CHEMOTAXONOMIC STUDY OF FEVERFEW

by

MARK DEREK BURFORD

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Supervisor: Dr R.M. Smith
Department of Chemistry

DECLARATION

This is to certify that I am responsible for the work submitted in this thesis, that the original work is my own except as specified in the acknowledgements or footnotes, and that neither the thesis nor the original work contained therein has been submitted to this or any other institution for a higher degree.

M.D. Burford

Dedicated to Andrew Brian Marsdon

"Beware of systematic errors"

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ABSTRACT

A chemotaxonomic identification of the herbal remedy *Chrysanthemum parthenium* (feverfew) and its reported adulterants *Chrysanthemum vulgaris* (tansy) and *Chamomile vulgaris* (German chamomile) has been undertaken. An initial survey investigated the distribution of phenolic compounds in the plants using a RP-HPLC diode-array system. A chemometric analysis of the data distinguished feverfew from its adulterant German chamomile, though tansy was ambiguous.

In the light of these findings, the GLC of essential oils and in particular the reported active ingredient parthenolide, were used as alternative fingerprinting markers. Conventional extraction techniques were investigated but were considered unsuitable. Supercritical fluid extraction (SFE) was extensively examined and successfully yielded the claimed active ingredient. The determination of the essential oil content of the plants enabled feverfew to be unambiguously identified from both adulterants. The technique was sufficiently sensitive to assign authenticity to feverfew products. During this study a detailed investigation was made of the SFE conditions required to give complete extraction and recovery of the terpenes from a model matrix and from the plant material.

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Aim of study

In the next few years the Department of Health is requiring herbal manufactures to provide bibliographic evidence of efficacy, safety and authenticity of their products. The aim of this study was to develop a technique which would ensure the authenticity of the medicinal herb, feverfew, so that it could be distinguished from its reported adulterants. A range of components in the plant were investigated as suitable taxonomic markers, to produce a fingerprint for the material. Several extraction procedures were assessed, including the conventional methods of steam distillation, organic solvent and Soxhlet extraction and the more recent supercritical fluid extraction technique.

Chapter 1

History of feverfew

1.0 Introduction

Feverfew, (*Tanacetum parthenium* L. Schultz bip.) has been used for many centuries as a folk medicine. It is a yellowish-green perennial, growing to a height of 14 to 45 cm with bipinnate leaves which have a strong camphor aroma [1] (Figure 1.1). The flowering head has a daisy-like appearance, the commercial variety having a single row of white rays and a yellow centre. A double variety is usually cultivated in gardens for ornamental purposes [2]. The plant is said to be plastic, which means that its appearance may differ according to growing conditions. For example leaves may sometimes be more or less serrated and the average size of the leaf may vary. The herb grows wild in British gardens, hedgerows and areas of wasteland. It is also cultivated throughout most of Europe and has been introduced into America. It flourishes particularly in semi shaded positions in well drained soil, though it will grow in virtually any conditions, producing flowers from June to September.

Featherfew, featherfoil, flirtwort, midsummers daisy, nosebleed, bachelors button, wermod and grande chamomile are all common names for the plant. Its botanical nomenclature is also varied. Feverfew has been known as *Tanacetum parthenium* [3], *Chrysanthemum parthenium* [2], *Pyrethrum parthenium* [4] and *Leucantemum parthenium* [5]. There are several varieties of feverfew, the *crispum* variety with curled leaf edges, the *aureum* variety where the leaves are noticeably yellow and a common wild variety with no rays just a yellow centre [6]. Feverfew is part of the daisy or Compositae family. This family is one of the largest plant families, comprising of 1000 genera and 15,000 species. However it is the source of relatively few products of economic or medicinal importance, with feverfew being one of these exceptions.

References to the medicinal use of feverfew go back more than 1900 years to the first century AD to the Greek writer Plutarch who reported the use of the herb [7]. The name feverfew is a corruption of the Latin febris meaning fever and fugure meaning to chase away, referring to the use of the herb in treating fevers. It was popular during the Middle Ages, sixteenth and seventeenth centuries. Bancke's herbal (1525) refers to feverfew under the

entry Febrifuga as "Good to assuage the access (ague or fever), quotidian (fever recurring daily) or cramp" [8]. John Gerard in 1597 considered it "very good for them that are giddie in the head, or which have the turning called vertigo, that is, a swimming in the head" [9]. In 1772, John Mill, MD, in his book "The Family Herbal", remarked of feverfew, "In the worst headaches thus herbs exceeds whatever else is known" [10]. It has been called the aspirin of the eighteenth century [7]. The plant is frequently quoted as having an action on the female reproductive system [2,8,9,11] most frequently being said to expel the placenta and still-born children and to induce abortion. Nicholas Culpeper in 1836 explained that it is a "general strenthener of their wombs and to remedy such infirmities as a careless midwife has there caused" [11]. Other uses have been as a decoction with sugar and honey in coughs and colds and to cleanse the kidney and bladder and expel stones; the distilled water is said to take away freckles and facial spots; the bruised herb heated with oil or fried with wine, and applied outwardly, helps wind and colic in the lower belly; as an infusion it was considered efficaceous against intestinal worms and in female hysteria [11].

As the science of medicine advanced, feverfew, together with many other traditional remedies, fell into relative obscurity. Feverfew was still used by those familiar with traditional remedies, but it is only during the last decade that a revival of herbal medicine has occurred. In the late 1970's, following a series of newspaper reports of successful responses in sufferers who were resistant to conventional medication [13-16], migraine and arthritis patients turned to feverfew as an alternative therapy. This re-emergence has been acknowledged by several scientific publications [17-24], two books [6,7] and the recent addition of feverfew to the British Herbal Pharmacopoeia [25]. However, it is still not seen as a totally bonafide herbal remedy, its absence being noted in the British Pharmacopoeia of which German chamomile is a member [12].

As part of a review of herbal remedies, the Department of Health is requiring manufacturers to provide bibliographic evidence of efficacy, safety and quality. Suppliers in Britain have encountered problems with regard to quality, as both German chamomile (*Matricaria chamomilla* L.) and tansy (*Tanacetum vulgare* L.) have been reported as being incorrectly supplied as feverfew [3]. In view of the possible large scale consumption of feverfew in the country and a need to identify feverfew from its adulterants, a systematic investigation of the plant was undertaken.

FIGURE 1.1. Feverfew (*Tanacetum parthenium* L. Schulzt bip.) in flower. This is the ornamental variety.

1.1. Herbal remedies and the law

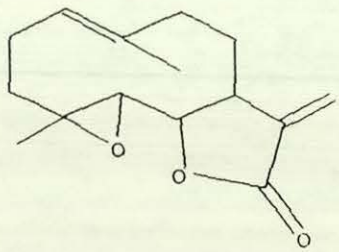
Feverfew is not officially recognised as a medicine as it does not possess a product licence. This means the herb has to be sold as a food supplement and as such no claims about its medicinal applications can be made on the packaging or in advertisements. Legislation introduced in 1968, following the thalidomide tragedy, has led to a far greater control over medicines. The Medicine Act made it illegal to manufacture or sell medicines unless they had been granted a product licence and this was only done when the Committee for the Safety of Medicines was satisfied that any ingredient of a medicine was safe and efficacious. When the Act became effective it was realised that there were many products on the market which could not be examined in this way and they were given a temporary licence, denoted as a "product licences of right". Seven years ago the Department of Health and Social Security (DHSS) established a Committee for the Review of Medicines and announced that the examination of all products having the product

licence of right would be completed by 1988. This has since been amended to 1991, probably in accordance with an EEC directive regarding herbal products in all the EEC countries. This decision has created a crisis among the manufacturers of herbal products. It came as a shock especially as the first indications were that herbal medicines would be subject to the same strict requirements as synthetic drugs. This has now been amended but it is still necessary for the manufacturers to supply evidence of efficacy, safety and quality. Due to political pressure by the herbal product manufacturers, the DHSS made concessions and appropriate bibliographies and similar publications would be accepted as evidence to prove the viability of the herbal remedies. No experimental data is required, but the applicant is obliged to indicate that there are no references in the literature to any toxicity in the plant. However, the question has been posed [19] , "if the active ingredient responsible for feverfew activity is identified, will such an identification transform feverfew from a health food into a drug?" Will regulatory bodies then require many years of laboratory and animal studies before it can reappear on the market? This is as yet still unclear.

1.2. Pharmacology of Feverfew

Fractionation of an extract of feverfew, followed by analysis of the fractions obtained, suggest that the sesquiterpene lactones were responsible for the medicinal properties of the plant [26,27], the major constituent being parthenolide (Figure 1.2) which possess cytotoxic activity *in vitro* [28]. All these sesquiterpene lactones contain an α-methylenebutyrolactone unit as an integral part of their chemical structure. It is this unit which is thought responsible for the antisecretory properties of feverfew, which help alleviate migraine and arthritis.

FIGURE 1.2. Structure of parthenolide.

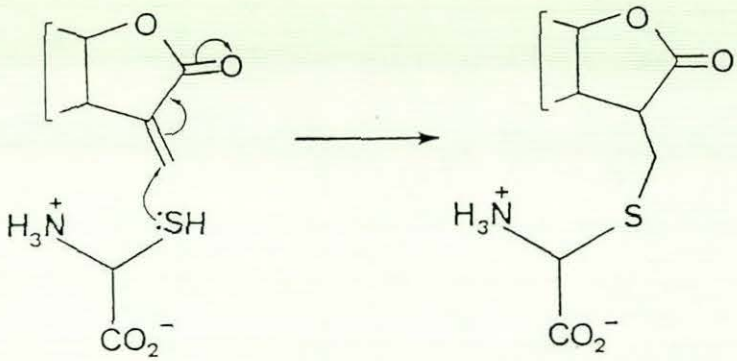


The basis for the ability of feverfew to give relief to migraine and arthritis suffers has come under recent investigation. Conventional treatment for such ailments, involves the use of anti-inflammatory agents, such as aspirin which inhibits prostaglandin and thromboxane synthesis [18]. Prostaglandins are hormone regulators and thromboxanes which regulate the activities of blood platelets, are produced from prostaglandins. Collier *et al.* tested aqueous extracts of feverfew for such anti-inflammatory ability [29]. The feverfew extract suppressed 86 to 88% of prostaglandin production but did not inhibit cyclo-oxygenation. It was concluded that feverfew contained a factor that inhibited prostaglandin biosynthesis, but that it differed from the salicylates, in that it did not inhibit cyclo-oxygenation by prostaglandin synthase (an enzyme functioning in the biosynthesis of prostaglandins from arachidonic acid).

Makheja *et al.* concluded from their research that the antiferbrile and antiplatelet activities of feverfew were due to a phospholipase inhibition which prevented the release of arachidonic acid by appropriate physiological stimuli [30]. Since arachidonic acid is the precursor of prostaglandins they suggest their observations could explain some of the diverse pharmacological activity ascribed to feverfew. It has been suggested that feverfew extracts may interfere with the initial step of thromboxane synthesis, i.e., the release of arachidonic acid from blood platelets [3]. Human blood platelet, labelled with ^{14}C-arachidonic acid, responded normally to thrombin (blood clotting agent) by releasing ^{14}C-arachidonic acid and ^{14}C-thromboxane. The addition of feverfew extracts prevented both the release of ^{14}C-arachidonic acid and formation of thromboxane, so inhibiting platelet aggregation.

This has important consequences as Jones *et al.* describe migraine as a blood disorder, suggesting that the transient blockage of small vessels by platelet aggregations, may cause a migraine attack [31]. The ability of feverfew extracts to inhibit platelet aggregation may explain its use in the treatment of migraine [32]. It has been suggested that the α-methylene-butyrolactone unit on the sesquiterpene lactones, may interact via Michael addition with biological nucleophiles such as sulphydryl groups present in the platelets (Figure 1.3) [33]. This reduces the number of soluble and protein sulphydryl groups in platelets, so reducing aggregation.

FIGURE 1.3. Michael-type addition of an α-methylenebutyrolactone to cysteine.



Feverfew is also a popular herbal remedy for many arthritis sufferers. Rheumatoid arthritis is characterised by the granular white blood cells (polymorphonuclear leucocytes), the secretions from which are implicated in tissue damage that give rise to the arthritis. Feverfew is thought to inhibit polymorphonuclear leucocyte release, this inhibition being much greater than has been achieved with high concentrations of non-steroidal anti-inflammatory agents [20]. Osteroarthritis and rheumatoid arthritis suffers are among those who found feverfew beneficial.

Feverfew has also been reported to possess anti-histamine properties [22]. An extract of feverfew produces a dose dependent inhibition of histamine release from rat peritoneal mast cells stimulated with anti-IgE or calcium ionophore A23187. A possible link has been suggested between the antihistamine properties of feverfew and its recorded uses for insect bites [7].

Feverfew appears to possess antimicrobial activity [34]. Parthenolide was the major antimicrobial constituent of crude chloroform extracts from seeds or leaves of the plant. It inhibited the growth of gram positive bacteria, yeasts and filamentous fungi in vitro. Parthenolide reduced infection when added to spores containing droplets of two pathogens on the feverfew petals and bean leaves. It was not phytotoxic except at very high concentrations. It has been suggested that the role of parthenolide is to protect the plant from pathogens [34].

Other less common uses have been reported in the Dictionary of Modern

herbalism [35]. It claims feverfew is an active stimulant and can be used in cases of sluggish menstrual flow and congestive dysmenhorrhoea (period pains). These painful menstrual symptoms, including nausea, vomiting, diarrhoea and headaches, have possible links with the production of prostaglandins, which feverfew is reported to inhibit. The use of feverfew as a uterine stimulant also links it with a traditional use as an agent for inducing abortion. Furthermore it has been suggested that the herb can aid hayfever suffers, skin disorders such as psoriasis and eczema and even overcome vertigo.

1.3. Dosage

There seems no firm rules for dosage of either the raw plant material or commercial preparations. The leaflet issued by the British Migraine Association advises one large or three small (3 x 3 cm) leaves a day [7,32]. This dosage works out at about 125 mg of dried and powdered feverfew. Commercial preparations vary from homoeopathic preparations to 250 mg capsules. Bio-Health make a 100 mg capsule containing the dried leaf. The Managing Director, David Smith stated that when calculating for dried feverfew, an estimated 500mg of dried plant was required. This was reduced to 100mg, as only the leaf was used, the leaf being seen as containing the highest levels of sesquiterpene lactones [7]. Potters Herbal Suppliers markets one of the largest feverfew tablets on the market at 200 mg. Timothy Whittaker, the company's chief chemist, stated that the 200 mg dose was based on information from users who were growing their own feverfew. It was suggested that there was scope for investigating higher doses of feverfew than is currently being used [7].

However, this is not a universal view among herbalists. Herbalist Claire Swann feels that the dosage should be low, and that the whole plant should be used [7]. Gerard House Limited take this view and produces essence of feverfew subject to a cold process of maceration in water and alcohol which acts as a preserving agent and solvent. Dosage is five drops twice a day. Welda (UK) Ltd take this low dose principle even further. They produce both a tablet and a liquid prepared homoeopathically. They use the traditional 6X potency method where the medicine is mixed one part herb to nine parts base material: then one part of the resulting dilution is mixed with nine parts of the base material and the process goes on until its been repeated six times. By conventional medical standards the amount of the herb in the medicine is barely traceable, but homoeopaths say this process of

potentisation produces a powerful healing force.

As the whole herb or parts of the whole herb are used in commercial preparations, the amount of active ingredient varies from plant to plant, batch to batch and season to season. Some manufactures have investigated the possibility of producing a feverfew tablet or capsule containing a standard amount of the active ingredient, namely the sesquiterpene lactones. Even this can give rise to difficulties, as there are many different sesquiterpene lactones in feverfew with different degrees of activity. R.P. Scherer is one pharmaceutical company which is convinced there is a future for a product with a guaranteed consistent level of active ingredient, a view also shared by Johnson *et al.* [32]. It has already developed Lomigram, which was reported to be standardised on the entire sesquiterpene lactone content by IR analysis [36]. This is intended to provide 0.1 mg of the active ingredient in a capsule which is equivalent to the average amount found in 25 mg of a dried leaf material. Standardisation is not a policy which all companies will be adopting. Timothy Whittaker of Potters has stated that to get a standard amount of sesquiterpene lactones in all the tablets, it may be necessary to vary the amount of herb in the product as the sesquiterpene lactone content of each batch of raw material would vary [7]. An alternative method would be to extract the active ingredient and to put it into a product. However, Potters do not see this as satisfactory, as the extracted sesquiterpene lactones may not be as stable as when they are in the leaf, with the result that the product could have a very short shelf life.

1.4. Efficacy and side effects

Johnson *et al.* carried out a survey of some 300 people who ate fresh feverfew leaves in sandwiches, crushed with honey, or as icing sugar pills to gain relief from the symptoms of migraine [10]. In 93% the condition had been diagnosed by a doctor, and 77% had never taken any other herbal remedy. The average duration of daily use was about two and a half years, and the average daily dose two to four small or one to two large leaves. Seventy per cent claimed that their migraine attacks were less frequent, less painful or both. The remaining 30% were not helped. One third had no further migraine attacks after the initial use of the plant, while four-fifth of all those who stopped taking it had a recurrence of severe migraine in two to three weeks. A further study using 17 patients who ate fresh feverfew leaves daily

were used in a double blind placebo controlled trial of the herb [32]. The mean frequency of migraine attacks in those who were given the placebo increased from the low level of 1.22 attacks each month during self-treatment with the herb, to three times this number on the placebo treatment. Far fewer severe and incapacitating headaches were recorded by the patients taking feverfew than by those taking a placebo. The patients taking feverfew also suffered a lower incidence of nausea and vomiting (39 reports compared with 116 in the placebo group) Furthermore, only 42% of attacks of migraine recorded by patients taking feverfew were associated with these symptoms compared with 79% of those experienced by patients taking the placebo. The global assessment made by the patients at the end of the trial showed that significantly more patients given feverfew thought that they had benefited from treatment.

Johnson *et al.* survey reveals that 82% of users of feverfew reported no unpleasant side effects, but about 12% encountered soreness of the mouth or tongue [10,32]. In 7%, oral problems were so troublesome that discontinuation of use occurred. In most cases the sore mouth was associated with recurrent "aphthous" ulceration, a condition thought to be due to the sesquiterpene lactones. It has been suggested that the allergy reaction of feverfew may been caused mainly by parthenolide [37]. A prerequisite for activity in this allergen appears to be a lactone moiety with an exocyclic α-methylenic functional group.

Sesquiterpene lactones have also been found to cause contact dermatitis. Some patients complain of an itchy skin, possibly accompanied by a rash. Indigestion or colicky stomach pains have also been reported [10]. In view of the association of feverfew with gynaecological treatment, it has recently been advised that pregnant women should not use the herb [6].

1.5. Conclusions

Feverfew, is at present remerging as a popular herbal remedy. More and more evidence is accumulating on the efficacy of the plant, though the mode of action is still unclear. It would appear from these findings, that a method to ascertain the authenticity of the medicinal herb, was required. This taxonomical method development is the aim of the study.

CHAPTER 2

Taxonomic identification

2.0. Introduction

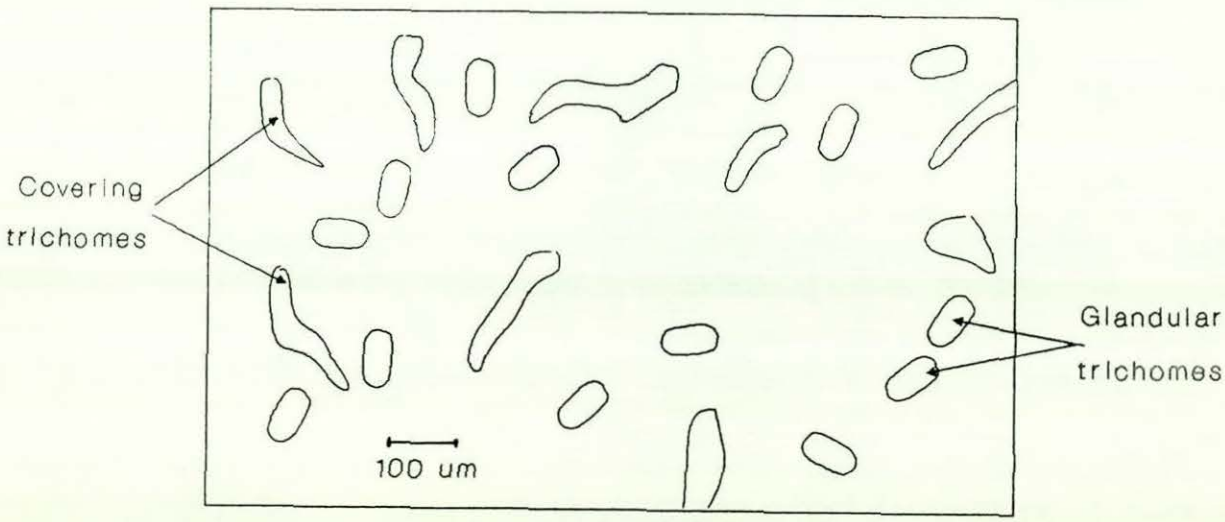
With the re-emergence in the last decade of the interest in feverfew as a herbal medicine of commercial viability, there has been an increase in the demand for the plant. Feverfew was not being grown anywhere on a large scale until the 1980s and companies developing feverfew products for widespread distribution found that their greatest problems was to locate sufficient supplies of the raw material. Feverfew plants were only being grown domestically and specialist herb growers could only offer a limited supply. The manufacturers had little alternative but to import plants from Eastern European countries, Hungary, Czechoslovakia and Bulgaria, or grow their own. Problems were encountered as both German chamomile (*Matricaria recutila* L. or *Matricaria chamomilla* L.) and tansy (*Tanacetum vulgare* L. or *Chrysanthemum vulgaris* L.) were incorrectly supplied from these Eastern European countries [3]. Even if manufacturers, such as Herbal Laboratories grew their own, the same problem of receiving the incorrect plant species were encountered when ordering the original stock [7].

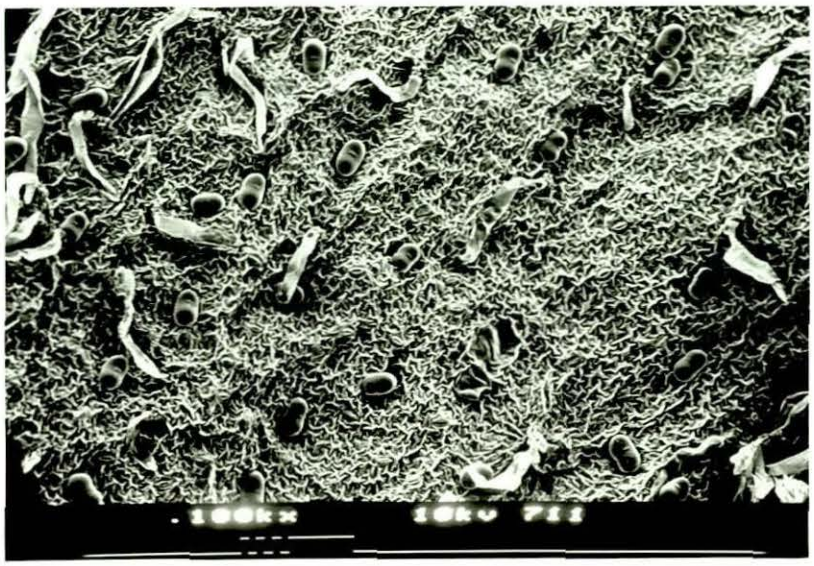
2.1. Microscopic analysis

In the light of such findings and with the announcement by the Committee for the Review of Medicines for the need of herbal remedies to comply with standards of efficacy, safety and quality by 1991, there appeared a need to establish a means of identifying herbal remedies. The analysis must cater for the identification of the whole plant and when it is in a pharmaceutical preparation.

The traditional means of identification is microscopy. Feverfew can easily be distinguished by the presence of glandular and covering trichomes of a type not found in the other species [34,38] (Figure 2.1). The non-glandular trichomes are large, multicellular, uniseriated with a dome-shaped basal cell which often has a striated cuticle. This bears from three to five smaller rectangular cells, the apical cell being very long, flat, strap-like and acutely pointed. The glandular trichomes are found on the surfaces of the leaves, being present mainly on the underside, and also

Figure 2.1. Scanning electron micrograph of trichomes present on the dehydrated surface of a feverfew leaf.

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exist between the ridges of the seeds. The active ingredients of feverfew, the sesquiterpene lactones, are reported to be present in the glandular structures. This confirms the work of Loomis *et al.* who state that the accumulation of sesquiterpenes in large quantities in plants is almost always associated with the presence of glandular structures [39]. In theory German chamomile show be easily distinguished from feverfew as the herb is usually supplied as dried flower heads and not the whole herb, as is the case for feverfew. However, care must be taken as experience has show that often leaf and stem fragments are present in the chamomile preparations [25].

Microscopic identification of the herb may also be applicable in pharmaceutical preparations, but, there are limitations. In some tablet formulations, , such as in Lomigram feverfew tablets, the plant is micromacerated to a green gel. In such instances, microscopic identification is no longer possible. A similar problem is also incurred with feverfew tinctures. However, microscopic examination of the herb can identify the majority of feverfew preparations, though such microscopic methods require considerable skill and years of experience. Some degree of computerisation has taken place in this field of expertise, for example with the data base handling programme called MICROID, which matches physical characteristics with species [40]. In a case of adulteration which leads to a case in a civil or criminal court of law, a second confirmatory test would normally be required. The most obvious choice would be that of chemical analysis, so that a chemical knowledge of the plant would be advantageous.

2.2. Chemical constituents of feverfew

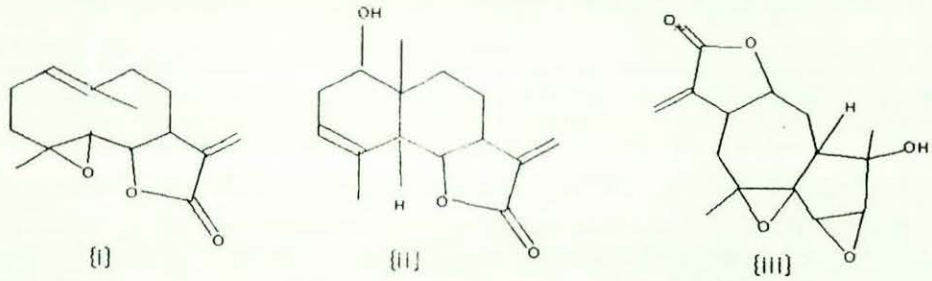
Although feverfew is a common plant, comparatively little phytochemical data is available, though the chemistry of the Compositae family has been reviewed [41]. This reports that probably every member of the family contains flavonoids and most contain volatile oils and triterpenes. The presence of unique structural types of sesquiterpene lactones and a variety of different groups of acetylenes further characterise the family, which also lacks any major classes of alkaloid or non-protein amino acids.

The tribe Anthemideae to which feverfew and its adulterants belong, is distinguished from neighbouring tribes by the presence of strong aromatic odours mainly based on high concentrations of terpenes. Camphor, borneol, and 1,8-cineole are the major and most widespread structural types in the

feverfew and tansy genera, *Tanacetum* [41]. The flavonoid content of this tribe has also been examined and the aglycones luteolin and apigenin are the most abundant, with the most common glycosides being 7-glucoside and 7-rutinoside. The relatively uncommon 7-glucuronide is thought to be systematic of the *Tanacetum* genera [41].

A limited survey on feverfew has been carried out by various research groups. The majority of the work has been focused on the principle sesquiterpene lactone, parthenolide, which was first isolated from feverfew by Romo *et al.* [42]. They also extracted the minor sesquiterpene lactones, santamarine and chrysartemin A and B (Figure 2.2).

FIGURE 2.2. Chemical structure of {i} parthenolide, {ii} santamarine and {iii} chrysartemin [42].



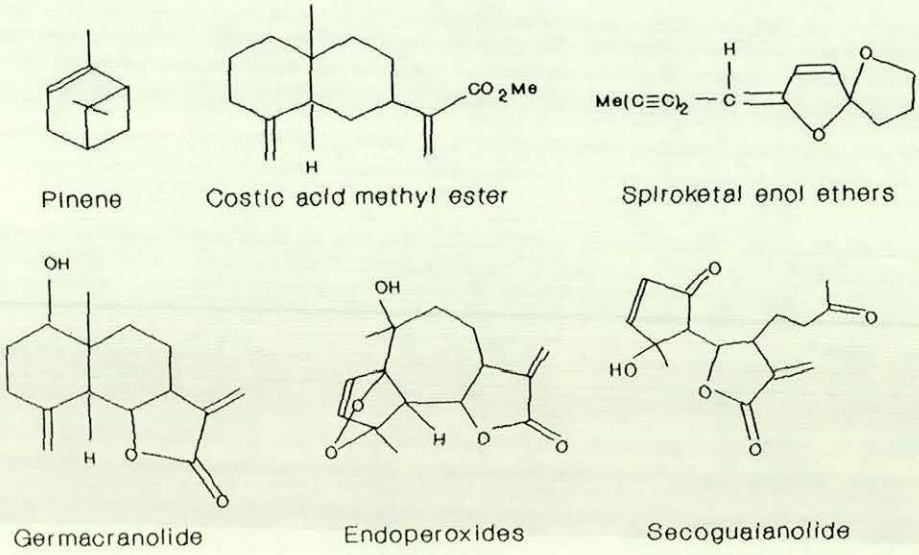
Drozdz *et al.* developed a thin layer chromatography (TLC) method for the identification of sesquiterpene lactones from *Chrysanthemum* species using selective spray reagents based upon resorcin, fructose and ferric acid solutions, which gave cherry, violet or lilac colours with parthenolide [43]. Fingerprint patterns were observed, based upon the content of other similar compounds in the plant extracts. The same research group also showed that species exhibiting a high bitter index, as calculated by the Polish pharmacopoeia method, had the highest percentage of sesquiterpene lactones. Such lactones were most abundant in feverfew (*C. parthenium*), tansy (*C. vulgare*) and balsamita (*C. balsamita*) [44]. The group provided a simple method for the quantitative determination of sesquiterpene lactones by infrared analysis [45]. For each *Chrysanthemum* species a suitable standard lactone was used, and these give sharp distinct absorption bands at 1770-1750 cm^{-1}, which are claimed to be easily distinguishable from the background absorbances, even in crude mixtures. Based on the parthenolide standard, feverfew was shown to contain 0.87% by dry weight of sesquiterpene lactones.

Groenewegen *et al.*, investigated the active anti-secretory extracts of

feverfew [46]. They were able to identify five of the active compounds that were present, parthenolide, 3-β-hydroxyparthenolide, seco-tanaparthenolide A, canin and artecanin, all of which are sesquiterpene lactones. Jessup also investigated the active ingredients of feverfew for spasmolytic activity in guinea pig ileum [47]. A number a sesquiterpene lactones were identified in the active extracts by GC-MS and NMR analysis. Parthenolide and chyrsartemin A were recognised as well as three novel components, partholide, chrysanthemolide and chrysanthemonin [27]. Recently a chlorine containing sesquiterpene lactone was identified from feverfew, though its biological activity was not ascertained [48] and it is suspected to be an artifact.

A more general analysis of the essential oils in feverfew was undertaken by Bohlmann *et al.* [49] (Figure 2.3). The roots of feverfew were shown to contain spiroketal enol ethers while the aerial parts contained a complex mixture of 36 different constituents. The aerial extract was separated on a silica column, the less polar fraction (petroleum ether eluent) contained β-farnesene, camphor, pinene derivatives, bornyl acetate, costic acid methyl esters, spiroketal enol ethers, and costunolide (a germacranolide). The more polar fraction (ether-methanol eluent) consisted mainly of parthenolide, though a variety of other sesquiterpene lactones of the germacranolide and guaianolide class were obtained. Reynosin, 3β-hydroxycostunolide, 3β-hydroxy parthenolide, artemorin, the hydro ketone, the ketone and the epoxide of artemorin were all isolated and identified. Furthermore, traces of 8α-hydroxyestafiatin, canin, artecanin, a further epoxide, two endoperoxides as well as secoguainolides were elucidated.

FIGURE 2.3. Examples of the major classes of essential oils identified in feverfew [43].

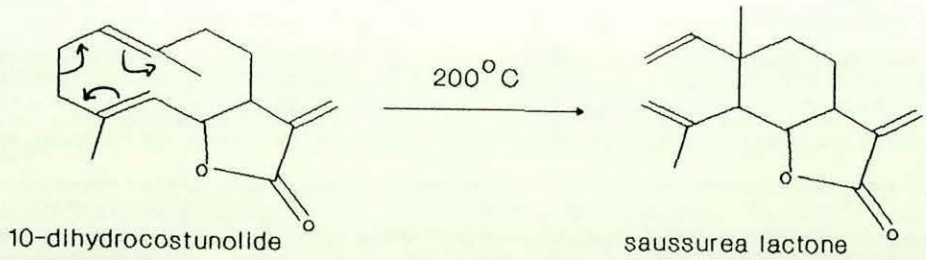


2.2.1 Stability of active ingredients in feverfew

As the sesquiterpene lactones are thought to be linked to the medicinal properties of feverfew [18,26], the long term stability of such components is of great concern. Some sesquiterpene lactones have been shown to be unstable [50], especially when subjected to high temperatures. For example, active aqueous extracts containing sesquiterpene lactones from feverfew, have been shown to inhibit prostaglandin biosynthesis in bull seminal vesicle. However, boiling the extract for ten minutes causes it to lose all activity [29]. Therefore, some manufactures of feverfew products try to avoid heating the plant material during the sample preparation. An American manufacture, Abco Laboratories, California produce feverfew tablets using a freeze-drying technique, in an attempt to minimise plant deterioration and extent the shelf life of the product [7].

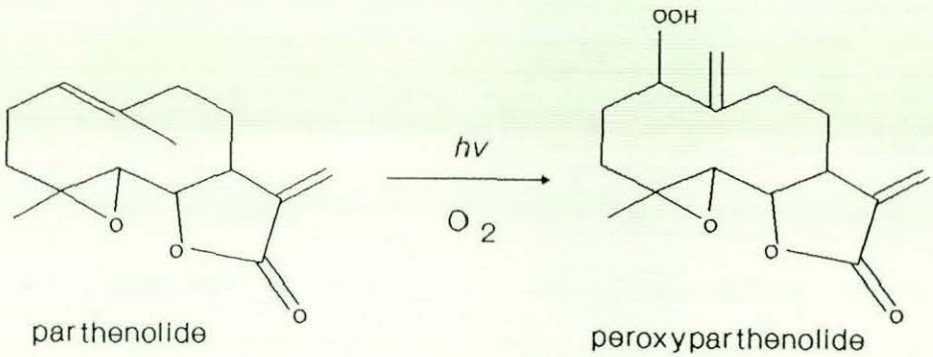
Furthermore, high temperatures may cause some sesquiterpene lactones to undergo Cope rearrangement, so that artifacts may be produced during the work-up of some plant extracts, for example during steam distillation or Soxhlet extraction. The germacranolide dihydrocostunolide undergoes Cope rearrangement to the elemanolide saussurea lactone on heating to 200°C for a few minutes [51] (Figure 2.4).

FIGURE 2.4. Cope rearrangement.



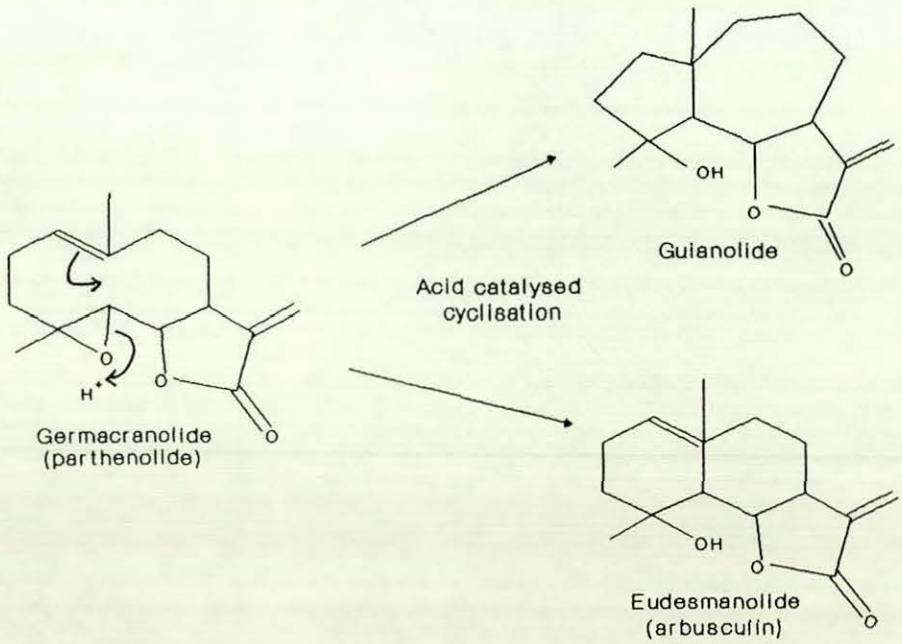
Another possible cause for sample deterioration could be due to photo-oxygenation. Photo-oxygenation of parthenolide extracted from *Magnolia grandiflora* L. yields peroxyparthenolide (Figure 2.5), this oxidised derivative being found naturally in the plant [52]. This photo-oxygenation of parthenolide is also thought to occur in the tansy variety *crispum* [53]. Therefore, if the closely related feverfew products were stored in clear glass bottles in sunlight, it is possible that such a degradation may take place, though at present no such degradation product have been found naturally in feverfew.

FIGURE 2.5. Photo-oxygenation of parthenolide.



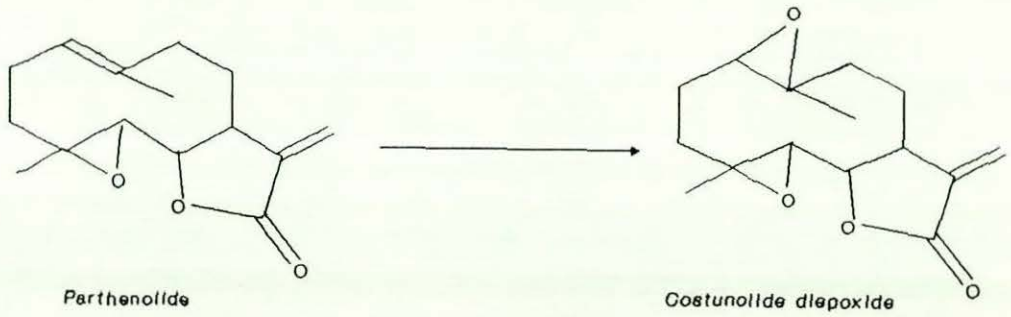
The germacranolides such as parthenolide are also prone to catalysed cyclisation (Figure 2.6), being the precursors of the eudesmanolides and guianolides in the Compositae family [41]. This cyclisation may be acid catalysed [27,54] as well as initiated by enzyme mediated processes [41]. It is possible that cyclisation could be induced in the extraction processes. Chloroform is a typical solvent used in essential oil extraction and always contains traces of hydrochloric acid [53]. Eudesmanolides and pseudoguianolides do exist in feverfew [49], though neither of the predicted structures in Figure 2.6 have been identified in feverfew extracts. However, the eudesmanolide, arbusculin is found in nature [54].

FIGURE 2.6. Cyclisation of parthenolide.



The instability of parthenolide has been demonstrated by El-Feraly *et al* [55]. Chloroform solutions of parthenolide were found to contain costunolide diepoxide when stored at room temperature for a few days. Within four weeks this transformation was nearly complete (Figure 2.7). This type of air oxidation has been reported for other sesquiterpenes [56]. Costunolide diepoxide is thought to be the precursor of a complex sesquiterpene chrysanthemonin, which has been isolated from feverfew [27]. Another artifact of storage is the polymerisation of parthenolide [55]. Govindachari *et al*. reported dimerisation of parthenolide after being stored for a few months [57].

Figure 2.7. Air oxidation of parthenolide.



2.3. Chemical constituents of tansy

Tansy is well known in Europe, Asia and North America. Tea preparations from the dried plant are used as an antiseptic, insecticide and anti-dandruff agent. The essential oils from the plant have long been used in medicine as an expectorant and a vermicide, in spite of its high toxicity, which is thought to be related to the thujones [58]. Commercial oils characteristically contain 50% thujone [59], though in Argentina, tansy plants have been identified as being particularly rich in thujone, which makes up 92% of the tansy oil [60]. In the 19th century, before the advent of more refined analytical techniques, camphor, borneol, pinene and camphene had been described as additional constituents [59]. Since tansy plants are widely diffused across Europe, more recently detailed studies have been made of the chemical composition of the essential oils [61-63]. Great differences were found in the composition of the essential oils, so that discrete, well defined chemotypes have been identified. At least 6 chemotypes were found in

German [63], 8 in Finland [64], 26 in Hungary [65,66], 1 in North Italy [59] and 4 in Holland [58]. Also seasonal variations in the volatile oils from tansy plants grown in Canada have been studied and variations in the content of the minor components were found in the very young plants [61].

In the Finnish study tansy was grown for three years in the same environmental conditions, it was considered that essential oil variations were independent of these conditions and thus may be genetically determined [64,67]. It appeared that in Finland, camphor was the main component, but in Canada and Central Europe it was thujone. This may indicate better adaption of the camphor types to the nordic environment. It is now thought that eight well defined chemotypes exist, depending on the concentration of sabinene, thujone, umbellulone, camphor, bornyl acetate, α-pinene, 1,8-cineole and germacrene D [67].

