Such techniques include microwave extraction, accelerated solvent extraction and supercritical fluid extraction.[1]

Since pharmacologically active compounds in herbal plants usually occur in low concentration, a great deal of research has been done to develop more effective and selective extraction methods for recovery of these compounds from the raw material. For conventional extraction methods, such as hydrodistillation (steam distillation) and solvent extraction, there are few adjustable parameters to control the selectivity of the extraction processes. Therefore, developing alternative extraction techniques with better selectivity and efficiency are highly desirable.

As a result, supercritical fluid extraction (SFE) as an environmentally responsible and efficient extraction technique for solid materials was introduced and extensively studied for separation of active compounds from herbs and other plants.[2] The extraction of natural compounds using a supercritical fluid as extraction agent is a non-destructive method of isolating valuable constituents from natural materials.[3]

Several investigations have been made in recent years on potential industrial applications of SFE which offer advantages over conventional methods, such as separation by extractive solvents or by distillation, especially in the food, pharmaceutical, chemical and oil industries.[4]

## *2.2 Developmental history*

It took approximately a hundred years from the discovery of the unusual solvent strength of supercritical fluids, especially for substances of low volatility, to their industrial use as extraction agents. In 1822 the observation of liquid phase disappearance when different liquids were heated in a closed vessel was first described. Upon cooling, the liquids were restored. This can be regarded as the first report on the phenomenon of the supercritical state.

The definition of the critical point was introduced in 1869. Even at that time it was possible to determine very precisely the critical temperature of 31°C and the critical pressure of 73 atm for carbon dioxide. In the following decades, several studies were published, especially about the solubility of inorganic and organic substances in condensed and supercritical gases.

The first industrial use of a compressed gas as a solvent for separation is considered to be the deasphalting of heavy mineral oil fractions by means of dense propane in the petrochemical industry in the late thirties. Since the fifties,

studies and development efforts have been focused on new ways of separating substances by utilising the extraordinary properties of supercritical fluids.

In 1978, the first decaffeinating plant was commissioned by Hag AG in Bremen with a capacity of 10 000 t/a, which has been increased considerably in the meantime. Following this, in 1982, again in Germany, a plant for hops extraction was constructed. In 1985 and 1988, facilities for hops (Pfizer) and coffee (General Foods) were commissioned in the U.S. The latter facility is said to have an annual throughput of 25 000 t and uses extractors more than 20 m high and fed in quasi-continuous mode.[5]

The use of supercritical fluids to enhance processes in the chemical industry dates back several decades. It stems from the relative high diffusivity, and high density of supercritical fluids, which allow for rapid mixing and high solubility, respectively. Furthermore, the ability to selectively dissolve substances by varying parameters like pressure and temperature, also favours the use of supercritical fluids in chemical processing. In addition, the environmentally benign nature of some supercritical solvents, e.g. carbon dioxide, encourages their use in industry.[6]

## 2.3 *Supercritical fluids*

A supercritical fluid (SCF) is a substance prevailing at temperatures and pressures beyond its critical point (CP).[7, 8] The critical point of a fluid is defined by its critical pressure ($p_c$) and critical temperature ($T_c$) as shown in **Figure 2.1**.[9] In this region, a substance exhibits unusual properties such as high compressibility, liquid-like density, high diffusivity, low viscosity, and low surface tension. Due to these properties, supercritical fluids show larger ability to diffuse into ultrafine structures than conventional organic solvents, thus improving extraction yield of desired materials from complex matrices.[10]

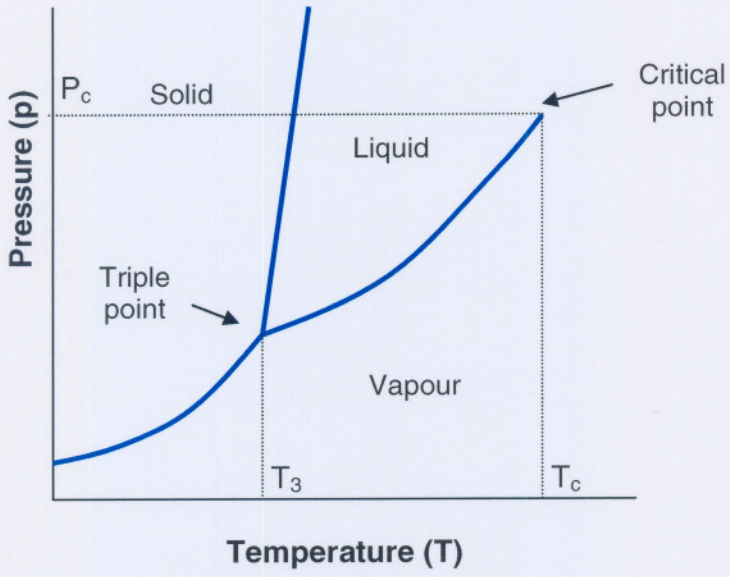


**Figure 2.1** Phase diagram of $CO_2$

There is a considerable number of substances that can be utilised as or converted into supercritical fluids but at the expense of extremely high pressures and/or temperatures, e.g. $H_2O$. **Table 2.1** lists just a small selection, together with a few substances of chemical importance with rather higher values of $T_c$.[7]

The CP for carbon dioxide is at $p_c$ = 72.8 bar and at $T_c$ = 31.1°C. The critical density $\rho_c$ is 0.468 g $mL^{-1}$.

**Table 2.1** Critical constants of selected substances.[9]

| Supercritical fluid | Critical temperature (°C) | Critical pressure (bar) | Critical density (g/mL) |
|---|---|---|---|
| **Carbon dioxide** | **31.1** | **72.8** | **0.47** |
| Nitrous oxide | 36.5 | 70.6 | 0.45 |
| Ethane | 32.3 | 47.6 | 0.2 |
| Propane | 96.7 | 42.4 | 0.22 |
| *n*-Pentane | 196.6 | 32.9 | 0.23 |
| Ammonia | 132.5 | 109.8 | 0.23 |
| Water | 374.2 | 214.8 | 0.32 |

Within the supercritical region, no phase boundary between the gas and liquid phases exists. There is thus continuity in physical properties between the gas and liquid states such that supercritical fluids have properties which are a curious hybrid of those normally associated with liquids and gases. At most conditions, the viscosities and diffusivities are similar to those of gases while the density is closer to that of a liquid.[7]

Below the CP, the liquid and gas phase can be clearly distinguished as shown in **Figure 2.2**. As CP is approached, the distinction becomes less visible. When the temperature and pressure are further increased, only one new phase appears, which is called the supercritical phase. The so-called fluid can be considered either as a highly mobile liquid or a highly condensed gas.[11]

A unique property of a SCF is the adjustability of its density and thus its solvent strength. If the temperature is constant, density can be adjusted from that of a vapour to that of a liquid without any discontinuity. It is common to focus on the region where the reduced temperature ($T_r = T/T_c$) and reduced pressure ($p_r$ =

$p/p_c$) are of the order of unity. In this region, considerable changes in fluid density and thus material solubility are observed for small changes in pressure. This characteristic makes SCFs very attractive as tunable process solvents or reaction media.[12]

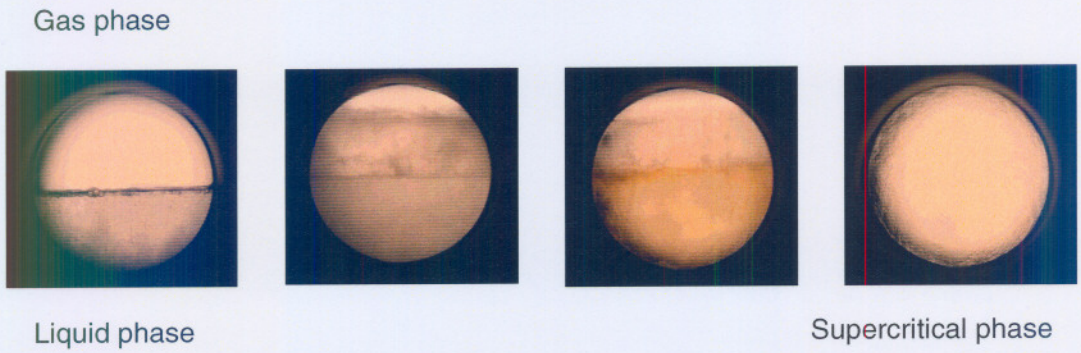


**Figure 2.2** Illustration of the conversion to supercritical state[11]

## *2.4 Basic principles of SFE*

The selectivity of SFE may be manipulated by the choice of solvent or by control of temperature, pressure, static or dynamic mode, flow rate, modifiers or co-solvents. The solvent strength of a supercritical fluid is directly related to its density and can thus be controlled by changing the extraction pressure (or to a lesser extent, the temperature). The low viscosities and high solute diffusible characteristics of supercritical fluids facilitate mass transfer during extraction.[13]

The relationship between pressure, temperature and density may be described by an equation of state. The equations of state derived by Pitzer and co-workers[14] have been incorporated in computer programs for the calculation of supercritical fluid parameters (SF-solver™, Isco, Inc., PO Box 5347, 4700 Superior Street, Lincoln, Nebraska, 68505, USA). The effect of pressure on density for $CO_2$ at various temperatures is shown in **Figure 2.3**.[14]

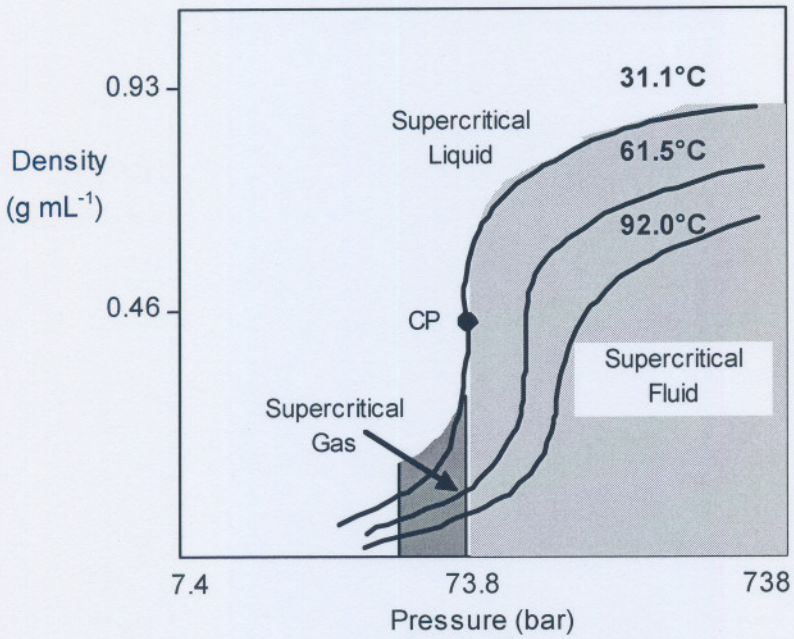


**Figure 2.3** Effect of pressure on fluid density (data for $CO_2$ from ref 14)

The general trend is that higher pressures (at a given temperature) increase density and solvent strength, while increasing temperatures at a constant pressure reduce density and hence solvent strength. These parameters (temperature, pressure and density) are therefore of prime importance in controlling an extraction process. Although a few relationships between these parameters and solubility and rate of extraction have been developed, it is generally not yet possible to predict ideal extraction conditions on a purely physicochemical basis.[14]

The high rates of diffusion of supercritical fluids permit faster extraction than with liquid solvents. This is a significant property as rates of extraction are ultimately limited by the rate at which analyte molecules are transported by diffusion from the sample matrix into the bulk fluid. **Figure 2.4** illustrates the supercritical fluid extraction of an analyte from a sample matrix. However, diffusion coefficients

are reduced as density increases, leading to less-favourable mass transfer at higher densities.[14]

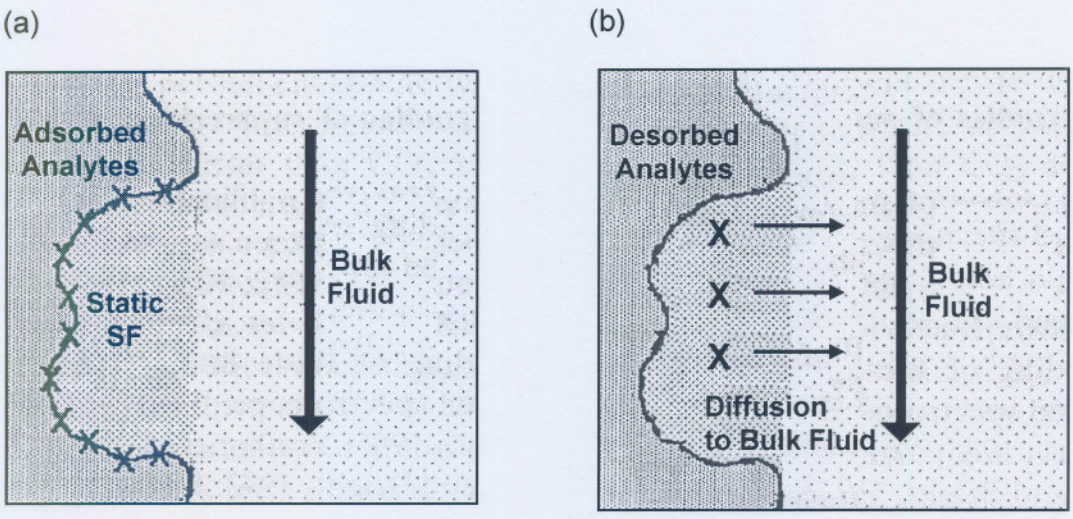


**Figure 2.4** Supercritical fluid extraction of an analyte from a sample matrix[14]
(a) Adsorbed analyte on matrix surface in static supercritical fluid; (b) desorption of analyte from matrix surface and diffusion into bulk supercritical fluid.

SCFs exhibit considerably lower viscosities than liquids, which facilitate favourable flow properties and allow SCFs to penetrate matrices with low permeability more readily than conventional solvents. Commonly used supercritical fluids, such as carbon dioxide ($CO_2$) and nitrous oxide ($N_2O$), are gaseous at room temperature and pressure and can be separated from the analytes by decompression.[14]

The extraction of any compound from a solid matrix requires two steps. Firstly, the compound must be detached from its original binding site in/on the sample matrix through either desorption by or dissolution in the supercritical fluid. Secondly, the compound should be eluted from the sample in a manner analogous to frontal elution chromatography (controlled by the thermodynamic partitioning coefficient $K_D$).[15] Depending on the extraction conditions and sample

matrix, either the rate of the initial desorption/dissolution step or the subsequent elution step may control the actual extraction rate. Several studies have focused on explaining and modelling release mechanisms occurring during supercritical extraction with carbon dioxide. The contribution of the desorption/dissolution step is usually (but not always) predominant.[15]

## 2.5 *Supercritical fluid extraction equipment*

SFE is performed with a supercritical fluid extractor suitable for either laboratory, pilot-plant or industrial scale operation and which may be either commercially available or custom-built in a workshop. A schematic flow diagram of a basic SFE instrument is shown in **Figure 2.5**.

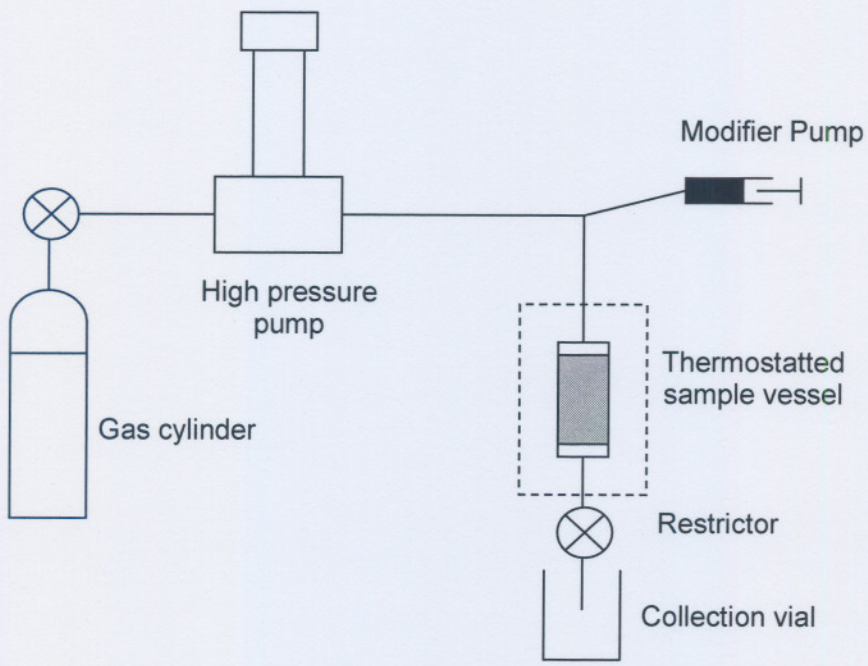


**Figure 2.5** A simple schematic flow diagram of a supercritical fluid extractor[9]

The basic operation of a supercritical extractor is quite simple. Samples are weighed into a vessel or thimble and placed in an extraction chamber or oven heated to a set value. The supercritical fluid is pumped through the extraction vessel to extract the target analyte from the sample vessel in either a static or dynamic mode (i.e. a fixed aliquot of fluid is used for the entire extraction time or fresh fluid is continuously sent through the vessel and its contents during extraction). The extract is subsequently released (static mode) or continuously exported (dynamic mode) through a restriction device, where ambient conditions are restored. The supercritical fluid expands rapidly to the gaseous state and the analytes are trapped in a collection device.[9]

## *2.6 sc-$CO_2$ as a solvent*

Supercritical fluids have a surprisingly long history as solvents for reaction chemistry, beginning with the high pressure polymerisation of ethylene. Environmental concerns are creating a distinct role for supercritical solvents, particularly *sc*-$CO_2$, which are currently regarded as potentially cleaner solvents for a variety of chemical reactions.[7]

The desire to obtain faster extractions with environmentally friendly solvents has led to a great deal of work with supercritical carbon dioxide (*sc*-$CO_2$).[15] It is the most frequently used supercritical fluid due to its favourable chemical inertness, its non-toxic and non-flammable properties and its availability in high purity at low cost. *sc*-$CO_2$ has a low critical temperature and pressure ($T_c$ = 31.1°C; $p_c$ = 72.8 atm), which makes it an ideal solvent for natural products, since these often suffer thermal degradation or are contaminated with solvent residues. In addition, it is easily removable from the extract following decompression.[8, 9, 16-20]

## 2.7 *Advantages of supercritical fluids*

Although the solvent strength of liquids is generally superior to that of supercritical fluids, the use of supercritical fluids as extraction media and mobile phases in chromatography has several important potential advantages.[14] These include shorter extraction times, potentially higher selectivity and increased sample throughput compared to conventional solvent extraction techniques.[9]

The motivation for developing extractive separation techniques with supercritical fluids can be summarised as follows:

- low temperatures, mild conditions;
- residue-free extracts due to simple and complete separation of solvent;
- substitution of problematic traditional solvents as a result of more restrictive environmental standards.[5]

## 2.8 *Applications of supercritical fluids*

SCFs have been widely used in extraction and recovery of high-value compounds. The experience accumulated in recent years in this field has reached the step that it is possible to explore and envisage the use beyond the common practice of extraction. A few more significant potential applications include:[21]

- preparation of ceramic filtration membrane using *sc*-$CO_2$;
- solvent recovery or separation of $CO_2$ from complex mixtures;
- $CO_2$ extraction linked with cross-flow filtration;
- $CO_2$ fluidification of viscous fluids during cross-flow filtration;
- membrane contactor/reactor operated under *sc*-$CO_2$ conditions.[21]

The use of supercritical fluids has been proposed for a wide range of industrial applications such as refining of lubricant oils, decaffeination of coffee, extraction of hop, and synthesis of polymers. Many studies have been carried out to extract carotenoids from natural sources.[10] In addition, many industrial applications on a smaller scale have been reported. These include extraction of spice (pepper), aromatic substances (aniseed, citrus fruit), fragrances (perfumes), pharmaceuticals, seed oils (soybeans, olives) and nicotine removal.[5]

The most important processes involving *sc*-$CO_2$ currently under development, are in the food, pharmaceutical and textile industries and in the production of nanopowders and polymers. Developments in environmental technology include extractive treatment of solids, separation of aqueous residues and supercritical water oxidation and conversion.[5]

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# Chapter 3

# Experimental principles and procedures

This chapter covers experimental aspects of the investigation such as selection of materials, sample preparation, performance of *sc*-$CO_2$ extraction runs, quantitative analysis of extracts, optimisation of extraction conditions, processing of data and process modelling (only theoretical principles). Acquisition, processing and interpretation of the experimental results are presented in the next chapter.

## *3.1 Materials and methods*

### *3.1.1 Chemicals and solvents*

Carbon dioxide (99,9%), helium (99,9%) and air (99,9%) required for the supercritical extractor was obtained from Afrox (South Africa). The lutein standard was purchased from Indofine Chemical Company, Inc. (New Jersey, U.S.A.). After every extraction run the flow line was rinsed with analytical grade acetone and HPLC grade ethyl acetate, both purchased from Merck Chemicals (Gauteng, South Africa), and dried under a gentle stream of nitrogen (99,9%) purchased from Afrox (South Africa). The soxhlet extraction was performed by using analytical grade *n*-hexane also purchased from Merck Chemicals (Gauteng, South Africa).

HPLC grade solvents used for chromatographic analysis were purchased from Merck Chemicals (Guateng, South Africa). These included acetonitrile, methanol

and ethyl acetate. Aluminium foil used for covering the collection vials was purchased from a local supermarket.

### *3.1.2 Plant specifications*

20 kg marigold plant material (**Figure 3.1**) was provided by Delphinius Distributions CC with a moisture content and oil content (dry mass) of 10% and 0,08-0,1%, respectively. The plant species was *Tagetes erecta*, cultivar Deep Orangeade, harvested in 2000 by Mr LJB Luus (farmer) in the Giyani district, Limpopo Province.

**Figure 3.1** Marigold plant material

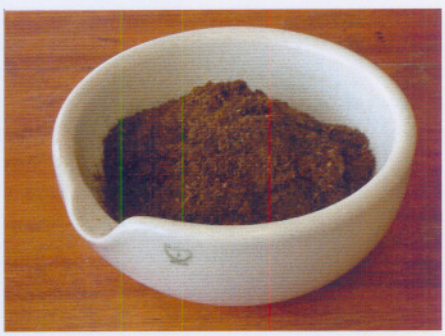

**Figure 3.2** Homogeneous petals

### *3.1.3 Sample preparation*

All extractions were performed using homogeneous freeze-dried (to minimize oxidative loss) marigold petals. The plant material was frozen using a Dura-Dry MP microprocessor controlled freeze-dryer (FTS systems) at -50°C for a duration of 36 hours and with vacuum set at 48 millitorr (mT). The freeze-dried plant material was homogenised (**Figure 3.2**) using an Optolabor commercial blender for 30 seconds and stored in commercially available Ziploc® bags at a temperature of -20°C in a refrigerator.

The samples were handled in diffused light to minimise photodegradation of lutein esters. The loading of samples into the extraction thimble is shown in **Figure 3.3**.

The lower end cap of an extraction thimble (sample holder) was screwed on and the thimble inserted into a supporting rack. Using a spatula, a Kimwipe plug (approximately ¼ of one Kimwipe sheet, folded in half) was placed into the bottom of the extraction thimble. A sample of 3.0 g on average of ground petals was then loaded into the extraction thimble for each extraction run using a scoop and funnel. Finally, the upper end cap was fitted, after which the thimble was inserted into the sample chamber of the supercritical fluid extractor.[1]

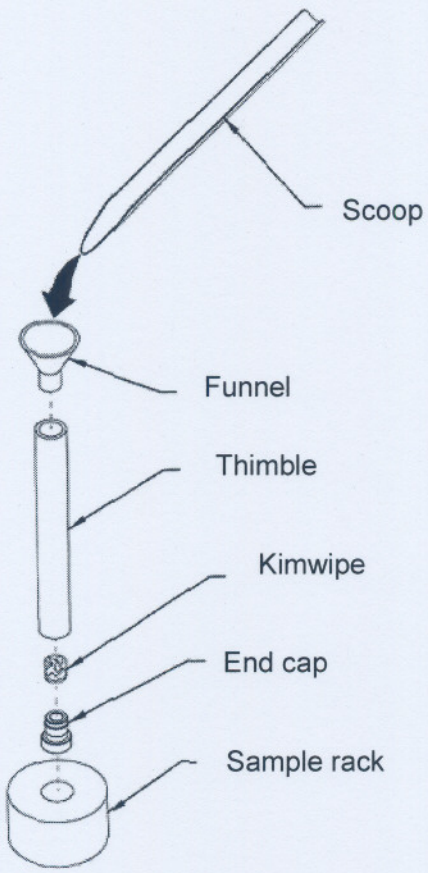


**Figure 3.3** Thimble preparation[1]

## 3.2 Extraction procedures

### 3.2.1 SFE apparatus

All extraction runs were performed on the laboratory scale supercritical fluid extractor (LECO TFE 2000) with auxiliary modifier pump (LECO/M2000) shown in **Figure 3.4** using *sc*-$CO_2$.


**Figure 3.4** LECO TFE/M2000

**Figure 3.5** Stainless steel thimbles

The instrument has two distinct advantages above similar units commercially available to date, viz.

1. high flow rates (L/min instead of mL/min range) making extraction less time consuming and more cost effective;
2. three separate flow lines with stainless steel thimbles (**Figure 3.5**) enabling three extractions to be performed simultaneously, each with its own separate restrictor.