One of the earliest detailed analysis of tansy investigated the essential oil content of the plant by packed column GC [61]. By this method around 25 different oil components were detected, of which about half were positively identified. The main components were camphor (25%) and thujone (15%), with α-pinene, β-pinene, 1,8-cineole, γ-terpinene, arteminisia ketone, umbellulone, borneol and humulenol being present as minor constituents. The most recent survey on tansy oil was able to distinguish 50 components of which all but 4 had been positively identified by GC-MS [58]. The majority of chemotaxonomic investigations on tansy have been concerned with the oxygenated sesquiterpenes in the plant, many of which are claimed to be novel. Appendino *et al.*, identified a new hydroperoxysesquiterpene lactone, crispolide, with a modified germacrane skeleton from the aerial parts from the tansy variety, *crispum* [68]. More recently the same research team identified several novel terpenoids in tansy, a guaianolide named vulgarolide [69] and sesquiterpene alcohols called tanacetols [70]. Chandra *et al.* afforded two new lactones from the plant, the germacranolides 8-oxo-2α-9-dihydroxy-*trans,trans*-germacr-1(10),4-dien-trans-6,12-olide and 8α,9β-dihydroxy-*trans,trans*-germacra-1(10),4-dien-*trans*-6,12-olide [71]. Ognyanov *et al.* elucidated a new terpenoid, *cis*-longipinane-2,7-dione, which was isolated from tansy flowers [72] and a new lactone, 1-epi-ludovicin [73].

Over 15 different known sesquiterpene lactones have been isolated from botanically non-specified forms or varieties of tansy. For example, dentatin [74], chrysantin, tamarin, tanacin, tabulin [75], tanacetin, 1-β-hydroxyarbusculin-A, reynosin and santamarin [76,77]. Tatridin-A and tatridin-B, costunolide dieoxyde, artemorin and even parthenolide, have been

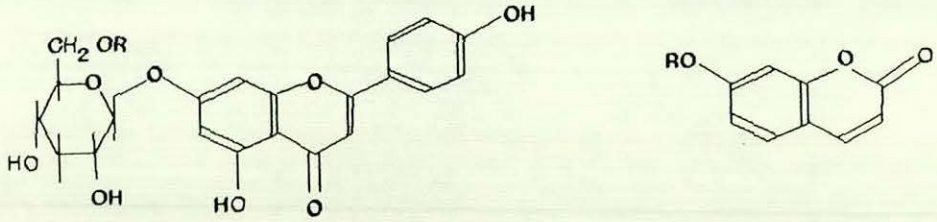
isolated from a tansy type specialising in germacranolides [78]. Two independent research groups claim that parthenolide is present in some of the tansy varieties [62,68], with one of the groups claiming that in *Tanacetum vulgaris* var. *crispum*, parthenolide is the main sesquiterpene lactone [68].

A series of additional components have been elucidated from tansy, with Chandler *et al.*, investigating the sterols and triterpenes in the plant [79]. By using TLC and MS the sterols, cholesterol, campesterol, stigmasterol and β-sitosterol were identified, with β-sitosterol being the major sterol. Similarly the triterpenes, α- and β-amyrin and taraxasterol were identified. Tansy has also been shown to contain the flavonoids, apigen, luteolin, chrysoeriol, diosmetin, isoharmnetin, quercetin and auxillarin [80], as well as jaceidin and jaceosidin [73].

2.4. Chemical constituents of German chamomile

The constituents of German chamomile are well documented as it is used therapeutically for its antiinflammatory and spasmolytic effects. It is notable for its relaxant properties, calming restlessness and tension, reducing allergic responses and the intensity of many gynaecological problems, in particular, dysmenorrhoea [81-83]. These pharmacological properties have been associated with the flavones and coumarins of the drug [84]. Apigenin, apigenin-7-glucoside and apigenin-7-acetylglucoside are the main constituents of the flavone fraction 85,86] and umbelliferone and herniarin are the main coumarin components [87] (Figure 2.8).

FIGURE 2.8. Structure of apigenin-7-glucoside (1), apigenin-7-acetylglucoside (2), umbelliferone (3), and herniarin (4).



1 R = H
2 R = Ac

3 = H
4 = Me

Dried flowers of German chamomile from Bulgaria contain 0.2-0.3% of apigenin and its glucosides as a mixture with other flavones, from which separation is claimed to be quite difficult [85]. The coumarin content of German chamomile flowers from various geographical locations in Egypt, Argentina, Italy and Bulgaria, were investigated [87]. The herniarin content was found to be almost constantly greater than that of umbelliferone, the ratio being about 5:1, with 7-14 mg of umbelliferone and 39-71 mg of herniarin being obtained from a 100 g of dried flowers.

Other flavonoids have also been distinguished namely luteolin and its glycosides, quercetin, quercimeritrin, rutin, hyperin, patuletin and its glycosides, isorhamnetin and its glycosides [88]. From these the chamomile flavones, apegenin, luteolin, patuletin, and quercetin have marked musculotropically and spasmolytic effects, with apigenin and its glycosides being by far the most active [89]. The plant phenolics, chlorogenic acid and caffeic acid have also been extracted from German chamomile [90]. Up to now the spasmolytic activity has been ascribed mainly to the hydrophilic compounds. An analysis of the lipophilic, essential oil components has been carried out in which (-)-α-bisabolol, the bisabol oxides A and B have been identified [89]. All demonstrate spasmolytic, musculotropic action, (-)-α-bisabolol being the most potent. A more recent investigation has shown that the terpenes, bisabolone oxide, β-farnesene and chamazulene are also present, these oils being obtained by supercritical fluid extraction [91].

2.5. Chemotaxonomy

With the rapid development of phytochemistry, there has arisen the hybrid discipline between chemistry and taxonomy, known as chemotaxonomy which is the use of chemical constituents as characteristics components in the identification of plants [40]. This discipline is to be applied to the present study of identifying feverfew in pharmaceutical preparations and to distinguish it from its adulterants. There are a variety of chemical classes used as potential taxonomic markers, notably, alkaloids, non-protein amino acids, terpenes, sulphur compounds, essential oils, carbohydrates and proteins. Perhaps one of the most useful class of compounds for such a study are the phenolics, with which numerous taxonomic classifications have been achieved [40]. It is this group which are to be used as taxonomic fingerprints for feverfew identification.

2.5.1 Phenolics

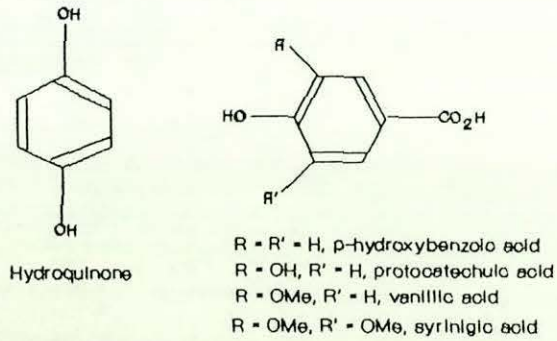
The phenolic compounds embraces a large array of chemicals, possessing an aromatic ring bearing one or more hydroxyl groups together with a number of other constituents. For convenience plant phenolics can be divided into three groups:-

Phenols and simple phenolic acids (C_6 and C_6–C_1 structures)
Phenylpropanoids (C_6–C_3 structure)
Flavonoids (C_6–C_3–C_6 structure)

{i} Phenols and simple phenolic acids.

Free phenols are relatively rare in plants, with hydroquinone (Figure 2.9) being probably the most widely distributed [107]. In contrast a range of substituted benzoic (C_6–C_1) acid derivatives are common in plants [107]. These phenolic acids usually occur in conjugated or esterified forms [107]. Universal among the angiosperms are *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid and syringic acid (Figure 2.9).

FIGURE 2.9. Structures of a phenol and phenolic acids.

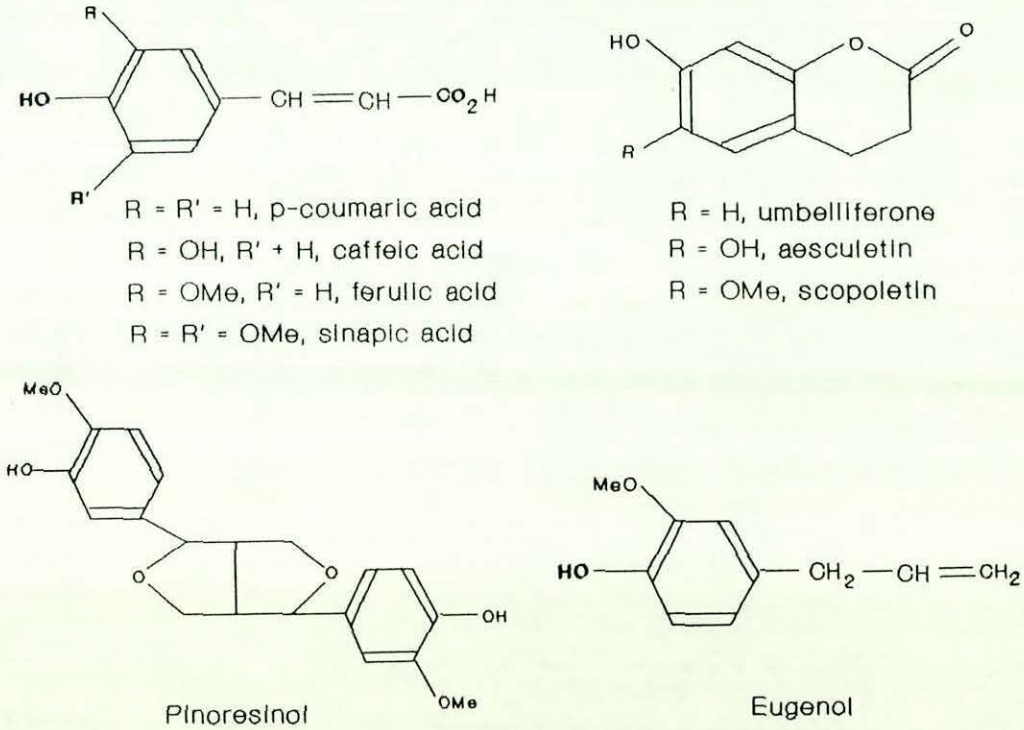


{ii} Phenylpropanoids.

Phenylpropanoids are naturally occuring phenolic compounds which have a basic C_6–C_3 structure and include hydroxycinnamic acids, coumarins, lignans and phenylpropenes (Figure 2.10). The most widespread are the hydroxycinnamic acids, the most ubiquitous being ferulic, sinapic, caffeic and *p*-coumaric acids. The most widespread plant coumarin is the parent compound, coumarin itself, which occurs in over 27 plant families [107]. Coumarins are often associated with the aromatic principle of new mown grass

and over 50 different hydroxylated coumarins are known [92]. These exist as a variety of sugar esters or glycosides and their distribution is restricted to a few plant families including the Compositae family [41]. Lignans, dimeric C_6–C_3 compounds such as pinoresinol, are mainly found in heartwoods [107]. The phenylpropenes are usually isolated in the "essential oil" fraction of plant tissues, together with the volatile terpenes. They are lipid-soluble as distinct from most other phenolic compounds. Some structures are widespread, such as eugenol, the major principle of oil of cloves. Others are restricted to a few families.

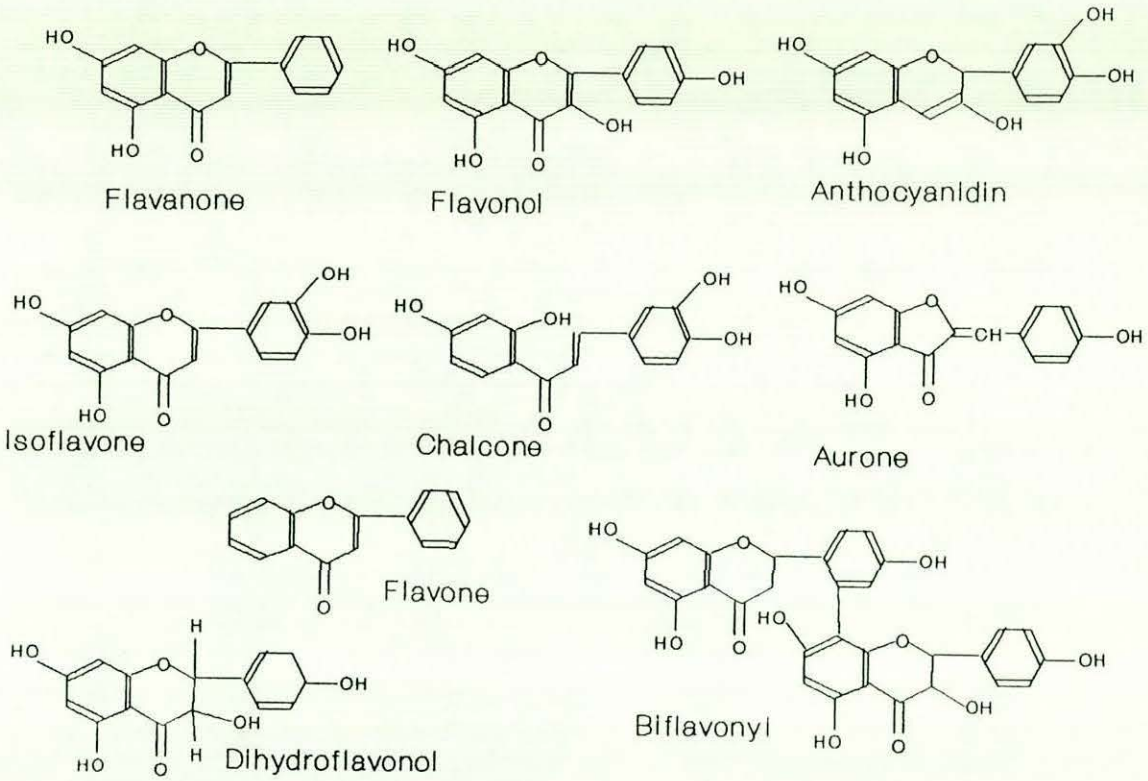
FIGURE 2.10. Structure of phenylpropanoids



{iii} Flavonoids.

This group of compounds share a basic C_6–C_3–C_6 structure and include by far the largest and most diverse range of plant phenolics. They may be classified into 9 main classes [107] (Figure 2.11). Most flavonoids occur as glycosides in which the C_6–C_3–C_6 aglycone part of the molecule is esterified with a number of different sugars. This linkage or glycosidation between a phenolic hydroxyl group and any one of a large array of sugars (over 50), renders the aglycone more soluble in the cell sap and may also confer stability.

FIGURE 2.11. Chemical structure of different classes of flavonoid.



2.5.2. Distribution of the flavonoids

Flavonoids are widely distributed in all species and parts of the vascular plant, though some classes are more widely distributed than others. For example the flavones and flavonols are universal in flowering plants while the isoflavones and biflavanols are often associated with particular plant families. Furthermore, the polymeric flavonoids are reported as only being present in wood and bark [92]. Some flavonoids coexist with others, the coloured anthocyanins in the petals are almost invariably accompanied by colourless flavones or flavonals. Recent research has established that the flavones are important co-pigments being essential for the full expression of anthocyanin colour in floral tissues [93]. A general trend to the distribution of the flavonoid classes in the vascular plants is given in Table 2.1.

TABLE 2.1. Distribution of the different flavonoid classes.

Flavonoid class	Distribution
Anthocyanins	scarlet, red, mauve and blue flower pigments, also present in leaf and other tissues
Proanthocyanidins	mainly colourless, in heartwoods and in leaves of woody plants
Flavonols	mainly colourless co-pigments in both cyanic and acyanic flowers, widespread in leaves
Flavones	same as flavonols
Glycoflavones	same as flavonols
Biflavonyls	colourless, almost entirely confined to the gymnosperms
Chalcones + aurones	yellow flower pigments, occasionally present in other tissues
Flavanones	colourless, in leaf and fruit (especially in citrus)
Isoflavones	colourless, often in root, only common in one family, the Leguminosae

2.5.3. Chemotaxonomic use of phenolics

The distribution of plant phenolics has been used extensively to support the morphological taxonomy of plants. Early applications of phenolics to taxonomic problems are found in the work of Bate-Smith who established a background knowledge of the their distribution [94,95]. In these studies it was concluded that the flowering pigments were generally too variable to be chemotaxonomic markers, so that more emphasis was placed on the phenols situated in the seeds and vegetative tissue. Bate-Smith ascertained that the presence of leuco-anthocyanins could be correlated with the woodiness of plants. Over 60% of the "woody" families examined contained leuco-anthocyanins, whereas they were only present at 15% in the "herbaceous" families. Initially such chemotaxonomic investigations were be based on a single compound. However, inevitably there are complications since most phenolics are found in more than one species, so that the majority of chemotaxonomic studies are now based on an array of components to fingerprint the plant and are frquently used in conjunction with numerical pattern recognition methods.

For example, it was demonstrated that there was sufficient differences in the total flavonoid content, to distinguish species or groups of species in the genus *Plectocima* [96]. In the case of *Cibadium*, *o*-methylated flavonoids provide the most meaningful taxonomic information and subsequently divide the species into two groups [97]. Glennie *et al.*. demonstrated a correlation

between flavonoid chemistry and plant geography in the *Senecio radicans* complex [98]. It was revealed that taxa from Madagascar, the Canary Islands and Kenya were markedly different in their flavonoid complement than those from South and South-West Africa. Harborne *et al.*, undertook a survey of aerial tissues from 42 European taxa of the genus *Teucrium* and indicated that at least one of the taxon *Teucrium compactum*, was misplaced within the genus based on its flavonoid content [99].

It must be noted that flavonoid evidence may prove ambiguous. In the case of the genus *Carmichaellia*, it was reported that the correlation between the flavonoids and present taxonomic characterisations were inconclusive [100]. With the *Stevia* species it was ascertained that no clear conclusions could be drawn from its flavonoid distribution [101]. A similar situation was reported for the Dilleniaceae family [102]. Therefore, the validity of plant phenolics as chemotaxonomic markers will very much depend on the plant species under investigation.

2.5.4. Isolation and identification of phenolics

No category of small molecules, which have been examined from a chemotaxonomic viewpoint, have proved so popular as that of the phenolic compounds. The main reasons for this popularity is that they are widely available, quickly and simply extracted from the plant material, easily separated by thin layer chromatography (TLC) and fairly readily identified by location reagents.

The primary choice for the extraction of these plant phenolics is via boiling alcohol. This normally prevents enzymic oxidation to which the phenolics are prone. Previous work suggests that with this extraction method the majority of the components are flavanoids, due to their retention times and UV-visible spectrum [103-105]. Selective extractions are possible, the aglycones being soluble in benzene to chloroform and the glycosides in acetone to water [106].

The classic procedure for detecting simple phenols is by means of the intense purple, blue or black colours many give in solution when 1% aqueous or alcoholic ferric chloride is added [107]. The majority of plant phenolics can be detected on TLC plates by means of their colours or fluorescences in UV light, the colours being intensified or changed on fuming with ammonia vapour [92,107].

Gas liquid chromatography (GLC) has not been widely used for phenol

separations, partly because most phenols have to be converted to suitable derivatives (trimethylsilyl ethers or acetates) to make them sufficiently volatile. However, it may be an important technique when complex mixtures of simple phenols occur in any one plant tissue. Cured tobacco leaf, for example, contains 38 phenols ranging from phenol itself to 4-methyl-2,6-dimethoxyphenol [108]. Separation of the acetates was obtained using three columns: polyphenol ether OS124 (PPE); trixylenyl phosphate (TXP); and the diethylene glycol succinate (DEGS).

Much of the recent work on phenolic analysis has been on reversed-phase high performance liquid chromatography (RP-HPLC), normal phase HPLC being avoided due to the possible danger of some of the highly polar substances becoming irreversibly retained [109]. A series of different systems in terms of eluent, column and detector have been reported, but due to the complex array of phenolics present in an extract, a gradient elution system is by far the most popular, coupled with a U.V detector, as all phenolics possess a strong chromophore. Neiman *et al.* reported that gradient elution with aqueous methanol containing 0.1% acetic acid on a μBondapak C18 column, provided a good general separation of phenolics [110]. Similarly a gradient elution system of 10-80% methanol-butanol in acetic acid and water on a Hypersil C18 column was used to separate a mixture of phenolic compounds from various Prunus tissue [111]. With such gradient systems it is possible to separate a potentially large number of plant phenolics, as was demonstrated by Casteele *et al.*, who separated 32 different flavonoids in a single run. A LiChrosorb RP-18 column was used with a linear gradient elution of 7 - 80% methanol in formic acid and water [109].

Elution systems for specific groups of flavonoid have also been devised. The separation of anthocyanins, anthocyanidins and proanthoyanidins has been achieved with a combination of isocratic and linear gradient systems (methanol/formic acid/water) [112]. A similar case can be found for the analysis of flavone glycosides in the Gentiana species [96] and the anthocyanins from fruit [113]. A gradient elution system with 15-60% acetonitrile in water containing 2% acetic acid has been used to separate the flavones and coumarins in German chamomile extracts [86].

Isocratic systems have to a lesser extent been able to separate the flavonoids. The polymethoxylate flavones in orange juice have been separated successfully using an isocratic phase of tetrahydrofuran-acetonitrile-water (22:6:72 v/v) [114]. The phenolics from grapes have been analysed via an isocratic system of water-acetic acid-methanol (65:5:70 v/v) [115].

Isocratic liquid chromatography has also been used for the simultaneous determination of *Passiflora incarnata* and *Crataegus monogyna* flavonoids using acetonitrile-water-acetic acid (18:82:1 v/v) [116].

So far all these HPLC systems have incorporated a UV detector, however electrochemical detection is also becoming increasingly popular [117-119]. Comparisons between the two means of detection suggested that the dual-electrode amperometric detector could be used to determine the degree and position of hydroxylation of the flavonoid, so providing additional structural information to the analyst [120,121]. It also claimed to have the advantage of greater sensitivity but is less sensitive to changes in the mobile phase conditions associated with gradient elution, than the corresponding UV detector.

Recently further improvements in the detection systems have allowed much faster data collection and consequently identification of the eluting compounds. This has come about with the introduction of the diode array [121-123] and programmable multiwavelength UV-visible detectors [124]. This simultaneous detection at different wavelengths and the measure of the UV spectrum of each compound during the elution, allows an easy and rapid means of detection. The flavonoids can be divided into a number of classes, based on their absorption maxima [107] (Table 2.2).

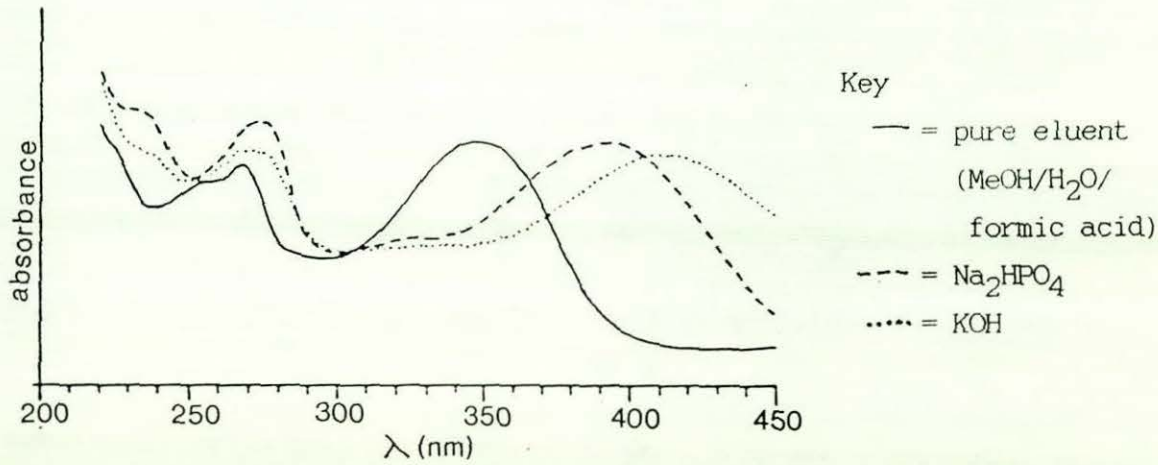
TABLE 2.2. Spectra characteristics of main flavonoid classes.

Principal maxima (nm)	Subsidiary maxima (nm) (relative intensities)	Indication
475 - 560	≈275 (55%)	anthocyanins
390 - 430	240 - 270 (32%)	aurones
365 - 390	240 - 260 (30%)	chalcones
350 - 390 250 - 270	≈ 300 (40%)	flavonols
330 - 350 250 - 270	absent	flavones + biflavonyls
275 - 290 ≈ 225	310 - 330 (30%)	flavanones + flavanonols
255 - 265	310 - 330 (25%)	isoflavones

Some of these compounds have such closely related structures that characterisation by their UV-visible spectrum could prove insufficient. Derivatisation with reagents inducing a shift of the U.V. absorption maxima may be required to furnishes additional structural information. The

application of such shift reagents (weak base, strong base, aluminium chloride) to flavonoids has been extensively described [103,106,124,125]. A weak base (sodium acetate or sodium monohydrogen phosphate) deprotonates only the more acidic phenolic groups, while a strong base (sodium methanolate or potassium hydroxide) reacts with all phenolic groups, except those forming a hydrogen bond with the keto function of the flavonoid (Figure 2.12). Aluminium chloride in neutral solutions forms complexes with ortho-dihydroxyl groups and or with keto functions having a hydroxyl group in the *para* position [92]. The former complexes are unstable when HCL is added.

FIGURE 2.12. UV spectra of isoorientin [124].



The diode array detector can also be used to assess peak purity and be a guide to identification, by means of absorbance ratios [126,127]. The absorbance ratio of two different wavelengths is independent of the concentrations of the compounds. If a peak is pure the absorbance ratio will be constant and form a horizontal plateau versus time throughout the entire elution of that peak. However, the wavelength ratios will not stay constant, but will vary with time, if two or more compounds showing spectral differences overlap. It is claimed that as the absorbance ratio is specific for each compound it can be used for identification (since the extinction coefficient at each wavelength, and therefore their ratio are intrinsic characteristics of each compound) [128]. This could have important implications for flavonoid analysis in which the complex mixtures often possess compounds which coelute.

Further identification may come from the cleavage of the glycosidic bond on flavonoid glycosides. Hydrolysis with hydrochloric acid and enzymes results in the formation of their monomeric aglycones as well as an increase of the peak area assigned to the monomeric compounds. This technique has been applied for the phenol analysis of purified spruce needle extracts of *Picea abies* species [123] and to even estimate the degree of glycosidation of flavanols [122].

CHAPTER 3

Chemometrics survey

3.0. Introduction

In this study, plant phenolics were investigated as potential chemotaxonomic markers. However, due to the large number of phenolics present in flowering plants, interpretation of their distribution is often complex and time consuming. Therefore a computerised pattern recognition method was assessed, to ascertain the potential of the phenolics to fingerprint the plant extracts. In recent years mathematical optimisation and classification techniques have been applied to a growing number of similar problems in analytical chemistry [129-135]. There are several reasons for this, including: increasing difficulties encountered in interpreting the results of analytical techniques which produce simultaneous information concerning a large number of parameters; and a recent adoption of pattern recognition techniques to chemical purposes (chemometrics). In many traditional classification methods, the potential of the data is not fully exploited as often only the most distinctive parameters are chosen, so often a certain amount of information is lost. In chemometrics, the optimal combination of all parameters leads to a better identification because more information is used.

Pattern recognition is usually used as a result interpreter, so as to classify samples on the basis of their measured parameters into classes or clusters. Some sort of feature extraction nearly always takes place. For example, the signal produced by a chromatograph is seldom used as a direct input into pattern recognition. The data entered into the pattern recognition analysis is usually represented by multidimensional data vectors. For complex mixtures such as plant extracts, the vector would have a dimension equal to the number of peaks in the chromatogram. The value for the vectors are then the peak heights or areas. To minimise quantitative variations between extracts, these values would be expressed relative to an internal standard.

The major task of pattern recognition is to define the criteria required to classify individual samples into groups. In this technique two different situations are considered according to whether the number and identity of

the classes are defined or not. In the first instance, one speaks of supervised learning and in the second of unsupervised learning. Initially in this study an unsupervised learning procedure was undertaken using principal component and hierarchical cluster analysis. These techniques determine the similarities and thus differences between the samples and whether they fall into natural groupings. In these analyses there is no prior knowledge of the data or of the type and number of groups. A semi-supervised learning technique was also investigated, called sums of squares analysis, which assumed that each object belongs to one of n predetermined number of classes so enabling a decision plane to be determined. In supervised learning the groups are predefined and the programme seaches for characteristic features by which membership can be identified.

3.2. Principal component analysis

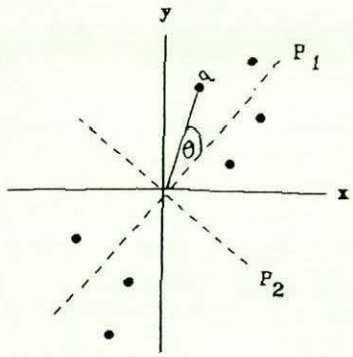
The aim of principal component analysis (PCA) is to summarise and to simplify the interpretation of large quantities of data. The original data sets are converted by a linear transformation into a much smaller set of characteristic principal component scores, which are chosen to reflect the most significant variations. This enables PCA to reduce the dimensionality of the problem under consideration to within human grasp. This reduced 2 or 3 dimensional space will hopefully maintain as far as possible the structure present in the original data.

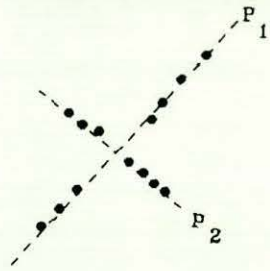
The first principal component may be regarded as the line of best fit (in the least squares sense) which represents the best weighted combination of the variations between the n-dimensional observations, to account for the maximum differences between the data sets from the samples (Figure 3.1). These variations which could be peak areas in a chromatogram etc, may be represented in one dimensional space by taking their projections onto an eigenvector P_1 line. If, as is usual, all the variations between the samples cannot be accounted for using one eigenvector, a second eigenvector is assigned that is orthogonal to the first and describes the maximum amount of the remaining variation. This process continues to give successive eigenvectors (equal in total, to the number of variables such as peaks in a chromatogram) of decreasing significance. It is usually desirable to use the first 2 or 3 eigen vectors which account for the majority of the differences, the later values being ignored.

FIGURE 3.1. Plot of original data with the first two eigenvectors superimposed.

P_1, P_2 = eigenvector axis which form a sub-space

Projection of points from 2 dimensions to 1 dimension





How well the linear transformation P_1 approximates the original n dimensional configuration, is determined by its corresponding eigenvalue. This eigenvalue is expressed as a percentage variance. In this example the P_1 line would have a percentage variance of about 80% and this accounts for 80% of the original variation in the data. The eigenvectors are expressed as eigenvector loadings. These loadings range from +1 to −1 and are the cosines (θ) of the angles between the eigenvector and the variable axes (i.e. point a in Figure 3.1). High loadings correspond to high correlations (large coefficients) where the angle between the eigenvector and a variable is very small {cos(0°) = 1} [136]. As the first eigenvector is presumed to account for the majority of the variation in the data, a high correlation indicates that the variable (i.e. a peak in the chromatogram) is highly discriminatory. Small loadings correspond to low correlations (small coefficients) where the eigenvector is orthogonal or nearly orthogonal to the variable {cos(90°) = 0}. The loadings are thus the contribution of that variable to the principal component score. The original data matrix has now been represented as :-

$$\begin{bmatrix} 1 & 1 \\ 1 & 1 \end{bmatrix} \Rightarrow \underbrace{\lambda \begin{bmatrix} a_1 \\ a_2 \end{bmatrix}}_{P_1} \quad \text{and} \quad \underbrace{\lambda \begin{bmatrix} a_3 \\ a_4 \end{bmatrix}}_{P_2} \quad \text{and} \ \ldots.$$

λ = large eigenvalue with corresponding eigenvectors

λ = smaller eigenvalue with corresponding orthogonal eigenvectors

The number of eigenvalues and their corresponding eigenvectors is determined by the number of variables present in each data set. Visualisation of the results of PCA is usually achieved by plotting pairs of the first few principal component scores (PCS), which are obtianed from the eigen values. So that for each data set there is a corresponding PCS (1st, 2nd etc) whose significance is defined by the percentage variance. In mathematical terms, the first principal component is the linear combination of the variances that has the smallest squared errors when used to estimate the original variables. The arithmetic value of each principal component is given by the equation [132] :-

$$PC = a_1 \frac{(x_1 - \bar{x}_1)}{sd_1} + a_2 \frac{(x_2 - \bar{x}_2)}{sd_2} + \ldots$$

PC = principal component score of sample (i.e. plant material)
x_1 = measurement of original variable (i.e. first peak area)
$\bar{x}_1$ = mean value for the corresponding variable (i.e. mean peak area of first peak)
sd_1 = standard deviation for corresponding variable (i.e. sd of first peak area)
a_1 = loading of the linear transformation (i.e. loading of first peak for the corresponding eigenvector)
x_2 = measurement of next original variable (i.e. second peak area) etc.

The data is usually represented as a plot of the scores for the first (largest) principal component against the second (second largest) principal component. It is possible to go onto a three dimensional plot using the first three principal components and these should account for the majority of variation in the data. Having established these principal components, a further analysis can be undertaken to ascertain what these variables have in common. The first two sets of principal components may be rotated in order to find a new set of components which distinguish the data better. The varimax method tries to find a new matrix where particular coefficients are relatively large or relatively small, compared to the original ones. The idea is that each variable should be heavily loaded on as few principal components as possible, so that a limited number of highly characteristic variables are identified.

3.2. Hierarchical cluster analysis

The other major unsupervised technique is cluster analysis, which seeks to separate a set of data into groups or clusters. The cluster analysis procedure is based on agglomerative (hierarchical) or divisive methods, the

former being more frequently used. The former method proceeds by a series of successive fusions of the data points into groups. The divisive method is the reverse procedure. Hierarchical cluster analysis is based on the concept of similarity. This is usually measured in Euclidean distance d_E.

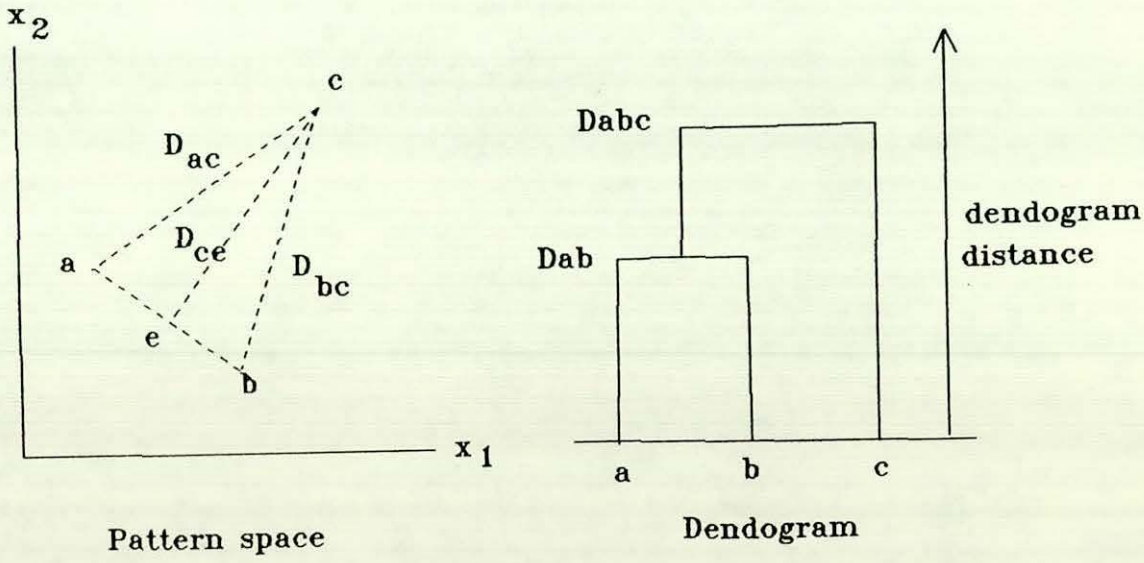
$$d_E = \left[\sum_{i=1}^{N} (a_i - b_i)^2 \right]^{1/2}$$

The a_i and b_i are the values of the i-th variable for the data sets a and b, respectively, and N is the number of variables.

This enables a distance matrix to be calculated, in which the mutual distances between all possible pairs of objects are determined. The distances are ranked according to their increasing values. In each step, the smallest distance is searched for and the two corresponding patterns are combined to a new point half way between the old ones. The number of patterns is thereby reduced by one. The distance matrix is recalculated for the reduced set and again the nearest pair of points is sought. This process is repeated until all the patterns have been combined (Figure 3.2).

Points a and b are the nearest in the original data set (a,b,c) and therefore are merged to a new point e. The new distance D_{ce} is obtained by averaging the distances D_{ac} and D_{bc}. These results are represented by an inverted tree structure or dendogram. This is a two dimensional diagram illustrating the "fusions" which have been made at each successive stage of the analysis (Figure 3.2).

FIGURE 3.2. Calculation of mutual distances between all the variables in the data set.



Euclidean distance is often less than satisfactory particularly when the variables vary in magnitude or are correlated. Therefore the results are usually expressed as the similarity, S_{ij} between the two points i and j as calculated from equation 3.1. The similarity values range from 0 (the two points furthest away) to 100 (coincident points). The grouping can be stopped at any stage to display the required degree of discrimination and can be used to determine the number of groups formed.

EQUATION 3.1.

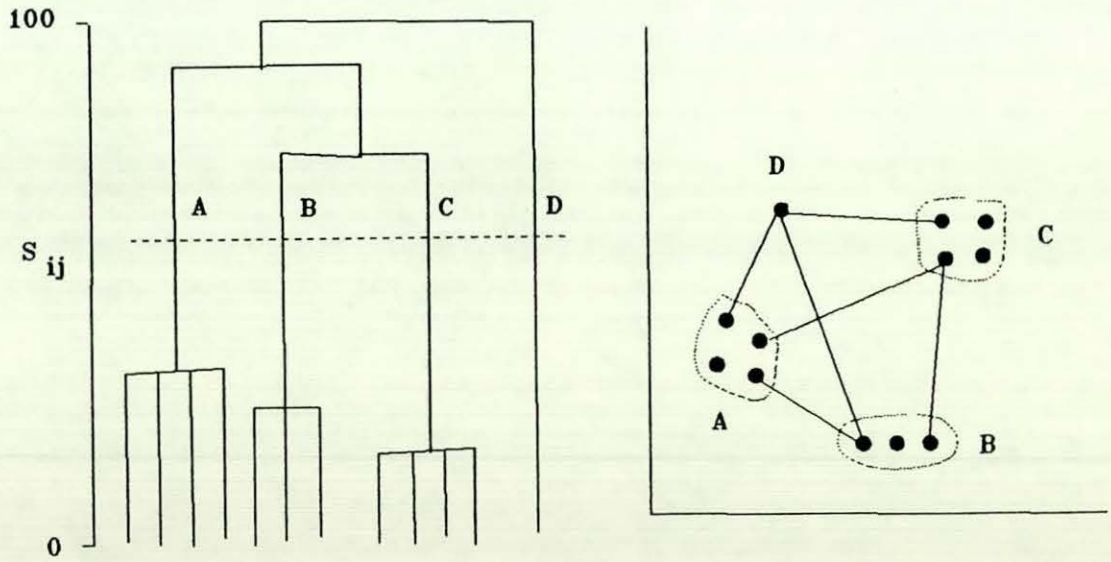
$$S_{ij} = 1 - d_{ij} / dmax$$

d_{ij} = the distance between the points i and j
dmax = the maximum distance between any two points.

3.2.1. Single linkage method

There are many methods of cluster analysis but perhaps the most popular is the single link or nearest neighbours method. In this method, the distance between the groups is defined as the distance between their closest members, so that the "connections" occur via a minimum total length of all line segments (Figure 3.3).

FIGURE 3.3. Single link minimum spanning tree.



The minimum spanning tree (Figure 3.3) displays all the groupings, from when the samples (i.e. plant extracts etc) are ungrouped to when the samples have all been grouped toegther. A line has been drawn on the tree at a similarity of 65 to demonstrate how the groups A,B,C,D are linked by their shortest distances. However, it is often clearer to use a dendogram that displays the similarities at set intervals of 100, 90, 80 etc, as this can often give a more distinctive classification.

The number of neighbours to be considered in the classification may also be varied. If only one neighbour is used in the classification (K=1, 1NN method) the class membership of the first (nearest) neighbour gives the class membership of the unknown. If more than one neighbour is used, a voting scheme is usually applied to determine the class of the unknown. Whether more than one neighbour should be used for classification or not depends on the classification problem.

3.2.2. Complete linkage method

The complete linkage or furthest neighbour technique evaluates the distance between two clusters as the longest distance that can be found between any pair of points from the corresponding clusters.

3.2.3. Average linkage method

In this method the distance between two groups is defined to be the average of the similarities between all pairs of individuals. The distance between two groups is therefore calculated as the average distance between all pairs of objects in the two groups I and J :-

$$D_{IJ} = 1 / (n_I \times n_J) \sum D_{ij}$$

where n_I and n_J are the number of elements in the corresponding groups and D_{ij} is the distance between two objects i and j in the groups I and J respectively. This method may be weighted so that the smaller cluster is always levelled with the larger one. This enhances the influence of a small distant cluster (outlier) if it is to join a larger group of objects.