**Figure 3.6** shows more details of the extractor main unit and **Figure 3.7** gives a flow diagram of its operation.

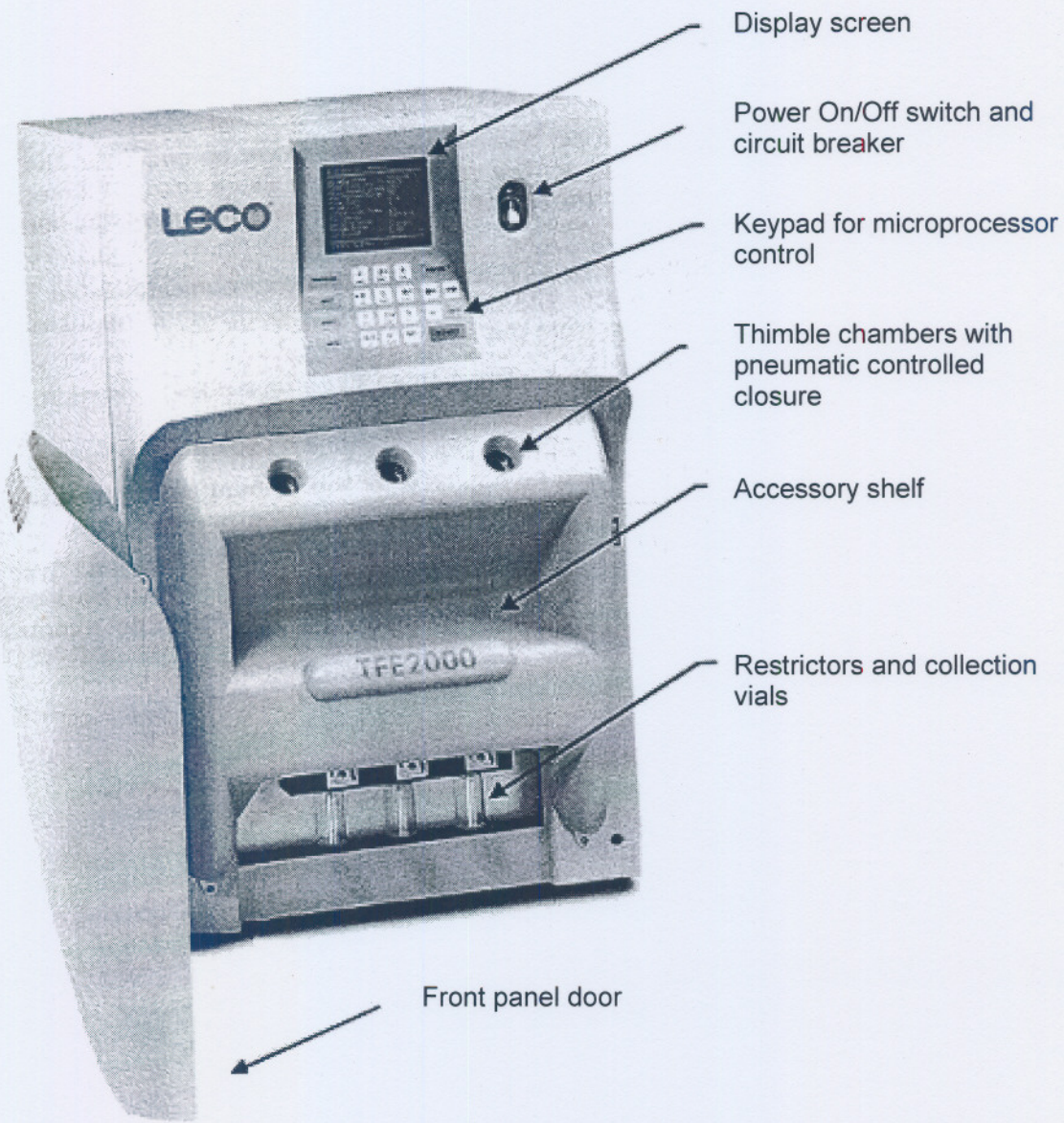


**Figure 3.6** LECO TFE 2000 supercritical fluid extractor[1]

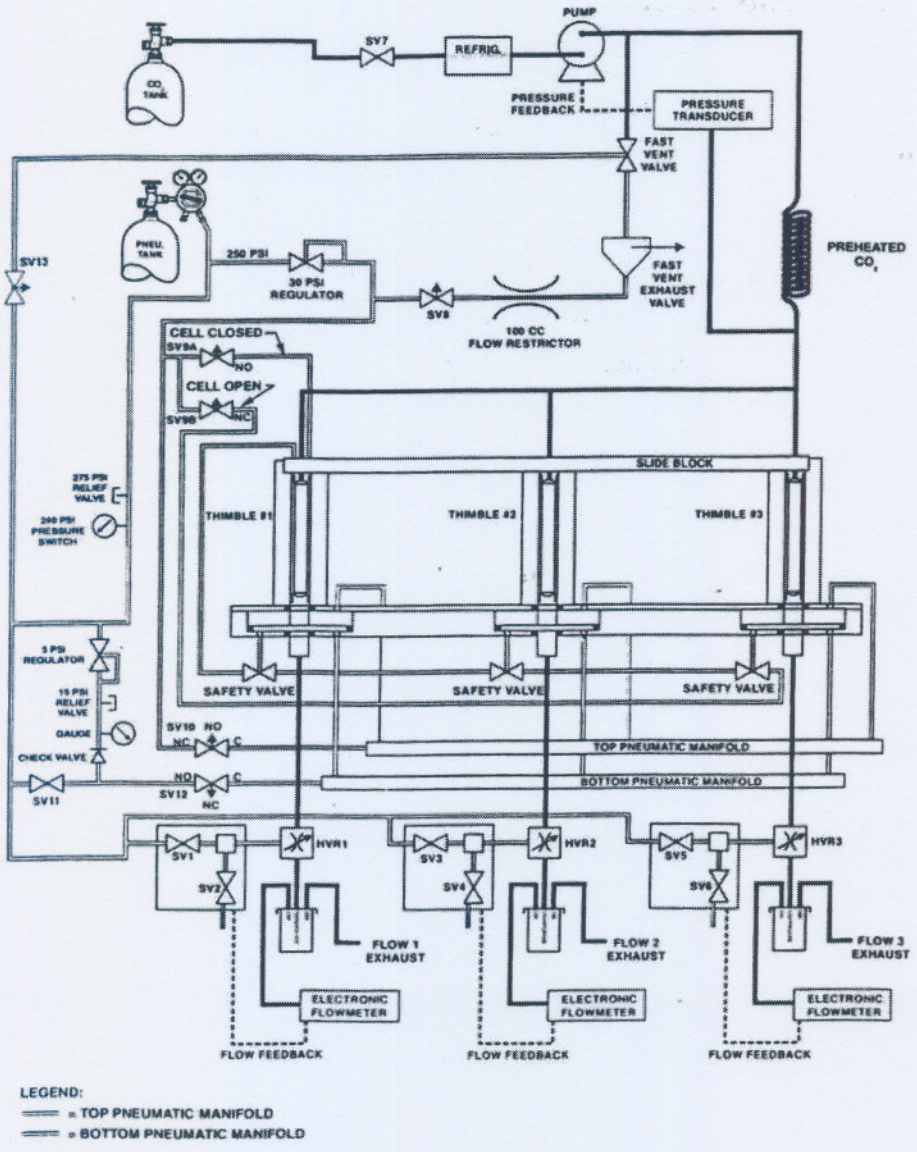


**Figure 3.7** LECO TFE 2000 flow diagram[1]

### *3.2.2 SFE extraction procedure*

Prior to each extraction run, the collection vial and the thimble (with Kimwipe plug) were weighed. The prepared samples were then loaded into the thimbles as described in **Paragraph 3.1.3** and weighed again.

After weighing, the thimbles were sealed and inserted into the thimble chambers. The collection vials were then secured to the instrument for collection of the extract and covered with commercially available aluminium foil.

The operator begins the extraction process by clicking "analyze" from the "samples" menu. Solenoid valves SV10 and SV12 (**Figure 3.7**) are activated and lowers the thimbles into position, after which solenoid valve SV9A is activated to close the chamber pneumatically with a pressure of 17.24 bar (250 psi). The thimbles are heated with cartridge heaters to a temperature default value of 100°C or any other temperature entered in the "analysis" menu.

After the thimbles are sealed off, solenoid valve SV7 is opened and the pump turned on to deliver the compressed $CO_2$. A pump cooler is also turned on to keep the pump and $CO_2$ to approximately 0°C and thereby enhance compression and increase the pump flow capacity.

Pump pressure ($CO_2$) continues to increase until the default pressure of 620.53 bar (9000 psi) is reached. This value can also be changed by entering a required value prior to the extraction run.

The pump pressure is regulated at the set value by variable restrictors (HVRs), electronic flow meters and solenoid valves SV1 through SV6. As an extraction run continued, marigold extract from the samples was collected in the collection vials.

After extraction expires, the flow of $CO_2$ is stopped and pressure is released. The HVR valves are fully opened and the quick vent valve opens when the $CO_2$ pressure decreases to 103.42 bar (1500 psi). After the pressure reaches ambient values, 0.34 bar (5 psi) of pneumatic pressure is applied to the bottom of the extraction cell piston to rise the thimbles for removal from the extraction chambers.

After an extraction run, the flow line was rinsed with acetone or ethyl acetate for 2 min at the same temperature, and dried under a gentle stream of nitrogen at 37°C. The collection vial with the marigold extract (**Figure 3.8**) was then weighed again. The mass of extract was calculated by subtracting the two measurements of the collection vials obtained prior and after every extraction.[1]

Numerous extraction runs where performed at various extraction times (0-99 min), temperatures (40-70°C) and pressures (100-500 bar) to determine optimum conditions. All extractions were done in triplicate.

**Figure 3.8** Vials containing marigold extract

### 3.2.3 Soxhlet extraction

A single extraction run was carried out for 2 hours at 70°C for the purpose of comparing the results with those obtained by *sc*-$CO_2$ extraction. Homogeneous freeze-dried marigold plant material (3.06 g) was extracted with 100 mL analytical grade *n*-hexane in a soxhlet apparatus (**Figure 3.9**) until the solvent was colourless. The extract was concentrated under vacuum to obtain a final mass of 0.5199 g. All operations were performed under light protection.

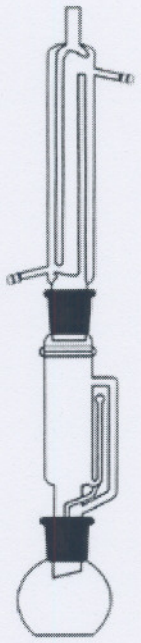

**Figure 3.9** Soxhlet apparatus

## 3.3 Analysis

Carotenoid analysis is inherently difficult and care is needed in carrying out this analysis. Precautionary measures to avoid artefact formation and loss of carotenoids during analysis (e.g. exclusion of oxygen, protection from light, avoiding high temperatures and contact with acids, use of high purity, peroxide-free solvents, completion of the analysis within the shortest possible time) need to be considered.[2]

There are two types of methods for quantitative chromatographic analysis of a sample, viz. external standard and internal standard methods. The calibration method entails preparation of standards of different concentration and analysing these by a developed method. Using linear regression analysis, a calibration line

can be constructed and the correlation coefficient ($r^2$) of the regression line can be determined.[3]

### *3.3.1 HPLC conditions*

High-performance liquid chromatography (HPLC) is considered to be the most reliable, efficient and reproducible method for carotenoid analysis[4, 5] and was therefore used for the analysis of the marigold extract.

An Agilent Technology 1100 Series HPLC System (**Figure 3.10**) equipped with a quaternary pump, a degasser, a thermostatic autosampler and a multi-wavelength detector system was used for identification of lutein in the extract. Separation of lutein was carried out on a Phenomenex Luna column (250 × 4.6 nm i.d.; particle size, 5 µm), with a $C_{18}$ guard column.

The binary mobile phase consisted of 85% acetonitrile and 15% methanol (solvent A) and 68% methanol and 32% ethyl acetate (solvent B). The flow rate was 1.0 mL/min and the column was kept at a constant temperature (40°C) for a total run time of 55 min.

The system was run with a gradient program of 100% solvent A for 12 min with a changeover to 100% solvent B in 2 min. 100% solvent B was used for the duration of the run. The injection volume was 20 µL. The detector was set at 445 nm and identification of lutein was achieved by comparing the retention time with that of pure (> 95%) lutein standard.

Lutein concentration was determined by using the onboard software to compare the corresponding peak area with the calibration graph referred to above.

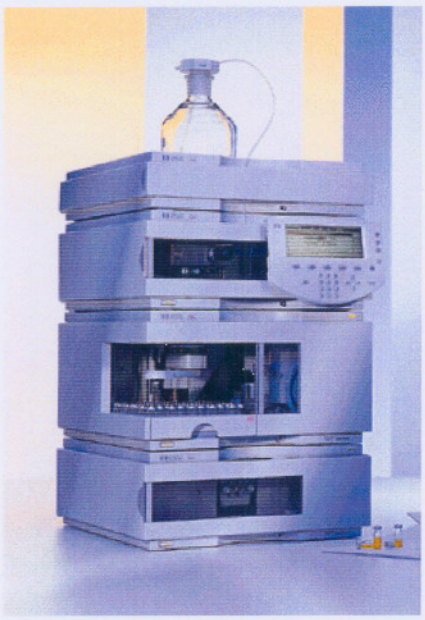

**Figure 3.10** Agilent 1100 HPLC system

### *3.3.2 Construction of calibration line*

The calibration line was constructed by dissolving 6.3 mg of standard lutein diluted in 1000 mL acetonitrile:methanol (9:1) mixture to give a stock solution of 6.3 µg/mL. Six different lutein concentrations were prepared by dilution of the stock solution to cover the concentration range expected in the extracts.

The peak areas of these solutions were measured in mAU (milli-absorbance units) and plotted against the concentration to construct the calibration line. Statistica 7.0 for Windows® was used to determine with linear regression the mathematical equation describing the calibration line.

## 3.4 Optimisation of extraction conditions

The extraction of lutein is usually evaluated by quoting the mass of extract obtained from a given mass of plant material and the lutein content of that extract. In order to compare the results of *sc*-$CO_2$ extraction of lutein with such data reported for hexane extraction, extraction conditions needed to be optimised in terms of extract and lutein yield. This was achieved by a statistical design based on the major process variables.

### 3.4.1 Experimental design

Optimisation of the conditions for lutein extraction from marigold was derived by computer assisted surface response analysis based on a statistical design which shows the effect of different process variables, such as temperature, pressure, density, flow rate, time of extraction, modifier content (independent variables) on the yield of extract of lutein (dependent variable). This approach required a minimum number of extraction runs to be performed to construct a multidimensional response graph from which the optimum values for the different variables can be derived.

A central composite design based on a statistical method is a scientific approach of planning the extractions to be performed in order to reveal the effects of the different process variables. An advantage of such a design is that a maximum amount of reliable information is obtained from a minimum number of observations.

A first step in surface response analysis of an extraction process is to list the variables which have an influence on the yield of extracted material. These could include pressure, temperature, density, time of extraction, fluid flow rate, modifier

content, nature of extractable substances, moisture content of plant material, and more. The design could be simplified by considering only those variables that are known or expected to be the major process controlling factors.[6]

The time dependence of the extraction process was studied first to establish the required extraction time for the acquisition of an optimum lutein concentration at typical (T = 45°C; p = 400 bar) extraction conditions. Extraction runs were done in triplicate at intervals of 10 minutes ranging between 10 and 100 minutes, and the optimum extraction time determined in this way was then applied to all other runs at different combinations of the remaining principal process parameters.

A further simplification of the design was done by selecting a fixed $CO_2$ flow rate (1.5 L/min) to ensure proper penetration of the extraction matrix. In addition, no modifier was used, as it was important to acquire a natural extract free from any solvent residues.

The independent variables mainly influencing the yield of extract were thus restricted to temperature and pressure. It is important to select the most significant factors to reduce the number of variables and to keep the number of experiments to a manageable number.

To study the effect of temperature and pressure combinations that may have a substantial influence on lutein extraction, Statistica 7.0 for Windows® was used to create the experimental design shown in **Table 3.1**.[7]

**Table 3.1** Central composite experimental design

| | Run | Independent variable 1 | Independent variable 2 |
|---|---|---|---|
| Factorial points | 1 | 1 | 1 |
| | 2 | 1 | -1 |
| | 3 | -1 | 1 |
| | 4 | -1 | -1 |
| Star points | 5 | -1.414 | 0 |
| | 6 | 1.414 | 0 |
| | 7 | 0 | -1.414 |
| | 8 | 0 | 1.414 |
| Center points | 9 | 0 | 0 |
| | 10 | 0 | 0 |

Runs 1 to 4 are known as the factorial points which constitute a 2x2 orthogonal design. Runs 5 to 8 are known as star points which are added to the design to calculate the quadratic components of the relationship among the variables without sacrificing the requirement of orthogonality and rotatability. The last two runs (9 and 10) are known as the center points and are added to test for a linear or polynomial model.[11] The information function of this second-order design is rotatable, i.e. constant on a circle around the origin as shown in **Figure 3.11**. Rotatability refers to the capability of the design to render the same information in all directions of the fitted surface.

### *3.4.2 Surface response graph*

The rotatable, orthogonal design can be displayed by a surface response graph, an example of which is shown in **Figure 3.12**. This graph presents the values of the variables in a three-dimensional fashion with the two horizontal axes (x and y) showing the independent variables 1 and 2 (temperature and pressure) and the vertical axis (z) the dependent variable (lutein concentration or mass of extract).

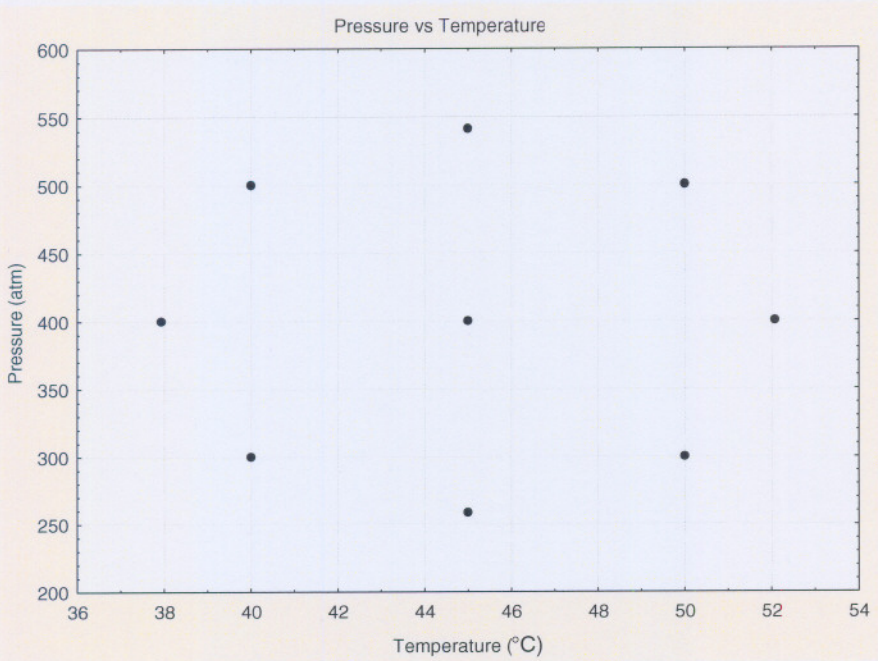


**Figure 3.11** Rotatability of second-order design

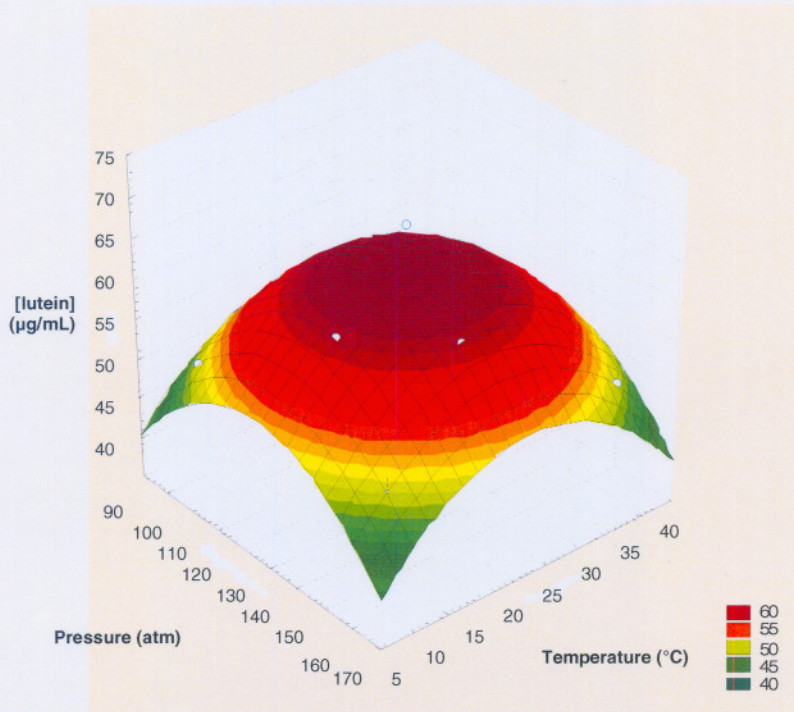


**Figure 3.12** Response surface 2nd-order model

STANDARD DESIGN SUMMARY:[8]

2**(2) cube plus star (central composite design)

Number of factors: 2

Number of blocks: 1

Number of runs: 10 nc=4 ns=4 n0=2

Alpha for rotatability: 1.4142 Alpha for orthogonality: 1.0781

| | | | | | | |
|---|---|---|---|---|---|---|
| Temperature (°C) | 40 | (Low) | 45 | (Center point) | 50 | (High) |
| Pressure (bar) | 300 | (Low) | 400 | (Center point) | 500 | (High) |

**Table 3.2** Runs suggested by central composite design.

| 2**(2) central composite, nc=4 ns=4 n0=2 Runs=10 | | |
|---|---|---|
| Runs | Temperature (°C) | Pressure (atm) |
| 1 | 40 | 300 |
| 2 | 40 | 500 |
| 3 | 50 | 300 |
| 4 | 50 | 500 |
| 5 | 38 | 400 |
| 6 | 52 | 400 |
| 7 | 45 | 259 |
| 8 | 45 | 541 |
| 9 (C) | 45 | 400 |
| 10 (C) | 45 | 400 |
| 11 (C) | 45 | 400 |
| 12 (C) | 45 | 400 |

The temperature and pressure values calculated according to the experimental design in **Table 3.1** are presented in **Table 3.2** and allow extraction runs to be performed with which a three-dimensional surface response graph such as the example in **Figure 3.12** can be constructed. The results of these runs and the real surface response plot following from these results are presented in the next (results) chapter.

The reliability of the mathematical model can be verified on account of the extent to which observed and predicted values correspond by plotting them on a common graph. If all data points fall on a straight line, the model can be considered perfect.[8]

## *3.5 Process analysis*

### *3.5.1 Yield determining factors*

The physical-chemical properties of the extraction process can be derived from its dependence on the different variables (temperature and pressure or density).

A strong dependence on pressure or density could suggest that the extraction of lutein results from a chemical dissolution process since the solvent strength of *sc*-$CO_2$ is determined by these two variables. Similarly, an independence of density could suggest a physical rather than a chemical extraction mechanism such as desorption of the desired substance from the plant matrix.

The joint effect of pressure and temperature on the yield of extracted lutein can also give insight into the extraction process because these two factors oppose each other in establishing the density, which in turn relates to the solvent strength of the fluid and thus to its capability to chemically dissolve lutein from the plant material.

### *3.5.2 Activation parameters*

The effect of yield determining factors provide information about the extraction process which is needed for mathematical modelling.

The rates of most reactions increase as the temperature is raised. An empirical relationship[9, 10] accounts for the increase in rate constant with increasing temperature, viz.

$$k = Ae^{-Ea/RT}$$

with $E_a$ the activation energy, R = 8.314 J $K^{-1}$ $mol^{-1}$ the universal gas constant, T the temperature in kelvin and A the pre-exponential factor (also known as the frequency factor). A plot of ln k against 1/T based on the logarithmic form

$$\ln k = \ln A - E_a/RT,$$

of the equation is a straight line as shown in **Figure 3.13** and allows determination of the activation energy ($E_a$) from the slope.

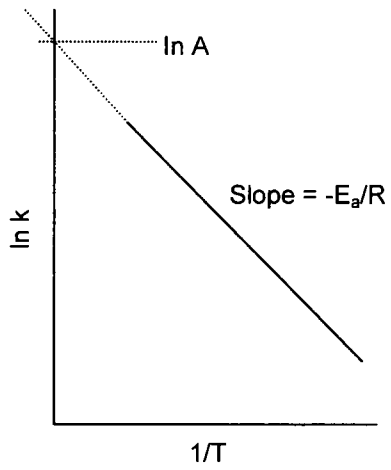


**Figure 3.13** Determination of $E_a$[10]

The rate constant k can be substituted by the yield of extract or concentration lutein without changing the magnitude of the slope, so that the correct value of $E_a$ can be obtained.

The empirical equation[11]

$$k = e^{-(\Delta^{\neq}V/RT)p}$$

can similarly be written in the logarithmic form

$$\ln k = -\frac{\Delta^{\neq}V}{RT}p + \text{constant}$$

to determine the volume of activation ($\Delta^{\neq}V$) from the slope of a plot of ln k versus p. Here p is the pressure of the extracting fluid and k the rate constant of extraction. Once again, k can be substituted by the yield of extract (concentration lutein) without changing the magnitude of the slope.

## *3.6 Mathematical modelling*

In mathematical modelling an attempt is made to derive a mathematical function which relates all process variables and which can serve as a "model" equation from which predictions about the process under different conditions can be made.

Consider a function of one independent variable

$$y = f(x)$$

and assume that four points have been deemed necessary to predict y over a suitable domain for x. When a second independent variable affects the situation under investigation, we have the function

$$y = f(x,z)$$

thus resulting in a total of 16 (i.e., $4^2$) experiments to be conducted. In general, $4^n$ experiments are required to predict y when n is the number of arguments of the function, assuming four points for the domain of each argument. Thus a procedure that reduces the number of arguments of the function f will considerably reduce the total number of required experiments. Dimensionless analysis is one such procedure.