3.2.4. Centroid method

In the centroid method the cluster of points is represented by a centroid which is a point somewhere in the middle of the cluster, like the centre of gravity. The distance between the clusters are the distances between the corresponding centroid points.

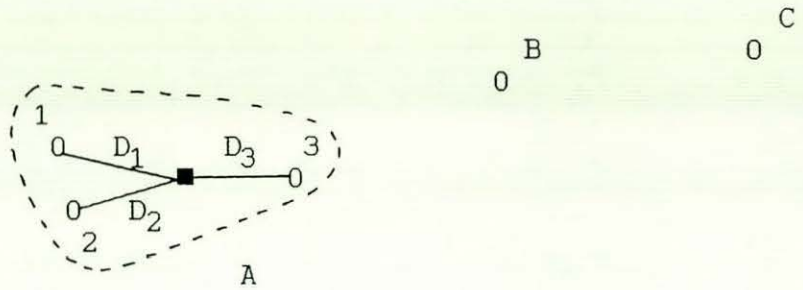
3.2.5. Median cluster analysis

The disadvantage of the centroid method is that if the sizes of the two groups to be "fused" are very different, then the centroid of the new group will be very close to that of the larger group and may remain within that group. This means the properties of the smaller group are then virtually lost. A way to avoid this situation is to weight both groups to give the smaller one a larger influence on the shift of the centroid point. To achieve this the number of objects in both clusters is considered to be equal, to give the median method. The median method is therefore only a special case of the centroid method.

3.3. Non-hierarchical cluster analysis

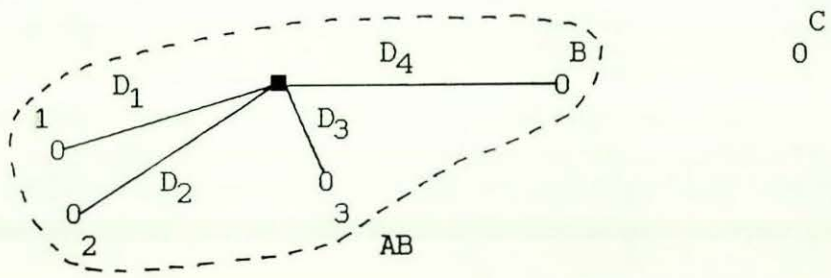
Non-hierarchical cluster analysis is another viable alternative to pattern recognition and is a pseudo-supervised learning technique, as it does not possess a true training set. In this study the Wards method was used. This method is based on statistical minimisation of clustering "expansion". This means that at any stage of an analysis the loss of information, which results from the grouping of the individuals into clusters, can be measured by the total sum of squared deviation of every point from the mean of the cluster to which it belongs. At each step of the analysis, union of every possible pair of clusters is considered and the two clusters whose combination results in the minimum increase of the error sum of squares, are combined. Therefore the distance between the two clusters has no meaning as a real geometrical distance, it is a purely statistically evaluated parameter (Figure 3.4).

FIGURE 3.4. Sum of squares analysis of original data.



■ = mean of group A
D = deviation of each point.
sum of squares for group A = $\sqrt{D_1} + \sqrt{D_2} + \sqrt{D_3}$

incorporating group B :-



■ = new mean calculated for group AB
sum of squares for group AB = $\sqrt{D_1} + \sqrt{D_2} + \sqrt{D_3} + \sqrt{D_4}$

In this instance the two groups to be merged are A and B as they give rise to the smallest sum of squares. The Wards method is normally regarded as a very efficient clustering method and favours the grouping of small clusters. In this study the method is used to classify the objects into a number of classes, the number being pre-determined by the user.

3.4. Applications of chemometrics

Chemometrics has been applied to a variety of analytical problems, the majority of which involve establishing the authenticity of complex materials, especially those associated with the food industry such as identifying wines [133], whiskies [135] and flavourings [132]. Kwan *et al.*, used principal component factor analysis to investigate the correlation

between objective chemical measurements and subjective sensory evaluations [133]. The pattern recognition analysis drew on the entire elemental and organic composition of 40 wines of *Vitis Vinifera* var. "Pinot Noir" from France and the United States. From this study a series of components were identified which appeared to be indicative of the flavour classification, so that an ad hoc relationship between sensory and chemical data was established.

The American Forensic Science Service have used pattern recognition techniques to detect counterfeit whiskies [135]. A blind assay was used in which the various constituents in the samples were determined by capillary GC, but no further identification was obtained. A range of chemometric methods were investigated including the unsupervised K-nearest neighbours analysis and the supervised statistical isolinear multicategory analysis (SIMCA). By using the relative areas under the peaks to provide a measure of relative quantities, these pattern recognition techniques were able to select the constituents relevant to the problem at hand. From these methods a classification was obtained between the Chivas Regal and non-Chivas Regal whiskies.

Tsimidou *et al.* investigated 45 authentic samples of Greek virgin olive oils for their fatty acid and triglyceride composition [132]. Using principal component analysis the olive oils could be separated into distinct groups with either the fatty acid or triglyceride data. The method showed considerable potential for both the characterisation of the oils and the detection of adulteration. Chemometrics has also been used to fingerprint weathered and unweathered crude oil samples [134] and even to established the authenticity of two ancient South Indian bronze statues using principal component and hierarchical cluster analysis [130].

These methods are also extensively used in the flavour and fragrance industry [131,137]. Fingerprint chromatograms are commonly used by perfumers to assess the essential oil qualities of the products. This may be an extremely tedious and time consuming process as fragrances may contain hundreds of components. Chien investigated the use of the K-nearest neighbours cluster analysis to identify Geranium Oil in a perfume sample [137]. With such complex mixtures it was not unusual that certain components were incompletely resolved from each other, even on capillary GC. Peak separation was, however, essential for a compound to be identified in a computer-automated process. A poor resolution in such an investigation caused the sample file to be incomplete and/or erroneous. To remedy this,

unresolved components were manually identified by mass spectral analysis and amended to the sample file. It was often necessary to use large sample sizes in order to observe trace components. Overloaded peaks affected the resolution of all nearby peaks and caused their retention times to deviate from the normal values. In these cases, an editing programme was used to correct the retention time data in the sample files. Errors were also caused by the instability of instruments. The column could deteriorate to a degree that the resolution is lost and the retention data become irreproducible. The oven temperature could be unstable, causing further error in retention times. All these experimental factors had to be carefully calibrated in order that an accurate identification was achieved. Overall, Chien reports that a successful identification of the essential oils was possible, though, some of the samples were very difficult to classify.

Further limitations of pattern recognition techniques have been discussed by Ramsey *et al.* who used principal component analysis to investigate the causes of noise and drift in inductively-coupled plasma/atomic emission spectrometry (ICP/AES) [138]. The effects of ten possible instrumental variables on the emissions of 24 elements were measured independently, to establish distinctive fingerprints. Principal component analysis showed that over 90% of the variance in routine analysis was correlated between elements, rather than being random. However, the results did not correspond to any of those established as suspected causes. In this application principal component analysis could not separate the true causes of the variability in the emission data. The reason for this failure was that two apparently independent causes had correlated multi-elemental effects. This exemplifies the erroneous conclusions which can be drawn from such techniques if verification is not sought. In this case, the error was obvious because of the improbability of the result. If the results were marginally in error, or had no independent validation, such errors would have been less obvious. It has therefore been suggested that simulation of the process under consideration be investigated, as a means of checking the interpretations made from pattern recognition methods [138].

In the light of these applications, it would appear that chemometrics is very applicable to interpreting the phenolic distribution of the plant species under investigation. Thus a range of pattern recognition techniques are to be used in this study, including principal component and hierarchical cluster analysis.

CHAPTER 4

Experimental for phenolic analysis

4.0. Introduction

In this chapter, general experimental details and chemicals are described for the extraction and identification methods for the plant phenolics.

4.1. Reagents

4.1.1. Solvents

Glacial acetic acid (SLR grade), hydrochloric acid (AR grade), ethanol (UV/IR grade), ethyl acetate (HPLC grade), diethyl ether (HPLC grade) and methanol (HPLC grade) were all supplied by FSA Laboratories Suppliers, Loughborough U.K. Water was scrubbed and deionised within the Department.

4.1.2. Standard compounds

Apigenin, quercetin, quercetin dihydrate, rutin trihydrate, hesperidin, 3,4-dihydroxy cinnamic acid, trans-cinnamic acid, hydrocinnamic acid and chlorogenic acid were supplied from Aldrich Chemical Company Ltd, Gillingham, U.K. Nylon 66 filters (0.45 μm) were supplied by Alltech, Carnforth, U.K.

4.1.3. Plant material

Feverfew, (*Tanacetum parthenium* L. Schultz bip.), was donated as the whole dried powdered herb by the British Analytical Control Company (B/no. A3783). An established feverfew seedling was purchased from Staunton Harold Nurseries. Home-grown feverfew was produced from Fisons seeds. The seeds were planted in the early spring and harvested in the late summer. Feverfew varieties were donated from Chelsea Physic Gardens. Four brands of feverfew tablets were purchased locally namely, Heath Heather, Seven Seas, Herbal Laboratories and Lomigram feverfew tablets.

Tansy, (*Tanacetum vulgare* L.) was supplied as the whole dried herb by Brome and Schimmer Ltd (B/no 9091). Tansy was also grown in the Department,

from established plants. German Chamomile, (*Matricaria chamomilla* L.), was obtained as dried flowers from Cathy of Bournemouth Ltd (B/no. C4285, origin Egypt) and from Brome and Schimmer Ltd (B/no. 9956). The plant was also grown in the department from Fisons seeds.

4.2. Scanning electron microscopy

Samples of air dried feverfew leaves (≈2 mm^2) from an 8 month old plant grown at the department (Fisons seeds) were mounted on stubs and sputter coated with gold (10 nm). Specimens were examined under an International Science Instrument (I.S.I.) SS40 scanning electron microscope and photomicrographs taken on 35 mm Kodak film.

4.3. Extraction procedure

Powdered plant material (1 g) was placed in an extraction thimble in a Soxhlet apparatus and extracted under reflux with methanol (150 ml) for one hour. The extract collected was evaporated to dryness using a rotary evaporator. The green residue was treated with methanol-water (1:1, 20 ml) and filtered (Nylon 66, 0.45 μm) to yield an orange filtrate for RP-HPLC.

4.4. Acid hydrolysis of methanolic extraction

The methanolic extract (3 ml) obtained in section 4.3., was heated in a steam bath (100°C) for forty minutes in the presence of 2M hydrochloric acid (3 ml). The sample was cooled and filtered (Nylon 66, 0.45 μm), then evaporated to dryness on a rotary evaporator. The residue was dissolved in methanol/water (1:1, 5 ml).

4.5. Sample preparation

The plant phenolic standards and extracts were stored in air tight containers wrapped in foil under refrigeration, to minimise sample deterioration. Solutions of reference phenolics were prepared by dissolving the appropriate amount of sample in methanol to give concentrations of about 1 mg/ml.

4.6. HPLC apparatus and conditions

The system consisted of a Kontron LC 414 pump, a Rheodyne 7125 injector with a 10 μl injection loop, an ODS Hypersil (5 μm, 4 x 250 mm) column packed in the Department and kept at 30°C by the use of a water jacket, a Pye Unicam PU4020 variable UV detector set at 254 nm, a Hewlett Packard 8451A diode-array detector scanning at 200 - 400 nm and a Hewlett Packard 3390A integrator. Retention times were expressed as relative capacity factors, and were calculated from the retention times of the void volume marker, sodium nitrate (T_1), the internal standard m-bromophenol (T_2) and the analyte (T_3).

$$\text{Relative capacity factor of analyte} = \frac{T_3 - T_1}{T_2 - T_1}$$

Two elution methods were investigated for the phenolics extracted from the plant material:-

{i} Methanol-sodium acetate buffer (30:70 v/v) eluent at pH 3.8. Flow rate 1.2 ml/min.

{ii} Acetonitrile-sodium acetate buffer (45:55) v/v) eluent at pH 3.8. Flow rate 1.2 ml/min.

4.7. Data handling of the results

Results obtained from the chromatogram were run on a Genstat V Mark 4.03 programme (1980, Lawes Agricultural Trust, Rothamsted Experimental Station) available on Multics at Loughborough University. The subroutines, principal component analysis, canonical variate analysis, least squares and cluster analysis were used.

4.8. Procedure used by Welèda UK

Feverfew and tansy tinctures were analysed by Welèda UK. Tinctures were prepared by placing plant material (1g) into cold EtOH/H_2O (100 ml 62:38) for 1 hour. The plant material was then discarded and the solvent evaporated to dryness in a rotary evaporator. The residue was resolvated in methanol

(10 ml) and filtered (Millipore 4μm).

The methanol extracts were analysed by RP-HPLC using a capped ODS-Merck 5 μm column and a gradient elution of 5 - 100% methanol over 20 minutes with a phosphoric buffer (0.5 ml phosphoric acid in 1 litre of distilled water). Detection at 325 nm.

CHAPTER 5

HPLC analysis of phenolics in feverfew and its adulterants

5.0. Introduction

An investigation was undertaken, to ascertain a distinction between feverfew, German chamomile and tansy. The traditional method of microscopic examination of the plant material is limited, as some commercial feverfew products are gel and tincture preparations. As an alternative, the presence of the sesquiterpene, parthenolide has been used to identify the plant [232], although objections to this have been raised [3,139]. The parthenolide has also been reported as being present in tansy [62,68]. Therefore, an easier means of identifying feverfew specimens was sought and in this initial study, plant phenolics were examined as possible chemotaxonomic markers, due to their abundance and ease of extraction and detection. As a large number of phenolics are known to exist in flowering plants [41,92], these components were seen as a potential means of fingerprinting the plant material, the UV-visible spectrum of the components providing possible additional discrimination and a class classification.

Traditional analysis techniques such as column, paper and thin layer chromatography have been used to separate and identify phenolics from plants. However, none of these methods are easily adapted to quantitative determination and high resolution separations. Thus the chosen technique in this study was HPLC, as it enabled an accurate quantitative determination and provided the resolution necessary to produce a fingerprint of the plant extracts. For polar substances such as the flavonoids, the reversed-phase technique is claimed to be far superior to the normal-phase method [109], since there is less danger that some of the highly polar substances become irreversibly retained, which could gradually change the separation characteristics of the column. The chromatographic behaviour of flavonoid compounds has been studied in detail [109,140-141]. The effect of stationary phase, acid modifier, solvent strength and of organic modifier on retention have all been analysed in order to define molecular structure-retention relationships and to find optimal separation conditions in isocratic and gradient elution. The elution profile of the flavonoids may be characterised according to the degree of unsaturation, hydroxylation or methylation pattern and type of sugar substituent [141]. Thus the elution sequence of

the individual compounds is usually typical reversed-phase mode with the most polar analytes eluting first and non-polar last [109].

Two reverse-phase isocratic elution systems were investigated to accommodate the wide range of components extracted. The components were detected with a photodiode-array spectrometer, as the UV spectra of the plant phenolics provide an important means of classification. To further try to elucidate the structure of the components, the plant extracts were acid hydrolysed in an attempt to cleave the sugar from the aglycones and thus ascertain the presence of glycosides. No attempt was made to isolate or identify individual components at this point as it was intended to use the phenolic content as the guide to plant identification.

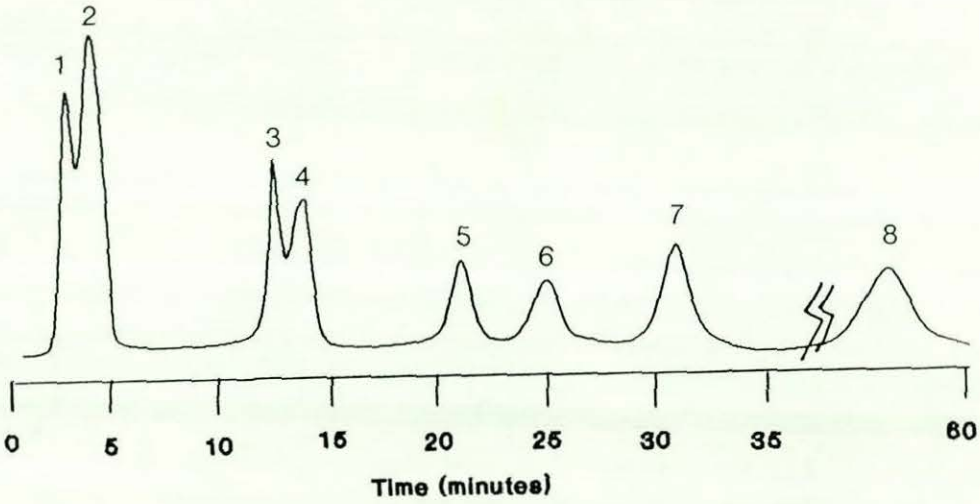
5.1. Method development

In this study 85 feverfew plants were investigated, including the common "main-line" feverfew and the rarer *Balkan Peninsula, Schneeball, Golden Veis, Boule de Neige* and *Flaeepleno* varieties. The majority of the plants were obtained from Chelsea Physic Gardens, but the common "main-line" plants were grown from seeds at the Department. 24 German chamomile samples were also investigated, the majority being plants grown in the department, as well as 2 commercial products which only contain the flowering heads. Unfortunately, due to a limited supply only 3 tansy samples were analysed, two home grown plants and a sample from a commercial herbal supplier. Unlike feverfew or German chamomile, no commercial products containing the plant could be found on the market and very few herbalists stocked the plant.

An initial investigation was carried out with an acidic acid-methanol eluent, which is a very common eluent for phenolic analysis on RP-HPLC. Each plant extract contained an internal standard (m-bromophenol) so that the retention times could be expressed as relative capacity factors and the areas as relative areas compared to the internal standard. The isocratic methanol-sodium acetate buffer (30:70 v/v, pH 3.8) proved capable of separating a wide range of plant phenolics standards (Figure 5.1), so giving an indication of the retention times of the different phenolic classes.

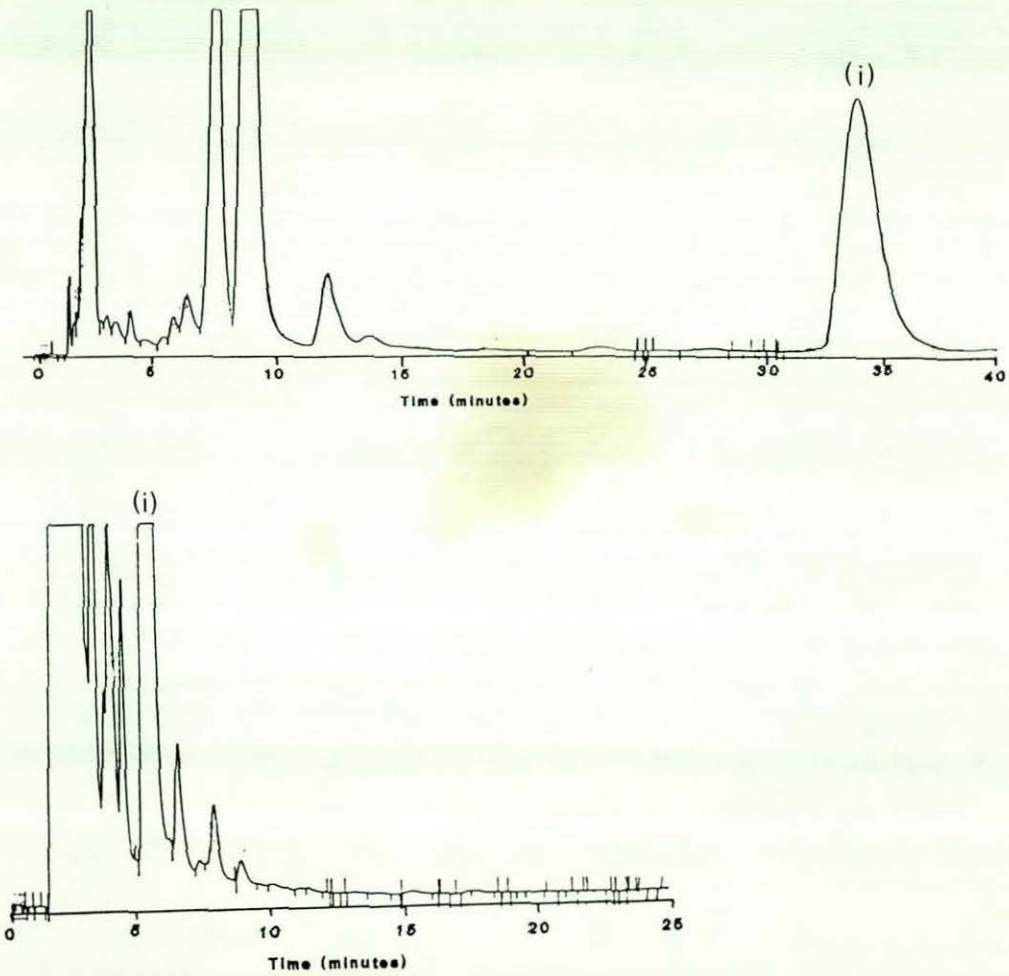
FIGURE 5.1. Plant phenolic standards run on an ODS Hypersil (5 µm, 4.6 x 250 mm) column with a methanol-sodium acetate (30:70, pH3.8) eluent. Detection at 280 nm (AUFS 1.28)

1 = chlorogenic acid (phenylpropanoid)
2 = 3,4 dihydrocinnamic acid (phenylpropanoid)
3 = rutintrihydrate (flavonol)
4 = hesperidin = (flavone)
5 = hydrocinnamic acid (phenylpropanoid) and myrectin (flavonol)
6 = transcinnamic acid (phenylpropanoid)
7 = quercetin (flavonol)
8 = apigenin (flavone)



However, the methanol eluent was unable to resolve all the components in the methanolic extracts from the plant material. These complex extracts appeared to required two isocratic eluents to obtain complete resolution. The methanol eluent resolved a large number of components though a small number were failing to be eluted or were slowly eluted after one hour, as small tailing peaks. To analyse these components an isocratic acetonitrile-acetate buffer eluent (45:55 v/v) was investigated. Using this system the majority of the components eluted within the first five minutes including the internal standard m-bromophenol, which on the methanol system had a retention time of 34 minutes (Figure 5.2). The acetonitrile eluent had the advantage that all the main components appeared to be eluted from the column, though only a small number were sufficiently resolved to be characterized.

FIGURE 5.2. HPLC separation of a methanolic extract of tansy with {a} a methanol-acetate eluent (30:70 v/v) and {b} an acetonitrile-acetate buffer (45:55 v/v) eluent on an ODS Hypersil 5μm column. i = m-bromophenol



5.2. Phenolic content of feverfew and its reported adulterants

5.2.1. Phenolic content resolved with methanol eluent

Methanolic extracts of feverfew, tansy and German chamomile were analysed by RP-HPLC, using the methanol-sodium acetate eluent (Figure 5.3.) to determine the phenolic content of each species. The plant extracts were reasonably resolved and appeared to contain a range of components which, from their UV spectra (Table 5.1), were assigned as plant phenolics. The spectra were compared to standard UV-visible absorption maxima (Section 2.5.4. Table 2.2) to enable a group classification to be obtained. Using just the retention times of the components extracted from the whole plant,

German chamomile could be distinguished from feverfew and tansy (Figure 5.3). German chamomile had a simpler phenolic distribution, failing to possess many of the well retained components present in the other plant species. Furthermore, it possessed components at 4.0 and 23.8 minutes (Figure 5.3, peak 3 and 8) which appeared absent or as minor components in feverfew and tansy. However, feverfew and tansy appeared to possess many similar components, and their chromatograms were often virtually indistinguishable (Figure 5.3).

To achieve a distinction between feverfew and tansy, the UV-visible spectra of the individual components were investigated, by means of a photodiode-array spectrophotometer. The two most prominent components in the plants have retention times of 7.9 (peak 4) and 9.5 (peak 5) minutes (Figure 5.3). The first of these components (peak 4) possessed the same UV spectrum in both plants (Table 5.1), which was tentatively identified as a phenylpropanoid. However, the second major component (peak 5) has different UV spectrum in feverfew and tansy (Figure 5.4 {i} and {ii}), both spectra being characteristic of flavones. Thus, although both plants possess a major component at 9.5 minutes, there appears to be sufficient variation in the UV-visible spectrum of the components to enable a possible classification of the plants. Furthermore, this component has a different UV-visible spectrum in German chamomile (Figure 5.4 {iii}), so that all 3 plant species can be distinguished. Other minor components in the plants extracts were also shown to possess different UV spectra (Table 5.1).

No obvious distinction could be found between the common "main-line" feverfew and the rarer feverfew varieties. All the feverfew plants possessed a qualitatively similar phenolic content, with subtle quantitative differences. However, there was as much intra as inter quantitative variation so no further inferences were drawn. One of the feverfew plants, the "Boule de neige" variety from Chelsea physic gardens, has been disputed on botanical grounds as a member of the feverfew species [36]. The plant is a lot smaller and a paler shade of green than the majority of feverfew plants so far encountered. However, the phenolic composition of the plant demonstrated that it has a "typical" feverfew chromatogram and so has been included in this survey.

FIGURE 5.3. (a) = feverfew (whole plant), (b) = tansy (whole plant). (c) = German chamomile (whole plant), using an ODS 5 μm column with a methanol-acetate eluent (30:70 v/v). Detection at 280 nm (AUFS = 1.28). For UV spectra of peaks 1-8 see Table 5.1.

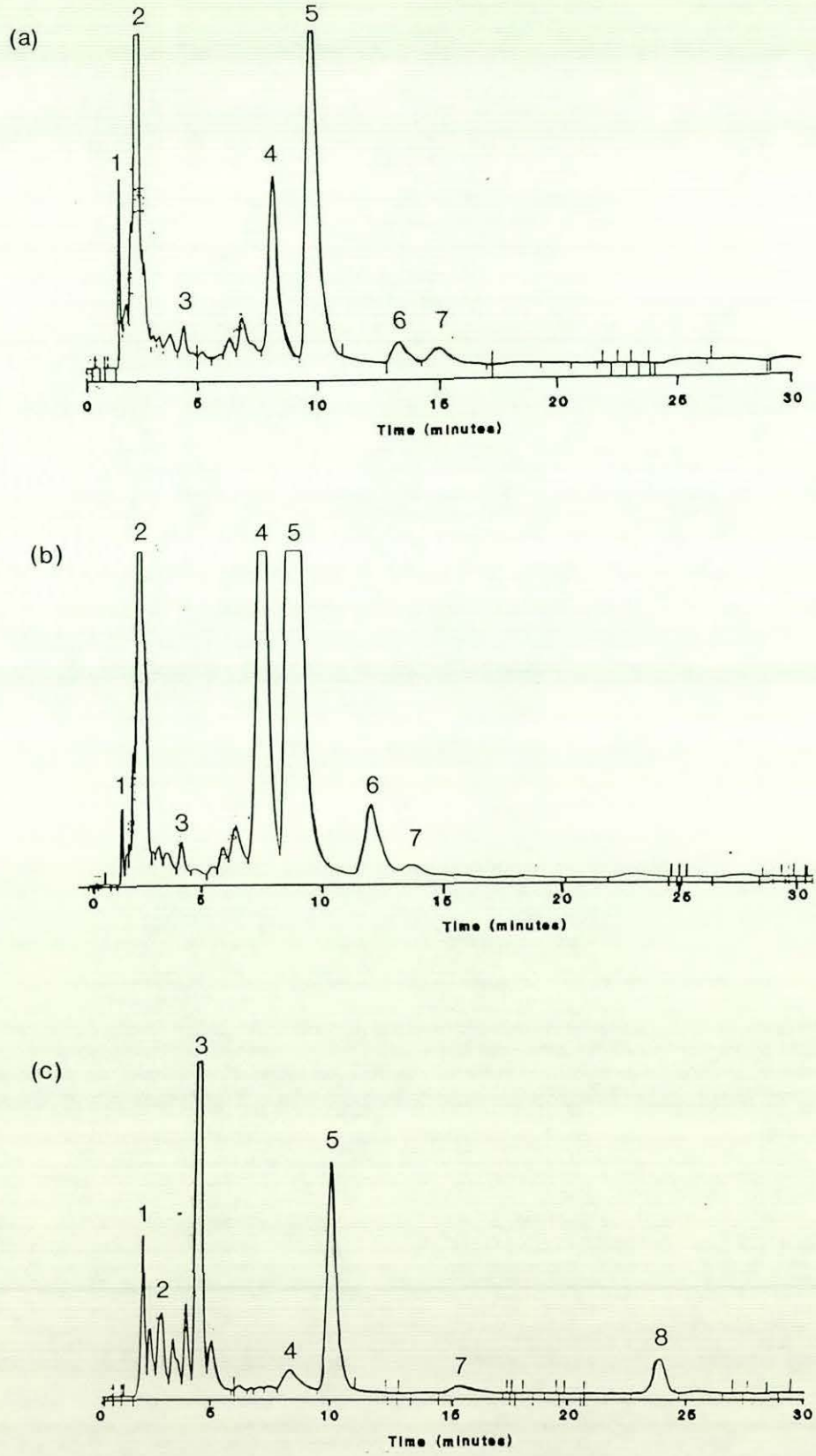


TABLE 5.1. UV-visible spectra of the major components in the plant extracts of Figure 5.3.

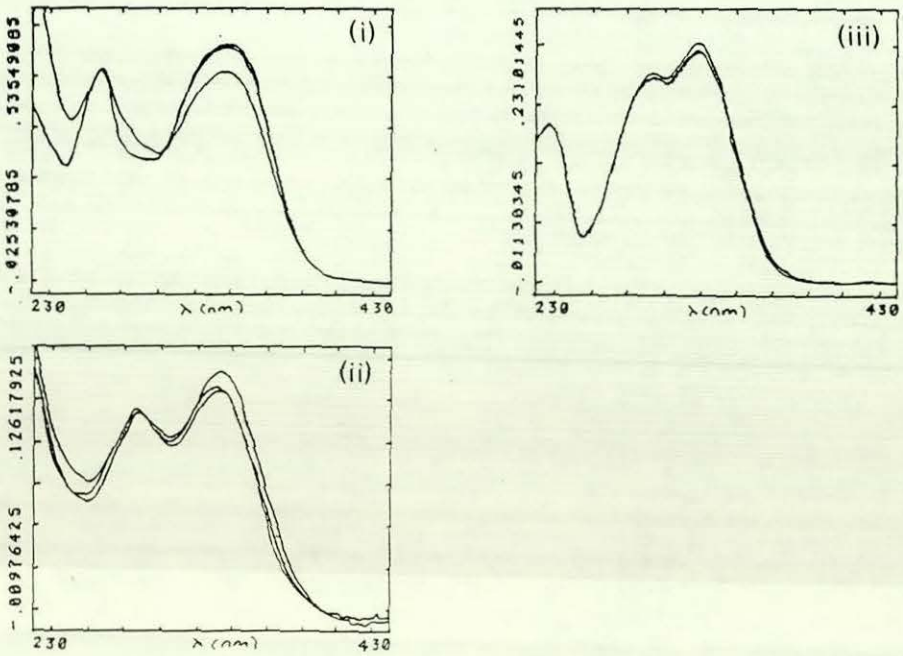
{i} Feverfew and tansy (a = feverfew, b = tansy)

Peak no.	Principal maxima (nm)	Subsidiary maxima (nm) (relative intensities)	Inference
1	270	220 (80%)	Unknown
2	multicomponent	–	Phenolics
3	335	280 (70%)	Flavone
4	320	300 (80%), 230 (50%)	Phenolic acid
5a	335	270 (80%)	Flavone
5b	330	285 (80%)	Flavone
6	330	290 (90%)	Phenolic acid
7a	260	355 (60%)	Flavonol
7b	340	255 (75%), 270 (60%)	Flavone

{ii} German chamomile

Peak no.	Principal maxima (nm)	Subsidiary maxima (nm) (relative intensities)	Inference
1	270	220 (80%)	Unknown
2	330	315 (80%), 240 (60%)	Phenolic acid
3	270	300 (90%)	Phenolic
4	330	300 (80%), 230 (50%)	Phenolic acid
5	320	290 (80%), 235 (60%)	Phenylpropanpoid
6	Absent	–	–
7	340	250 (80%), 270 (70%)	Flavanol
8	320	–	Flavanone

FIGURE 5.4. UV-visible spectrum of peak 5 in Figure 5.3 for {i} feverfew, {ii} tansy, {iii} German chamomile. Three spectra were obtained for each component to ascertain peak purity. See Table 5.1 for inferences.



5.2.2 Phenolic content resolved with acetonitrile eluent

Using the acetonitrile-acetate buffer eluent (45:55 v/v) a range of new components have been detected which were not seen with the methanol eluent. (Figure 5.5). From the UV-visible data (Table 5.2) the majority of these components were assigned as flavones, flavanones, flavanonols and isoflavones. Feverfew and German chamomile possessed similar ranges of components, so that there appeared to be insufficient variation in the extracts to enable a distinction to be made. The tansy extract contained fewer components, one of the components (peak 1 Figure 5.5) only being detected in tansy, suggesting that there may be sufficient chemotaxonomic variation to enable tansy to be distinguished from the other plant species. However only tansy leaf material was available at the time of the study. Therefore, the whole plant may possess a similar range of components which would make it indistinguishable from the other plant species.

TABLE 5.2. UV-visible spectra of the major components in the plant extracts of Figure 5.5.

Peak no.	Principal maxima (nm)	Subsidiary maxima (nm) (relative intensities)	Inference
1	330	285 (80%)	Flavone
2	Signal too weak	-	-
3	Signal too weak	-	-
4	280	-	Isoflavone
5	315	235 (40%)	Flavone or isoflavone
6	Signal too weak	-	-
7	320	240 (50%)	Flavone or isoflavone
8	325	240 (40%)	Flavone or isoflavone

5.2.3. Phenolic content resolved with gradient elution

Both the methanol and acetonitrile eluent were unable to resolve all the components in the complex extracts. A possible alternative is to use a gradient elution system which would be able to resolve all the components present in the extracts, rather than rely on these two separate isocratic systems, each of which has its limitations. Welda U.K. generously offered to analyse feverfew and tansy on a gradient HPLC system (Figure 5.6). Alcoholic tinctures of the whole plant material were investigated (See Chapter 4 for sample preparation). The majority of the components extracted were present in both plant species. However, variations did occur with the more highly

Figure 5.5. HPLC separation of methanolic extracts of the plant material with an acetonitrile-acetate buffer (45:55 v/v) eluent on an ODS Hypersil 5 µm column. a = feverfew (whole plant), b = tansy (leaves), c = German chamomile (whole plant) i = internal standard (m-bromophenol). UV-visible spectra of peaks given in Table 5.2.

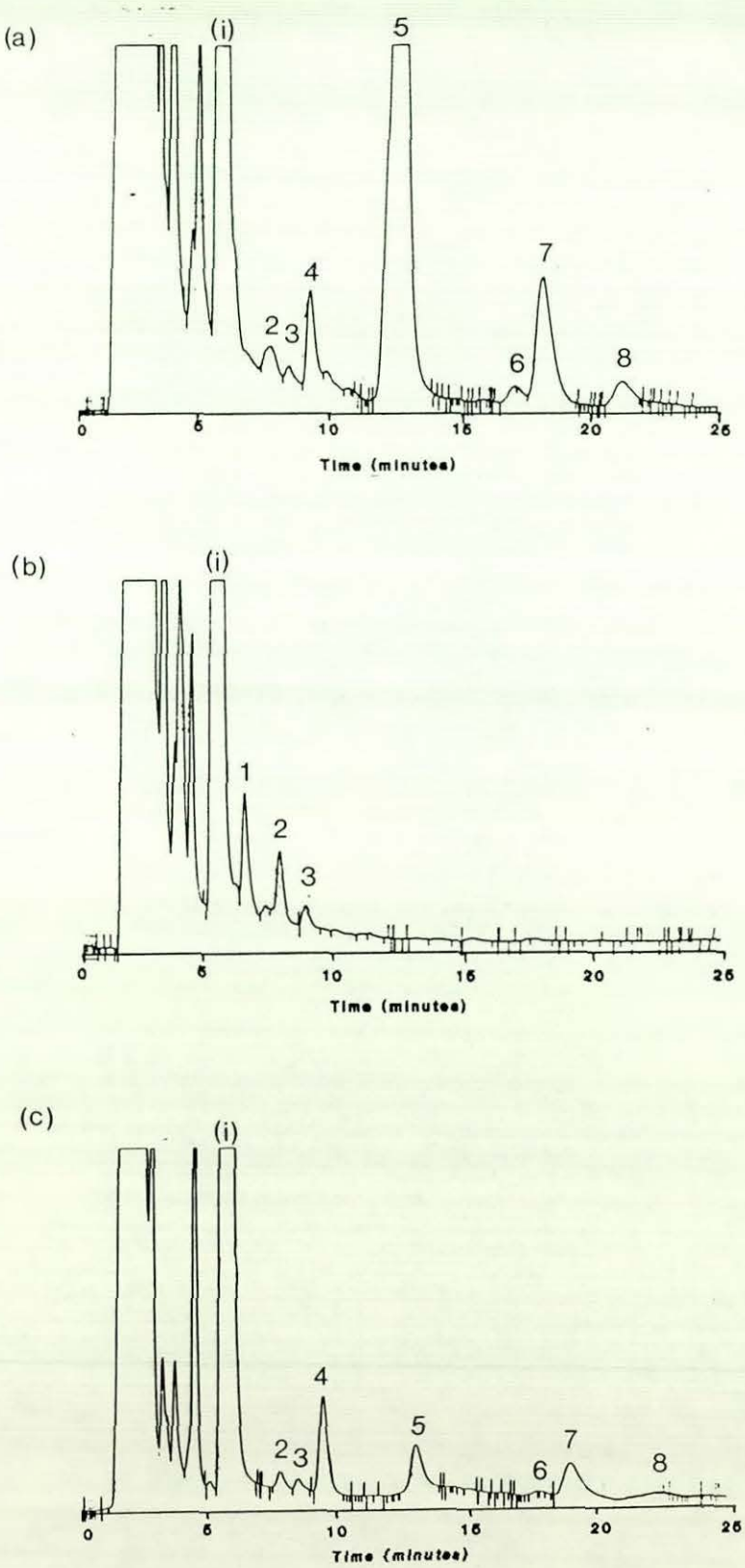
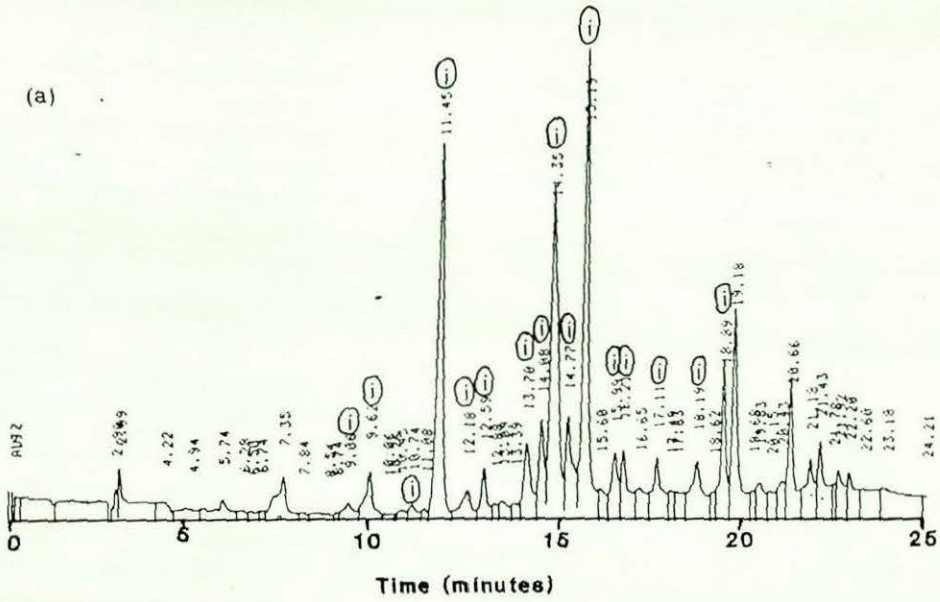
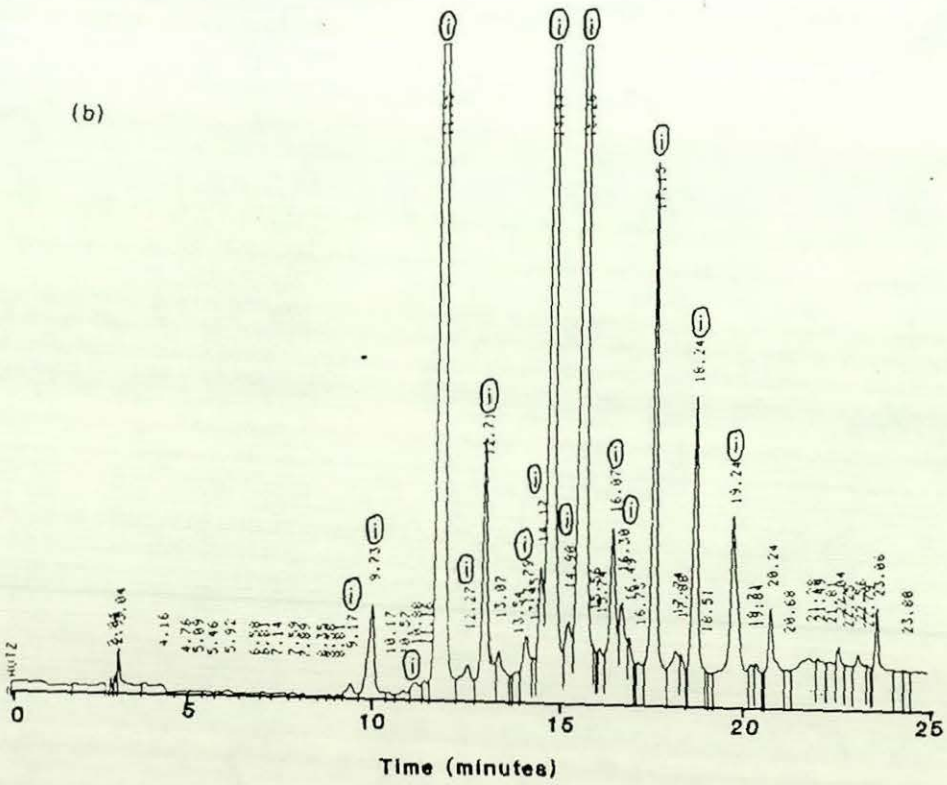


FIGURE 5.6. HPLC separation of alcoholic tinctures of plant material with a gradient elution of methanol-phosphoric acid eluent on an ODS Merck 5 μm (4.6 x 250 mm) column. Gradient elution of 5 - 100% methanol over 20 mins. Detection at 325 nm (AUFS = 0.1) a = whole feverfew plant, b = whole tansy plant. (i) = present in both feverfew and tansy.





retained components, from which it is thought an identification could be obtained. The latter part of the HPLC chromatograms in Figure 5.6 can be seen as the "fingerprint" region, and would appear to confirm the results obtained with the study using acetonitrile-acetate eluent.