The experimental work to be done depends more heavily on the number of arguments of the model function than on the true number of independent variables originally selected by the modeller. For example, consider a function of two arguments, say y = f(x,z). The discussion concerning the number of experiments necessary would not be altered if x were some particular combination of several variables, i.e. x could be uv/w, where u, v, and w are the variables originally selected in the model. In this way, experiments should still be conducted to make predictions, but the extent of experimental effort required may be reduced significantly.[12]

Supercritical fluid extraction of a botanical component can be presented by a function that consists of the following variables:

σ = amount of waxes and oil in plant material

t = extraction time

T = extraction temperature

w = moisture content of plant material

p = extraction pressure

ρ = density of fluid

r = amount of extract

f = flow rate of fluid

Each of these factors may play a role in the extraction. The extraction yield y may then be presented by a function

$$y = F\left\{\frac{f^2\rho}{T^3t^4}, \frac{p}{T}, r, w, \sigma\right\}$$

which can be expanded into

$$y = m_0 + m_1\frac{f^2\rho}{T^3t^4} + m_2\frac{p}{T} + m_3 r + m_4 w + m_5\sigma$$

were $m_0$, ..., $m_5$ are constants.

If the physical units of the different variables are considered, it is possible to express these variables in such a way as shown in the table below that they become dimensionless.

| **Variables** | f | p | T | ρ | t |
|---|---|---|---|---|---|
| **Dimension** | $\frac{M}{T}$ | $\frac{M}{LT^2}$ | $\frac{M}{LT^2}$ | $\frac{M}{L^3}$ | T |

Here M is the mass in kg, L the length in m, t the time in s and T the temperature in K. A product of the variables can be written in the form

$$f^a p^b T^c t^d \rho^e$$

which will be dimensionless (i.e. without units) if

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

$$- b - c - 3e = 0$$

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

Two independent solutions for these equations are

a = 1; b = 0; c = -3/2; d = -2; e = ½, so that

$$\Pi_1 = \sqrt{\frac{f^2 \rho}{T^3 t^4}}$$

and

a = 0; b = 1; c = -1; d = 0; e = 0, so that

$$\Pi_2 = \frac{p}{T}$$

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

$$f(\Pi_1, \Pi_2, \ldots, \Pi_n) = 0$$

where f is a certain function with a number of arguments presenting a complete set of dimensionless products. $\Pi_2$ stays fairly constant for the extraction in this investigation as p and T conversely affect the density; therefore only $\Pi_1$ needs to be considered. The relationship between y and $\Pi_1$ is given by the function f(y, $\Pi_1$) = 0, which can be solved to give y = g($\Pi_1$). Thus ln y = $m_2$ $\Pi_1$ + $m_1$ with $m_1$ and $m_2$ being dimensionless constants. This equation can be presented by a graph (**Figure 3.14**) which, if a straight line is obtained, proves that all important process factors have been taken into account by the particular model.

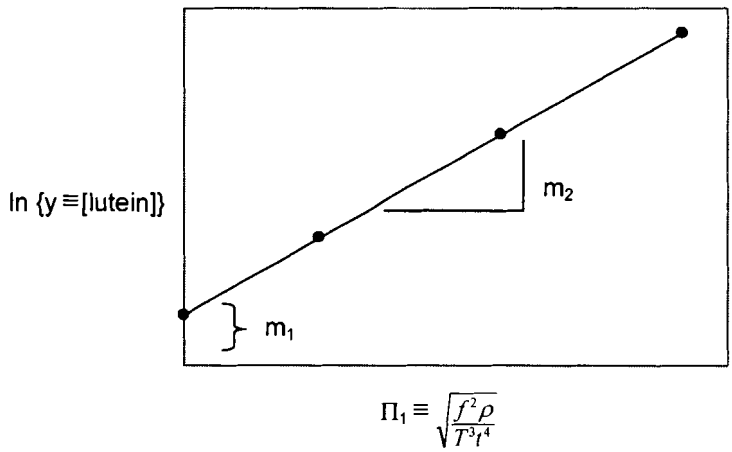


**Figure 3.14** Relation between ln [lutein] and dimensionless grouping of variables

The straight line is described by the equation

$$\ln [\text{lutein}] = m_1 + m_2 \sqrt{\frac{f^2\rho}{T^3 t^4}}$$

Which implies that a graph of ln [lutein] as a function of $\sqrt{\frac{f^2\rho}{T^3t^4}}$ can be used to validate the model concerned if the experimental extraction data can be fitted to a straight line.[13]

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# Chapter 4

# Data acquisition, Processing and Interpretation

This chapter covers the data acquisition and processing as well as the interpretation of the experimental results obtained during the study. The concentration lutein extracted with *sc*-$CO_2$ from marigold petals at selected conditions was quantitatively determined by virtue of a calibration line constructed by using a commercially available lutein standard. The optimum extraction conditions for a maximum concentration lutein were determined according to a statistical design and computer assisted surface response analysis. The validity of the underlying model was proven by a satisfactory data fit of observed versus predicted values. Activation parameters were calculated using the extraction data to elucidate the characteristics of the process mechanism. Finally, an attempt was made to model the extraction process mathematically by virtue of a function comprising a dimensionless grouping of process variables. The above mentioned aspects are all discussed in the paragraphs below.

## *4.1 HPLC chromatogram*

The HPLC chromatogram of the lutein standard exhibited a single, well-defined peak as shown in **Figure 4.1**. The corresponding retention time was determined as (5.63 ± 0.03) minutes from the profile overlays of several solutions of the substance. Lutein is one of the components present in a *sc*-$CO_2$ extract of marigold flower as shown by the HPLC chromatogram in **Figure 4.2**. The remaining peaks were mainly those of lutein esters, but these were not identified as the extraction of specifically lutein was the principal objective.

### *4.1.1 Overlay of calibration range*

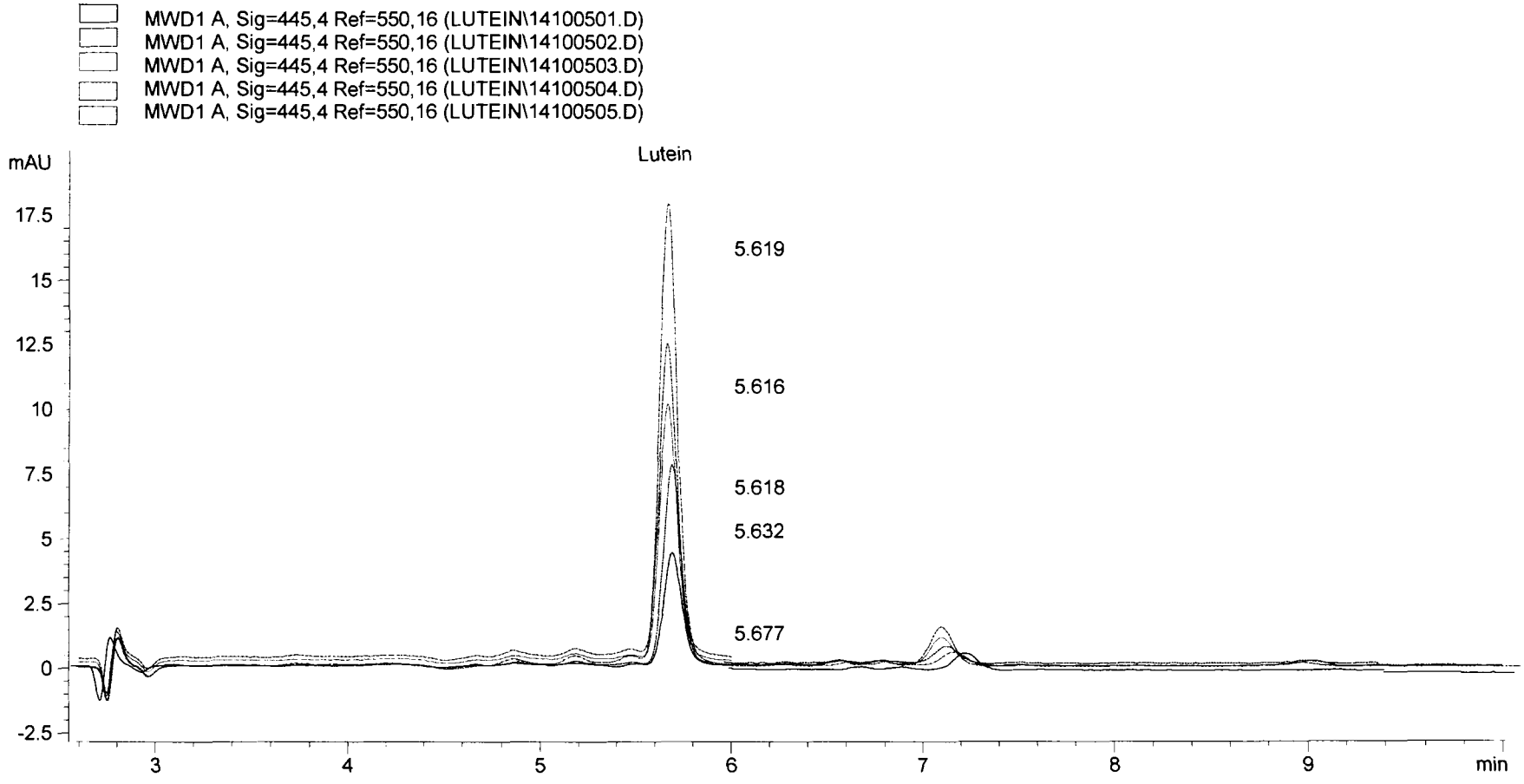


**Figure 4.1** HPLC profile of lutein standard overlay

### *4.1.2 SFE marigold extract*

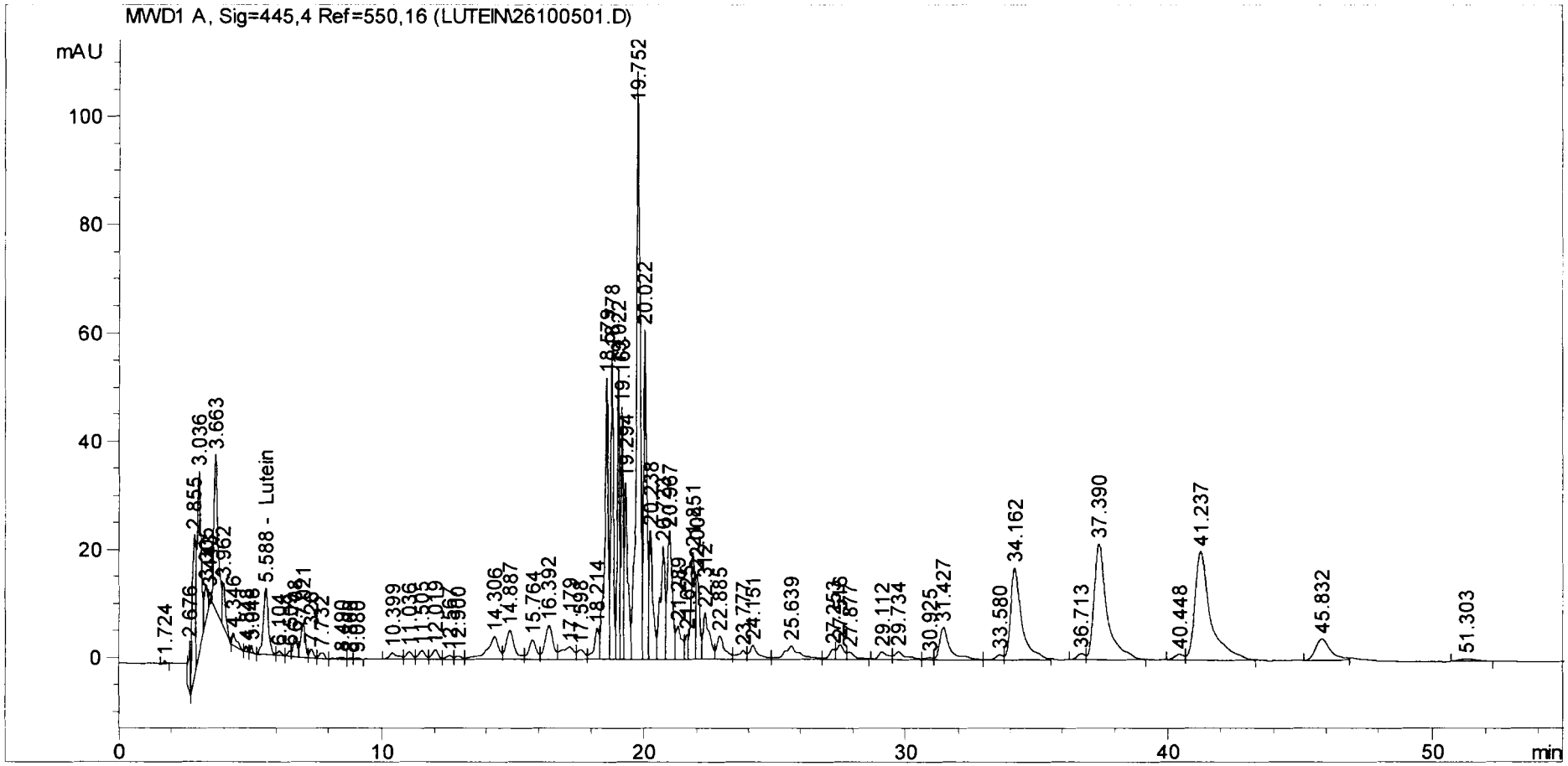


**Figure 4.2** Typical HPLC chromatogram of marigold flower extract

## 4.2 *Calibration line*

The calibration line in **Figure 4.3** was used to determine the concentration lutein extracted with *sc*-$CO_2$. The procedure followed to construct the calibration line was explained in **Paragraph 3.3.2**. The area of the HPLC peak of lutein in the chromatogram of a given extract could be used to read off the corresponding lutein concentration from the calibration line. The calibration line has a correlation coefficient of 0.999 and covers the concentration range within which lutein occurred in the extracts.[1]