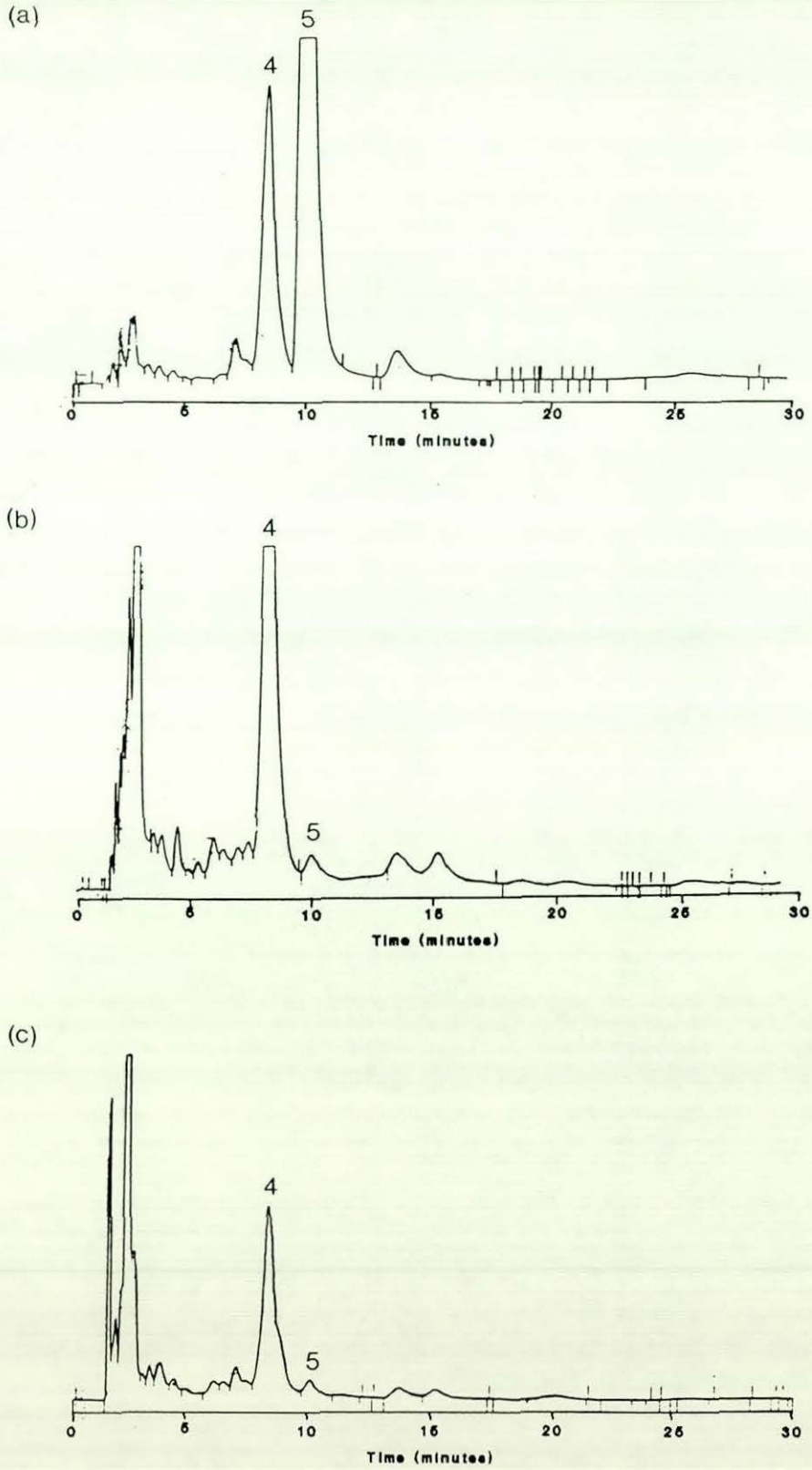
5.3. Distribution of phenolics within the plant structure

In trial studies the majority of phenolic compounds appeared to exist throughout the plant, though a minority were present in localised regions such as the flowering heads. This has important implications, as the plant leaves are a waste product in the manufacture of German chamomile tea, and are therefore a potential adulterant. As samples in pharmaceutical preparations could be based on different plant parts, a series of samples consisting of just the leaves, stems, or flowering heads were compared. Both the methanol and acetonitrile eluents were required to assess all the components present in the extracts.

5.3.1. Localised phenolics resolved with methanol eluent

For feverfew, the components with the retention times of 8 and 9.5 minutes (peak 4 and 5, Figure 5.7) appeared to vary the most in relation to the origin of the plant extract. From the UV-visible spectrum (Table 5.1), these components were identified as a phenylpropanoid and a flavone, respectively, and were the major components in the flowering head. The phenylpropanoid appears to be the main component of the yellow centre of the flower and is also present within the leaves and stems. The majority of the flavone appears to be present in the white petals which lacks the early eluting peaks present in the stems, these absent peaks possibly being glycosides and phenolic acids. The presence of a flavone in the petals is not unexpected, as flavones are reported as invariably accompanying the coloured anthocyanin pigments in flowers [107], though no anthocyanins were detected using this eluent. Therefore it should be possible to distinguish feverfew plants or tablets composed of just leaves or of the whole plant, including flowers. Other variations within the components also exist between feverfew extracts, but these are all minor in comparison.

FIGURE 5.7. Distribution of plant phenolics in feverfew (home grown from Fisons seeds) using the methanol eluent. HPLC conditions as in Fig. 5.3. Peak 4 = phenylpropanoid, 5 = flavone
a = white petals from the feverfew flower
b = yellow floral centre from the feverfew flower
c = leaves and stem of feverfew



5.3.2. Localised phenolics resolved with acetonitrile eluent

Some of the components resolved with the acetonitrile eluent appeared highly localised in particular plant parts. Components 5 and 7 (Figure 5.8) appear to be mainly situated in the flowering heads of the plant, being only minor components in the leaves of the material. Both components were classified as flavones or isoflavones (Table 5.2) from their UV-visible spectrum. This distinction may enable the origin of the plant parts to be determined, and as stated earlier this could be important when assessing the authenticity of the commercial preparations of the plants.

5.4. Acid hydrolysis of extracts from feverfew and its reported adulterants

Flavonoids are generally present in plants bound to sugar as glycosides and any one flavonoid aglycone may occur in a single plant in several glycosidic combinations. For this reason when analysing flavonoids, it is often beneficial to examine the aglycones present in hydrolysed plant extracts, so reducing the complexity caused by the glycosides present in the original extract. In this study the standard procedure for hydrolysis of O-glycosides was adopted using hot diluted acid [142]. Flavonoids commonly occur as flavonoid O-glycosides in which one or more of the flavonoid hydroxyl groups is bound to a sugar or sugars by an acid liable C-O bond. However, a few flavonoids exist as C-glycosides, the sugar unit being attached directly to the benzene nucleus by a C-C bond, which is acid resistant. Therefore in this study, only the presence of O-glycosides or acid labile aglycones would be ascertained.

5.4.1. Acid hydrolysed extracts resolved with a methanol eluent

The acid hydrolysed extracts were initially analysed with the methanol-acetate eluent (30:70 v/v) which revealed that a number of the phenolics in the extracts were either glycosides or acid labile (Figure 5.9). On acid hydrolysis a number of new components ({i}, {ii}, {iii} and {iv} Figure 5.9) presumably aglycones, appeared in the plant extracts. Most of these new peaks in the chromatograms were multicomponent so that no inferences could be obtained from the UV-visible spectra, the exception being peak {iii} (Figure 5.9) which was classified as a flavone. Tansy extracts produced the largest number of these new components ({ii}, {iii} and {iv} Figure 5.9), so

FIGURE 5.8. Distribution of plant phenolics in feverfew (home grown from Fisons seeds) using the acetonitrile eluent. HPLC conditions as in Figure 5.5. For peak identification see Table 5.2.
a = Aerial part of feverfew (stem, leaves, flowers)
b = Flowering heads of feverfew
c = Leaves of feverfew

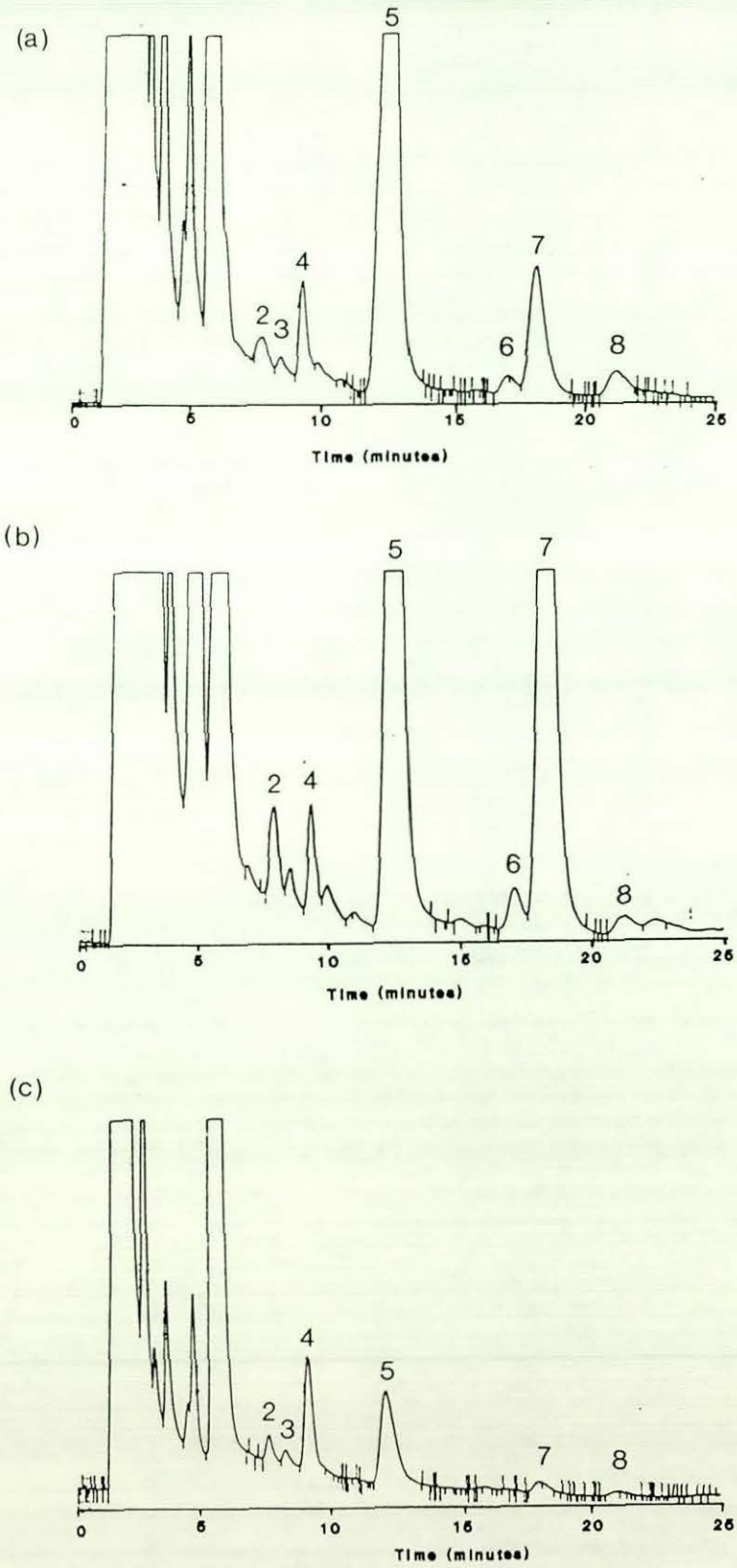
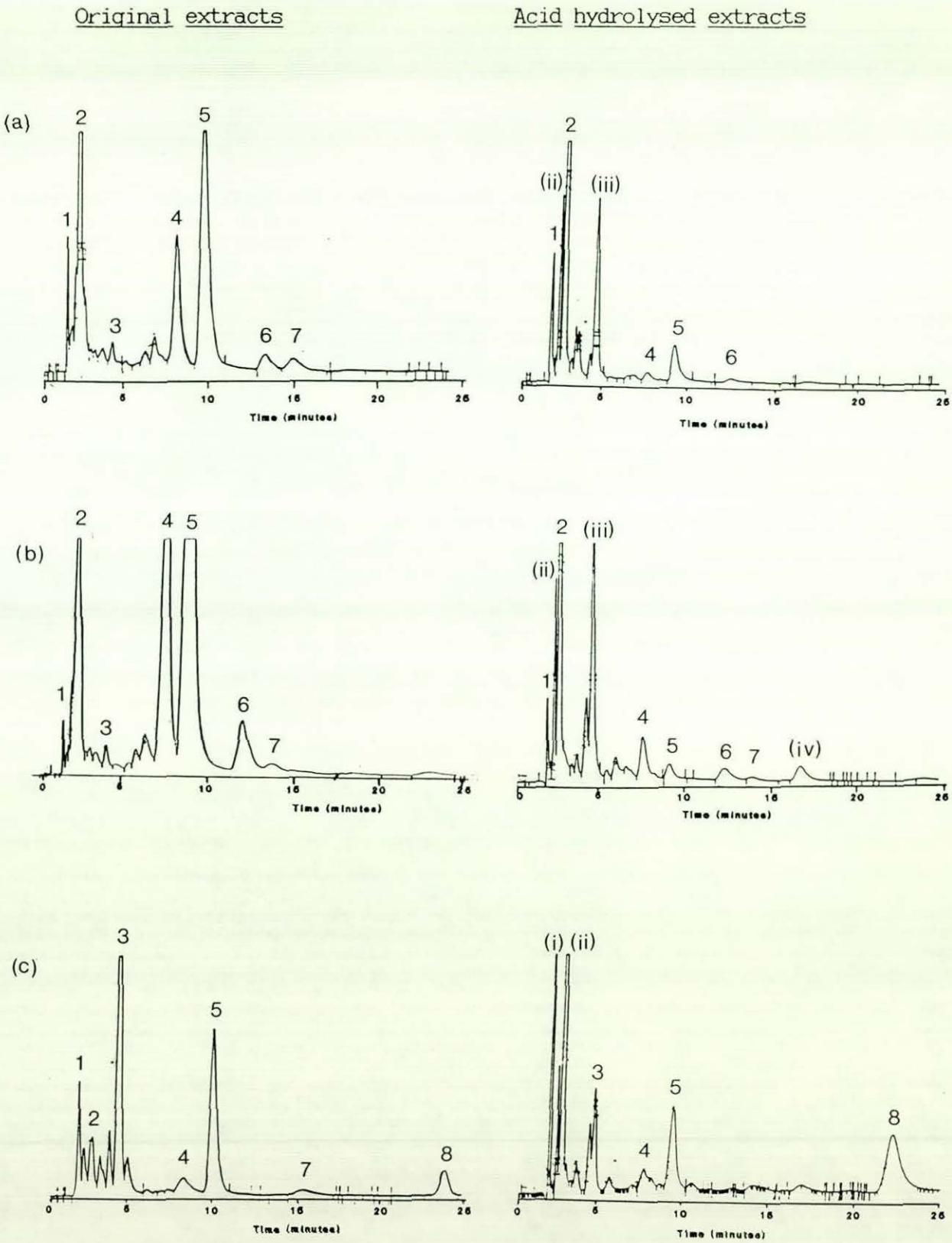


FIGURE 5.9. HPLC separation of acid hydrolysed plant extracts with a methanol-acetate buffer eluent. HPLC conditions as in Figure 5.2. a = feverfew, b = tansy, c = German chamomile



that a distinction was obtained between tansy and the other plant species. German chamomile appeared to possess fewer glycosides or more acid resistant phenolics than feverfew and tansy, though the major component in the German chamomile extract (peak 8, Figure 5.9) was affected by acid hydrolysis.

These hydrolysed extracts may therefore have the potential to distinguish feverfew from tansy and German chamomile. However, the sample preparation is time consuming, each extract requiring over an hour to prepare. Thus, the technique would not be recommended for routine analysis, though it may prove worth while in cases of ambiguity, where the original extracts were indistinguishable, even with gradient elution HPLC.

5.4.2. Acid hydrolysed extracts resolved with an acetonitrile eluent

The acid hydrolysed extracts were also investigated with the acetonitrile-acetate eluent (45:55 v/v). The majority of the components eluted in this eluent appeared to be acid labile, components 4,5,6,7 and 8 in Figure 5.5 being absent in the acid hydrolysed extracts. The exception was German chamomile as component 5 appeared acid resistant. Therefore, this technique enabled feverfew and German chamomile to be distinguished, which before acid hydrolysis, was not possible when using this eluent.

5.5. Pharmaceutical preparations of feverfew

The distribution of phenolic compounds in four commercial preparations of feverfew (R.P. SchererLtd, Herbal laboratories, Seven Seas and Heath Heather) were analysed by the same procedure as the plants. In order to obtain adequate detection, the technique required two to three tablets or capsules, representing between 75 and 375 mg (depending on the manufacture) of plant material. Both the methanol and acetonitrile eluent were required to obtain sufficient chemotaxonomic information to identify the origin of the commercial preparations.

5.5.1. Feverfew products analysed using a methanol eluent

Three of the brands (R.P. Scherer Ltd, Herbal laboratories and Seven Seas) possessed phenolic compounds similar to the feverfew plants (eg Figure 5.10), with no obvious loss of any components during the pharmaceutical preparation of the plant. Some of the minor components in the pharmaceutical preparations were not resolved, but this is probably due to problems in detecting such low quantities of plant material. The tablets or capsules also possessed a low concentration of the flavone (peak 5, Figure 5.10), suggesting that the majority of the plant material used in the pharmaceutical preparation, consisted of the leaves.

The fourth brand (Heath Heather Ltd) of feverfew tablets possessed an odd array of components, some of which could possibly be present in feverfew, and others which appeared unique to that extract (Figure 5.10). The tablets also lacked the characteristic component, peak 5, which in this study has always been found in feverfew, even when just leaf material was present. Problems were encountered with the detection of the individual components. The Heath Heather feverfew tablets only contained 25 mg of plant material per tablet, consequently the diode-array detector lacked sufficient sensitivity, so that the UV-visible spectra failed to be of any discriminatory use. To compensate for this 6 tablets were co-extracted to increase the yield of plant phenolics. However, this proved unsatisfactory as the binder and packing constituents started to be a problem, producing a cloudy extract, with even more spurious peaks present. It may be that the tablet has deteriorated with age, as some plant phenolics have been reported to degrade on exposure to temperature and light [92]. To ascertain if this is occurring, a long term study would be required in which a series of extractions would be obtained over a period of months or years, as the plant material ages.

An additional component was noted in two brands of the feverfew tablet (Herbal Laboratories and Seven Seas) with a retention time of 11.3 minutes (peak {x}, Figure 5.10 {b}), this being absent in the Lomigram gel capsules. Therefore, it would appear that the additional component may be due to a packing or binder constituent in the tablets. To investigate this further, British Analytical Control provided a feverfew tablet and all the individual constituents to make it (Table 5.3). None of the tablet constituents corresponded to the additional peak, however, the British Analytical Control tablet did not contain the additional component either.

FIGURE 5.10. Pharmaceutical preparations of feverfew investigated using the methanol eluent (HPLC conditions Figure 5.3) Detection at 280 nm.
a = Whole feverfew plant (home grown from Fisons seeds) AUFS = 1.28
b = Feverfew tablet (Seven Seas Health Care Ltd) AUFS = 0.32
c = Feverfew tablet (Heath Heather Ltd) AUFS = 0.04

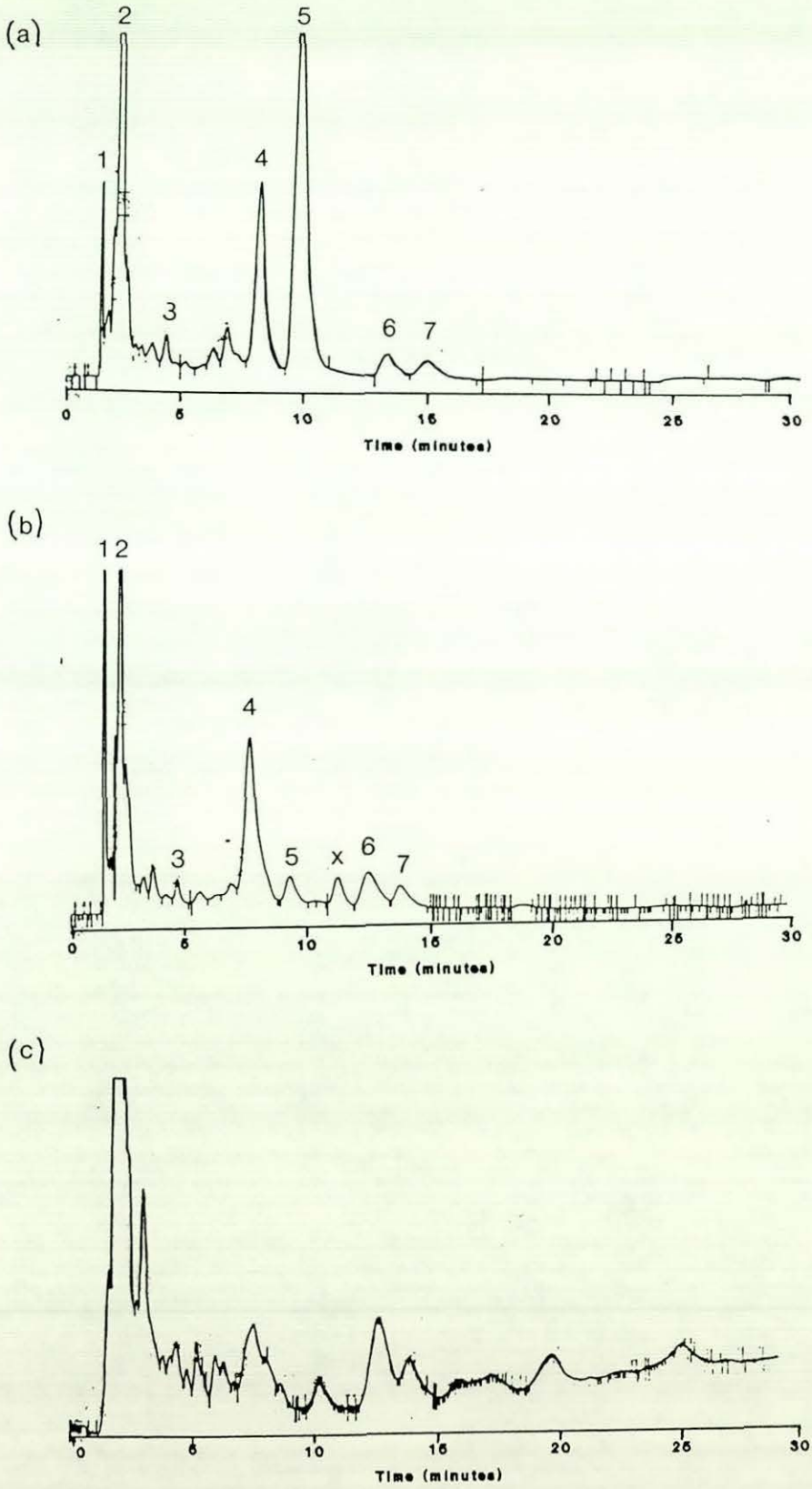


TABLE 5.3. Feverfew tablet composition (British analytical control):-

Dried powdered Feverfew (25mg)
Stearic acid
Acacia
Syloid 244
Magnesium sterate
Sodium starch glyconate
Calcium sulphate, dried

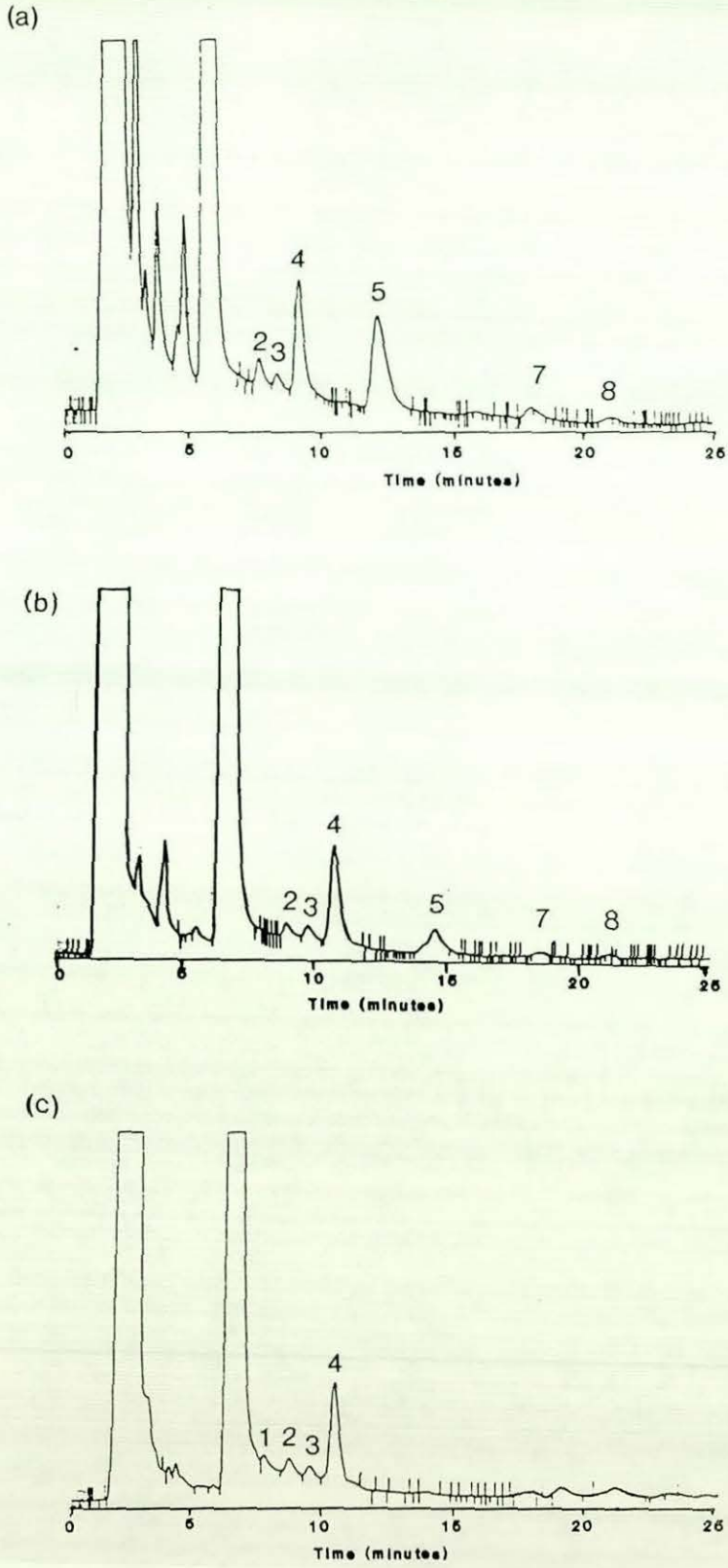
The effect of the pharmaceutical preparation on the plant material was also investigated. The dried finely chopped feverfew was analysed before tablet manufacture and after, with the methanolic extracts obtained appearing indistinguishable by HPLC. This suggests that the plant phenolics under-went little degradation or change with the pharmaceutical processesing and storage.

5.5.2. Feverfew products analysed using an acetonitrile eluent

To further elucidate the components in the commercial feverfew preparations, the methanolic extracts were analysed using the acetonitrile-acetate eluent (45:55 v/v). Three of the brands (R.P. SchererLtd, Herbal Laboratories and Seven Seas) possessed similar components to the feverfew plant, suggesting the preparations contain the authentic plant material (eg Figure 5.11). The components 5 and 7 in the commercial preparations (Figure 5.11 {b}) were only minor constituents suggesting that the plant content consisted mainly of leaf material. No additional components were seen with this elution system, as the binder and packing constituents were probably eluted near the solvent front.

The fourth brand (Heath Heather) of feverfew tablets, possessed few components, the majority of which were found in feverfew. However, the absence of the other components is of some concern. The origin of this plant therefore, appears uncertain. The plant material may have aged so possibly causing the degradation of some of the components, or it may contain a mixture of plant species.

FIGURE 5.11. HPLC separation of methanolic extracts of the plant material with an acetonitrile-acetate eluent (45:55 v/v) on an ODS Hypersil 5 μm column. Detection at 280 nm (AUFS 0.32).
a = Feverfew leaves (*grown from Fisons seeds*)
b = Herbal Laboratories feverfew tablets
c = Heath Heather feverfew tablets



5.5.3. Acid hydrolysis of feverfew products

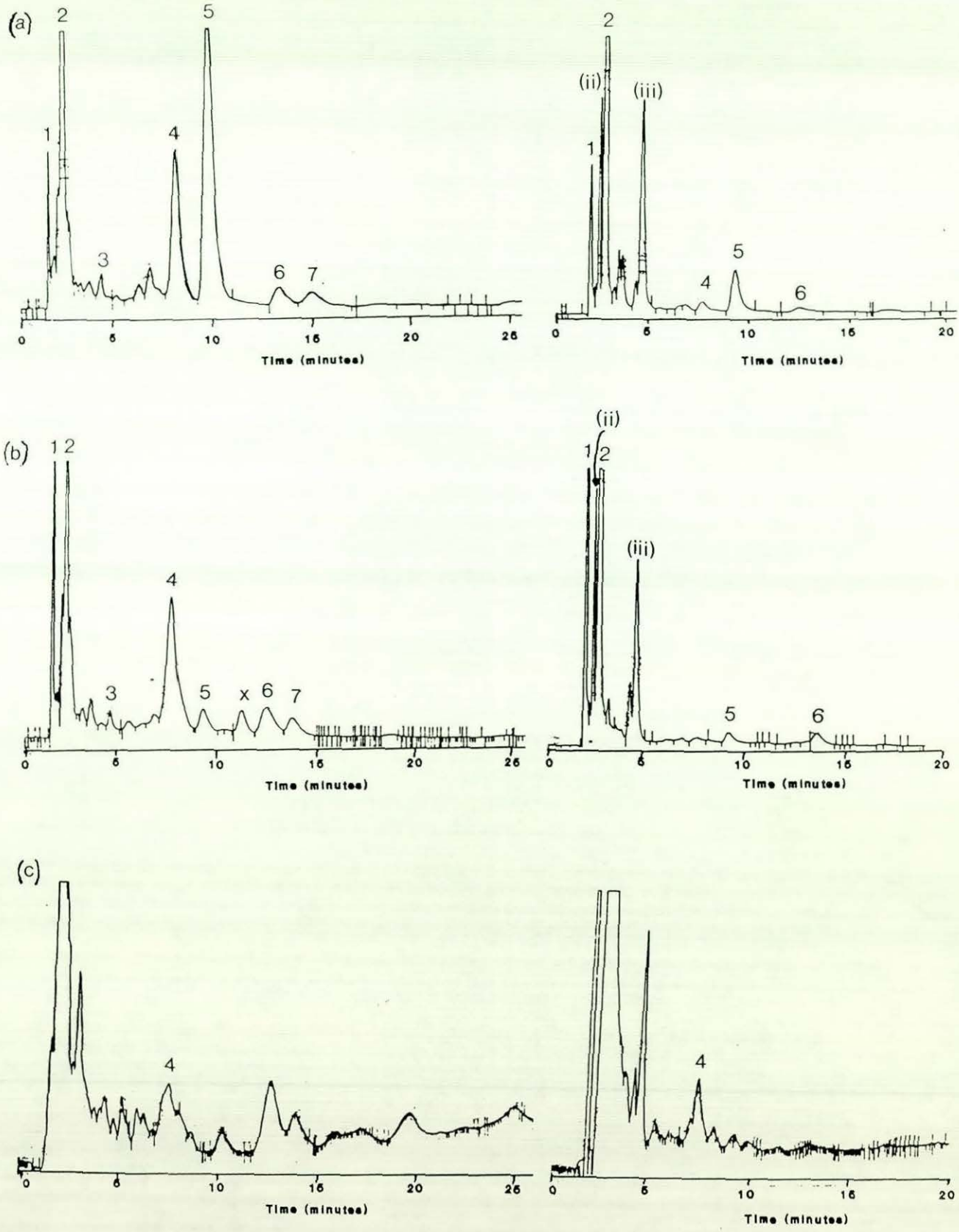
The pharmaceutical preparations of feverfew were also acid hydrolysed in an attempt to further distinguish the samples. The treated methanolic extracts were initially analysed using the methanol-acetate (30:70 v/v) eluent. Three of the brands (R.P. SchererLtd, Herbal Laboratories and Seven Seas) appeared to contain similar glycosides or acid labile components which were found in feverfew, with tablets and plant extracts producing new components (eg {ii} and {iii} Figure 5.12) on acid hydrolysis. The additional component {x} found in the feverfew tablets was also acid hydrolysed.

The fourth brand (Heath Heather) of feverfew tablets possessed a range of components some of which corresponded to those in feverfew and others which were incompatible with the feverfew phenolic distribution (Figure 5.12). For example component 4 in the Heath Heather product (Figure 5.12 {c}) appeared acid resistant, whereas, the component with a similar retention time in the other feverfew preparation and the feverfew plant material were acid hydrolysed. The origin of the Heath Heather product is therefore unknown. Analysing the extracts with the acetonitrile-acetate (45:55 v/v) eluent, furnished no additional discriminatory information.

5.6 Chemometrics study

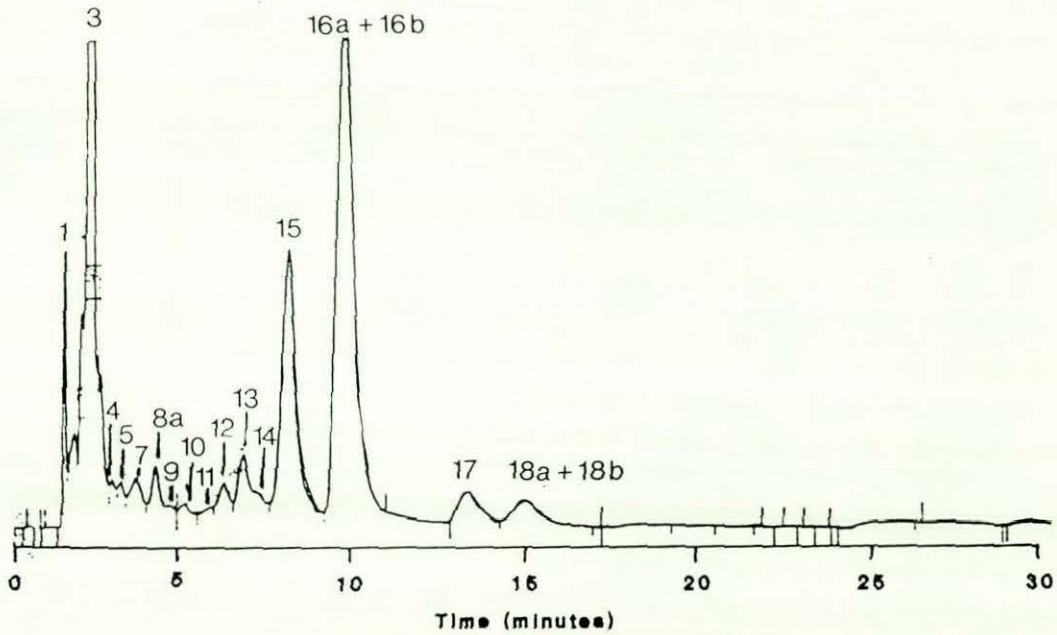
Due to the complexity of the phenolic distribution in the plant extracts, a chemometrics analysis was investigated in an attempt to obtain an unambiguous distinction between the plant species. The chemometrics study was undertaken using the Genstat programme available on a Multics computer at Loughborough University. Genstat is a multivariant analysis programme which was used to ascertain if the species were substantially different so as to enable individual clusters or groups to be formed from the data. To determine this a series of statistical approaches has been attempted within the Genstat programme. The study investigated 85 feverfew plants including the varieties discussed in Section 5.1, 24 German chamomile plants and 3 tansy plants. The data was obtained from the methanol-acetate eluent system in Section 5.2 and the results generated from this elution system were entered as relative areas of the individual components compared with the internal standard m-bromophenol. The components were distinguished by their relative capacity factors and UV-visible spectra (Figure 5.13). Each component in the extracts was numbered (Figure 5.13) and where components

FIGURE 5.12. HPLC separation of acid hydrolysed plant extracts with a methanol-acetate buffer eluent. HPLC conditions as in Figure 5.2. a = feverfew, b = Seven Seas feverfew tablet, c = Heath Heather feverfew tablet.



with the same relative capacity factor could be distinguished by their UV-visible spectrum, an assignment of {a} or {b} etc was used. Thus a list of components used in this chemometric survey was obtained (Table 5.4). A full list of the input data for the chemometric survey is given in the Appendix (A.1).

FIGURE 5.13. Components used in chemometrics analysis (see Table 5.4).
{i} Typical feverfew or tansy chromatogram.



{ii} Typical German chamomile chromatogram.

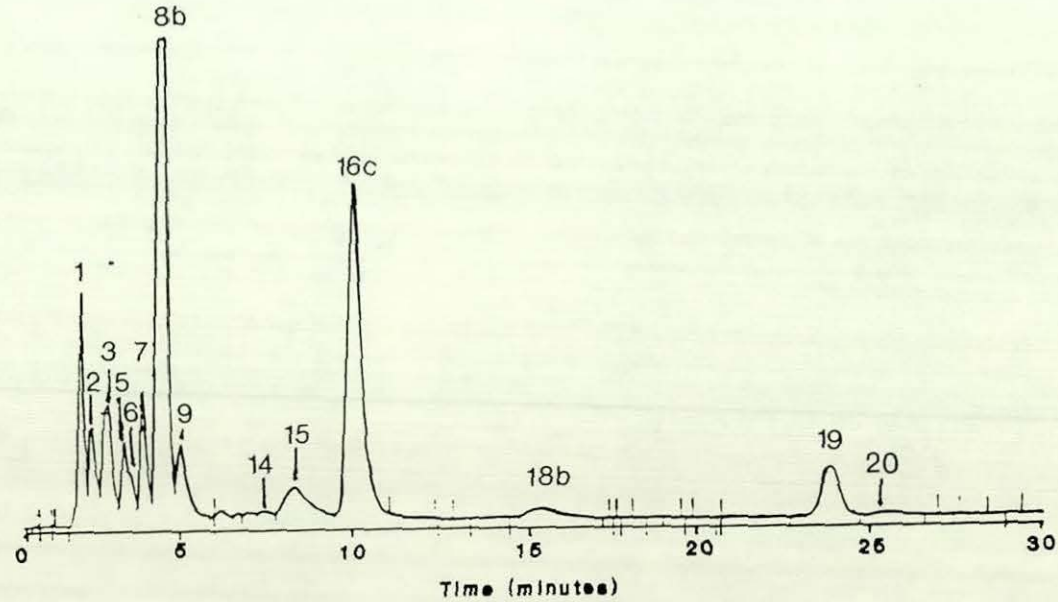


TABLE 5.4. Components used in chemometric survey (See Figure 5.13)

Peak no.	retention time (mins)	max if used (nm)
1	1.4	-
2	1.6	-
3	2.0	-
4	2.4	-
5	2.7	-
6	3.0	-
7	3.8	-
8a	4.0	335
8b	4.0	320
9	4.2	-
10	4.5	-
11	5.3	-
12	5.9	-
13	6.7	-
14	7.0	-
15	8.1	-
16a	9.6	330
16b	9.6	335
16c	9.6	320
17	12.9	-
18a	14.9	260
18b	14.9	340
19	23.4	-
20	26.1	-

From this survey it was ascertained that a number of components were specific to a plant species, so that a table identifying the origin of the components was constructed (Table 5.5).