**Table 4.1** Calibration line data

| Standard no. | Concentration (µg/mL) | Peak Area (mAU) |
|---|---|---|
| 1 | 0.252 | 25.9 |
| 2 | 0.504 | 52.0 |
| 3 | 0.756 | 76.7 |
| 4 | 1.01 | 109 |
| 5 | 1.26 | 127 |
| 6 | 1.51 | 155 |

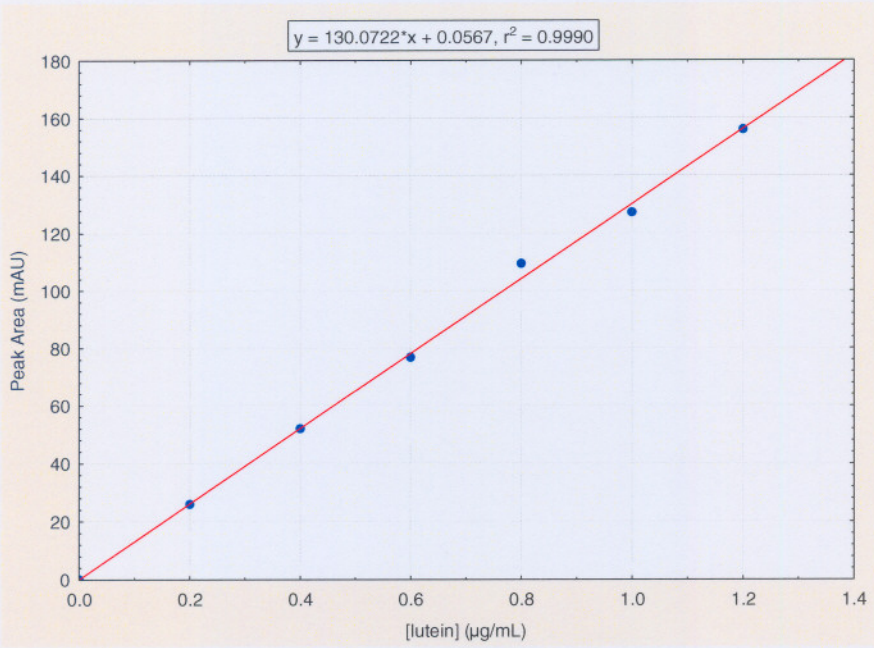


**Figure 4.3** Calibration line for quantitative lutein analysis

## 4.3 *Optimisation of extraction time*

The duration of an extraction run with *sc*-$CO_2$ to warrant a maximum lutein yield at typical extraction conditions was determined before a bulk of extraction data was accumulated in order to reduce the number of variables for a statistical optimisation of the extraction conditions. The reason for this was that every additional variable requires additional extraction runs to be performed, and the objective of statistical surface response analysis indeed was to acquire the maximum information from a minimum of extraction runs.[2]

The extraction conditions were kept constant at values (T = 45°C, p = 400 bar) approximately in the center of the ranges over which these conditions could be varied experimentally. A relatively high flow rate of 1.5 L/min was selected to deliver high yields of extract and to facilitate effective removal of extracted material from the supercritical fluid extractor. The duration of the runs was varied between 10 and 99 min. The optimum extraction time was determined by plotting the average value of several measurements of the yield of lutein as a function of time and drawing a smooth curve through the calculated standard deviation limits of the scattered data as shown in **Figure 4.4**.[3, 4] It follows from this curve that the maximum lutein concentration was obtained after approximately 60 min. The curve reached a plateau at this value and did not change beyond that point as the plant material was exhausted in terms of extractable material and continued extraction would not lead to any further gain in lutein concentration. The duration of all further extractions was therefore limited to 60 min.

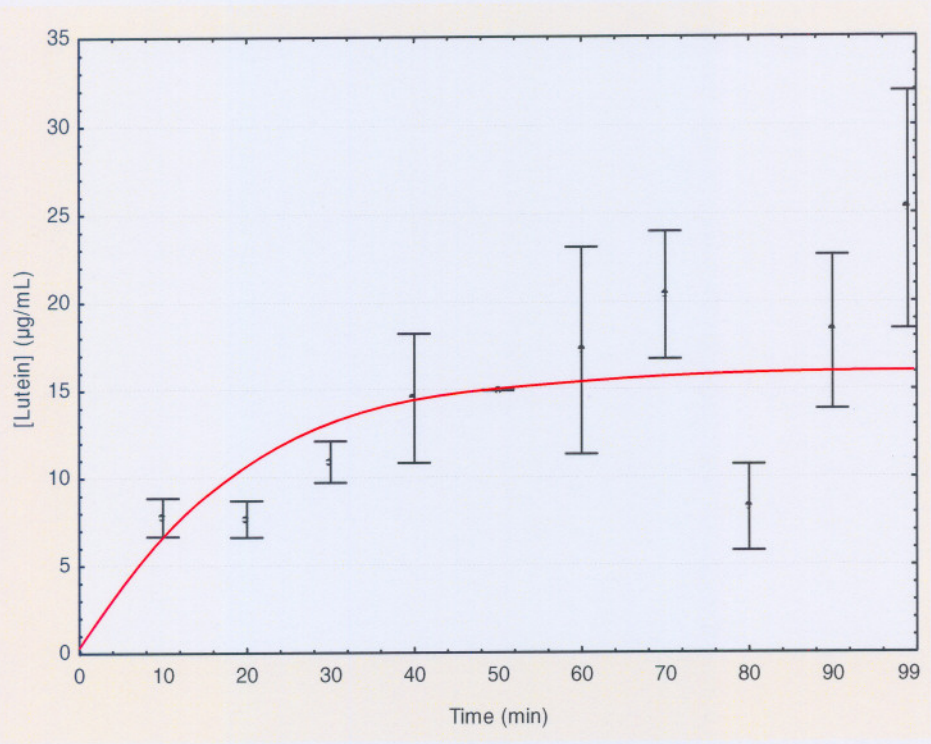


**Figure 4.4** Optimum extraction time for *sc*-$CO_2$ extraction of lutein

lutein concentrations given as average ± standard deviation of several measurements

The course of an extraction run could also be monitored in terms of the decrease in mass of the plant material in the thimble and the mass of extract collected at the restrictor. **Figure 4.5** shows these two quantities plotted on the same graph against the extraction time. The coincidence of the two mass versus time profiles proves that a perfect mass balance and thus effective removal of the extract from the flow lines of the extractor was achieved. Moreover, the profiles confirm the result obtained in the previous figure that the optimum extraction time is approximately 60 minutes.

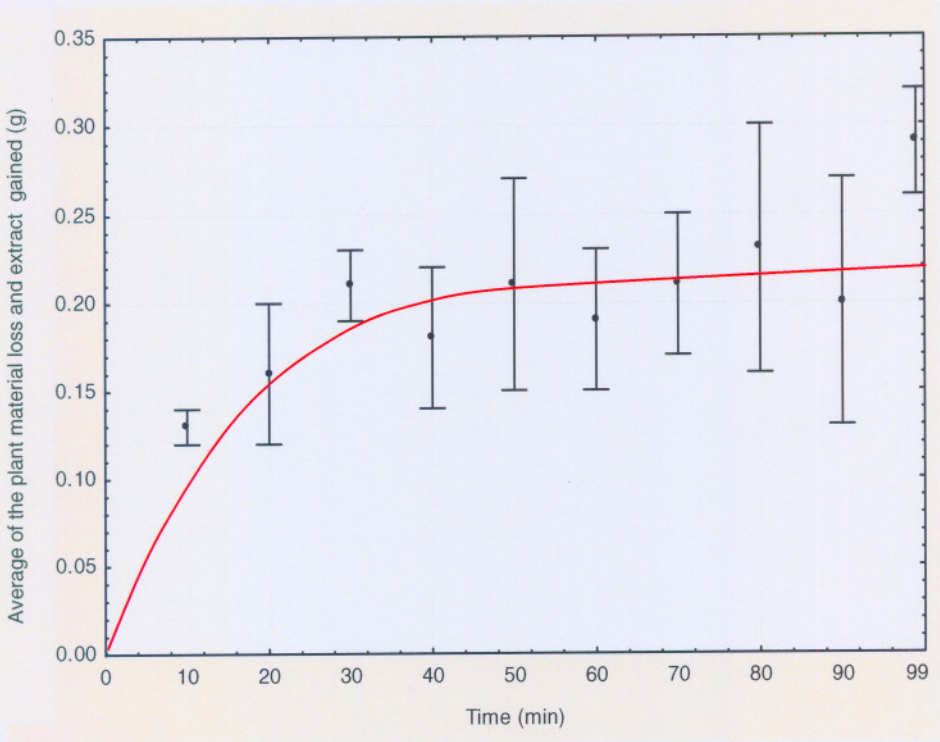


**Figure 4.5** Optimum extraction time for plant material loss/extract gained

(Plant material loss and extract gained given as average ± standard deviation of several measurements.)

## 4.4 *Optimisation of temperature and pressure*

The experimental design presented in **Table 3.2** was used to determine the influence of temperature and pressure on the concentration lutein extracted. The assigned values and corresponding lutein concentrations are listed in **Table 4.2** along with the densities resulting from the temperature/pressure combinations. The runs were performed in triplicate by utilising the three separate flow systems of the supercritical fluid extractor (**Paragraph 3.2.1**), and the corresponding lutein concentrations reported in three adjacent columns in **Table 4.2** illustrate an acceptable level of reproducibility.

**Table 4.2** Results of experimental design runs

| Number of run | Pressure (atm) | Temperature (°C) | [lutein] (µg/mL) | [lutein] (µg/mL) | [lutein] (µg/mL) | Density (g/mL) |
|---|---|---|---|---|---|---|
| 1 | 300 | 40 | 23.6 | 27.6 | 25.3 | 0.921 |
| 2 | 500 | 40 | 64.3 | 60.5 | 60.3 | 1.002 |
| 3 | 300 | 50 | 26.6 | 35.2 | 30.8 | 0.882 |
| 4 | 500 | 50 | 56.5 | 56.5 | 54.8 | 0.965 |
| 5 | 400 | 38 | 42.8 | 37.9 | 36.8 | 0.971 |
| 6 | 400 | 52 | 68.3 | 78.8 | 95.5 | 0.923 |
| 7 | 259 | 45 | 17.0 | 17.5 | 18.4 | 0.875 |
| 8 | 541 | 45 | 73.4 | 76.8 | 73.6 | 1.002 |
| 9(C) | 400 | 45 | 48.3 | 42.8 | 45.3 | 0.947 |
| 10(C) | 400 | 45 | 29.6 | 45.3 | 18.7 | 0.947 |
| 11(C) | 400 | 45 | 45.4 | 50.3 | 52.5 | 0.947 |
| 12(C) | 400 | 45 | 28.2 | 47.6 | 52.8 | 0.947 |

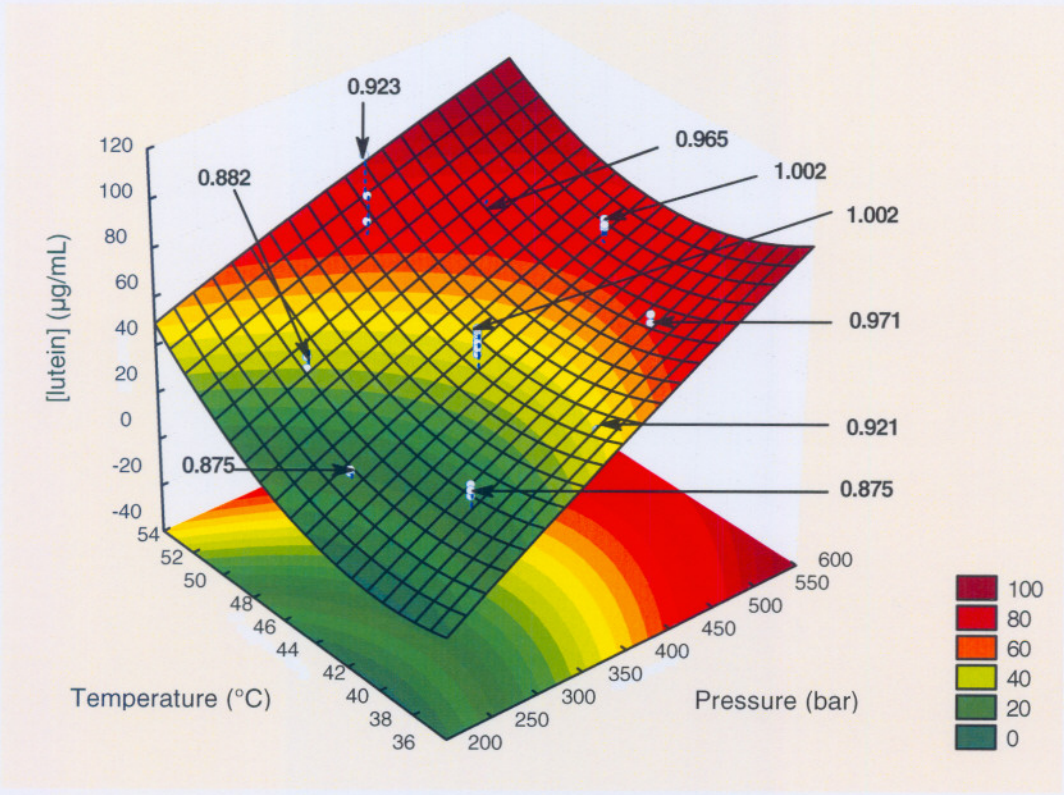


**Figure 4.6** Surface response graph of extraction of lutein as function of temperature and pressure

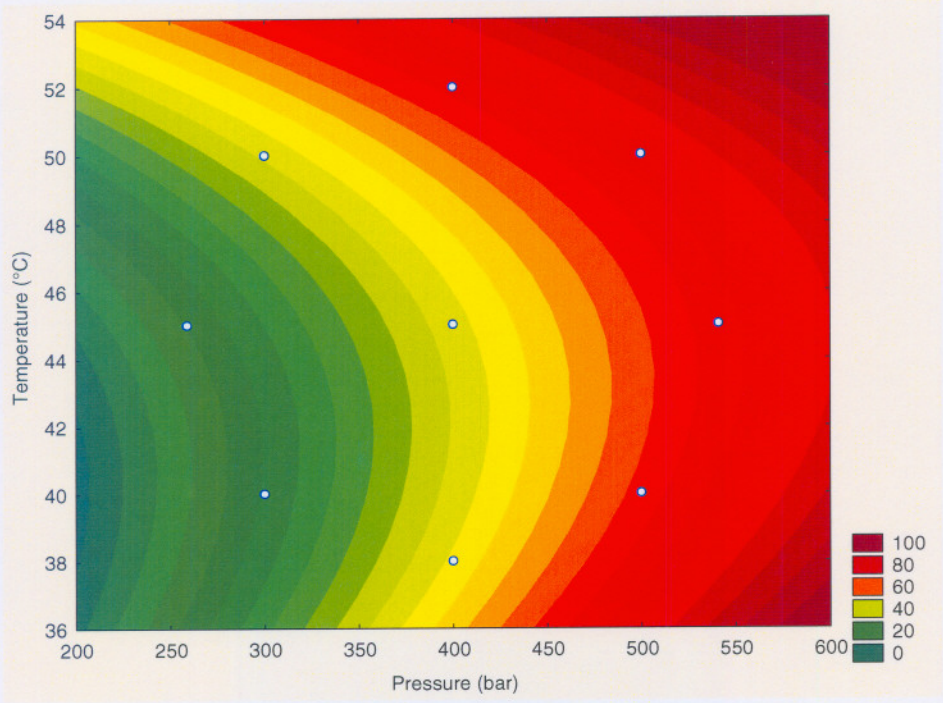


**Figure 4.7** Contour plot showing rotatability of statistical design

### *4.4.1 Surface response graph*

The surface response graph in **Figure 4.6** allowed the experimental conditions at which a maximum lutein concentration was extracted to be read off directly. These optimum conditions turned out to be 52 °C, 400 bar and 0.92 g/mL, at which a lutein concentration of (81 ± 14) µg/mL was obtained. The contour plot in **Figure 4.7** confirms the rotatability of the design, and close inspection of the plot enables the optimum conditions and yield mentioned above to be checked instantly.

The surface response graph illustrates that the lutein concentration depends on both temperature and pressure, though pressure seems to be more important for good recovery of lutein from the plant material. The reason for this is that a rise in extraction pressure at a constant temperature leads to higher fluid density,

which in turn increases the solvent strength of the fluid and thus improves the solubility of lutein in the fluid. The influence of temperature is less significant. A change in temperature either increases or decreases the yield of lutein as a result of two opposing effects. A rise in temperature results in a lower activation energy barrier and thus in improved yield, but at the same time the density of the fluid is reduced and thus less lutein is dissolved. The nett effect of temperature at a given pressure thus depends on which of the two opposing effects dominates and outweighs the other effect.

The reliability of the statistical model underlying the surface response graph in **Figure 4.6** was proven by plotting the observed lutein concentrations against the values predicted by the mathematical model in **Figure 4.8**. It can be concluded from the reasonable fit of the data that a reliable optimisation of the extraction conditions was obtained by the statistical design.

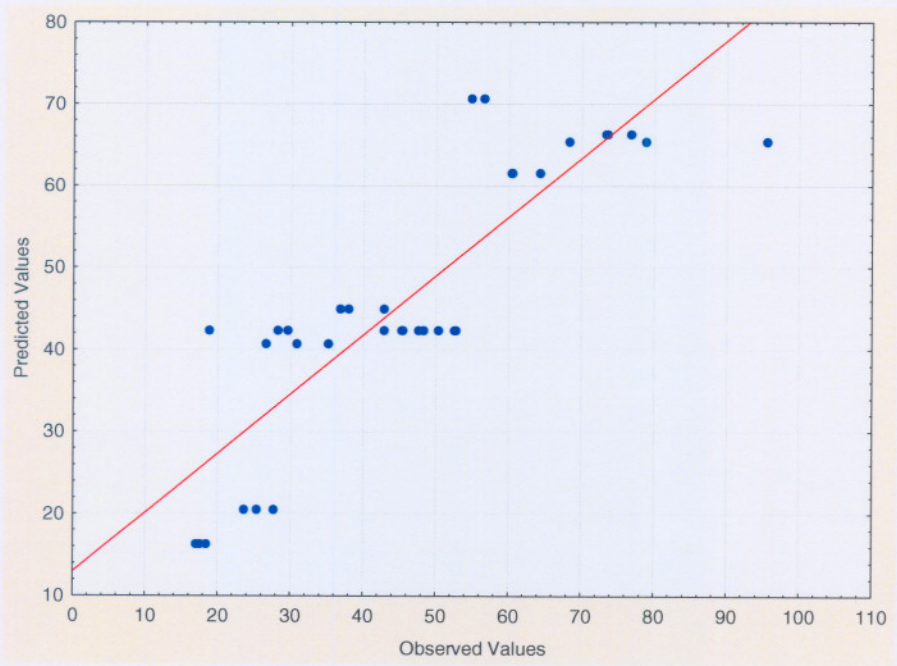


**Figure 4.8** Accuracy of model underlying surface response graph

### 4.4.2 *Solubility profile*

The opposing effects of temperature and pressure referred to in the preceding section jointly establish the density, which is the real factor that determines or limits the extraction process. The extraction runs listed in **Table 4.3** were performed in addition to the extraction runs in **Table 4.2** based on the statistical design in order to give a more complete picture of the effect of density on the lutein concentration by virtue of the solubility profile in **Figure 4.9**.

**Table 4.3** [lutein] at additional temperature/pressure or density values.

| Number of run | Pressure (atm) | Temperature (°C) | [lutein] (µg/mL) | Density (g/mL) |
|---|---|---|---|---|
| 13 | 140 | 70 | 0.529 | 0.463 |
| 14 | 160 | 70 | 0.895 | 0.550 |
| 15 | 200 | 70 | 1.98 | 0.662 |
| 16 | 200 | 60 | 3.73 | 0.733 |
| 17 | 300 | 70 | 19.9 | 0.793 |

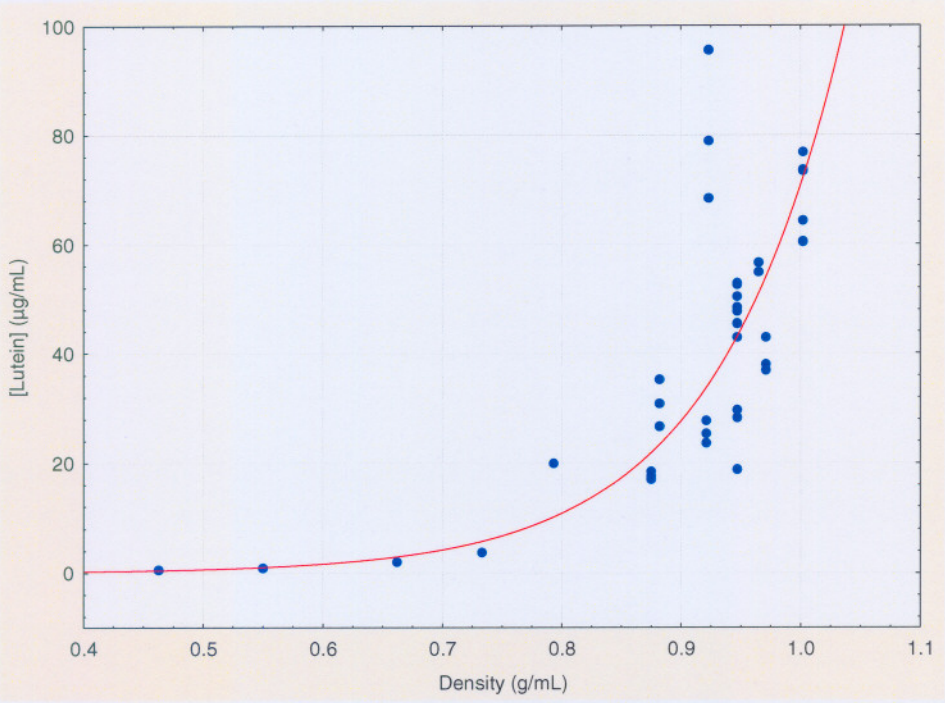


**Figure 4.9** Solubility of lutein in *sc*-$CO_2$

A principal feature of the plotted relationship is the almost exponential increase in lutein concentration as the density approaches liquid-like (or solvent-like) values ($0.8 < \rho < 1.0$ g/mL) at which *sc*-$CO_2$ acts as a solvent capable of dissolving material from the plant matrix. At gas-like densities ($0.4 < \rho < 0.7$ g/mL) the corresponding lutein concentration is almost zero, indicating that chemical dissolution is the only mechanism by which lutein is extracted from the plant matrix by *sc*-$CO_2$.

## 4.5 *Activation parameters*

Activation parameters, such as the enthalpy of activation $\Delta^{\neq}H$ and the volume of activation $\Delta^{\neq}V$ are useful mechanistic indicators, and both these could shed light on the mechanism of lutein extraction by *sc*-$CO_2$.[5, 6]

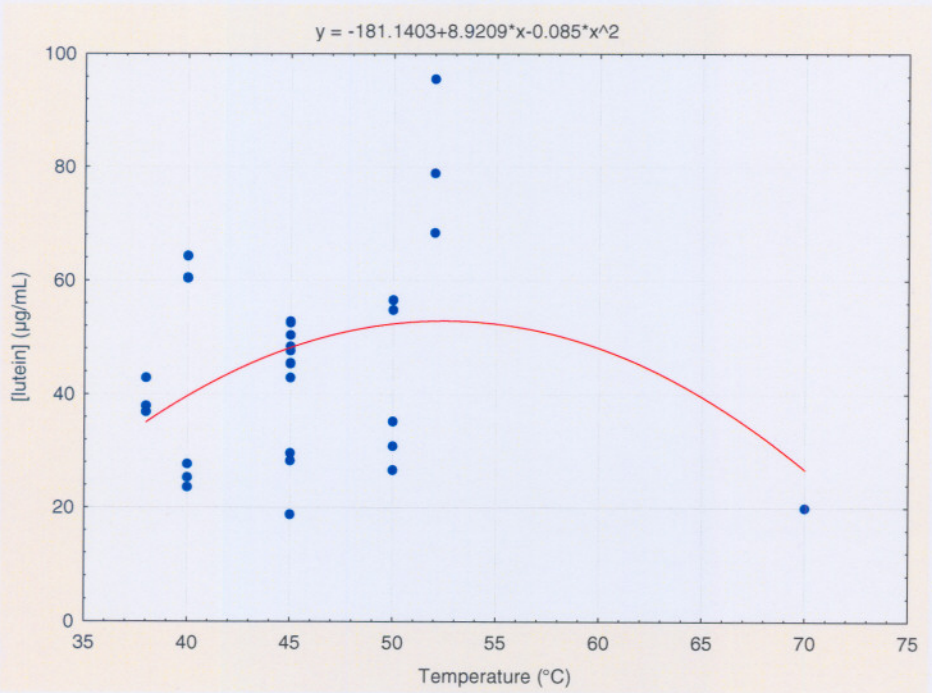


**Figure 4.10** Graph of [lutein] versus temperature

The polynomially fitted data of the graph of [lutein] versus temperature in **Figure 4.10** echoes the temperature dependence of the yield of extracted lutein shown by the surface response graph in **Figure 4.6**. This temperature dependence has both positive and negative attributes, and neither of these is suitable for calculation of the enthalpy of activation since the data is the nett result of opposing tendencies that cancel each other to such an extent that the true enthalpy of activation is hidden.