Table 5.5 Components present in plant species (Figure 5.13).

Plant species	Peaks identified in plant
Feverfew	1, -, 3, 4, 5, -, 7, 8a, 9, 10, 11, 12, 13, 14, 15, 16b, 17, 18a, 19, -
Tansy	1, -, 3, -, -, -, 7, 8a, -, -, -, 12, 13, 14, 15, 16a, 17, 18b, -, -
German chamomile	1, 2, 3, -, 5, 6, 7, 8b, 9, -, -, -, -, 14, 15, 16c, -, 18b, 19, 20

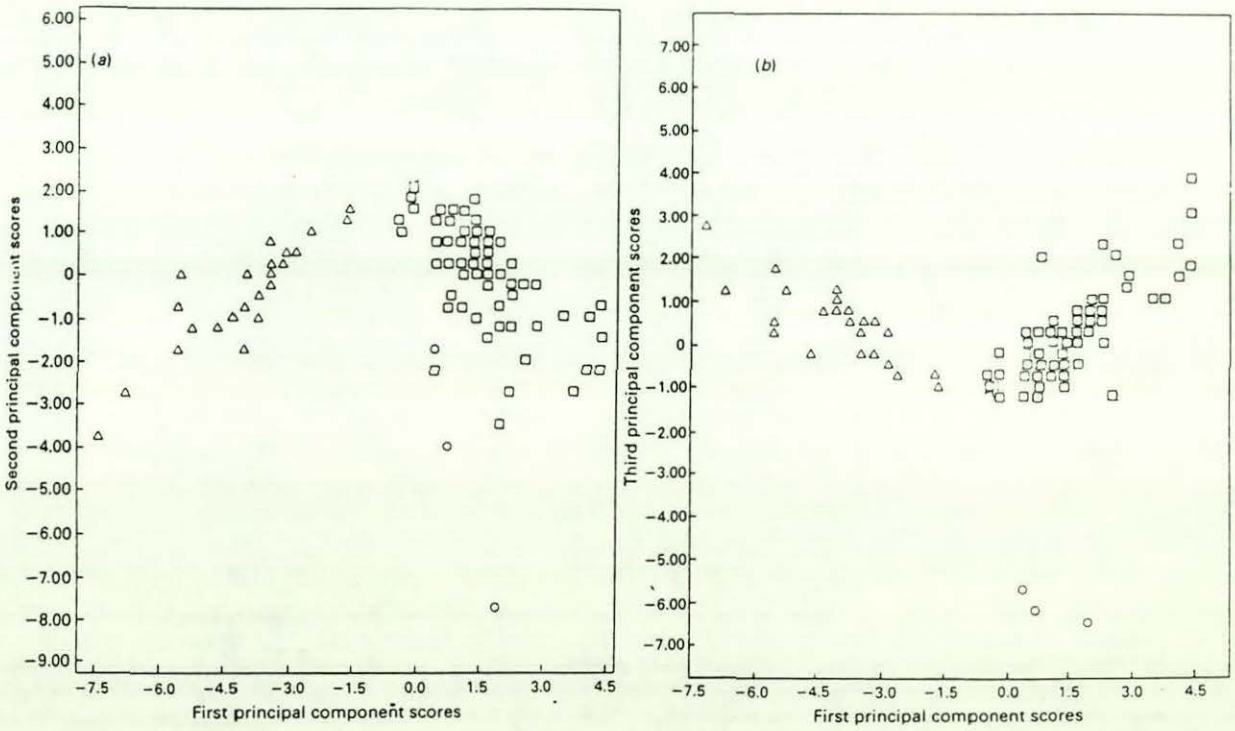
5.6.1. Principal component analysis

The initial means of interpreting the results was via principal component analysis (PCA). The first group of data to be analysed was that obtained from just feverfew and its possible adulterants. No tablet extracts were present as their composition was unknown. The input data was normalised to produce a correlation matrix (Appendix A.1), in which each of the variables

(i.e. peak in a chromatogram) was equally weighted. This should ensure that the minor components are emphasised and avoids the principle components becoming more a function of the units of measurement than of the underlying trend in the observations.

The first and second principal component scores (Figure 5.14 {a}) appeared to distinguish feverfew from German chamomile, both forming discrete clusters. Tansy was placed on the outer edges of the feverfew cluster. A good proportion of the feverfew samples were distinguished from tansy, but some of the feverfew varieties overlapped with the tansy population. However, overall a reasonable degree of what could be termed "appropriate" clustering has occurred.

FIGURE 5.14. Plot of principal component scores. Feverfew = □ , tansy = O German chamomile = △ .



One of the problems of interpreting principal components as representing the original data, is in assessing the importance of the loadings. By examining the first two loadings it can be seen that the variables (i.e. peak areas) 2, 3, 5, 6, 7, 8a, 8b, 9, 14, 15, 16a, 16c, 17 and 18b have a significant loading relative to the other variables (Table 5.6). In particular, the variables 2, 6, 8b and 16c are only present in German

chamomile and all possess large negative loadings. This corresponds with the first and second principal component scores for German chamomile which are mainly negative, so placing the species as a discrete cluster to the left of the feverfew and tansy cluster in Figure 5.14 {a}. Conversely, the variables 8a, 16a and 17 are only present in feverfew or tansy and these consist of both positive and negative loadings, so contributing to the positive and negative first and second principal component scores of these populations (Figure 5.14 {a}). Variables 3, 5, 7, 9, 14,15 and 18b also have large loadings even though they are present in more than one plant species. However, variables 5, 7, and 9 are more quantitatively prevalent in German chamomile, whereas variables 3, 14 and 18b are more abundant in tansy and/or feverfew. Thus, although these variables are not unique in any one plant species, there would appear to be sufficient quantitative variation between the plant populations to enable these variables to be classified as characteristic for a plant species.

The first two principal scores used to obtain the two dimensional plot, only account for about 37% of the variation in the original data. It therefore appeared that a great deal of the variation had still not been accounted for. Examining the third loading revealed that a great number of the variables possessed large loading values, in particular variables 5, 8a, 10, 11, 16a and 18b (Table 5.6). This demonstrates the importance of investigating the first 3 principal component scores which now account for about 47% of the variation. For example, it was not until the third loading that variables 10 and 11 were classified as being potentially characteristic of a plant species, even though they were only present in feverfew (Figure 5.13). Furthermore, variable 16a has the largest loading in the data set and it would appear that this variable is very important in distinguishing tansy from feverfew (Figure 5.14 {b}). However, variable 16b present in feverfew, did not possess a particularly high loading and this is probably due to the large variation in peak area for this variable within the feverfew population (See Appendix A.1). These results demonstrate the importance of obtaining the UV-visible spectrum of the components, as these were the variables (i.e. 8a, 8b, 16a, 16c and 18b) with some of the largest loadings, and which aided in the distinction of the plant species.

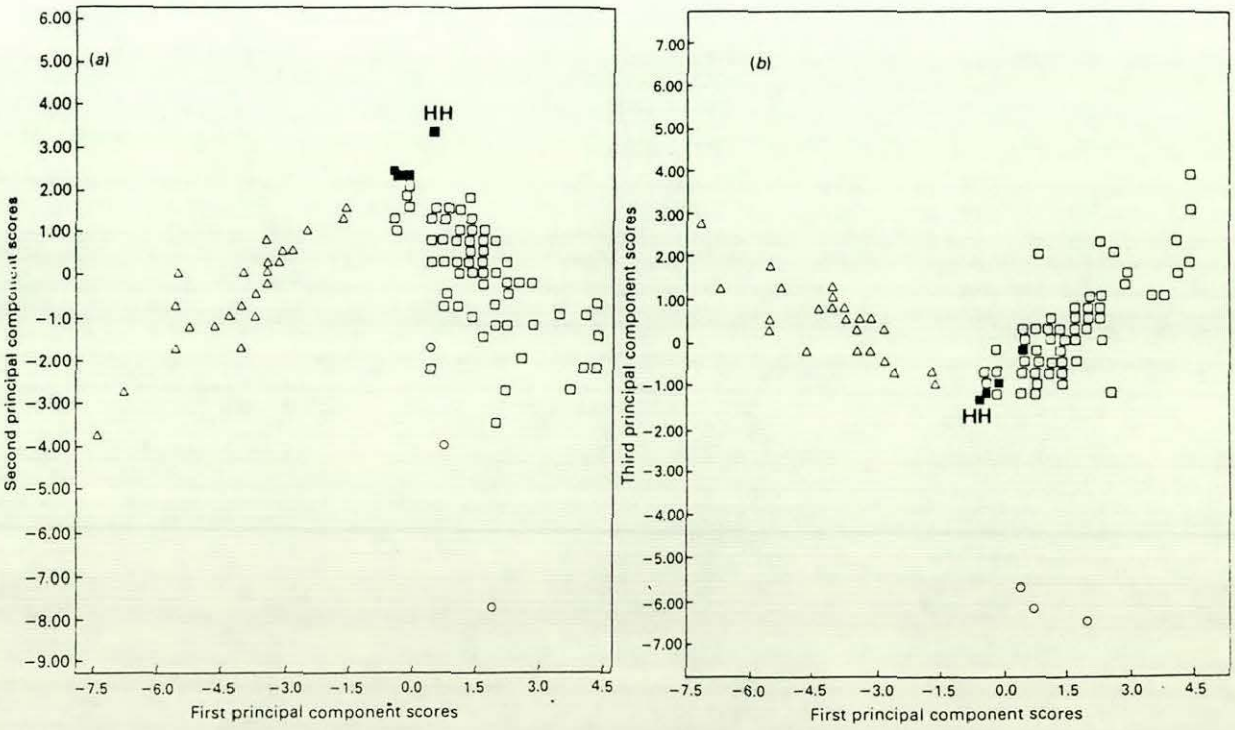
TABLE 5.6. The first 3 loadings (latent vectors) of the variables shown in Figure 5.13. Note ___ = large loading.

Variable	Loading 1st	2nd	3rd
1	0.1726	-0.1821	-0.2599
2	-0.2934	-0.1646	0.1309
3	0.1736	-0.2804	0.2339
4	0.0929	0.0852	0.0880
5	-0.0456	-0.3658	0.3394
6	-0.2657	-0.1485	0.1280
7	0.0103	-0.4566	0.0046
8a	0.0935	-0.2902	-0.3422
8b	-0.3328	-0.1571	0.1068
9	-0.2976	-0.1936	0.1765
10	0.1651	-0.0972	0.2999
11	0.1871	-0.0773	0.3185
12	0.2015	-0.1959	-0.1208
13	0.2468	-0.0395	0.0264
14	0.1293	-0.2840	0.0055
15	0.2978	-0.1760	0.1220
16a	0.0138	-0.2580	-0.4665
16b	0.2194	0.0806	0.0455
16c	-0.2592	-0.0909	0.1001
17	0.2524	-0.1138	0.0397
18a	0.2008	-0.0388	0.1716
18b	-0.1967	-0.2834	-0.2812
19	-0.1292	-0.0047	0.0166
20	-0.1430	-0.0073	-0.0024

Even with 47% of the variation being accounted for by the first three scores, this still leaves much of the variation unaccounted for. This is partially due to the feverfew population being so diverse, containing a wide range of botanical varieties, and possibly due to "seasonal or climatic" variations in the phenolic content of the plants. It is also probably related to the fact that only a small number of tansy plants were investigated, which do not necessarily fully represent the tansy population. There was also the limitations of the chromatography system. The extracts contained such a wide variety of components, that the isocratic elution system was unable to fully resolve the plant extracts. The less well retained components eluted near the solvent front as co-eluting peaks, whereas the more highly retained components were eluted as small severely tailing peaks or were not eluted at all. Therefore, the elution system failed to account for all the variations within the plant species. A possible alternative would be gradient elution HPLC, which would resolve all the components in the extracts but this equipment is considerably more expensive.

The data from the pharmaceutical preparations of feverfew were incorporated into the PCA analysis (Figure 5.15). All four brands (R.P. Scherer Ltd, Seven Seas, Herbal Laboratories and Heath Heather) appeared on the edge of the feverfew cluster. This may be due to the commercial feverfew preparations containing less plant material than was present in the 1g of whole feverfew plant material being analysed in the study. To compensate for this, the data from the tablet or capsule extracts were increased by an appropriate factor to make the data comparable to the other plant extracts. However, the values obtained for the pharmaceutical preparation were still low suggesting that some of the phenolics may have deteriorated with time. Furthermore, due to the low levels of plant material present in the pharmaceutical preparations, some of the minor phenolics present were not detected so that the data obtained only exhibited the major components. One of the brands (Heath Heather) is situated away from the other feverfew preparations (marked HH in Figure 5.15), but could still be judged as being within the feverfew population, due to the spread of samples in the principal component plot. This classification of Heath Heather tablets by PCA does not agree with the inferences drawn by studying the chromatograms by eye, when the origins of this tablet were seen as ambiguous.

FIGURE 5.15. Plot of principal component scores. Feverfew = □, feverfew tablet = ■, German chamomile = △, tansy = ○, HH = Heath Heather.



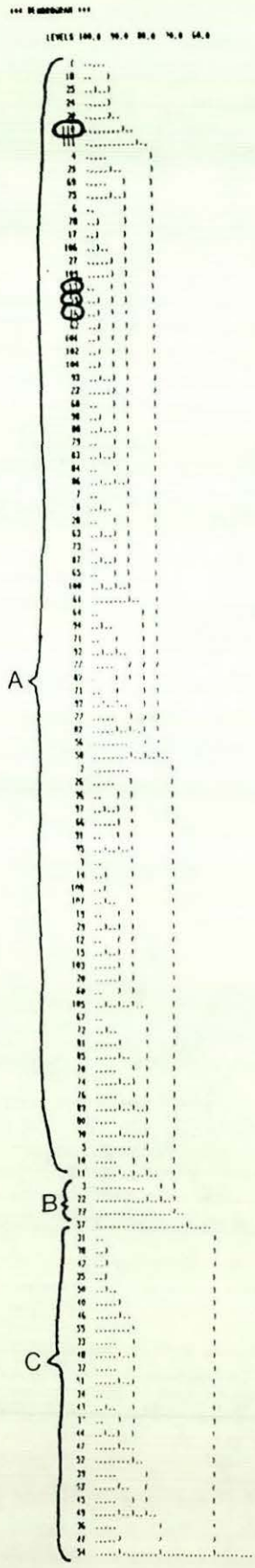
5.6.2. Hierarchical cluster analysis

Hierarchical cluster analysis was also used to interpret the phenolic distributions in feverfew, tansy and German chamomile. A variety of cluster analysis methods were investigated in this study. The single linkage cluster analysis classified tansy as a sub-group of feverfew and German chamomile as two sub-groups one belonging to feverfew the other being unique to German chamomile. This classification proved unsatisfactory. Four other cluster analysis techniques were investigated and most of these gave better discrimination. Three of the techniques (furthest neighbour, average link and median cluster method) classified the data into two groups (Figure 5.16), the large and varied feverfew group and the reasonably homogeneous German chamomile group which possessed at least 10% dissimilarity from the feverfew group. Tansy was placed on the periphery of the feverfew group as a possible sub-set. The fourth technique (centroid method) failed to clearly distinguish any of the groups. The K (nearest neighbours) value can also be varied, though no significant additional discrimination was achieved. It was found that using low K values (K = 1-3) was more advantageous due to the relatively small sample sizes involved.

The phenolic distribution in the pharmaceutical preparations of feverfew were incorporated into the survey. Again poor discrimination was obtained with the initial single linkage cluster analysis. However, the other four techniques represented by the furthest neighbour analysis (Figure 5.16), incorporated the tablets into the feverfew group. Three of the brands were grouped together, the fourth brand (Heath Heather), was placed on the edge of the feverfew group.

The major problem with these clustering procedures was that they were good at detecting spherical clusters but not so good when dealing with other shapes such as the elongated groups present in this data [129]. These techniques are particularly sensitive to outliers and it is these outliers or possible chemotypes that are probably causing the difficulties. This may become less of a problem if a larger study was to be undertaken with hundreds of plants, so that these outliers become well defined sub-clusters in the main cluster.

FIGURE 5.16. Furthest neighbour cluster analysis of the phenolics in feverfew, feverfew products, tansy and German chamomile.



A = feverfew plants

O = feverfew products

B = tansy plants

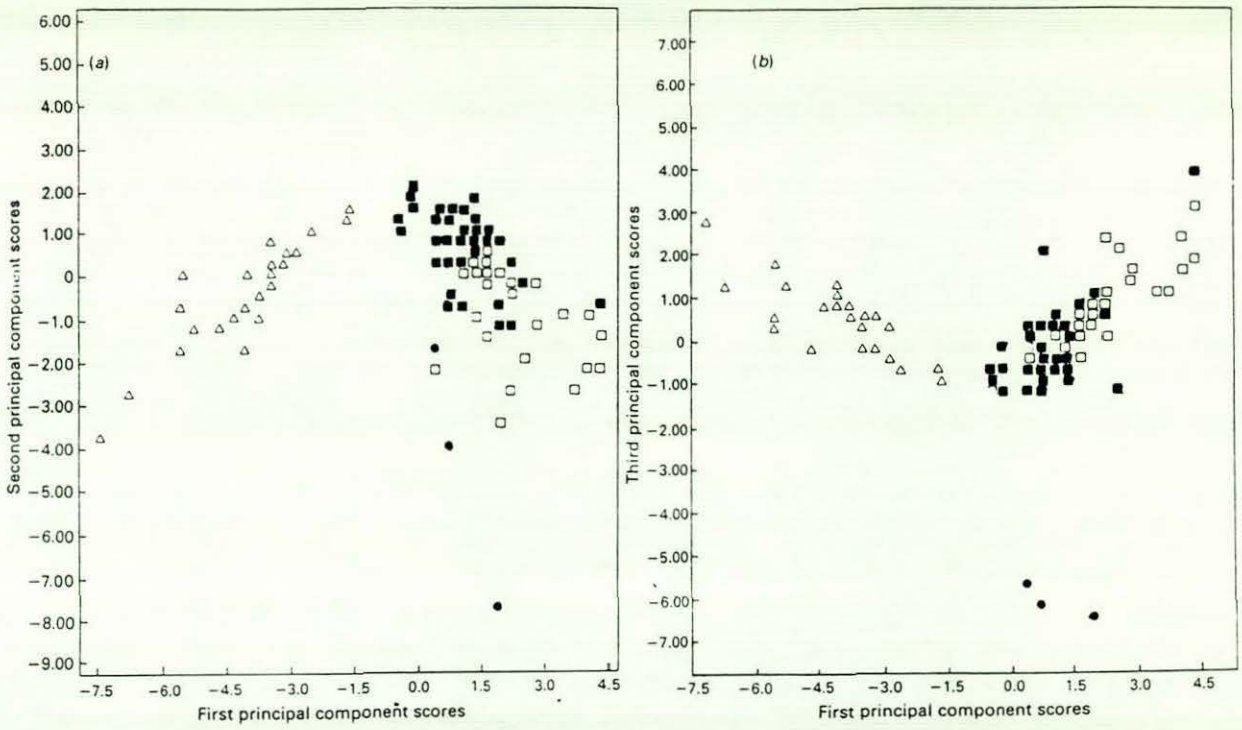
C = German chamomile plants

5.6.3. Sum of squares programme

So far the techniques used in this study have been unsupervised, in that no preconceived ideas have been placed on the data. However, a "semi-supervised" technique is available on Genstat, which classifies the data into n specified classes using the sum of squares criterion. With this technique reasonable discrimination was achieved between the plant species, German chamomile being perceived as a homogeneous group (group 1), compared to feverfew which seemed to possess two groups (groups 2 and 3) one of which also encompassed the tansy samples (group 2). The data generated from the semi-supervised technique was superimposed on the plot of the unsupervised principal component scores generated in Section 5.6.1 (Figure 5.14). It would appear that the two groups designated for the feverfew population (groups 1 and 2) in the sum of squares analysis are situated at either end of the feverfew cluster generated by the principal component analysis (Figure 5.17). The inability of many of the techniques to distinguish feverfew from tansy suggests there is as much variation within the feverfew population as there is between the feverfew and tansy population. When the data from the commercial feverfew preparations was incorporated into the sum of squares criterion, all four brands were classified as belonging to the feverfew population (group 2).

Supervised learning techniques such as SIMCA are available, in which the data is classified into groups by the user and the unknowns are assigned to a class according to the best statistical fit. However, it could be argued that the whole point of principal component analysis is to try and prove that the plant species are substantially different so as to appear as discrete groups. There is also the problem that it would be very difficult to obtain a complete phenolic distribution of a plant species, to form the training classes which would embrace all the different plant varieties and chemotypes. The training sets would have to be continually updated with new data. Therefore, this approach was seen as unsuitable for this study.

FIGURE 5.17. Sum of squares data incorporated into the principal component analysis. Feverfew = ■ (group 2), feverfew = □ (group 3) tansy = ● (group 2), German chamomile = △ (group 1).



5.7. Conclusions

The results demonstrate that there may be sufficient variation in the plant phenolic distribution to distinguish feverfew, German chamomile and tansy. The limitations arise when trying to differentiate feverfew from the closely related tansy species. To enable such a classification when using the methanol-acetate (30:70 v/v) eluent, the UV-visible spectrum of the phenolic compounds were required and this lowers the limit of detection (≈ 100 mg plant material). Unfortunately these few UV-visible differences often appear insufficient for chemometric techniques such as hierarchical analysis, to distinguish the species adequately. Furthermore, it may be impractical to rely solely on variations in UV-visible spectrum of the components, as the pharmaceutical preparations of feverfew often possess low levels of plant material, so that poorly resolved spectrum are obtained. Three brands of feverfew tablet were confirmed to contain feverfew, with the

fourth brand appearing to possess plant material of unknown origin, though the chemometrics analysis classified the sample as feverfew. However, as these techniques have identified the most distinctive components in the phenolic content of the plants, a more effective classification may be achieved, if in future analysis only these distinctive components are used in the chemometrics survey.

The acetonitrile-acetate (45:55 v/v) eluent resolved a small number of components which were not eluted using the methanol eluent. These components appeared to possess the potential to distinguish tansy from German chamomile and feverfew, but without the need to determine their UV-visible spectra. However, to ascertain the full potential of this eluent a much larger tansy population needs to be investigated. By contrast the gradient HPLC system was able to resolve all the components in the complex extracts and was therefore envisaged as the most practical way of fingerprinting the plant extracts. Further elucidation of the plant phenolics may be achieved with the acid hydrolysis of the extracts. However, these methods are time consuming.

Some of the plant phenolics are highly localised in their distribution and may be able to give an indication as to what part of the plant is present, such as the flowers or the leaves. This has important implications, as the plant leaves are a waste product in the manufacture of German chamomile tea, and are therefore a potential adulterant. However, quantification of the plant material based on the phenolic content of the sample may prove complicated, as the levels of phenolics was found to vary greatly, depending on the variety, age and composition (leaves or flowers) of the plant.

Due to the limitations of using the phenolic contents of the plant to distintinguish the plant species, alternative chemometric markers were investigated. All three plants are known to be rich in essential oils, especially feverfew, which has a high percentage of sesquiterpene lactones. Thus the rest of the study is involved in assessing the ability of the essential oils to fingerprint the plant species. Because the oils were found to be unstable in the preliminary study, supercritical fluid extraction (SFE) is to be investigated as this is milder than steam distillation or solvent extraction.

Chapter6

Supercritical fluid extraction

6.0 Introduction

In the light of a continually shrinking list of safe solvents, ever tightening environmental concerns, rising cost of energy and thermal instability of many ingredients, alternatives to conventional solvent extraction methods has been sought. One such alternative is supercritical fluid extraction (SFE). SFE refers to the extraction of a material with a solvent above its critical point. The solvent power of a supercritical fluid (SCF) was discovered by Hannay and Hogarth [143] in 1876, but it was not employed usefully to any extent until the middle of this century. Efforts in recent years have centred on separation methods that are clean and efficient and do not cause thermal degradation of the products. This has led to the extension of SFE into new areas of food and biomaterial separations.

The extraction of vegetable oils represents an extensive area of application [144-147] where the use of dense CO_2 gas can replace several steps of classical procedures and so largely reduce the total expenditure for the edible end product. In the patent literature, there are a series of publications concerning the detoxification of tea [148], coffee [149] and tobacco [150] with unique methods differing in the details of the process technology. Various patents exist for extraction of soft resins from hops using sub- [151] and supercritical CO_2 [152]. The extraction of flavours [153,154], spices [155] and volatile oils [156] is of special interest to the food sector. Furthermore, there are a number of special applications, such as the separation of alcohol-water mixtures [157] and the regeneration of absorbents [158].

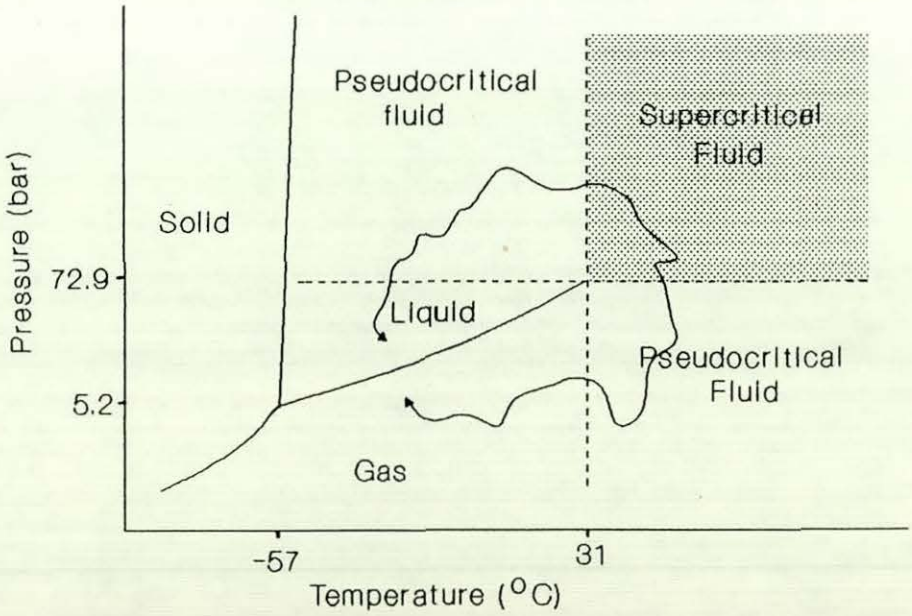
It is in this context that SFE is to be used to isolate the terpenes in feverfew, which will be investigated as potential chemotaxonomic markers.

6.1 Supercritical fluids

A series of potential supercritical solvents are available, but by far the most popular is carbon dioxide. This is due to its good solvating properties, low viscosity, large diffusion coefficient, accessible critical point, non toxicity and low cost (being the cheapest solvent next to water [159]).

A SCF is a substance which has been raised above its critical temperature (T_c) and critical pressure (P_c), as seen in the pressure - temperature phase diagram for carbon dioxide (Figure 6.1). The phase boundaries indicate the coexistence of two phases. The liquid - gas line extends from the triple point where all three states of matter coexist, to the critical point where both a dense gas and molecularly diffuse liquid coexist and which are indistinguishable from each other. At temperatures above T_c it is not possible to liquefy the gas no matter how much pressure is applied. Conversely P_c is the lowest pressure which will liquefy the gas at T_c.

FIGURE 6.1. Phase diagram of CO_2. The arrows demonstrate going from a liquid to a gas with no observable phase change.



For SCF solvents, density dependent properties such as solubility parameters [160,161], dielectric constants [162] and transport properties such as diffusion coefficients [163], may be changed appreciably by slight changes in the solvent pressure or temperature. This adjustability in the solvent strength is the basis of SFE. In the supercritical and *pseudo* - critical regions (Figure 6.1) these variations in fluid properties are continuous in nature, so that as long as the fluid temperature or pressure is above the critical values, no abrupt changes are to be expected. Such variations are desirable during the SFE process. Therefore SFE should not be restricted to the supercritical region alone but should also include the *pseudo* - critical regions. Where discontinuous variations occur, crossing phase boundaries, or where the solubility of the solvent tails off as it approaches an ideal gas, then precipitation of a solute may occur. This is desirable for the collection of solutes after extraction.

The physical properties of a SCF are generally intermediate between those of a gas and a liquid (Table 6.1). The SCF possess a liquid like density over much of the range of industrial interest and generally has viscosities which are an order of magnitude smaller, and diffusivities an order of magnitude larger than a liquid. This provides appreciable penetrating power into the solute matrix. Furthermore, the supercritical solvent presents no surface tension or wetting problems as associated with polar liquids, so that SCF loadings may exceed liquid loadings, as the higher temperatures in SFE conditions result in higher solute vapour pressures and thus mass transfer.

TABLE 6.1. Typical physical properties associated with different fluid states of carbon dioxide.

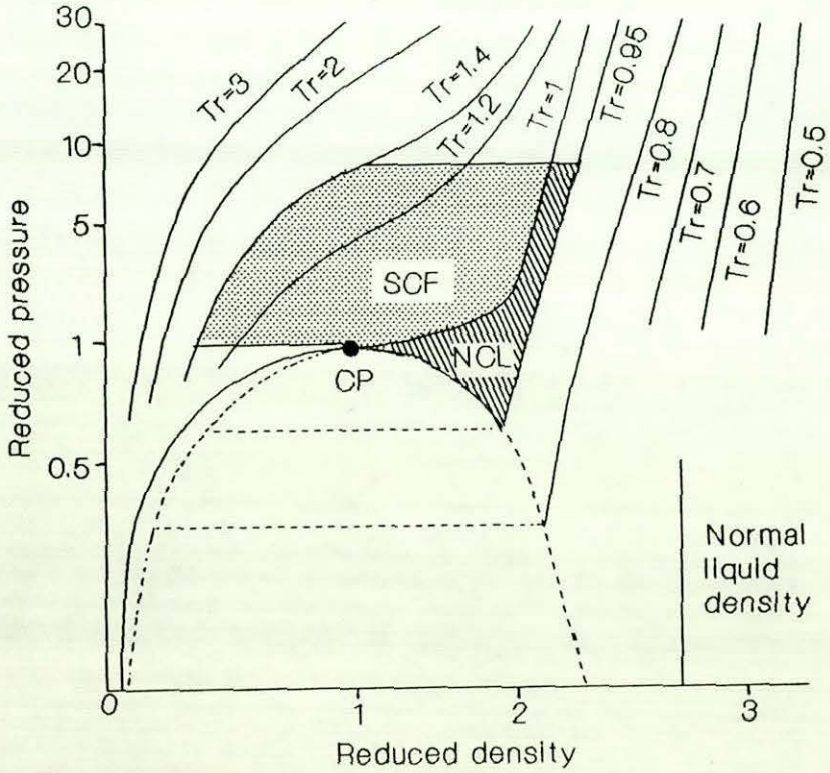
Phase	Density (g/cm^3)	Diffusivity (cm^2/s)	Viscosity (g/cm sec)
Gas P=1atm, T=15-30°C	$(0.6-2)\times10^{-3}$	0.1 - 0.4	$(1-3)\times10^{-4}$
Liquid P=1atm, T=-15-30°C	0.6 - 1.6	$(0.2-2)\times10^{-6}$	$(0.2-3)\times10^{-2}$
Supercritical Fluid P=73-300atm, T=31°C	0.2 - 0.9	$(0.2-0.7)\times10^{-3}$	$(1-9)\times10^{-4}$

6.2 Solubility

6.2.1 Effects of temperature and pressure

In theory, two properties are responsible for determining the solubility of a solute in a supercritical solvent, namely, the volatility of the solute and the intermolecular forces in the SCF phase [164]. The latter may be manipulated in order to increase extraction efficiency. Since the ability of a solute to dissolve in a SCF is generally thought to be proportional to solvent density [165], the extraction of a solute can be controlled as a function of temperature and pressure. Typical relationship between density and pressure at different temperatures are shown in Figure 6.2.

FIGURE 6.2. Pressure-density isotherms for carbon dioxide [157].
Note, Tr = reduced temperature (temperature/critical temperature).

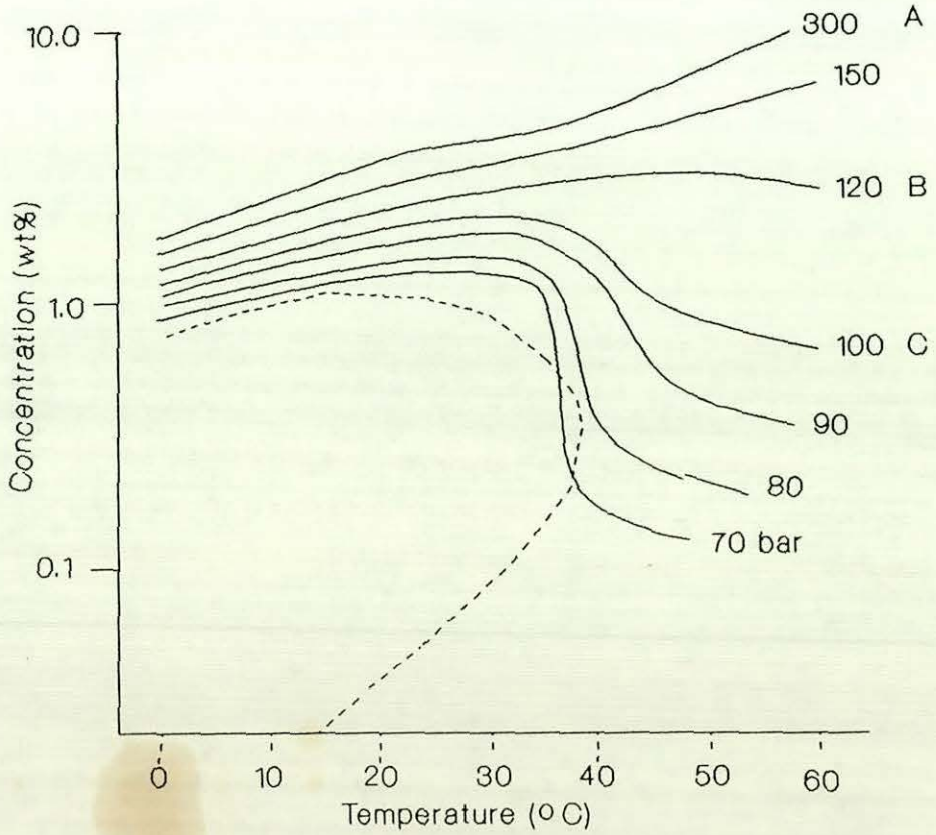


The region of greatest interest is near the critical point (CP) marked SCF and NCL (Figure 6.2). In these regions relatively small changes in temperature and pressure produce large changes in density. At higher temperatures the pressure-density isotherms are very much steeper so that

meaningful density changes require substantial pressure changes. Therefore supercritical extractions are usually carried out at not more than 100°C above T_c. Conversely, the greatest density changes occur between 70 and 400 bar in the supercritical region, at higher pressures the pressure-density isotherm becomes prohibitively steeper. Thus the majority of SFE processes occurs in the SCF and NCL region of Figure 6.2.

The importance of these density changes with temperature and pressure can be seen in the CO_2/Naphthalene system [163]. The two major factors controlling solubility are solute vapour pressure and solvent density. A rise in temperature from 20 to 60°C at constant pressure leads to the vapour pressure of the solute increasing which tends to increase solubility and conversely the SCF density decreases which tends to decrease solubility. The consequences of these two competing effects can be seen in Figure 6.3. In line "A" density is less sensitive to temperature so that vapour pressure effects dominate. At line "B" the two competing effects balance each other and at line "C" density effects dominate.

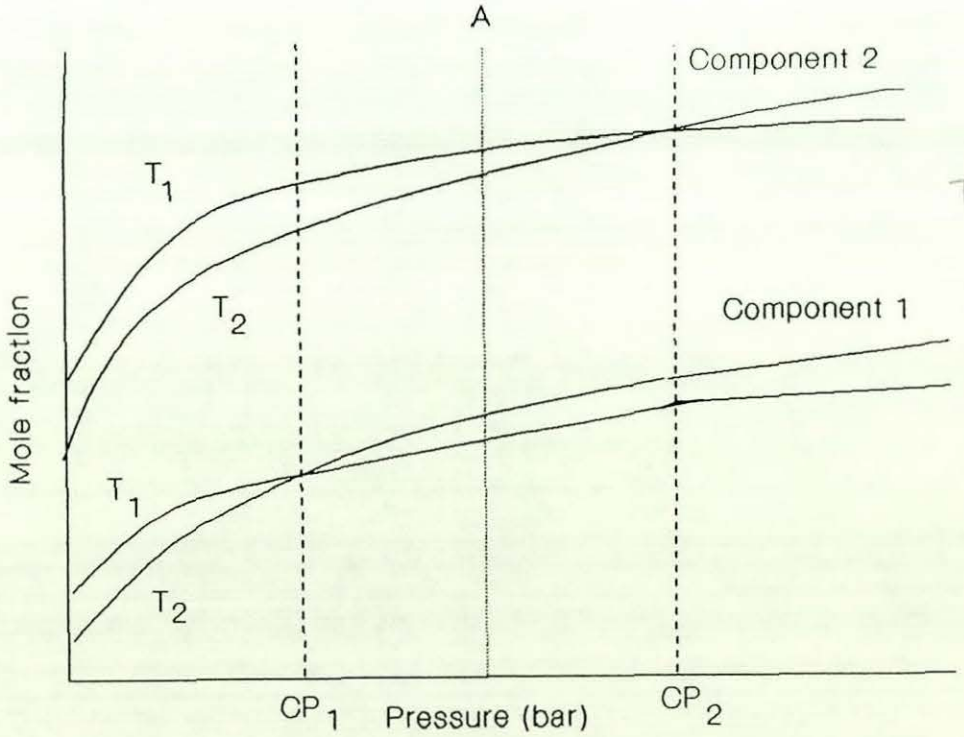
FIGURE 6.3. Solubility of naphthalene in carbon dioxide as a function of temperature and pressure [162].



6.2.2 Crossover region

An interesting feature of the solubility of a solute in a SCF, is the existence of crossover points [167,157] (Figure 6.4). For a solute the crossover point is unique with respect to temperature. Below the crossover pressure an increase in temperature causes a decrease in the solubility in the SCF, while above the crossover pressure the opposite effect occurs. This is thought to be due to the competing effects of solute vapour pressure and solvent density. It is an unusual phenomenon that does not exist in liquid solvents at usual liquid extraction conditions. The crossover pressure for a specific solute can vary considerably with different supercritical solvents [168].

FIGURE 6.4. Separable crossover points with two solutes dissolved in a supercritical solvent [167]. T_1 = low temperature, T_2 = high temperature.



The importance of crossover points is that they exist in multicomponent mixtures, individual constituents possessing their own unique crossover point. This has been applied to SFE to enhance selectivity [167,169,170]. If a mixture is heated isobarically at a temperature (point A) which is below the crossover point of one solute (CP_1) but above the crossover point of another (CP_2), then selective precipitation could be achieved. At this

pressure, increasing the temperature will cause the solubility of component one to increase and the solubility of component two to decrease. Presently, data in the crossover region for multicomponent systems is limited, as previous work has generally been directed at the higher pressure regions where solubilities are higher and this phenomenon is absent. Schaeffer *et al.* utilised this cross-over point to obtain a selective extract of the alkaloid monocrotaline from the seeds of *Crotalaria spectabilis* [170].

6.2.3 Solubility parameter

The concept of a solubility parameter (Hilderbrand and Scott, 1950 [171]), has been known for liquids for many years and is used extensively in interpreting and predicting thermodynamic behaviour of solutions in a semiquantitative way. Defined as the square root of cohesive energy density, the solubility parameter (δ) of a liquid is usually calculated from the heat of vaporisation and molar volume of liquid. For SCFs, the heat of vaporisation is no longer meaningful as it becomes zero at the critical temperature, therefore an empirical correlation was developed by Giddings *et al.* [172,173].

$$\delta = 1.25\ P_c^{1/2}\ (p/p_{liq.})$$

P_c = critical pressure
p = density of solvent
$p_{liq.}$ = reference density in liquid state

The solubility parameter can be separated into two terms, $1.25P_c^{1/2}$ and $p/p_{liq.}$. The first is referred to as the chemical effect. It depends upon the identity of the gas, specifically the intermolecular forces of that compound. The second term is a state effect. As $p \rightarrow p_{liq.}$, a maximum value is obtained for δ. Hence, while there is a maximum value at high pressures, the solubility parameter can assume a whole range of values below that maximum. The maximum value of δ is determined by solvent identity (ie the chemical effect), while all other values depend upon the physical state. There is a threshold density below which a solute is not soluble in the dense solvent.

The solubility parameter δ varies from 0 up to liquid-like values of 10 at high densities [174]. A plot of solubility against density [174] resembles that of density-pressure isotherms (Figure 6.2) demonstrating the direct relationship between density and solubility. The δ for gaseous carbon

dioxide is essentially zero whereas the value for liquid carbon dioxide is comparable with that of a hydrocarbon like hexane (δ = 7.3). This can be seen with a large increase in δ upon condensation from a vapour to liquid as occurs at -30°C. Above the critical temperature, a range of solubilities can be obtained with a small isothermal pressure change and to a lesser degree by a small isobaric temperature change. The ability to adjust the solvent strength of a supercritical fluid is its unique feature, and it can be used to obtain selectivity in extraction processes.

Carbon dioxide is seen as a non-polar solvent, being symmetrical and having no permanent dipole moment. However, CO_2 can possess an induced dipole moment, this being determined by the polarizability of the molecule. The dielectric constant of CO_2 can be changed by varying the pressure [162], with the greatest changes occurring between 70 and 300 bar.

It is interesting to note that very dense supercritical CO_2 has a density and dielectric constant similar to liquid CO_2, both of these having a dielectric constant in the region of pentane [175,176]. This observation would appear to contradict the idea that the supercritical fluid is intrinsically different as a solvent from the liquid phase. Supercritical CO_2 does offers advantages over liquid CO_2 as a solvent due to higher diffusitivity and mass transfer values, but it has not been demonstrated that the solvent behaviour is essentially different between the two phases. In fact, Alwani has shown that the solubility of α-tocopheryl acetate in both subcritical and supercritical CO_2 increases with increasing pressure and that no significant changes in the solubility occur between the phases [177]. Wright *et al.* found that supercritical and liquid CO_2 possessed comparable extraction efficiencies for analytes from absorbent material, but supercritical CO_2 had an order of decrease in extraction time [178]. Therefore the supercritical region is of such interest because useful temperatures, pressures, and solubility levels lie there, not because supercritical CO_2 is intrinsically a more polar solvent than subcritical CO_2.

To confirm this perspective on CO_2, solubility test data has been obtained for a range of organic compounds in sub- and supercritical CO_2 [179]. In general the difference in solubilities between sub- and supercritical CO_2 was a matter of degree, often an order of magnitude. Seldom was a material found to be completely insoluble under subcritical conditions yet soluble in the supercritical phase.

6.2.4 Solvatochromic probes in CO_2

The ability of a solvent to induce a chemical or physical change is usually rationalised in terms of the solvents "polarity", where polarity includes the sum of all solvent-solute interactions: coulomb, inductive, charge transfer, and hydrogen bonding. In general, the dielectric constant does not give a good measure of solvent polarity being a bulk or macroscopic property, whereas solvent-solute interactions occur at the molecular level. An alternative approach is the use of spectroscopic solvatochromic scales to correlate and in some cases to predict solubility phenomena [179-185].

The solvent strength or solvatochromic scale is defined as the transition energy, $E_T = hc/\lambda_{max}$, where h is Planck's constant, c is the speed of light and λ_{max} is the wavelength of maximum absorption. An alternative scale is the Kamlet and Taft π^* scale which is suggested to be more appropriate for use with supercritical fluid solvents [186]. It is an empirical relationship between measured solute absorption maxima in a solvent and the polarity/polarizability of that solvent:

$$v = v_0 + s\pi^*$$

v = absorption maximum in test solvent
v_0 = absorption maximum in reference solvent
s = solute dependent parameter
π^* = measured solvent polarity/polarizability parameter

Hyatt [180] compared liquid and supercritical CO_2, using two UV-visible solvatochromic probes. There was little difference between the E_T in the liquid and supercritical state, with CO_2 being in the same low polarity range as hexane. This view is confirmed by Deye *et al.* [186] whose used data from three probes and found liquid CO_2 comparable to liquid pentane. Frye *et al.* [187] used a wider range of non-polar solvents and found sub- and supercritical CO_2 to have λ_{max} values in the range between those of the perfluoro-alkanes to that of the n-alkanes (n-pentane, etc.). Caution must be taken here, as many investigators have implied that the solvent strength of a supercritical fluid approaches that of a liquid at the point where the density approaches that of a liquid. This can be misleading. At 50°C and 130 bar, the density of CO_2 approaches that of n-hexane (density ≈0.68 g cm^{-3}), yet at these conditions CO_2 is a weaker solvent for aliphatic and aromatic hydrocarbons [188]. This may be because that at these conditions the

polarizability of CO_2 is less than that of hexane. To obtain a similar δ value the pressure of CO_2 must be increased to 350 bar (density $\approx$ 1g/cm^3). At this pressure, the polar/ polarizability value π^* is a little more than that of n-hexane, which suggests that there are other molecular interactions present such as electron-acceptor forces [189].

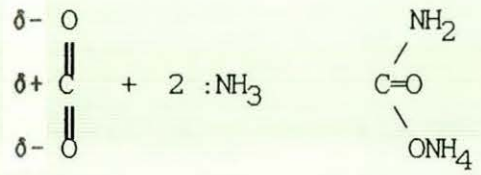
So far the solvatochromic probes have been used to investigate the non-specific forces in CO_2 namely the polar/polarizability of the solvent. However, they have also been used to investigate the specific acid-base and hydrogen bond forces which are claimed to exist. Sigman et al [190] determined the Kamlet-Taft β scale of HBA (hydrogen bond acceptor) basicities for supercritical CO_2 as a function of density. The values ranged from about -0.10 for gas; -0.05 for liquid; and -0.08 for supercritical CO_2. Since all the values are essentially zero, β, unlike π^*, does not show any correlation with the density of the CO_2 and suggests a non-hydrogen bonding solvent. (Note cyclohexane, acetone and methanol have β values of 0.0, 0.48 and 0.71 respectively [191]).

Hyatt [180] investigated the acidity of liquid CO_2, measuring shifts in the IR C=O stretching frequency of acetone and cyclohexanone. The acidity of CO_2 was insignificant as it gave the same results as nonpolar non-acidic liquid solvents. The ability of CO_2 to be involved in H bonding was also investigated, by studying the solvent induced frequency shifts of the N-H stretch in pyrrole [180]. CO_2 has two carbonyl oxygens that compete with other hydrogen bond acceptors for the hydrogen bond donors in solution. For pyrrole in solution, CO_2 behaves like a solvent in the ether to ethyl acetate range. Carbon dioxide therefore appears to exhibit properties typical of hydrocarbon solvents; however, for acidic molecules, such as pyrrole, CO_2 provides more H bonding basicity than do the hydrocarbon solvents.

R-X-H	+	:Y-R	$\longrightarrow$	R-X-H...Y-R
Electron pair acceptor (Lewis acid)		Electron pair donor (base)		Hydrogen bond

This may explain the enhanced solubility of acridine in supercritical CO_2 compared to that in supercritical ethane [192]. It has been suggested that an acid-base complex forms between the basic nitrogen of the acridine and supercritical CO_2. However the acidity of CO_2 becomes even more important in the presence of strong bases such as ammonia and amines, as solid

carbamates are formed [183].



Lewis acid (weak acid)	Lewis base (strong base)	Lewis acid-base pair

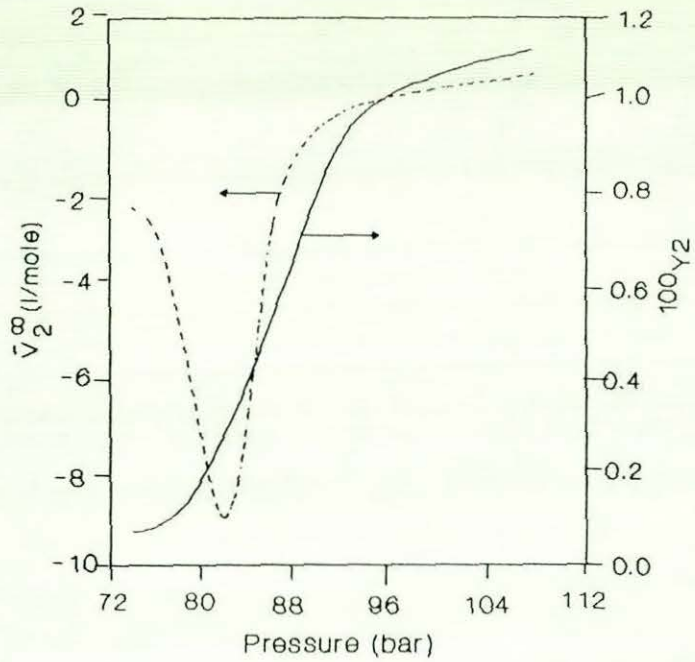
Phillips *et al.* [189] suggest that supercritical CO_2 possesses Lewis base and H bonding properties which give it selectivity properties not observed in hexane. It has been claimed that benzoic acid, phenol and methanol all undergo Lewis acid-base and H bonding interactions with CO_2.

6.2.5 Clusters

A variety of complex interactions exist between the solvent and solute some of which are nonspecific, occurring between any solvent-solute pair, while others interactions (such as H-bonding) are dependent upon the specific solvent and solute. As the strengths of such interactions depend on intermolecular distances, the relative importance of each contribution is density dependent. However, much of the unusual behaviour in supercritical fluids is now being related to the formation of loose aggregates or clusters. The existence of these clusters may explain phenomena including enhanced solubility and cosolvent effects.

A strong indication of the unusual behaviour in supercritical fluid solutions was seen with the measurement of the partial molar volume (V^{∞}) of napthalene at infinite dilution in supercritical CO_2 [193], (Figure 6.5). At high pressures, the solvent is sufficiently dense such that the addition of solute will give a partial molar volume approaching a positive value, due to repulsive forces. A very sharp negative dip in V^{∞} was observed for the solute in the compressible region of the solvent, which is near the solvent critical point. In this region the attractive forces between the solvent and solute cause fluctuations in the structure of the solvent fluid by compressing the molecules into energetically favourable locations, known as clusters. This can be envisioned as the collapse of the solvent shell about the solute. The minimum in V^{∞} corresponds to the greatest rate of increase in solubility.

FIGURE 6.5. Partial molar volume (V^{∞}) and solubility (Y_2) vs. pressure for naphthalene in CO_2 at 35.23°C [193].



Since clusters are caused by the same types of solute-solvent intermolecular forces (i.e. dispersion, induction and dipole-dipole forces) that influence solubilities, solvatochromic probes may be used to determine the presence of clusters [194]. The degree of clustering may be determined by a direct comparison of the experimental value of the solvatochromic parameters, for example, E_T, with the value which is calculated for a homogeneous polarisable dielectric solvent which has no specific interactions such as H-bonding [195]. At high densities where SCF are relatively incompressible the two parameters coincide. However, near the critical region where the fluid has high compressibility the two parameters diverge. This is related to the formation of clusters.

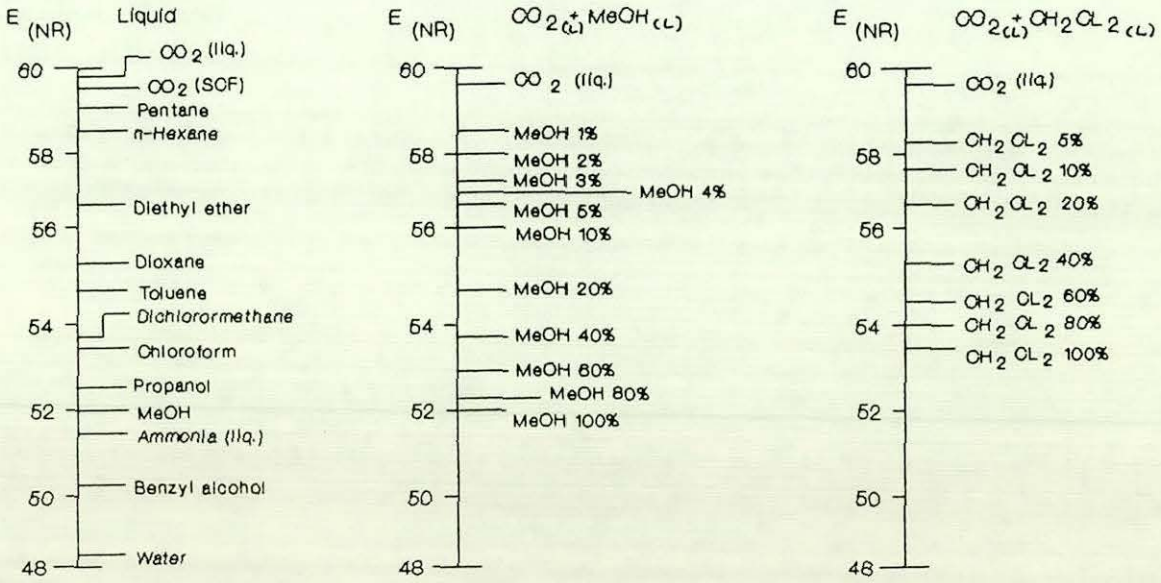
A number of other experimental studies using spectroscopy have addressed the formation of clusters in SCF solutions. Kajimoto *et al.* have used both absorption and fluorescence to look at (N,N-dimethylamino)benzonitrile in supercritical CHF_3 [196]. Fluorescence spectra have been obtained for naphthalene and pyrene in supercritical CO_2 and ethylene, [197]. The technique provides information concerning solute-solvent clustering at extremely low concentrations. Solute-solute clustering, as well as solute-solvent clustering, has been observed recently using computer simulation and integral equation calculations of radial distribution functions [195,196].

6.2.6 Entrainer effects

In the chemical engineering literature, clustering of polar modifier molecules around polar solute molecules in large excess of a supercritical fluid is largely accepted [184,191,198-200]. Such clustering appears to become more pronounced as the difference in polarity between the supercritical fluid and the modifier increases [184]. It has also been shown that the modification in the solvents density due to the addition of the cosolvent contributes only slightly to the solubility enhancement [199].

Deye et al undertook a comparison of three solvatochromic dyes in normal fluids, CO_2 and CO_2 with co-solvents [186]. This enabled a comparison of the determined solvent strength of CO_2 with and without co-solvents, to that of conventional liquid solvents. A schematic of the results is shown in Figure 6.6. Very little change in solvent strength is observed when the pressure of CO_2 is increased from 76 bar to 241 bar at temperatures from 25 to 40°C. At low levels of co-solvent (≈ 1%) there is little observable effect on the solvent strength of CO_2. It would appear about 5% of the polar modifier methanol is needed before an appreciable increase in solvent strength is discernable. A similar result has been found with the absorption spectrum of mesityl oxide in the presence of 1% methanol in supercritical CO_2 [185].

FIGURE 6.6. Comparison of transition energies calculated from the wavelengths of maximum absorption of Nile Red solvatochromic dye in a number of solvents [186].



A detailed investigation of the effects of pressure on solvatochromic probes in pure and modified CO_2 [184], was carried out by Johnston *et al.* It is apparent that adding methanol increases solvent polarity and that modified CO_2 is affected by increasing pressure, similar to pure CO_2. However, above 9.5% methanol, pressure no longer seem to have an effect. It is thought that at the higher methanol concentrations, the solute co-solvent clusters are enriched in methanol relative to the bulk concentration and that this methanol rich cluster is not highly sensitive to pressure effects, as would be the case for pure methanol.

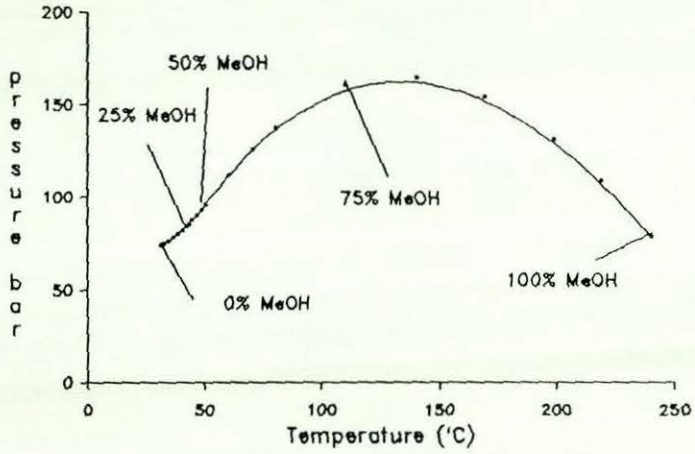
Most cosolvent induced solubility enhancements can be explained qualitatively by using the dispersion and acid base solubility parameters of the solute and cosolvent [199]. For example, the solubility enhancement of benzoic acid (δ^A = 9.3 $\{cal/cm^3\}^{1/2}$) is significantly greater for methanol (δ^B = 8.3 $\{cal/cm^3\}^{1/2}$) than for the weaker base acetone (δ^B = 3.0 $\{cal/cm^3\}^{1/2}$) [199]. Lira claims that these acid-base interactions are a secondary cosolvent effect superimposed on a primary effect determined by cosolvent concentration [201]. Entrainer effects are not always observed in supercritical CO_2. Walsh et al state that the solubility of fluorenone (Lewis acid) is not enhanced by methanol in supercritical CO_2 [197]. It is thought that the CO_2 competes with fluorenone to form H bonds with methanol, the combined effect of carbon dioxide's weaker H bond accepting tendency but higher concentration masks the entrainer effect.

Non-polar, polarisable hydrocarbon cosolvents have claimed to be as effective as polar cosolvents for enhancing solubilities in CO_2 [199]. However, it is claimed that the effect of such cosolvents in supercritical ethane is negligible, as ethane is more polarisable than CO_2 [201]. Dispersion forces are stated as the major contribution to the interaction between non-polar solute and cosolvent [164]. Solubility increases with chain length, for the straight chain alkane cosolvent while it decreases as the degree of branching increases.

Cosolvent studies must be carefully performed. Addition of the cosolvent shifts the critical properties from the pure solvent critical properties. These conditions must be known to assure that the mixture remains homogeneous throughout the range of experiments. The critical curve of CO_2/MeOH can be seen in Figure 6.7 [202]. Special care should also be taken in the modifier storage. It has been shown that two values for a solvatochromic dye in methanol have been obtained [186]. The less polar

value was obtained from a freshly opened bottle of methanol, and the more polar value from an older, used bottle. The two values represent the extremes observed over several months using methanol with no special preparation. Small concentrations of water purposely added to the modifier gave small spectral shifts similar to those observed with "old" bottles of modifier.

FIGURE 6.7. Critical curve of CO_2 and cosolvent methanol [202].



6.2.7 Synergistic effects

Solute/solute/solvent studies have been reviewed [201] and it has been suggested that a solute may enhance the solubility of another solute in the sample in supercritical fluids. For example, in the naphthalene/ phenanthrene/CO_2 system, naphthalene increases the solubility of phenanthrene and the ternary system selectivity is substantially below the selectivity predicted from the ratio of binary solubilities. Thus substantially soluble solutes can act as cosolvents for less soluble compounds.

Dobbs demonstrated that the solubility of pure hexamethylbenzene is influenced very little by the cosolvent methanol or by benzoic acid [164]. However, at 350 bar in the presence of benzoic acid and 3.5% methanol, the solubility of hexamethylbenzene increased by 30% [164]. Methanol appears to act indirectly by raising the solubility of benzoic acid to such an extent,

that it acts as a cosolvent for hexamethylbenzene.

Conversely, Schaeffer *et al.* showed that the presence of lipids in the seeds of *Crotalaria spectabilis* reduce the solubility of the alkaloid monocrotaline in supercritical CO_2 [170]. Shimshick investigated the extraction of aqueous sodium carboxylate solutions with SCFs [203]. In this case supercritical CO_2 reacted with the sodium salts in the aqueous phase to form a carboxylic acid which was extracted into the SCF phase.

6.2.8 Limiting factors in SFE

So far, extraction has been discussed in terms of maximum solubilities, such correlations being quite useful when the target analytes represents a large percentage of the bulk sample (e.g., the extraction of fats from meat products). However, for many extractions involving complex matrices, the analyte is present in minor or trace amounts, so that the concentration of the analyte in the SCF is well below the solubility limit. For such samples, maximum solubility is no longer of concern as the analyte need only be soluble enough in the SCF to be transported out of the extraction vessel. Thus solubility parameters appear to only address part of the extraction problem. As extraction of the analyte may depend on its distribution between the SCF and the sorptive sites on the sample matrix, the ability of the SCF to compete with the analytes for the sorptive sites may be more important than solubility considerations [204]. The rate of extraction will therefore not be determined principally by solubility, but by the rate of mass transfer out of the matrix.

If the rate determining step of the extraction process is the actual mass transfer of material from the matrix to the SCF, then it has been suggested that the structure of the matrix will have a direct influence on the process [205]. For example, the gas-like diffusivity of a SCF will enhance diffusion in an extraction of an analyte when present on the surface of a solid. If, on the other hand, the extraction is from a liquid phase into a SCF phase, then the gas-like diffusion characteristics of the SCF solvent will not have an enhancing effect on the overall mass transfer rate. Similarly, if the extraction is occurring from within a non-porous particle, such as a polymer, where internal solid-phase diffusion will probably govern the overall rate of mass transfer, then the external phase diffusion of the SCF

will have little or no effect on the rate of mass transfer.

Spiro suggests there are three main factors which determine the rate of extraction, namely: the diffusion of the analyte through the matrix; its transfer across the matrix/solvent interface; and its diffusion away through the Nernst layer [206]. With the extraction of [6]-gingerol from ginger with supercritical CO_2, Spiro suggests that the rate determining step is the diffusion of the analyte through the matrix [207], which by using steady state theory [208] predicts that the diffusion coefficient is given by:

$$D = \frac{k_{obs}\, r^2}{12} \Bigg/ \left(1 + \frac{m}{pVK}\right)$$

k_{obs} = first order rate constant
r = radius of ginger particle
m = mass of ginger particles
p = density of ginger particles
V = volume of supercritical CO_2
K = partition constant (volume concentration) of [6]-gingerol between the ginger particles and the supercritical CO_2

However, the results do not entirely exclude the possibility of the rate being determined by diffusion through the Nernst layer surrounding each particle, though it has previously been shown in ginger extraction with acetone [209] that Nernst layer diffusion was not rate determining, a major contributing factor being the much smaller diffusion coefficient of the soluble constituents inside the ginger matrix than outside it.

The extraction rate of [6]-gingerol from ginger using supercritical CO_2 in the static mode [207] can be represented by a first order equation of the kind:

$$\ln \left(\frac{C_\infty}{C_\infty - C}\right) = k_{obs}\, t$$

C = concentration of extracted [6]-gingerol
C_∞ = equilibrium value of extracted [6] gingerol
k_{obs} = first order rate constant
t = time

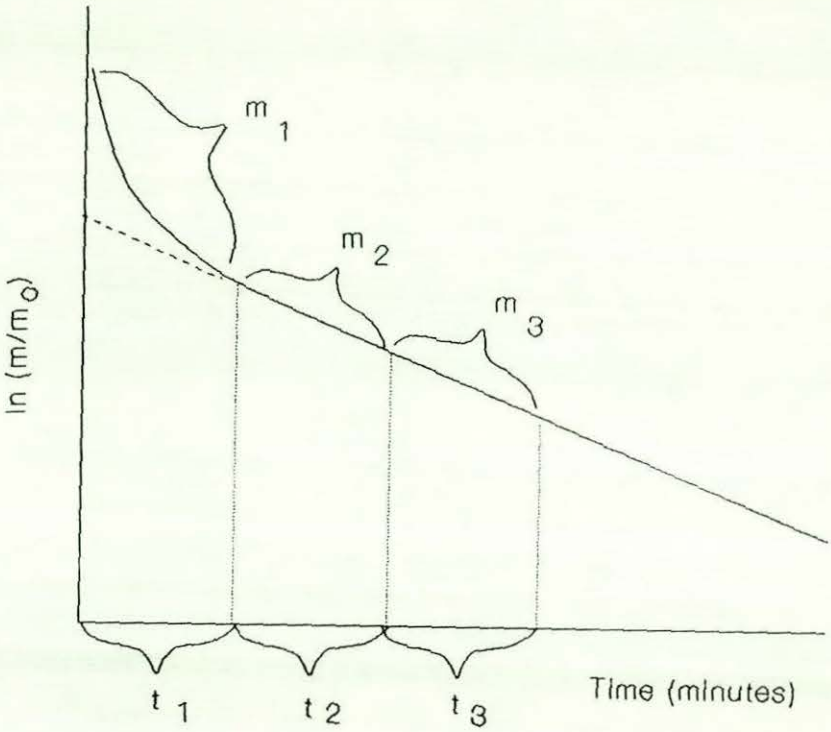
A first order plot shows two approximately linear sections, the first corresponds to a relatively fast initial extraction stage while the last 20% or so of the gingerol was extracted in the much slower subsequent stage. Reducing the ginger particle size by a factor of 3 increased the rate constant of the fast stage nine-fold. Despite the low viscosity of the SCF, the rate constants of the fast stage were much smaller than in extractions with organic solvents. Only when the CO_2 density was increased to 0.775g cm^{-3} did the rate constant of the fast stage rise to a value comparable with, but still smaller than, those in organic solvents of similar density.

Bartle *et al.* have developed a similar theory called the "hot-ball" model which sees the rate determining step as the mass transfer out of the matrix [211]. A characteristic of such extractions is that the majority of the analyte is removed during a short period at the beginning of the extraction to give a steep curve, but subsequently the extraction rate tails off dramatically, becoming approximately linear (Figure 6.13). The physical explanation of the shape of the extraction curve is that initially the analyte present on the surface of the particle will diffuse very rapidly into the SCF and as SCFs have gas-like diffusitivity, the diffusion step will probably be rarely, if ever, the rate determining step. As the extraction continues this step becomes eroded, but nevertheless the concentration gradient near the surface is large, and diffusion, which is proportional to the concentration gradient continues to be at a high rate. Eventually, a smoother concentration profile is established over the whole sphere and the diffusion loss becomes a simple exponential decay.

Although the initial steeper fall (corresponding to the curve) in Figure 6.8 appears as a relatively small feature, it represents the loss of the majority of the material from the particle, usually in the region of 60%. To obtain 99% removal of the analyte requires an extraction time typically ten times that needed to remove the first 50%. The exponential behaviour of the extraction enables the extrapolation of the data to yield a theoretical value for the total mass of analyte in the sample, without requiring an exhaustive extraction. If the extraction is carried out over the initial non-linear period to obtain a mass m_1, followed by extraction over two subsequent equal time periods to obtain masses m_2 and m_3, then the total mass in the sample (m_0), is given by:

$$m_0 = m_1 + m_2^2/(m_2 - m_3)$$

FIGURE 6.8. A characteristic plot of $\ln(m/m_0)$ vs. time for a supercritical extraction. Note, m = mass of analyte at time t in particle and m_0 = mass of analyte at zero time (t_0) in particle.



Two simple variations in the distribution of the analyte within the particle have been suggested [211]. If the analyte is more concentrated on the surface of the particle then the initial extraction would be expected to be faster, so giving a steeper curve. On the other hand, the regions close to the surface of the particle may be depleted in analyte and thus the initial fall would be smaller, giving a shallow curve.

For other matrices the mass transfer out of the particles may not be a matter of diffusion within a homogeneous medium but involve such processes as diffusion out of pores, migration from one adsorption site to another or replacement of analyte molecules on adsorption sites by SCF molecules. King *et al.* suggests one such possibility when investigating the extraction of rape seed with liquid CO_2 [212]. At constant extraction flow rate, the oil content in the SCF phase (i.e. the "loading") remained approximately constant until about 60 or 70% of the oil present has been extracted. With further extraction of the oil, the oil content in the SCF phase falls

sharply and is independent of flow rate. It has been suggested that this effect is probably due to the presence of the oil in two forms, a "free" form , the extraction of which is completed at the end of the first stage, and a second form which is in some way "bound". Some evidence for this comes from the fact that the change in loading is associated with some change in the composition of the extract. Thus the mechanism of mass transfer from the bed of rape seed is probably complex, involving diffusion through pores in the solid as well as across the Nernst layer.

It would appear that the solvating power of supercritical CO_2, does not provide sufficient information to estimate an analyte's extractability from a complex matrix. This is undoubtedly due to the way the analyte is attached to the matrix. In the plant material the analyte may be present as salts, hydrogen bonded, or even microencapsulated [182]. For example, the binding strength of nicotine varies in raw tabacco, some of it is removed very easly in SCF extraction, but the rest is thought to be present as a complex salt with tobacco constituents, such as chlorogenic acid and citric acid. To remove this bound form the cosolvent, water is required.

Alternatively, it has been suggested that differences in extraction yields may be due to the various degrees to which the analytes are dissolved in the lipid or aqueous phase in the plant matrix [213]. In a plant with a high lipid content, all non-polar volatile material may be dissolved in lipid globules. Therefore, a mixture of volatiles in two different matrices might be the same qualitatively and quantitatively, but might produce two different extracts due to the distribution of components within the matrix.

From these suggestions it can be seen that sample preparation may have a direct influence on the extraction yield. Homogenising the matrix to increase the surface area, or rupturing the matrix with a sudden release of pressure, could greatly aid the mass transfer step. The use of ultrasonic sound during the SCF extraction of coffee beans was shown to enhance the extraction yield, by inducing convection through the internal pores of the macro-porous material [214]. It has even been suggested to use trichloroacetic acid in methanol, as it is claimed this will break down cellulose bonds and possibly aid diffusion of the SCF into the matrix [215]. It would appear that the adsorption effects of the matrix, need to be incorporated into the solubility equation to give a realistic interpretation of SFE.

6.3 Solubility of organic compounds in carbon dioxide

Supercritical CO_2 is a particularly effective solvent for medium molecular weight and relatively low polarity substance, being especially useful in the recovery of heat liable substance of low volatility from an insoluble matrix. For CO_2, an insoluble matrix may consist of cellulose, starch, organic or inorganic high molecular weight polymers, sugars, glycosidic compounds, proteins, metals or metal salts. Some general conclusions about the solubility of various compounds in sub- and supercritical CO_2 can be drawn from the literature [179,180,182]. CO_2 behaves like a hydrocarbon solvent, with a few notable differences such as methanol miscibility etc. It has a strong homogenizing action, so that many pairs of immiscible or partially miscible liquids form a single phase when mixed with sub- or supercritical CO_2. It is a good solvent for aliphatic hydrocarbons up to at least the low C_{20}s and for most small aromatic hydrocarbons. Few polycyclic hydrocarbons show appreciable solubility. Low to medium molecular weight halocarbons, aldehydes, eaters, ketones and low alcohols are freely soluble in CO_2. Fatty acids and their glycerides have low solubility, however, monoesterification enhances fatty acid solubility considerably [182]. Phenols show poor solubility, as do most anilines. Hydroquinone and other polyhydro aromatics are essentially insoluble. Polar compounds, such as amides, ureas, urethanes, and azo dyes, exhibit poor solubility in CO_2. Carboxylic acids are soluble if the molecular weight is very low. Chlorophylls, cartenoids, and amino acids are usually insoluble. Few materials of any structural type with molecular weights above around 500 are soluble.

A number of structural features appear to greatly influence the solubility of analytes in CO_2, such as chain length, branching, acidity, bascity, aromaticity, and position and type of substitution. For example, branched alkanes are comparativily more solube than the corresponding striaght chained alkane in sub- and supercritical CO_2 [179]. This is explained by Hildebrand [216] in terms of smaller intermolecular interactions in branched alkanes, so aiding solubility. When determining solubility, it has been suggested that these differences in structure, aromaticity or unsaturation are of secondary importance in comparison to differences in the size or polarity of the analyte [217].

To highlight some of these rules a few examples of extraction with supercritical CO_2 at 40°C are given in Table 6.2 [182].

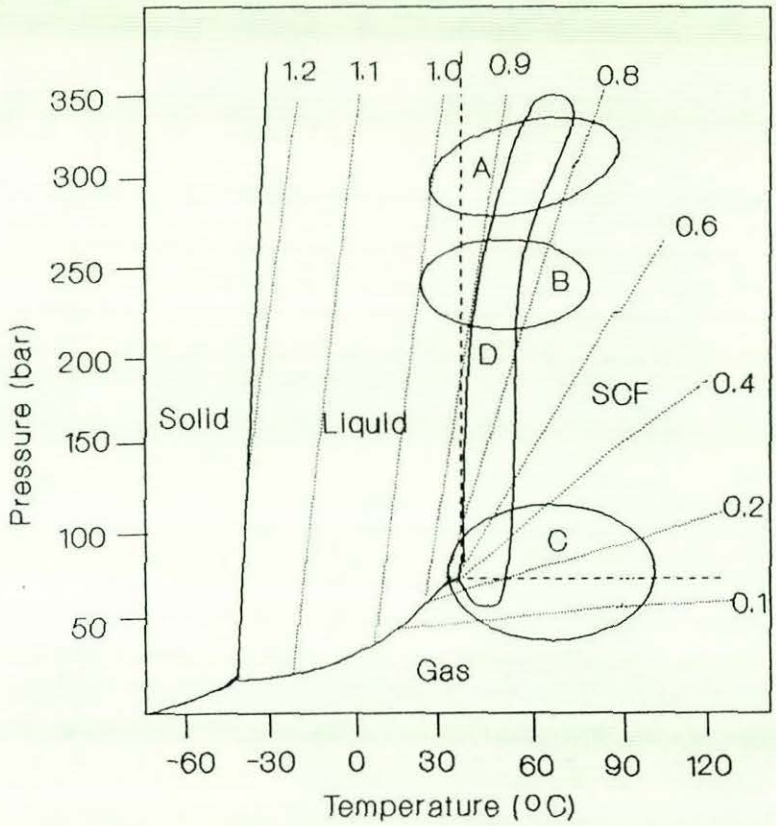
TABLE 6.2. SCF extraction of components from plant material.

Material	Principal constituents	R.M.W.	Formula	M.P. (°C)	B.P. (°C)	Extraction starts at (bar)
Caraway fruit	Limonene	136	$C_{10}H_{16}$	−74	178	70
	Carvone	150	$C_{10}H_{14}O$	–	231	70
	Triglyceride	>600	–	–	–	190
Peppermint leaves	Methone	156	$C_{10}H_{18}O$	–	209	70
	Menthol	158	$C_{10}H_{20}O$	44	216	70
Chamomile flower	Herniarin	176	$C_{10}H_8O_3$	177	–	70
	α-Bisabolol	222	$C_{15}H_{26}O$	–	155	70
	Ene-yne dicyclo ether	200	$C_{13}H_{12}O_2$	–	–	80
	Matricin	306	$C_{17}H_{22}O_5$	–	–	85
Sunflower	Squalene	410	$C_{30}H_{50}$	−20	280	80
	Cholesterol	386	$C_{27}H_{26}O$	148	360	85
	Triglyceride	600	–	–	–	90

6.4 Selectivity of SFE in plant material

Most botanical samples contain a wide spectrum of compounds that can be dissolved in supercritical CO_2. However, usually only a certain group of components are required from the plant matrix, so that selectivity is desirable. Selectivity occurs in the pressure and temperature gradient where the greatest differences in solute volatility and solvent polarity exist. This fractionation region coincides with the sharp rise in the density and dielectric constant of supercritical CO_2 (Figure 6.9)

FIGURE 6.9. Areas chosen for optimal results in some applications of extraction using supercritical CO_2 [166]. Density (g/cm^3) is the third dimension. A = Total extraction, B = Total extraction (pale), C = Essential oils and deodourisation, D = Fractionation.



As illustrated in Figure 6.9, the high pressure region is generally used for processes where total extraction of the target analyte is required, producing extracts which are mostly dark in colour. If such pigments are not desirable, then a slightly lower pressure must be used. For example, hop extracts produced with supercritical CO_2 at 300 bar are green, while those produced at 140 bar are yellow [166]. Increasing the temperature at a given pressure has a variable effect depending on the solute vapour pressures and frequently there is a trade off between extraction efficiency and selectivity (see crossover region Section 6.2 {b}).

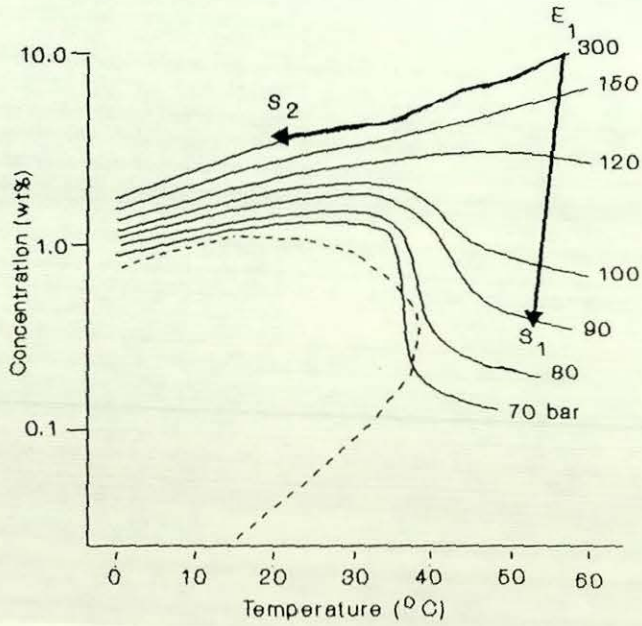
In the operating region closest to the critical point, the process becomes somewhat selective for the most soluble components in the mixture. If a material containing components of wide range of solubilities is exposed to CO_2, then the low pressure region can be suitable for deodorization. For fractionation, extraction commences in the gaseous phase near the critical point and is increased to higher temperatures and pressures according to the solubility of the least soluble component to be extracted. This suggests

that low solvent power has high selectivity and high solvent power, low selectivity. For example, a simple two stage fractionation can be obtained using "dry" CO_2 followed by "wet" CO_2. Cinnamon is extracted at 300 bar and 55°C. The first stage occurs with dry CO_2 to remove the essential oils responsible for the aroma and odour of the spice. The second extraction, using CO_2 saturated in water, extracts the flavour components [162].

6.5 Methodology of selectivity in SFE

Selectivity in supercritical CO_2 may be achieved by varying the temperature and pressure of the system, so that the corresponding change in the solubility of the analytes will be favourable for the fractionation of the sample. The concept behind these separation processes can be seen from the solubility data of naphthalene in CO_2 as a function of temperature and pressure [219] (Figure 6.10). E_1 represents conditions in the extraction vessel (300 bar, 55°C) and S_1 conditions which exist in the separation vessel (90 bar, 43°C). Using this isothermal path, the equilibrium solubility has been reduced from about 5 to 0.2% during the pressure reduction step, naphthalene precipitating from solution. The alternative separation is done isobarically to S_2, cooling the extract to 20°C, though this separation is not as efficient as the path E_1–S_1.

FIGURE 6.10. Operating paths for SFE [219].



If the extraction pressure is denoted by P_1 and the separation pressure by P_2, with the corresponding temperatures T_1 and T_2, then 4 different cases of separation can be distinguished [155]:

Case 1.	$P_1 > P_c > P_2$ $T_1 > T_c > T_2$	Most common method of separation on an analytical scale
Case 2.	$P_1 > P_c > P_2$ $T_1 \geqq T_2 > T_c$	P_2 below critical pressure, isothermal conditions for industrial scale.
Case 3.	$P_1 > P_2 > P_c$ $T_1 \geqq T_2 > T_c$	P_2 between extraction pressure and critical pressure, isothermal cond. for industrial scale.
Case 4.	$P_1 = P_2 > P_c$ $T_1 \gtrless T_2 > T_c$	Isobaric separation, disadvantageous with thermally labile components.

The most selective extractions are often achieved by using a series of separation vessels, where the temperature and/or pressure are changed in a stepwise fashion [162]. This configuration is sometimes referred to as a multistage fractionation. The dissolved components drop out of the supercritical solvent at the point in the system where they are no longer soluble and collect in the appropriate vessel. If the relative volatility of the components is sufficiently different and the conditions maintained in the separation vessel mirror those differences, a high degree of separation is theoretically possible. In a mixture of more closely related components, it may only be possible to change the relative distribution of individual components in the extract composition. One draw back of such an approach is that the system is only capable of separating the original mixture into as many fractions as there are separation vessels. Furthermore, the system is complicated and costly.

A more cost effective way of obtaining selectivity on an analytical scale, is by the use of adsorption columns, often referred to as "traps" as they trap the required analyte. The most common adsorptive material used is silica gel, which retains the polar constituents in the extraction so that they may be eluted at a later stage under more polar solvent conditions. Saito *et al.* used a silica gel column to selectively trap and concentrate

tocopherols from wheat germ [220]. Miller Schantz *et al.* used a LC guard column packed with C_{18} material for the collection of extracted Aroclors in sediment [221]. Less successful traps include the use of a hollow tube placed in an ice bath and packed with copper shot or stainless steel balls [222]. Alternatively, a large sample loop [222] or cryogenic trap [223] can be used to concentrate the sample and then separated on an analytical column, the fractions being collected at the end. Further selectivity may be achieved by linking SFE to TLC, GC, HPLC and SFC, all these techniques being thoroughly reviewed by Vannoort *et al.* [224].