The volume of activation was calculated from the slope of the straight line in **Figure 4.11** obtained by plotting ln [lutein] versus p as described in **Paragraph 3.5.2**.

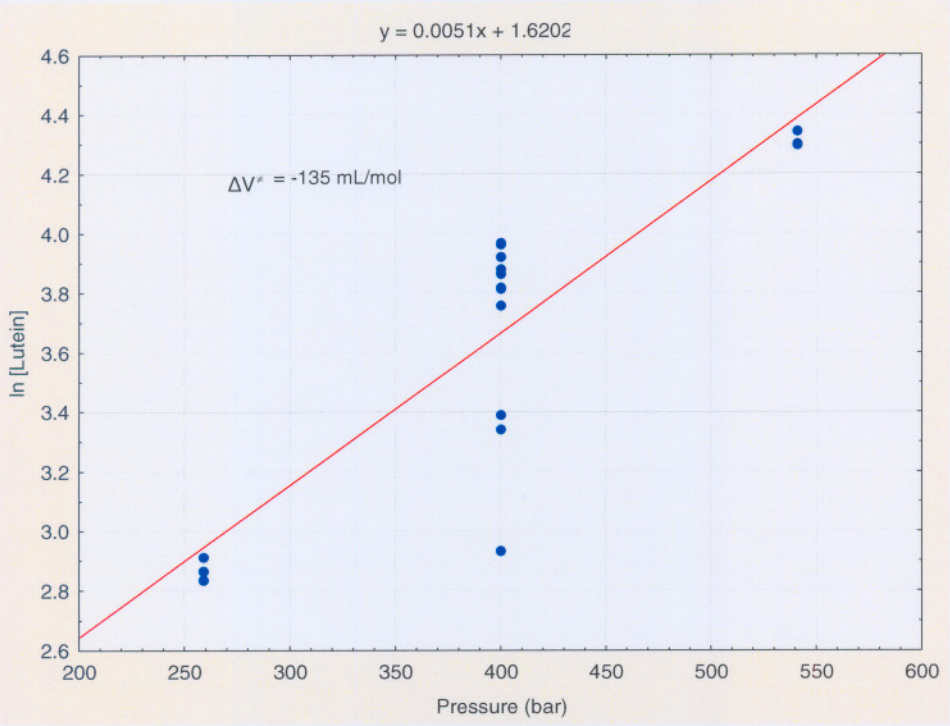


**Figure 4.11** Graphic determination of volume of activation at 45°C

The volume of activation turned out to be $\Delta^{\neq}V$ = -135 mL/mol. This large negative value should be understood in terms of the fact that chemical dissolution comprises two consecutive steps, viz. melting and solvation, and that $\Delta^{\neq}V = \Delta^{\neq}V_{melting} + \Delta^{\neq}V_{solvation}$.[6] Lutein is a solid which, on dissolution in *sc*-$CO_2$, is

converted into the liquid phase during the first step (melting). The value of $\Delta^{\neq}V_{melting}$ is negative as a result of the "collapse" of the lutein molecule (bond/angle rupture) during melting, though it is relatively small negative (typically -10 mL/mol)[6] since the slope of the solid/liquid boundary line of a typical phase diagram is always large[5] even at the high pressures associated with *sc*-$CO_2$. The lutein molecule is solvated by highly compressed $CO_2$ in the second step (solvation), which results in a much larger (can be 10 times as large) negative value of $\Delta^{\neq}V_{solvation}$. The nett value of $\Delta^{\neq}V$ thus stems from two contributions which are both negative.

## 4.6 *Mathematical modelling of extraction process*

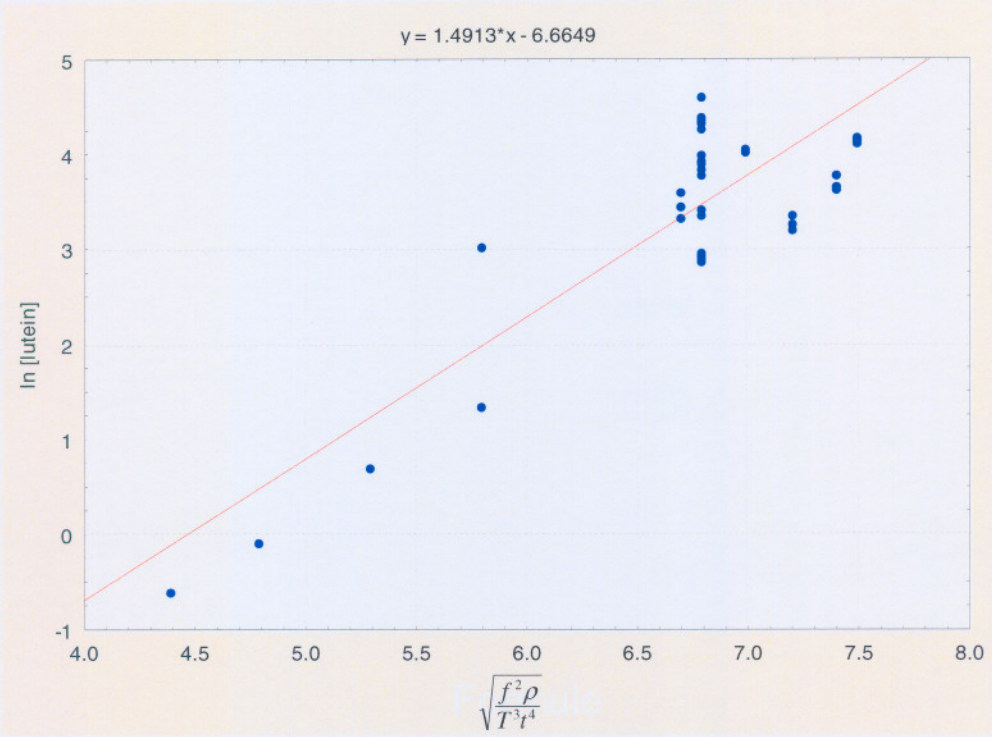


**Figure 4.12** ln [lutein] versus dimensionless grouping of variables

A function based upon a dimensionless grouping of process variables was suggested (**Paragraph 3.6**) as a possible way of describing mathematically the *sc*-$CO_2$ extraction of lutein. The validity of the proposed model is confirmed by the linear relationship between ln [lutein] and the dimensionless grouping of variables in **Figure 4.12** on fitting the extraction data obtained in this investigation. The multivariable function thus offers a means of modelling the extraction and predicting the concentration of lutein extractable by *sc*-$CO_2$ at a given set of conditions.[7]

## *4.7 Hexane versus sc-$CO_2$ extraction*

A single extraction run using n-hexane as extracting solvent was performed on a soxhlet apparatus in order to compare the results obtained by the two methods (**Paragraph 3.2.3**). **Table 4.4** lists the results of two comparable runs performed by these two methods.

**Table 4.4** Comparison of *sc*-$CO_2$ and hexane extraction

| Quantity | *sc*-$CO_2$ | | | *n*-hexane |
|---|---|---|---|---|
| Mass of plant material (g) | 3.0111 | 3.0188 | 3.0688 | 3.0600 |
| Mass of extract (g) | 0.3313 | 0.2456 | 0.3026 | 0.5199 |
| µg lutein / sample | 68.3 | 78.8 | 95.5 | 146 |

The average mass of the *sc*-$CO_2$ extract obtained was 0.2932 g per 3.0329 g of plant material or 9.67%, and the lutein content of such an extract was 80.9 µg per 0.2932 g or 0.028%. The mass of the soxhlet extract obtained was 0.5199 g per 3.0600 g of plant material or 16.99 %, and the lutein content of such an extract was 146 µg per 0.5199 g or 0.028 %.

Although more extract was obtained with n-hexane, the percentage of lutein extracted was quite similar for the two extraction methods. The lutein content of the used plant material seems to be low compared to the figure of 2000 ppm of carotenoids reported in the literature[8], but the percentage lutein within this

amount is unknown. Moreover, since the plant material has been kept for quite some time in storage, a substantial part of the active component could have undergone decomposition with time.

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# Chapter 5

# Conclusion and Perspectives

In this chapter an evaluation of the study is done by considering successes and shortcomings measured against the objectives stated in Chapter 0. A few perspectives for further research are presented.

## *5.1 Successes*

This study demonstrated the viability of extracting lutein from marigold flower (*Tagetes erecta*) using *sc*-$CO_2$. The extract consisted of quite a few more components, mainly lutein esters, but these were not identified as lutein was the target compound. The lutein content of the extracts was determined by HPLC analysis based on a calibration line constructed by plotting the peak area of a series of solutions of a commercial lutein standard against the respective concentrations.

The extraction was optimised in terms of yield of extracted lutein by determining the most favourable conditions (52 °C, 400 bar, 60 min) by means of surface response analysis. The density, and thus the solvent strength of the supercritical fluid, turned out to be the limiting factor controlling the mechanism of extraction. An exponential increase in the yield of extracted lutein at densities where *sc*-$CO_2$ becomes liquid-like and exhibits extraordinary solvent strength confirmed that extraction occurs as a result of chemical dissolution.

The dependence of the yield of extracted lutein on various process variables enabled activation parameters to be calculated and conclusions to be drawn about the mechanism of extraction. An attempt to describe the extraction process by a mathematical function derived by a dimensionless grouping of variables proved to be successful.

A typical *sc*-$CO_2$ derived extract amounted to closely 0.1 g per gram of plant material used, and such an extract contained roughly 25 µg of lutein. These figures are in close agreement with those obtained for a single *n*-hexane derived extract.

## *5.2 Shortcomings*

It could not be concluded on the basis of the results obtained in this study that *sc*-$CO_2$ extraction is superior to or can replace traditional solvent extraction to obtain lutein containing extracts from marigold flower, but the study proved that *sc*-$CO_2$ extraction has advantages over solvent extraction such as shorter extraction times, milder extraction conditions and solvent free extracts while yielding component rich extracts comparable to those obtained by solvent extraction.[1, 2]

Another shortcoming was the often encountered lack of reproducibility and thus unsatisfactory scatter of data in spite of the care taken to fully recover extracted material from the flow lines of the supercritical extractor. This could be ascribed to several factors, including differences in the three separate flow systems of the extractor, inability to exactly maintain conditions during extraction, extent of homogeneity of samples of the plant material, and others.

Although all experiments were done in subdued light, exposure to white fluorescent light and elevated temperatures could not be fully avoided. Experimental work should have been done in air-conditioned and darkened laboratories in view of the sensitive nature of lutein.

The quality of *sc*-$CO_2$ extracted lutein could not be evaluated since specifications for benchmarking of lutein for pharmaceutical or medical purposes are not readily available.

This study demonstrated the feasibility of *sc*-$CO_2$ extraction of lutein from marigold flower, but the batch type of extraction rendered the process commercially unpractical due to low yields and high capital cost. A continuous type of extraction catering for high throughput of plant material needs to be developed and implemented before industrial scale operation may become reality.[3]

## *5.3 Future perspectives*

The scope of lutein analysis can be improved by applying other suitable techniques, such as UV/visible spectrophotometry (in view of the characteristic absorbance of lutein at 445 nm)[4] and GC-GC/TOF-MS (in view of its capability to identify components with overlapping peaks).[5]

Efforts should be made to evaluate the quality of *sc*-$CO_2$ derived lutein in future studies since benchmarking is essential if lutein is to be used in the pharmaceutical or medical sectors.

A more comprehensive comparison between *sc*-$CO_2$ and soxhlet extraction of lutein should be conducted in which UV/visible spectra and/or chromatograms are critically compared in order to establish more specifically similarities and differences in composition and to obtain a more complete picture of the advantages and disadvantages of the two extraction methods.

Finally, a few unresolved matters need to be attended to. These include, among others, the usability of plant material after prolonged storage (3, 6, 12 months), the necessity to pretreat plant material (drying, freeze-drying, grinding) and the effect of different plant materials (species, origin, cultivation).

## *5.4 References*

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# Acknowledgements

*"Two are better than one, because they have a good return for their work: If one falls down, his friend can help him up. But pity the man who falls and has no one to help him up! Though one may be overpowered, two can defend themselves. A cord of three strands is not quickly broken."*

*Ecc. 4:9-10, 12*

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