6.6 SFE of essential oils

Essential oils are usually a complex mixture containing tens and sometimes hundreds of components [225,226]. In the narrow sense these components are seen as terpenoids, based on the isoprene molecule $CH_2{=}C(CH_3){-}CH{=}CH_2$. Terpenoids are built up from the union of two or more of these C_5 units, to produce the mono- and sesquiterpenes (C_{10} and C_{15}) and their oxygenated compounds [225]. However, sometimes this classification is unsatisfactory, some essential oil constituents containing nitrogen or sulphur [107]. Aromatics such as the phenylpropanoids are often associated with plant odours, a phenylpropene, eugenol, being the principle constituent in the oil of cloves [225]. The essential oils may be classified into five groups according to their differing properties, typical examples are:

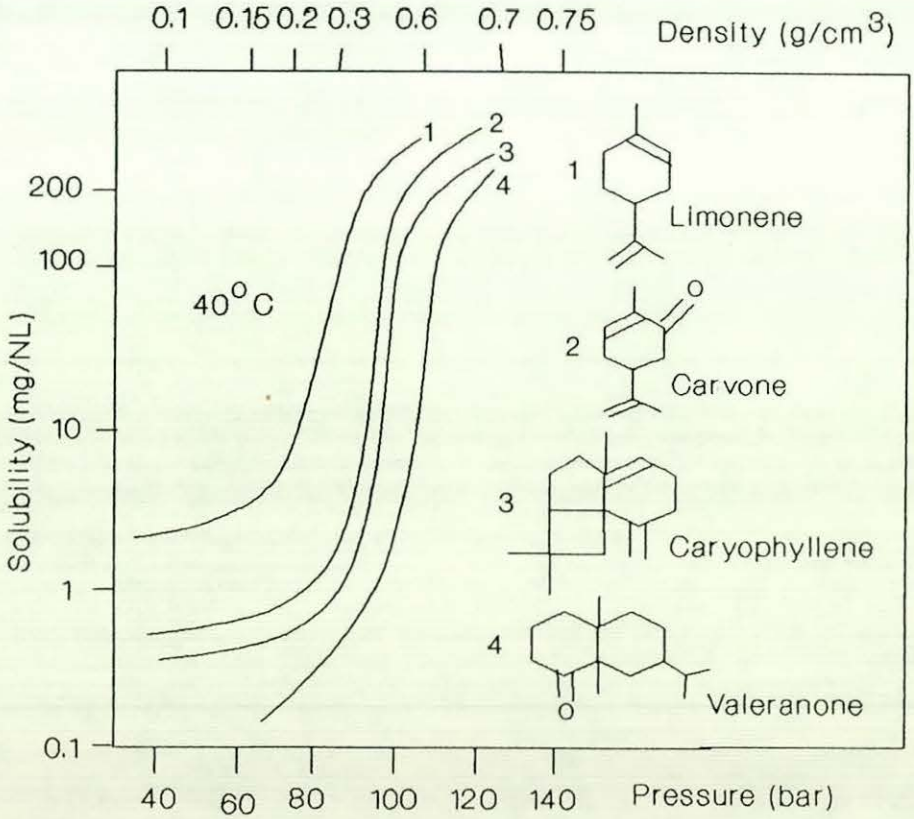
{i} Hydrocarbon monoterpenes (limonene)
{ii} Oxygenated monoterpenes (carvone)
{iii} Phenylpropanoids (eugenol)
{iv} Hydrocarbon sesquiterpenes (caryophyllene)
{v} Oxygenated sesquiterpenes (valeranone)

Essential oils are highly soluble in liquid and supercritical CO_2 [227], as they are low molecular wieght lipophilic substances with relatively high vapour pressures. The problem arises in the choice of extraction conditions that enable a quantitative recovery but prevent the coextraction of undesirable material such as fatty oils, waxes and carotenoids. To enable as complete a recovery as possible, the solubility characteristics of the essential oils,

needs to be understood. Vapour pressure and polarity appear to have the greatest influence on the solubility behaviour of essential oils in dense CO_2.

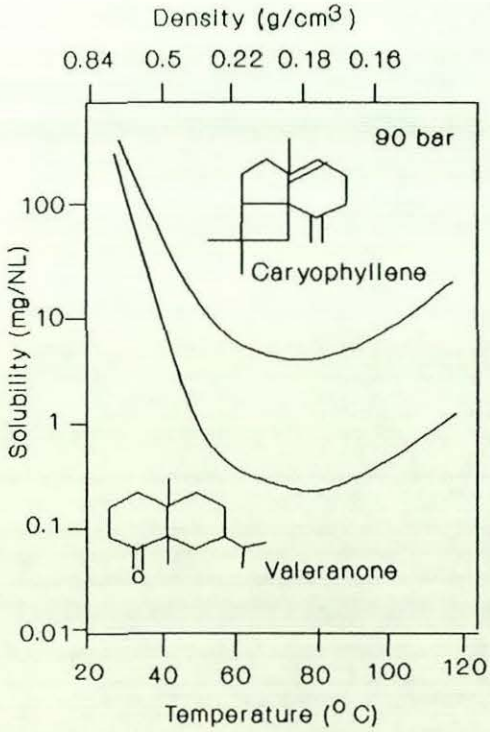
It can be shown that the highest solubilities are obtained for the essential oils with the least polarity, the smallest molar mass and the largest vapour pressure, namely the monoterpene hydrocarbons (see figure 6.11) [227]. With the strong increase in density of CO_2 in the pressure range between 70 and 100 bar at 40°C, solubilities increase exponentially. Carvone and caryophyllene have almost the same solubility in supercritical CO_2 at 40°C, having correspondingly very similar vapour pressures. Valerone has a solubility curve which is markedly lower than the other components, being in the higher pressure range. This phenomenon enables relative selectivity to be achieved, with the lower pressures preferentially extracting the monoterpenes and the higher pressures extracting all the other essential oils.

FIGURE 6.11. Solubility isotherms of essential oils in dense CO_2 at 40°C [227]. 1 limonene, 2 carvone, 3 caryophyllene, 4 valeranone.
1 NL CO_2 = 1000cm^3 at 1 bar and 20°C = 1.78g NL = Normal litre



To separate essential oils of differing volatility or polarity, the solubilities of the compounds in the dense gas should vary as much as possible. The solubility isobars of caryophyllene and valeranone (Figure 6.12) show that at CO_2 densities above $0.8 g/cm^3$, that is at temperatures below 27°C at 90 bar, fractionation is impossible [227]. With isobaric temperature increases the solubility curves pass through a minimum. This is due to two opposing factors, namely the decrease in solvent density and the increase in solute vapour pressure. Therefore at the higher temperatures (≈80°C) the difference in volatilities, together with the solvating capacity of the supercritical CO_2 produces markedly different solubilities, so that a relative fractionation can take place. Similar solubility curves exist at higher pressures but even higher temperatures are required to enable a comparable fractionation. Thus the separation should be done at low pressure and high temperature.

FIGURE 6.12. Solubility isobars for caryophyllene and valeranone as a function of temperature and density of CO_2 [227].

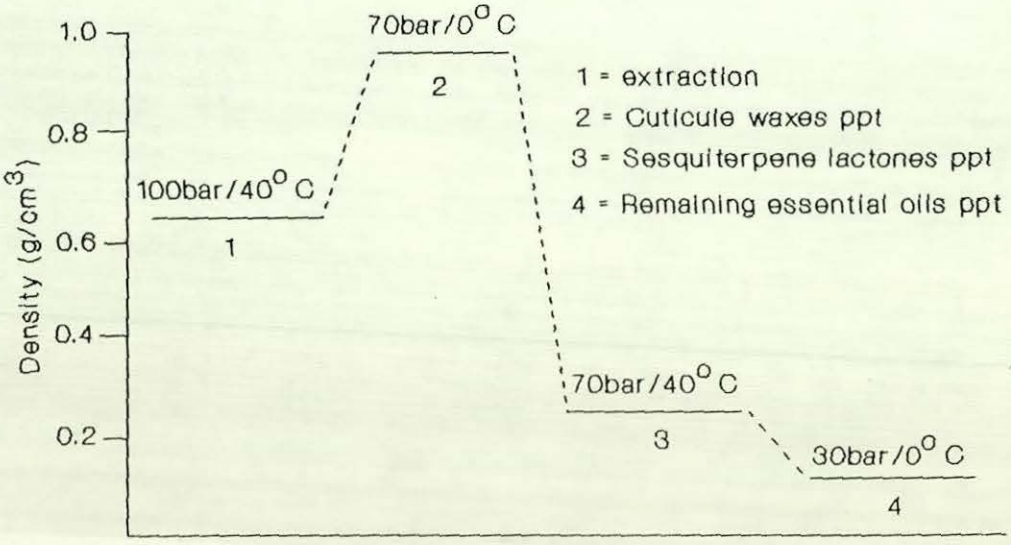


The separation of sesquiterpene hydrocarbons and oxygenated monoterpenes is difficult as their solubility behaviour in CO_2 is alike and their vapour pressures are almost the same. However, the selectivity of the extraction may be enhanced by increasing the polarity with the saturation of CO_2 with

water [227]. Within a certain pressure range, this causes a solubility increase of the polar components and a decrease of the hydrocarbons, so that selectivity may be achieved.

With supercritical CO_2 extractions, the ubiquitous cuticular waxes and long chain hydrocarbons are in general, coextracted with the desired components from the raw drug [228]. For this reason, the extract has a waxy, greasy consistency. An extraction scheme for wormwood has been developed and enables selective precipitation to take place [229]. Wormwood is valued for its bitter taste, which is largely caused by the sesquiterpene lactone, absinthine. However, the plant also contains β-thujone which is responsible for its toxicity [230]. Thus a selective separation was required. The cuticular wax, sesquiterpene lactones and all the other essential oils are extracted at 100 bar and 40°C (Figure 6.13). Precipitation of the extract then takes place in three stages. In the first stage, the temperature is reduced to 0°C which results in a large increase in density and liquefication of the CO_2, when pure white cuticular wax precipitates. The precipitation of the sesquiterpene lactones and the remaining essential oils takes place in the second and third stages respectivily, by stepwise pressure reductions. The purity of the essential oil extracts obtained by this method compared favourably to those obtained by steam distillation [231].

FIGURE 6.13. A schematic representation of the fractional separation of the dense gas extract of wormwood [229].



6.7 Conclusions

It is clear from these examples that SFE is an active area of development, especially in the important field of natural products, Its attractions include: its flexibility in terms of the ways in which the properties of the solvent can be altered; the selectivity that can be achieved; the rapidity of phase separations and the reduction in the contamination of the products with the solvent. However, except for a few notable commercialisations, to date SFE is still very much in the early stages of research and development.

Chapter 7

Experimental for the extraction, separation and identification of essential oils

7.0 Introduction

In this chapter, general experimental details and chemicals are described for the extraction and identification methods for essential oils. The components and operation of the supercritical fluid instrumentation will be described in detail in Chapter 8.

7.1 Reagents

7.1.1 Solvents

The carbon dioxide used was industry grade (99.98%) supplied by BOC Ltd, Middlesex, U.K., carbon tetrachloride was UV/IR grade from Blackford Wells Ltd., acetonitrile was Far UV grade supplied by Romil Ltd, Shepshed, Leics, U.K. and chloroform (LR grade), toluene (LR grade), methanol (HPLC grade), ethanol UV/IR grade), tetrahydrofuran (HPLC grade) dichloromethane (HPLC grade) and hexane (HPLC grade) were supplied from FSA Laboratory Supplies, Loughborough, U.K. Water was scrubbed and deionised within the Department.

7.1.2 Standard compounds

Eugenol, santonin and α-cellulose (non-assayed) were from Sigma Chemicals, Poole, U.K. carvone, quercetin, anisaldehyde (98%) and lead (II) acetate trihydrate were from Aldrich Chemical Company Inc. Gillingham U.K., xylene, aluminium oxide active neutral (Brockmann grade I) and sodium sulphate (anhydrous) were from BDH, Poole, U.K., limonene and caryophyllene were from Koch-Light Laboratories, Colnbrook, Bucks, U.K., Tansy oil (no. 2779400) from Carl Roth, KG, Karlsruhe Germany, pinene, glacial acetic acid (SLR) and sulphuric acid (AR) were from FSA Laboratory Supplies, Loughborough, U.K., TLC plates (20 x 20cm) Kieselgel $60F_{254}$ (0.2mm) were from Merck, Darmstadt, Germany, and Nylon 66 filters (0.45μm) were supplied

by Alltech, Carnforth, U.K. Parthenolide standard was donated by Dr Hylands of Chelsea College, London.

7.1.3 Plant material

Feverfew varieties were obtained from Chelsea Physic Gardens. Home grown feverfew were produced from Fisons, and Johnson seeds. The seeds were planted in potting compost in the early spring and harvested a year later, in the late summer. Air-dried powdered feverfew was supplied by the British Analytical Control Company (B/no. A3783).

Tansy was supplied as a whole dried herb by Cathy of Bournemouth Ltd (B/no. C4206, origin France) and from Brome and Schimmer Ltd (B/no. 9091). Tansy was also home grown in the department. German chamomile was obtained as dried flowering heads from Cathy of Bournemouth Ltd (B/no. C4285 origin Egypt) and from Brome and Schimmer Ltd (B/no. 9956). The plant was also home grown in the department.

7.2 Sample preparation

The essential oil standards and extracts were stored in air tight containers wrapped in foil under refrigeration, to minimise sample deterioration. Solutions of reference essential oils were prepared by dissolving the appropriate amount of the sample in dichloromethane to give concentrations of about 2 mg/ml. The flavonoid standard, quercetin, was dissolved in THF at 5 mg/ml. The essential oil extracts from plants were solvated in 1 ml methanol and 1 ml dichloromethane, to ensure complete solvation of all components present.

7.2.1. Cellulose plant model

A synthetic plant model was constructed. The essential oils were solvated dichloromethane (10 ml) so that in total 50 ml of solvent were added to the cellulose (25 g). This was dried at room temperature and pressure until the solvent vapour could no longer be detected. The spiked cellulose was stored in an air-tight container, which was wrapped in foil and refrigerated at $-10^{o}C$.

TABLE 7.1. Spiked cellulose matrix.

Essential oil	% oil added by dry weight of cellulose
Limonene (60 mg/10 ml)	0.24
Carvone (60 mg/10 ml)	0.24
Eugenol (60 mg/10 ml)	0.24
Caryophyllene (60 mg/10 ml)	0.24
Santonin (125 mg/ 10 ml)	0.50

7.3 Extraction of plant material

7.3.1 Head space analysis

Plant material (10 g) was placed in a conical flask (100 ml) and a "suba-seal" stopper used to make the flask air tight. The flask was kept in a water bath (40°C) and warmed for 4 hours. A headspace sample was extracted with a SGE syringe (250 μl) inserted through the "suba-seal".

The GC analyses were performed using a Carlo Erba Vega 6000 series gas chromatograph, in a splitless/split injection mode (split ratio 20:1). Samples (250 μl vapour) were injected in the splitless mode. The splitter was opened after 10 seconds of the run-time had elapsed. Injection port was set at 120°C. The syringe was scrupulously washed with acetone, followed by methanol, by filling and emptying 10-20 times between injections. The syringe was dried in a stream of nitrogen gas. A fused SGE capillary column was used (12 m x 0.33 mm i.d.), coated with 5 μm film of dimethyl polysiloxane (BP-1). The temperature programme was 60 to 300°C at 8°C min^{-1}, then isothermal at 300°C for 8 minutes. Flame ionisation detection (FID) was used with hydrogen (30ml min^{-1}) and air (300 ml min^{-1}). The detector was maintained at 280°C. The carrier gas (helium) flow was set at 2 ml min^{-1}. The chromatograms were recorded on a Opus PC III computer via a Nelson interface and integration package.

7.3.2 Steam Distillation

The determination of volatile oil in plant material was carried out by steam distillation, in accordance with the British Pharmacopoeia method [12]. The plant material (50 g) and distilled water (500 ml) were placed in a round bottom flask (2 litre). The flask was heated until equilibrium

began and then the rate of distillation was set at 2 or 3 ml per minute for 4 hours. The distillate was collected in a graduated tube and the aqueous phase was automatically recirculated into the distillation flask. The volatile oil was collected in xylene (1ml) with the recovery being expressed as a percentage volume of oil/weight of plant material. The oil collected in the xylene was diluted in dichloromethane to 10 ml. Volatile oils from feverfew and German chamomile were obtained by this method.

7.3.3 Liquid extraction

Four methods for the extraction of parthenolide from feverfew were investigated:

{i} French Pharmacopoeia method [232].

Air-dried feverfew supplied by British Analytical Control Ltd. (10g) was extracted with methanol (100 ml) at 45°C for 30 minutes. The plant material was then further extracted with fresh methanol (40 ml) at 45°C for 20 minutes. The extracts were combined, filtered, evaporated and then the residue resolvated in methanol (50 ml).

{ii} Marchand *et al.* method [233].

Air-dried feverfew supplied by British Analytical Control Ltd. (10 g) was extracted in chloroform (100 ml) for 30 minutes. The filtrate was evaporated under vacuum till dry, the residue dissolved in methanol (10 ml) and filtered again.

{iii} Bloszyk *et al.* method [44].

Air-dried feverfew supplied by British Analytical Control Ltd. (10 g) was extracted with three aliquots of methanol (200 ml) at 40°C. The extracts were combined and evaporated under vacuum to 10 ml. Distilled water (50 ml) was added and the remaining methanol was evaporated off under vacuum. A saturated lead acetate solution was added to the aqueous solution until encumbering substances were completely precipitated. The solution was left to stand for 1 hour at room temperature and then centrifuged. The supernatant was separated and extracted with three aliquots of chloroform (100 ml). These organic extracts were combined, dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was dissolved in chloroform (2.5 ml).

{iv} Govindachari *et al.* method [57].

Air-dried feverfew supplied by British Analytical Control Ltd. (10 g) was extracted with two aliquots of hexane (100 ml). The extracts were combined, filtered and evaporated under vacuum to 3ml. The hexane extract was purified by chromatography on a column of neutral alumina (Brockmann grade I) with toluene (130 ml). The eluent was evaporated under vacuum to dryness and resolvated in hexane/dichloromethane (1:1, 2 ml).

7.3.4. Supercritical fluid extraction

{i} Analytical scale extraction.

A Jasco extraction vessel (2 ml) was packed with plant material (≈ 0.5 g) and exposed to various temperatures, pressures and flow rates of sub- and supercritical CO_2. To obtain subcritical extractions at -10°C, a length (1 m) of equilibration tubing and the extraction vessel were placed in a bath of acetone and ice (1:1). The instrumentation is described in detail in Section 8.5.1.

{ii} Preparative scale extraction.

A Jasco extraction vessel (40 ml) was packed with air dried plant material (≈ 10 g) and extracted with supercritical CO_2 at 250 bar, 45°C and 0.85ml min^{-1} (liq. CO_2 flow rate at pump) for 1 hour. The instrumentation is described in detail in Section 8.5.1.

7.4. Scanning electron microscopy

Air dried feverfew leaves (≈ 2 mm^2) from an 8 month old plant grown at the department (Fisons seeds) were mounted on stubs and sputter coated with gold (10 nm). Specimens were examined under an International Science Instrument (I.S.I.) SS40 scanning electron microscope (SEM) and photomicrographs taken on 35 mm Kodak film. Prior to SEM the leaves were exposed to various SFE conditions, including:-

{i} CO_2 at 1 ml/min, 250 bar and 40°C for 1 hour.

{ii} CO_2 at 1 ml/min, 250 bar, 40°C and 10% methanol for 1 hour.

7.5 Separation methods

7.5.1 TLC of essential oils

{i} Analytical scale TLC.

Silica gel TLC plates (Kieselgel $60F_{254}$, 10x10cm) were used to separate the essential oil extracts. The plates were placed in a TLC tank lined with filter paper and run with the appropriate eluent:

Steam distillation extracts:- Chloroform/toluene (3/1).

Conventional liquid extracts:- Chloroform/acetone (95/5).

The components were visualised by spraying with anisaldehyde-sulphuric acid reagent [234] (anisaldehyde (0.5ml) was mixed with glacial acetic acid (10ml), followed by methanol (85ml) and concentrated sulphuric acid (5ml) respectively). The TLC plate was then heated at 120°C for 7-10 minutes in a drying cabinet. The plate was viewed at 254 nm, 366 nm and in daylight.

{ii} Preparation scale TLC.

Prep-TLC was used to obtain the parthenolide fraction for the spectroscopic studies carried out in Chapter 10. The SFE extracts and a parthenolide standard were run on large glass TLC plate (20 x 100 cm) coated with Kieselgel $60F_{254}$ (≈ 0.25 mm thick). The eluent was chloroform/acetone (95/5). The spots were visualised by spraying the edge of the plate with the anisaldehyde reagent and viewing at 254 nm. The appropriate band was scraped off the plate and extracted with methanol and filtered (0.45μm filter).

7.5.2 HPLC of essential oils

The system consisted of a Kontron LC 414 pump, a Rheodyne 7125 injector with a 10μl injection loop, a Pye Unicam PU4020 variable UV detector set at 220nm, a Hewlett Packard 8451A diode-array detector scanning at 200-400nm and a Hewlett Packard 3390A integrator. Two methods were investigated for the analysis of essential oils in plant material by HPLC:-

{i} French Pharmacopoeia method [232].

This HPLC method is the recommended separation technique for parthenolide in feverfew. The liquid extractions obtained in Section 7.4.3 were analysed by this method. An ODS Spherisorb 5 μm (4 x 250 mm) column supplied by Thames Chromatography at 30°C was used with an acetonitrile-water (55:45) eluent at 1 ml/min flow rate.

{ii} Normal phase HPLC.

A silica Spherisorb 5 μm (4 x 250 cm) column supplied by Thames Chromatography was used with different ratios of a hexane/methanol (0 - 3.2%) eluent under isocratic conditions at 30°C). The essential oil standards and a flavonoid standard were analysed on this system.

7.5.3. GC and GC-MS analysis of essential oils

The GC analyses were performed using a Carlo Erba Vega 6000 series gas chromatograph, in a split injection mode (split ratio 20:1). Samples (0.5 μl) were injected using a 10 μl SGE syringe. Injection port was set at 180°C. The syringe was scrupulously washed with acetone, followed by methanol, by filling and emptying 10-20 times between injections. A fused SGE capillary column was used (12 m x 0.33 mm i.d.), coated with 5 μm film of dimethyl polysiloxane (BP-1). The temperature programme was 60 to 300°C at 8°C min^{-1}, then isothermal at 300°C for 8 minutes. Flame ionisation detection (FID) was used with hydrogen (30 ml min^{-1}) and air (300 ml min^{-1}). The detector was maintained at 280°C. The carrier gas (helium) flow was set at 2ml min^{-1}. The chromatograms were recorded on a PC III computer via a Nelson interface.

The GC-MS analysis was kindly carried out by Dr A.W. Payne at Kodak, Harrow on a Hewlett Packard 5890 GC and a V6 Trio-2 mass spectrometer. The samples were run on a Hewlett Packard Ultra II column (5% methlyphenyl silicone 12 x 0.02 mm i.d.). The temperature programme was 50°C for 1 minute then increasing to 8°C min^{-1} to 300°C. The data base system used to compare with the mass spectral data, is the National Bureau of Standards (NBS) library.

7.5.4. SFC of essential oils

The chromatographic system was a Jasco SFC/SFE modular system which comprised of: a cooling system (Haake KT2) attached to the pump designated for CO_2; two Jasco 880 solvent delivery systems; a Gilson 811b dynamic mixer; a Jasco 812 back pressure regulator; a Jasco 820 absorbance detector set at 220 nm; and a Rheodyne 7125 injector with a 10μl injection loop. Three columns were used: a Spherisorb 5 μm ODS (4.6 x 250 cm) supplied by Thames Chromatography; a Spherisorb 5 μm silica (4.6 x 250 cm) column supplied by Thames Chromatography; and a Polymer Labs. 5 μm PLRP-S (4.6 x 150 mm). The mobile phase was CO_2 (industry grade supplied by BOC.). This mobile phase was modified by the addition of methanol, from the second Jasco 880 HPLC pump. The time equivalent (t_o) to the void volume (V_o) was determined by THF. Retention times are recorded as capacity factors (k') and are calculated from the retention time of the solute (t_r) and the retention time of the unretained peak (t_o).

$$k' = (t_r - t_o) / t_o$$

7.6. Spectroscopic analysis of parthenolide

A comparison was made between the authenticated parthenolide and that extracted from feverfew by SFE. The following techniques were used:-

{i} UV analysis. Samples were analysed on a Shimadzu UV-160 spectrophotometer. The samples were prepared by dissolving 2 mg of material in UV grade ethanol (2 ml).

{ii} IR analysis. Samples were analysed using a Perkin Elmer 1600 series FTIR as a KBr disc (2 mg parthenolide in 200 mg KBr).

{iii} NMR analysis. Samples were analysed on a Bruker FT-NMR 250MHz spectrometer to produce carbon and proton NMR spectrum. The sample (1-2 mg) was dissolved in deuterated chloroform (1 ml).

7.7. Calculations

7.7.1 Quantitative analysis of essential oils

Quantitative analysis of the extracted oils from the spiked cellulose matrix, were determined by both external and internal standards (0.25, 0.5, 1.0, 2.0, 3.0, 4.0 mg/ml solutions of standards required to construct calibration plot). Each essential oil standard had its corresponding external calibration graph, to compensate for detector response variations. An internal standard (safrole 1 mg/ml) was present in the solvent used to resolvate the extracts, this compensated for injection volume variations. For calibration data for external and internal standards see Table 7.2.

TABLE 7.2. Calibration data of essential oils standards on capillary GC. (GC system described in Section 7.5.3).

External standards:-

	Limonene,	Amount = $(3.84 \times 10^{-5}) \times$ area Correlation (R squared) = 0.994
	Caryophyllene,	Amount = $(4.593 \times 10^{-5}) \times$ area Correlation (R squared) = 0.998
	Carvone,	Amount = $(4.33 \times 10^{-5}) \times$ area Correlation (R squared) = 0.998
	Eugenol,	Amount = $(4.83 \times 10^{-5}) \times$ area Correlation (R squared) = 0.995
	Santonin,	Amount = $(6.58 \times 10^{-5}) \times$ area Correlation (R squared) = 0.998

Internal standard:-

	Safrole,	Amount = $(5.03 \times 10^{-5}) \times$ area Correlation (R squared) = 0.993

To determine the percentage parthenolide extracted from the feverfew plant material, calibration graphs were constructed. Authenticated parthenolide standards (0.1, 0.5, 1.0, 1.5 and 2.0 mg/ml solutions) were run on a HPLC system (Section 7.5.2) and capillary GC system (Section 7.5.3). The calibration data generated is given in Table 7.3.

Table 7.3. Parthenolide standards analysed on GC and HPLC to construct calibration plots.

GC system:- Parthenolide standard, Amount = $(4.36 \times 10^{-4}) \times$ area
Correlation (R squared) = 0.995

HPLC system:- Parthenolide standard, Amount = $(2.21 \times 10^{-5}) \times$ area
Correlation (R squared) = 0.996

7.7.2 Carbon dioxide density

Numerous equation of state have been proposed to describe pressure-volume-temperature (PVT) data for CO_2 in the literature [235-238]. For the calculations in this study, the analytical IUPAC equation of state has been exclusively used (Equation 7.1) [236,237]. This equation closely describes PVT data for CO_2 apart from the region very close to the critical point [238].

$$Z_m = \frac{PV_m}{RT} = \frac{PM}{\rho_m RT} = 1 + \rho_r \sum_{i=0}^{9} \sum_{j=0}^{6} b_{ij} \left(\frac{1}{T_r} - 1 \right)^j (\rho_r - 1)^i \quad (7.1)$$

ρ_m = density of CO_2
Z_m = compressibility coefficient of CO_2
P = pressure
T = temperature
V_m = molar volume of CO_2
R = gas constant (0.0821 1 atm mol^{-1} K^{-1}
M = molar weight of CO_2 (44.009 g/mole)
ρ_r = reduced density (ρ/ρ_c)
T_r = reduced temperature (T/T_c)
b_{ij} = coefficient for the equation of state

The majority of the values for the density of CO_2 were obtained from a table which used equation 7.1 [237]

7.7.3 Carbon dioxide flow rate

Carbon dioxide flow rate was measured using a rotameter to give a volume flow rate (l min^{-1}). However, it is more convenient to express the CO_2 flow rate as the mass flow rate (g min^{-1}), as this enabled a direct comparison with the modifier concentrations.

$$CO_2 \text{ flow rate (g/min)} = \frac{60 \times \text{R.M.W. of } CO_2}{\text{flow (l/sec)} \times R \times \text{temperature of } CO_2}$$

R.M.W. of CO_2 = 44.01g

R = gas constant (0.08205, 1 atm mol^{-1} K^{-1})

Temperature of CO_2 ≈ 293K

60 = conversion factor to minutes (SI units)

7.7.4. Modifier concentration

The modifier concentration in SFC or SFE was calculated from the mass flow rate of CO_2 and the modifier flow rate indicated on the modifier pump. Modifier mass flow rate (g min^{-1}) was calculated as:

modifier flow rate	=	flow rate at pump	x	density of modifier at RTP
(g min^{-1})		(ml min^{-1})		(g ml^{-1})

The modifier concentration was then calculated as:

$$\text{modifier concentration (\%w/w)} = \frac{\text{modifier flow rate (g min}^{-1}\text{)} \times 100}{CO_2 \text{ flow rate (g min}^{-1}\text{)} + \text{modifier flow rate (g min}^{-1}\text{)}}$$

Chapter 8
Method development for the extraction and separation of essential oils

8.0 Introduction

Numerous methods have been used for the isolation of essential oils from plant material, including headspace analysis, steam or water distillation, solvent extraction, extraction with hot or cold fat and cold expression [107,225,226]. These methods are usually used to fingerprint a plant, by extracting a range of components. These traditional methods were investigated and compared to the new alternative extraction method, SFE.

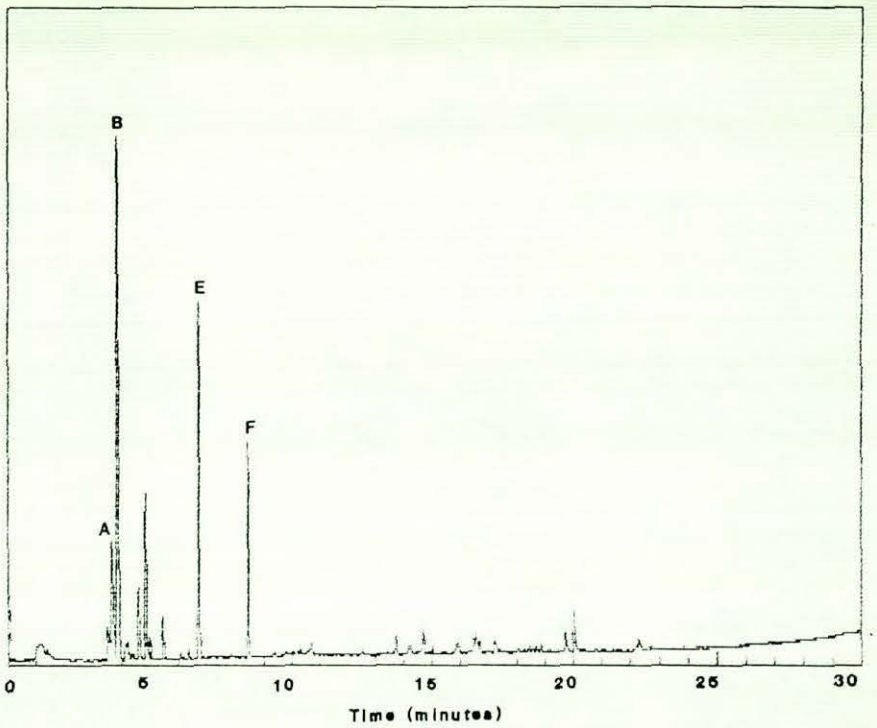
8.1 Headspace analysis

Headspace analysis is a relatively simple and quick technique, avoiding tedious sample preparation procedures, and is non-destructive which ensures that neither the sample composition nor the structure of the components under examination are altered. It produces a fingerprint which normally corresponds to the odour or fragrance of the plant. This analysis was carried out on feverfew and its reported adulterants tansy and German chamomile (Figure 8.1 {i-iii}). The major components present in feverfew and tansy have been identified by GC-MS (Chapter 11), so that some of the components obtained in headspace analysis have been assigned an identification. See Table 8.1.

Feverfew, tansy and German chamomile all possessed a qualitatively distinct set of components. This corresponds to the odours of the plants, feverfew and tansy having a similar "camphor type" odour (camphor being the main component in feverfew, Figure 8.1 {i}), while German chamomile exudes a more pungent, sweeter aroma. However, the chemotypes in tansy may make any qualitative variations between feverfew and tansy less distinctive. Furthermore, the technique may require large amounts of sample depending on the age of the stored plant material, as the volatile constituents are very vulnerable to degradation and evaporation with time. This loss may also cause changes in the distribution of the oils, thus complicating the identification of the plants.

FIGURE 8.1. Headspace analysis of plant material. GC conditions: non-polar (BP1) column. Temperature 60 to 300°C at 8°C min^{-1}, then isothermal at 300°C for 8 minutes. See Table 8.1 for peak identification.

{i} Feverfew (home grown from fisons seeds, dried and stored for 1 week).



{ii} Headspace analysis of tansy (Cathy of Bournemouth Ltd).

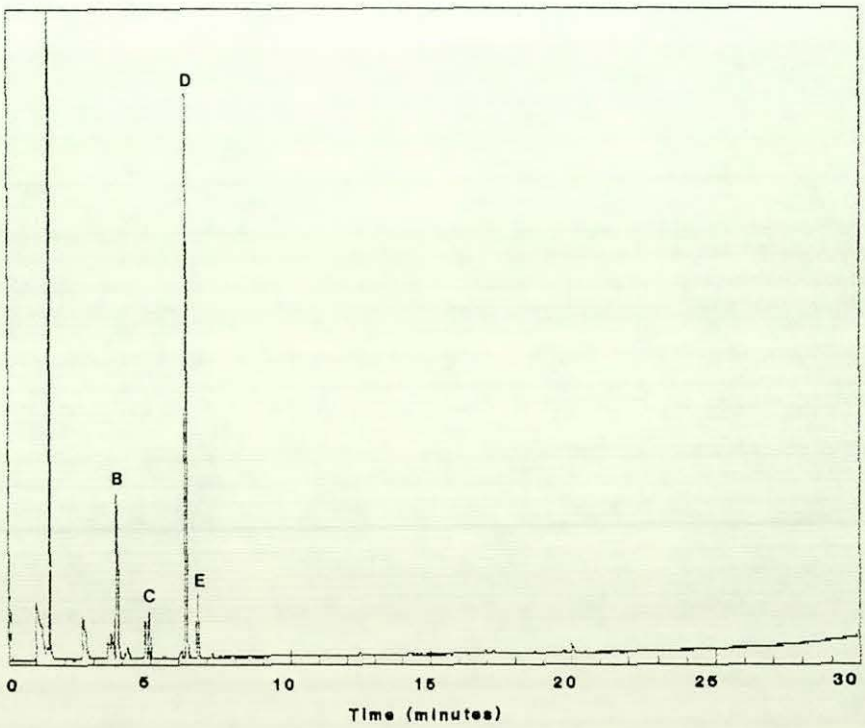


FIGURE 8.1 {iii} Headspace analysis of German chamomile (Brome and Schimmer Ltd).

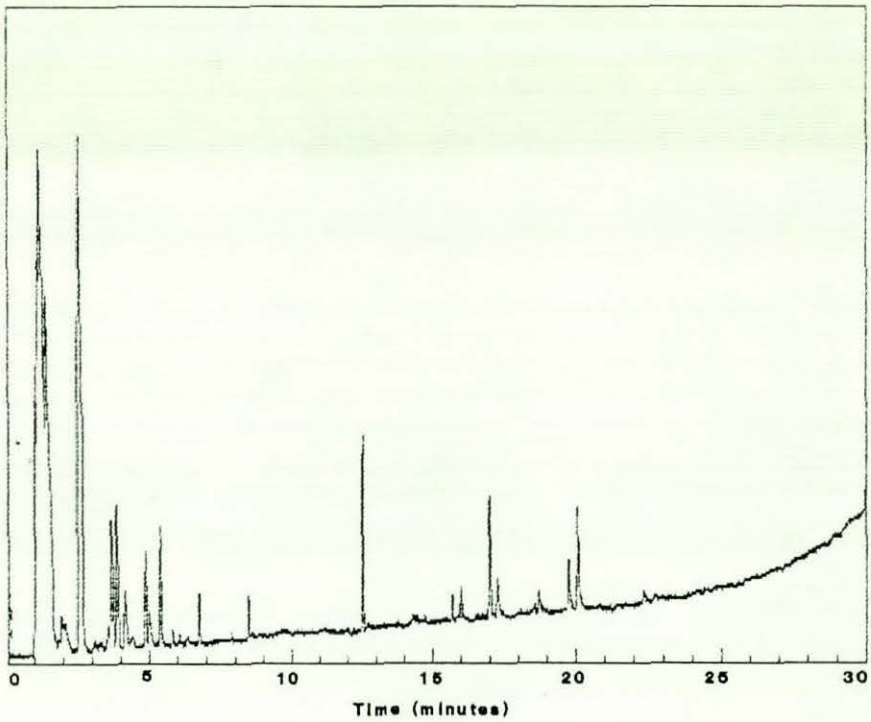


TABLE 8.1. Major volatile components present in feverfew and tansy.

Peak no.	Compound
A	α-pinene
B	camphene
C	cineole
D	thujone
E	camphor
F	pinene acetate

8.2 Steam distillation

This is by far the most common technique for the extraction of essential oils from plant material. Both feverfew (50 g) and German chamomile (50 g) produced about 0.1ml of essential oil after steam distillation for four hours. Tansy oil was obtained commercially. The oils were analysed by GC to show a complex array of components (Figure 8.2 {i-iii}), namely the mono- and sesquiterpene hydrocarbons and oxygenated monoterpenes (GC-MS results Chapter 11). However, none of the larger, polar components of the plant such as the oxygenated sesquiterpenes are obtained. Steam distillation appeared to yield some of the less volatile components which were not obtained in headspace analysis.

An initial examination of the steam distillates suggested that the components could be used as chemotaxonomic markers. All three species appeared to possess distinguishable chromatograms. However, many of the major components in feverfew and tansy were similar and so the identification would have to rely on the numerous minor components. Unfortunately, many of these minor components appeared to be artifacts from the steam distillation process, as they were not found in any other extraction methods.

Such steam distillation artifacts are well known, with the water acting as a chemical agent causing the hydrolysis, intramolecular rearrangement, thermal decomposition, polymerisation and cyclisation of essential oils [225]. For example, linalool was found to undergo cyclisation to terpineol during the steam distillation process [241]. For a long time the only known active principle of German chamomile was the blue azulene compound chamazulene, which was produced from matricin (a guianolide) during steam distillation [27]. The alcohol, hedicaryol (V) found in the leaves of *Hedicarya angustifolia*, underwent Cope rearrangement to elemol (VI) during its extraction [225]. So a whole host of artifacts can be produced due to the rigorous conditions during distillation, these varying in concentration depending on the distillation time. These quantitative variations in minor components could make a chemotaxonomic identification between feverfew and tansy quite complicated.

8.3 Conventional liquid extraction methods

Several organic solvent extraction methods for the isolation of essential oils from plant material were investigated (for experimental detail see Chapter 7). Three of the methods were specifically designed for the extraction of parthenolide, namely the French Pharmacopoeia [232] (a methanol extraction); Bloszyk *et al.* [44] (initial methanol/water extraction, re-extracted into chloroform); and Govindachari *et al.* [57] (a hexane extraction) methods. The fourth method, Marchand *et al* [233] (a chloroform extraction), was a general method for the isolation of sesquiterpene lactones. The extracts were initially qualitatively identified by TLC, then quantified by HPLC and GC.

FIGURE 8.2. Steam distillation of plant material, GC conditions in Figure 8.1. {i}Feverfew (British analytical Control Ltd.)

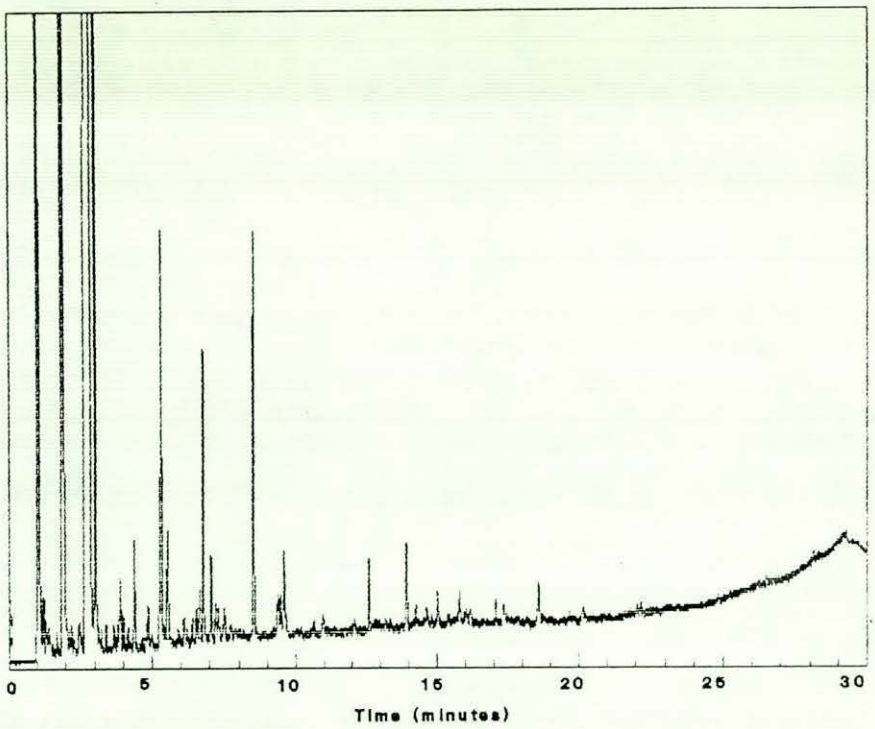


FIGURE 8.2 {ii}. Steam distillation of tansy (commercial preparation from Carl Roth, KG).

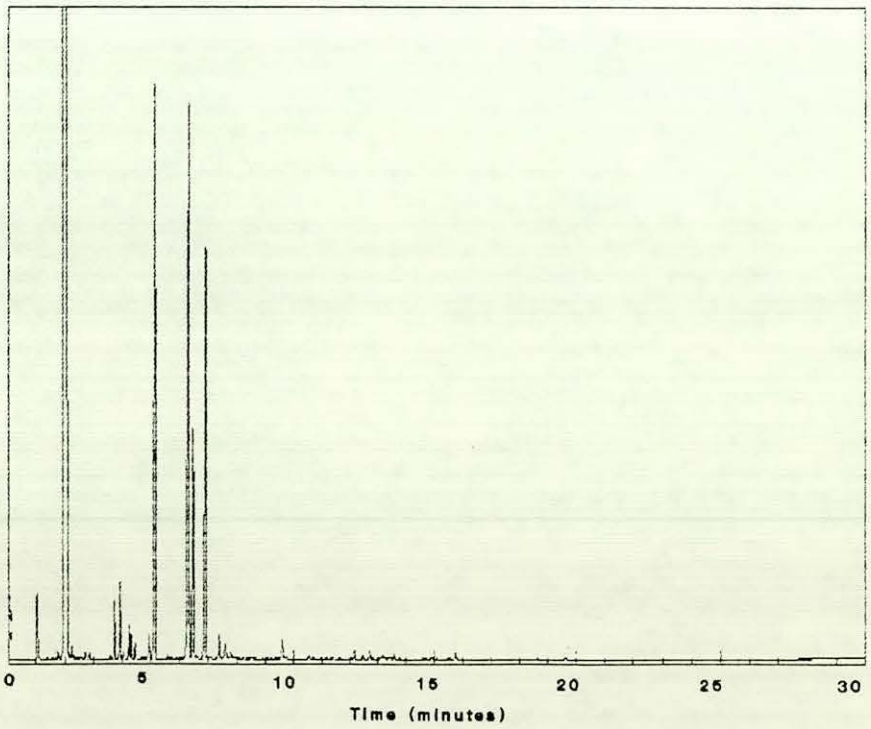
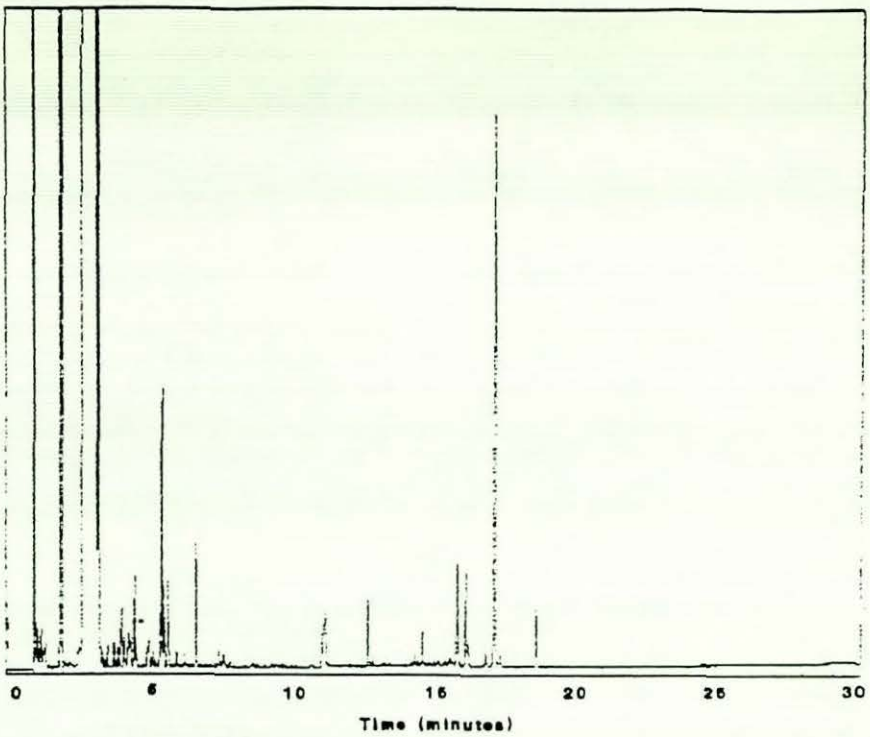


FIGURE 8.2 {iii}. Steam distillation of German chamomile (Cathy of Bournemouth Ltd).

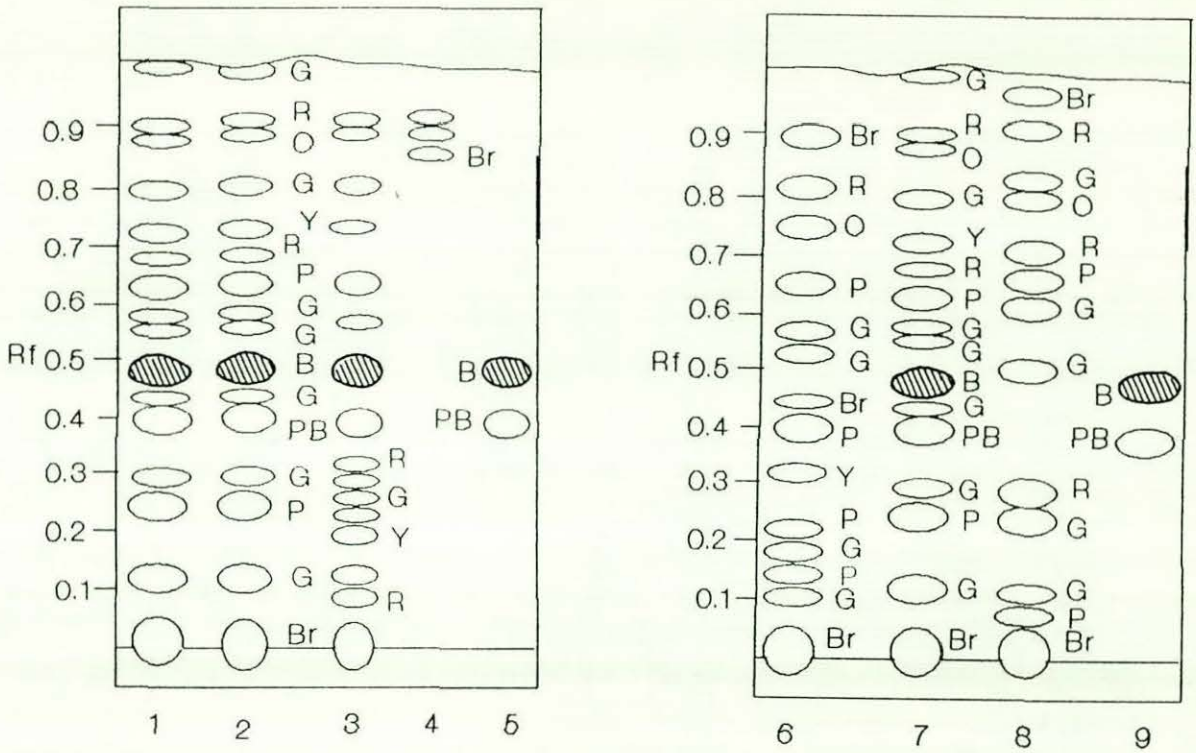


8.3.1 TLC analysis

The most common method for the quality control of plant material in the herbal remedy industry is TLC. The extracts of feverfew from the four extraction methods and a parthenolide standard were analysed by TLC (Figure 8.3 {i}). This technique proved to be a good screening method for the presence of parthenolide, as the compound gave a characteristic dark blue spot when sprayed with anisaldehyde-sulphuric acid reagent. This could easily be distinguished from the other components which tend to give red, yellow, green or brown spots. It would appear that three of the methods extracted parthenolide, but the fourth method by Govindachari *et al.* failed to do so. The latter method was the only one to use an alumina column. Alumina columns have been reported to cause the ring opening of sesquiterpene lactones [242] and this may explain why no parthenolide was seen in the TLC analysis. This is also the only method to use a very non-polar solvent, namely hexane, to extract the medium polar sesquiterpene lactone. However, parthenolide is insoluble in water but very soluble in methanol and dichloromethane. It would appear that the wrong solvent has been recommended.

FIGURE 8.3. TLC analysis of plant extracts. TLC plate (Kieselgel $60F_{254}$) with an eluent of chloroform/acetone (95/5) and a anisaldehyde-sulphuric acid spray reagent.

{i} {ii}



1 = French Pharmacopoeia method [232].
2 = Marchand *et al.* method [233].
3 = Bloszyk *et al* method [44].
4 = Govindachari *et al.* method [57].
5 = parthenolide standard
6 = German chamomile (Cathy of Bournemouth Ltd) extracted by French Pharm. method [232].
7 = Feverfew (British Analytical Control Ltd) extracted by French Pharm. method [232].
8 = Tansy (Cathy of Bournemouth Ltd) extracted by French Pharm. method [232].
9 = parthenolide standard

Key

P = pink
Br = brown
G = green
R = red
Y = yellow
B = dark blue/purple
O = orange
PB = pale blue

German chamomile and tansy were also investigated using the French Pharmacopoeia method of extraction, the extracts being analysed by TLC (Figure 8.3 {ii}). From the results it was quickly ascertained that these plants did not contain parthenolide, so that a distinction between feverfew and its adulterants could be drawn. However, some tansy varieties are reported to possess parthenolide [78] and if this is the case then the distinction between the species would be less obvious. In such instances further identification by capillary GC would be recommended.

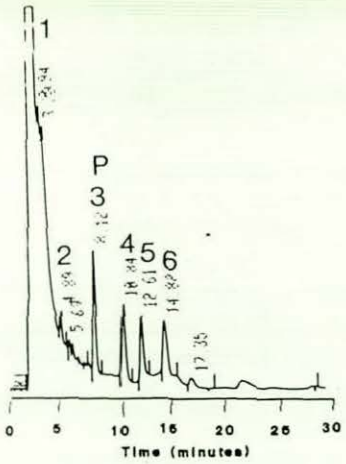
8.3.2 HPLC analysis

TLC is a good qualitative technique but is not very easy to quantify. The French Pharmacopoeia recommends HPLC to determine the concentration of parthenolide [232]. The extracts from the four extraction methods were therefore analysed by RP-HPLC (Figure 8.4 {i-iv}). The chromatograms suggest that all four extracts could contain parthenolide. However, the resolution of the system is limited, with parthenolide co-eluting with an unknown compound. The UV spectrum of the components (Table 8.2) was investigated by means of a diode-array detector. It would appear that the majority of the pigments and plant phenolics eluted at the solvent front, the parthenolide peak being fairly well resolved from this initial broad peak. A series of components are eluted after parthenolide and these are mainly flavonoids, probably flavones or flavanones (see Chapter 5), the amount present depending on the extraction method. The amount of parthenolide in each extract (Table 8.3) was determined from a calibration graph of parthenolide standards (Chapter 7). The most efficient method for extracting parthenolide was that from the French Pharmacopoeia [232], though it is also one of the least selective.

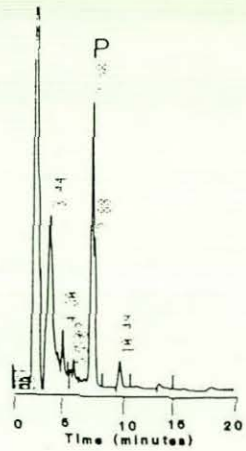
German chamomile and tansy were also analysed by the French Pharmacopoeia method [232]. The results (Figure 8.4 {v,vi}) are inconclusive, as it is unable to ascertain with certainty the presence or absence of parthenolide. This is due to the initial broad peak tailing quite badly and obscuring the potential parthenolide peak. However, it can be established that if parthenolide is present, it is so at a very low concentration.

FIGURE 8.4. HPLC analysis of solvent extraction from feverfew, using a spherisorb ODS column with an acetonitrile/water (55:45) eluent at 1ml min^{-1} and UV detection at 220nm (AUFS 0.08). P = possible parthenolide component.

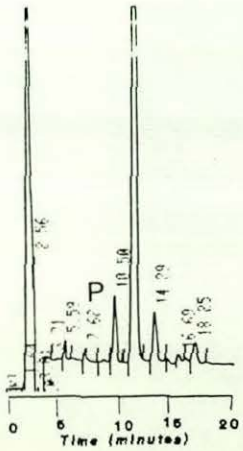
(i) Feverfew (British Analytical Control Ltd) extracted by the French Pharm. method [232]. (table 8.2)



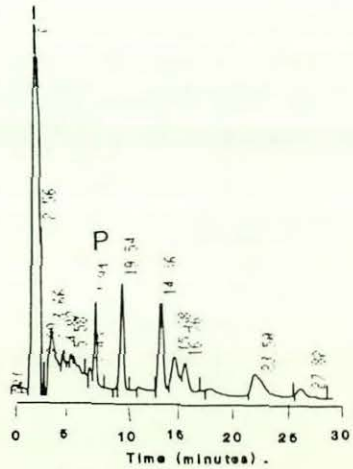
(ii) Feverfew (British Analytical Control Ltd) extracted by the Bloszyk *et al.* method [44].



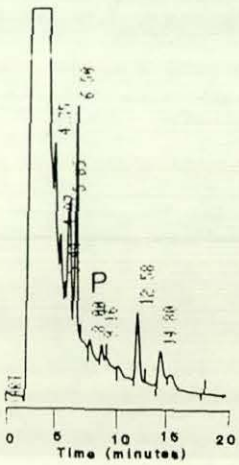
(iii) Feverfew (British Analytical Control Ltd) extracted by the Govindachari *et al.* method [57].



(iv) Feverfew (British Analytical Control Ltd) extracted by the Marchand *et al.* method [233].



(v) Tansy (Cathy of Bournemouth Ltd) extracted by the French Pharmacopoeia method [232].



(vi) German chamomile (Cathy of Bournemouth Ltd) extracted by the Pharmacopoeia method [232].

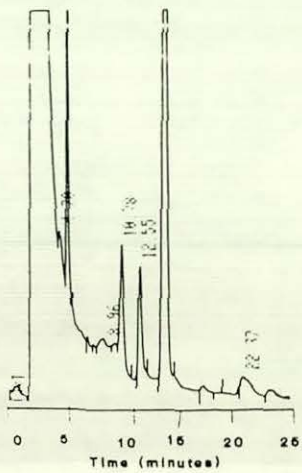


TABLE 8.2. UV spectral data of the components in feverfew (British Analytical Control Ltd) extracted by the French Pharmacopoeia method [232].

Peak no.	Principal maxima (nm)	Subsidiary maxima (nm) (with relative intensities)	Indications
1	205	220 (66%), 280 (37%) 335 (25%)	Pigments and plant phenolics
2	210	-	Essential oil
3	210	-	Essential oil
4	315	225 (58%)	Flavonoid
5	320	230 (62%)	Flavonoid
6	320	230 (75%)	Flavonoid

8.3.3. GC analysis

The extracts from the four extraction methods were analysed by GC to ascertain their essential oil content (Figure 8.5 {i-iv}). The French Pharmacopoeia method [232] and the Marchand *et al.* method [233] showed a high degree of selectivity (Figure 8.5 {i} and {iv}), whereas the Bloszyk *et al.* method produced an extract with a wide range of components (Figure 8.5 {ii}). It is interesting to note that in the extract from the Bloszyk *et al.* method, both the parthenolide and dihydroparthenolide are present, these two oxygenated sesquiterpenes possibly co-eluting to give the split peak in the HPLC chromatogram in Figure 8.4 {ii}. The Govindachari *et al.* method [57] appears to have extracted all the mono- and sesquiterpene hydrocarbons which are particularly soluble in hexane (Figure 8.5 {iii}), but has failed to extract the sesquiterpene lactones. The tansy and German chamomile extracts obtained by the French Pharmacopoeia method [232] were also analysed by GC, though as expected no parthenolide was detected (Figure 8.6 {i} and {ii}). With these solvent extractions, there appeared sufficient variation in the essential oil content of the plants to be able to distinguish the 3 plant species. A more in depth analysis of the essential oils is undertaken in Chapter 11.

The amount of parthenolide present in all the extracts (Table 8.3) was determined from a calibration graph of parthenolide standards (Chapter 7). All the results show quite a low level of parthenolide, probably due to the age of the feverfew samples (stored for about 2½ years) and not the extraction processes. The GC and HPLC results show the same general trend, in that the French Pharmacopoeia method has the highest extraction efficiency. However, the results also show that the HPLC method produces consistently higher values for the concentration of parthenolide, than GC.

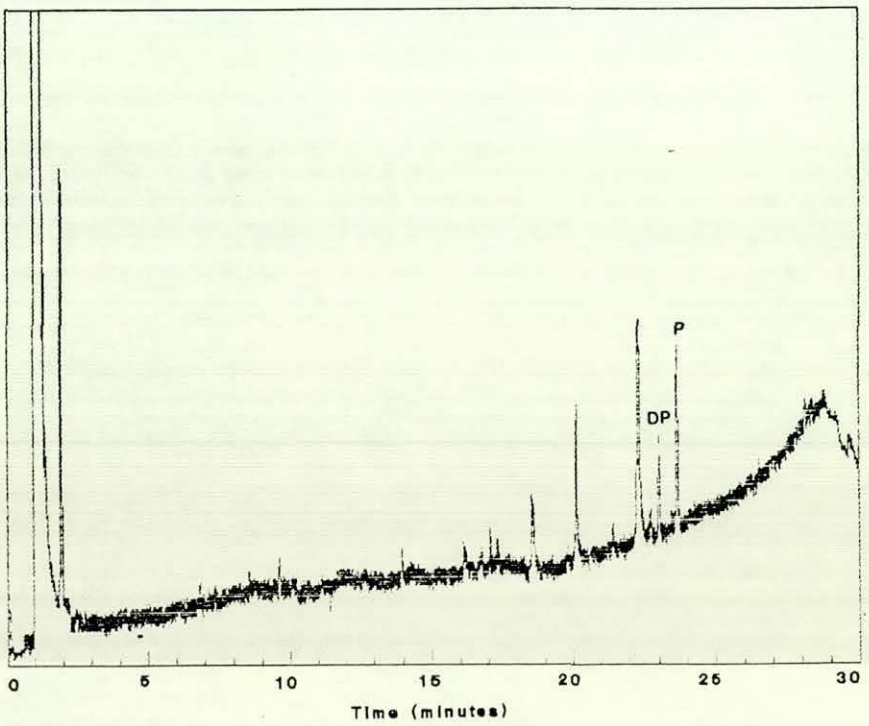
This could be due to the parthenolide peak in the HPLC chromatogram comprising of a number of co-eluting essential oils. This is not to be unexpected, as the HPLC column does not have the same resolution for the separation of essential oils as a GC capillary column. This is further exemplified by the HPLC method which suggested the possible presence of parthenolide in tansy and in the feverfew extract obtained by the Govindachari *et al.* method, whereas GC analysis proved this was not to be the case.

TABLE 8.3. Assessment of extraction efficiency for the various isolation methods.

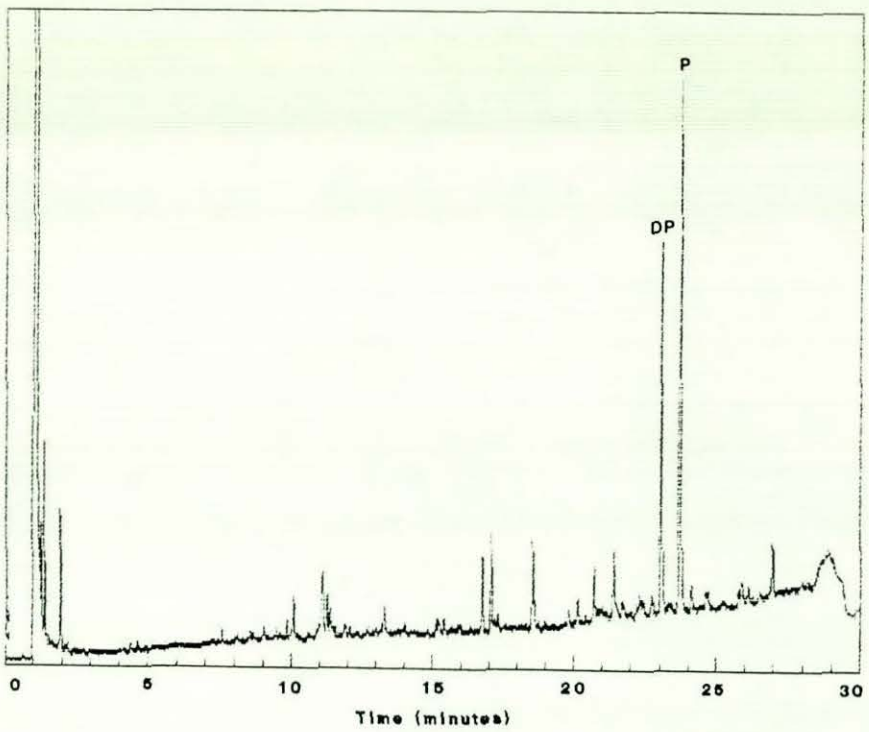
Plant	Extraction method	% parthenolide in dried plant material	
		HPLC	GC
Feverfew	French Pharm. [232]	0.07	0.04
Feverfew	Bloszyk *et al.* [44]	0.05	0.02
Feverfew	Marchand *et al.* [233]	0.01	0.006
Feverfew	Govindachari *et al.* [57]	0.01	0.0
Feverfew	SFE[a]	–	0.03
Tansy	French Pharm. [232]	0.01	0.0
German cham.	French Pharm. [232]	0.0	0.0

a = SFE at 250 bar, 45°C and 0.8 ml min^{-1} CO_2 (at the pump head) for 30 minutes. Feverfew supplied by British Analytical Control Ltd.

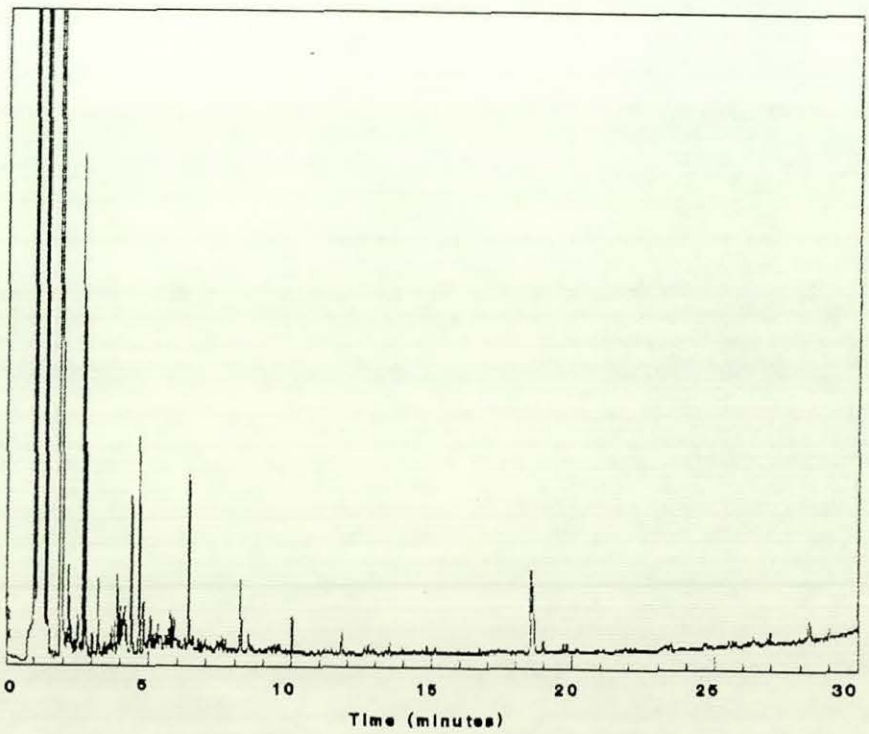
FIGURE 8.5. GC analysis of the extracts obtained by the four extraction methods. GC conditions given in Figure 8.1.
{i} GC analysis of feverfew (British Analytical Control Ltd) extracted by the French Pharmacopoeia method [232]. P = parthenolide, DP = dihydroparthenolide.



{ii} GC analysis of feverfew (British Analytical Control Ltd) extracted by Bloszyk *et al.* method [44]. P = parthenolide, DP = dihydroparthenolide.



{iii} GC analysis of feverfew (British Analytical Control Ltd) extracted by the Govindachari *et al.* method [57].



{iv} GC analysis of feverfew (British Analytical Control Ltd), extracted by the Marchand *et al.* method [233]. P = parthenolide, DP = dihydroparthenolide.

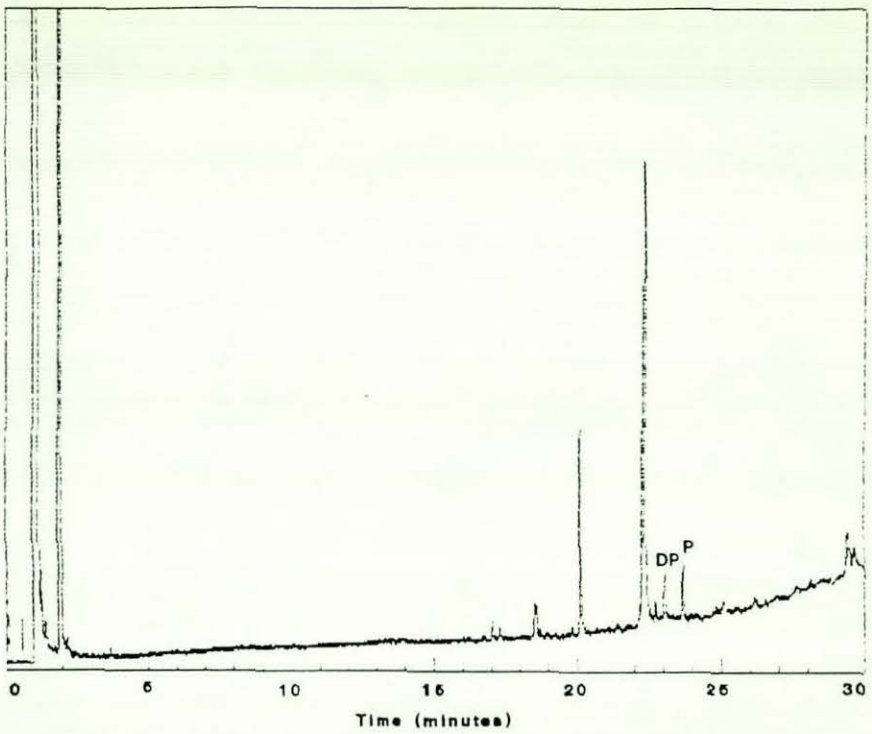


FIGURE 8.6 {i}. GC analysis of tansy (British Analytical Control Ltd) extracted by the French Pharmacopeia method [232].

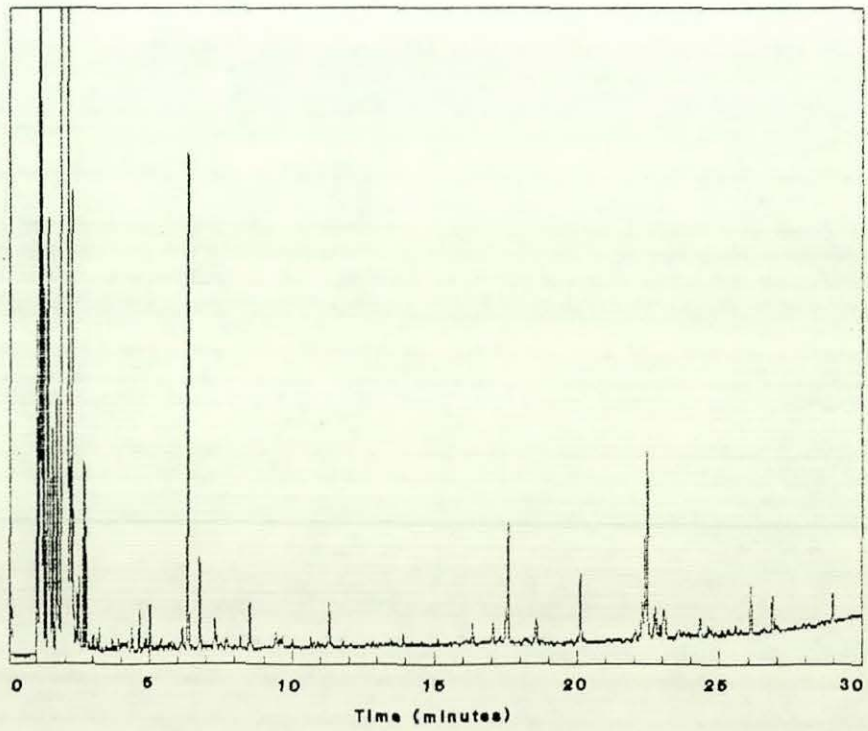
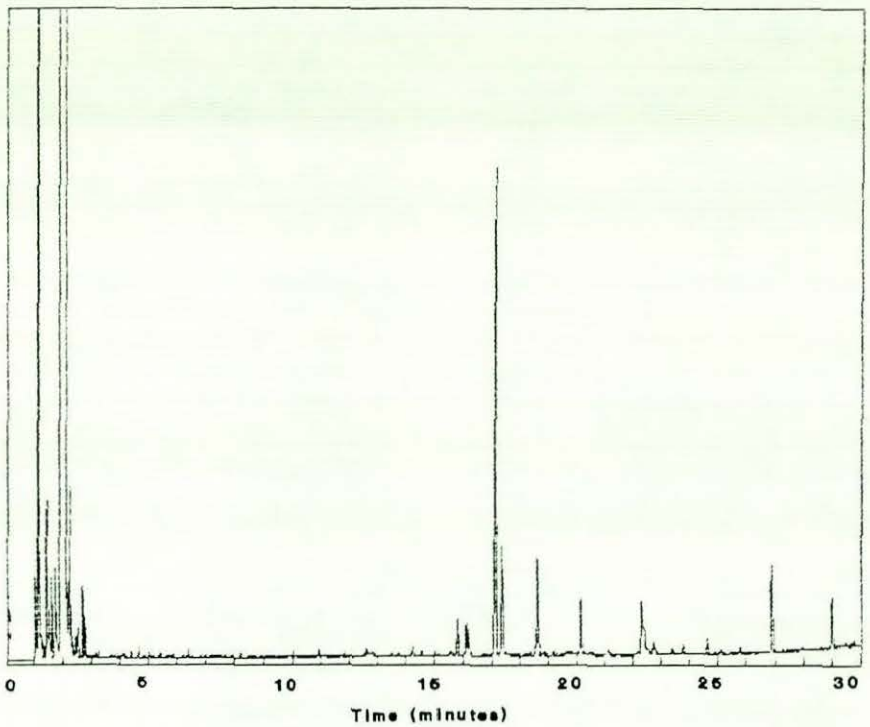


FIGURE 8.6 {ii}. GC analysis of German chamomile (British Analytical Control Ltd) extracted by French Pharmacopoeia method [232].



8.4. Supercritical fluid extraction

In the light of these findings from solvent extractions, an alternative extraction method, SFE, was investigated to try and obtain the thermally labile components present in the plant, which are often only isolated as rearranged or secondary products by conventional methods. A comparison of an initial SFE extract to conventional extraction methods suggests that this method is most comparable with that of the organic solvent extraction (i.e. Bloszyk *et al.* method [44]) (Figure 8.7). The recovery yields are also comparable (Table 8.3). The supercritical extraction method was not that similar to the hexane extraction (Govindachari *et al.* [57]) as some of the literature suggests [179,180,186]. The headspace analysis removed more of the volatile monoterpenes compared to SFE, however, this is probably due to poor collection efficiencies rather than lack of solubility in the SCF. The steam distillation extract appears to possess more components, but some are probably artifacts. A similar study was carried out by Bicchi *et al.* who found that SFE gave the most complete extraction profile, the conventional methods having certain components absent [243]. It has also been shown that

FIGURE 8.7. Comparison of SFE to conventional extraction methods.

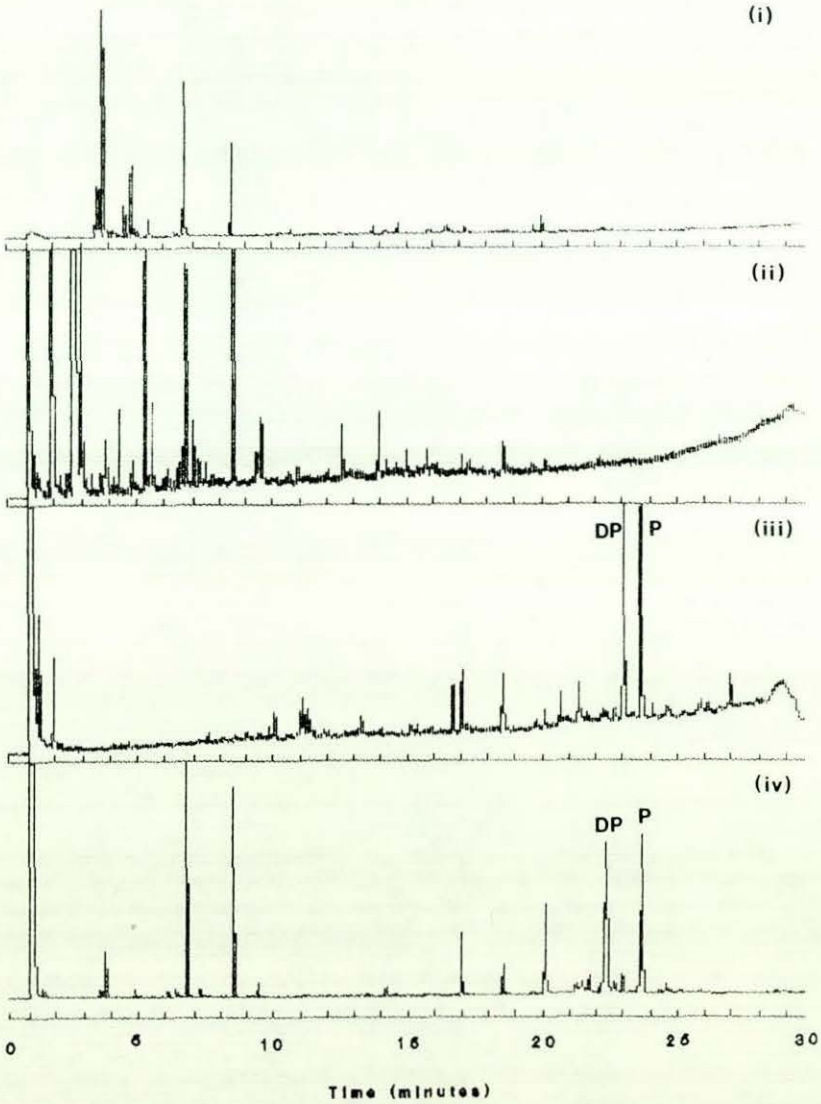
P = parthenolide, DP = dihydroparthenolide

{i} = Headspace analysis of feverfew

{ii} = Steam distillation of feverfew

{iii} = Solvent extraction of feverfew (Bloszyk *et al.* method [44])

{iv} = SFE of feverfew (200 bar, 45°C, pumping at 1 ml min^{-1} CO_2)

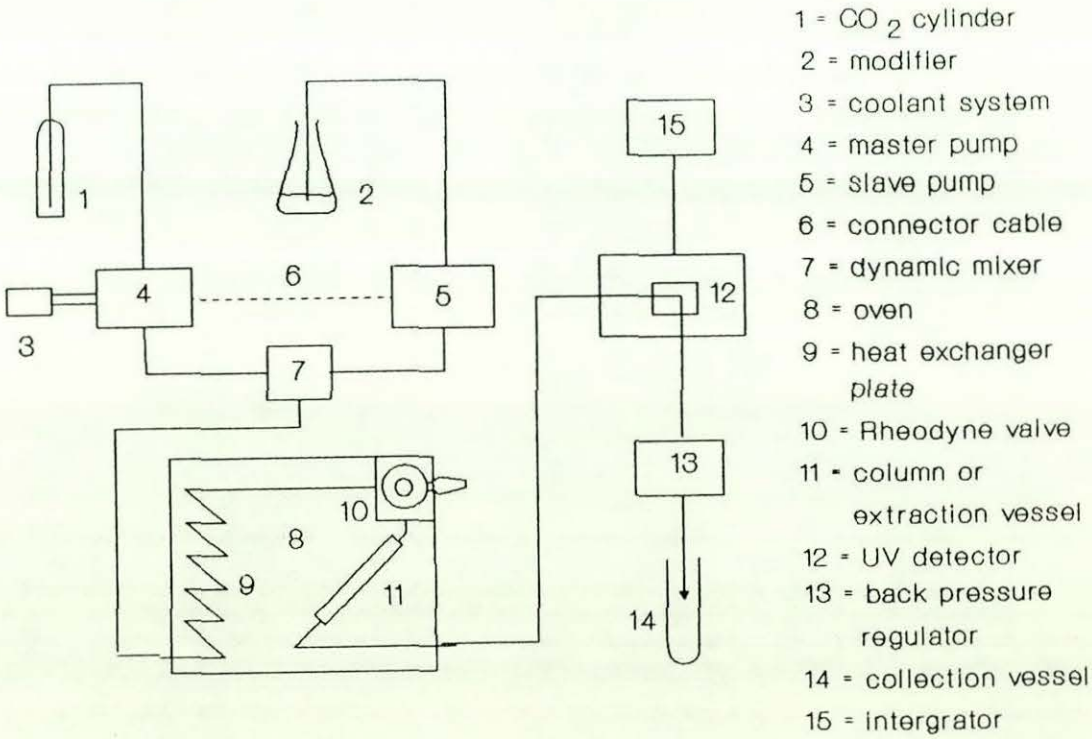


SFE is as quantitatively efficient as soxhlet extraction [178]. The initial success of the SFE technique has lead on to a more extensive study of the application of SFE for feverfew analysis (Chapter 9).

8.4.1 Instrumentation

The components fundamental to every SFE instrument are a solvent delivery system, an extraction vessel, a temperature controller, a back pressure regulator (BPR), a solute detection device and a collection vessel. Most of the components were from the Jasco LC-800 series with the exception of the dynamic mixer which was a Gilson 811B. A schematic diagram of the SFE system is shown in Figure 8.8.

FIGURE 8.8. Jasco modular SFE system.



Liquid carbon dioxide was obtained from a standard dip tube cylinder. It was then passed through a valve, a frit filter, and another valve before it was passed through a 7μm in-line filter (Nupro). The filters helped purify the CO_2 from any solid materials before it entered the pump, thus minimising the accumulation of impurities on the pump check valves. Pressures higher

than cylinder pressure (≈ 50bar) were achieved with a modified Jasco 880-PU HPLC pump. The first pump head was cooled to between -12 and -8°C depending on the temperature of the laboratory. The organic modifier (if used) was added to the pressurised CO_2 using a second Jasco 880-PU pump and a dynamic mixer. The fluid was then passed through a heat exchanger plate situated at the back of the oven. Samples of plant material were placed in a Jasco extraction vessel (2, 10 or 40 ml) which was situated on the Rheodyne valve, in place of the injection sample loop. To initiate an extraction the Rheodyne valve was turned from the "load" to the "inject" position so exposing the sample to the preheated CO_2. The solvent pressure prior to and after the extraction vessel were monitored by pressure sensors situated on the pumps and BPR. The extracted solutes were monitored with a Jasco 875-UV detector. These extracted solutes then passed on to the Jasco 880-81 BPR, where the CO_2 pressure was reduced to atmospheric conditions and the precipitating solutes were collected in a standard Jasco collection vessel (10 ml tapered centrifuge tube).

8.4.2 Method development

In the course of this study a series of modifications had to be incorporated into the commercially available Jasco SFE system (Chapter 7), to enable better extraction efficiencies to be achieved. The problems encountered fall into four categories:

{i} Leaks.

The initial problem encountered with the system was that of leaks. This is undoubtedly due to using HPLC fittings to contain a supercritical fluid. As the extraction vessel must continually be removed for refilling, it was found that after about twenty extractions, the ferrules were no longer leak proof and had to be replaced. This problem with leaks was the major reason for the down time on the system. An alternative may be in the use of a cartridge extraction vessel with lever type compression fittings which enable quick change over times and the possibility of automation, without the need to use the conventional nut and ferrule arrangement.

The other problem area was with the check valve seating seals on the pumps. In 6 to 12 months use these lost their plasticiser and became discoloured and brittle. This allows very subtle leakage past the check

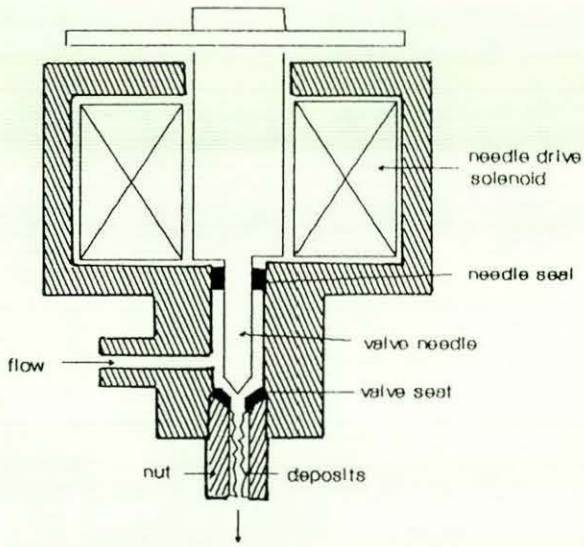
valves and the pumps then give a varying delivery. The symptoms are being unable to maintain the pressure and an uneven baseline, but without necessarily seeing CO_2 physically escaping from the pump heads. Coupled with this was the problem that the inlet check valve needed to be cooled so that it was operating with liquid carbon dioxide rather than a mixture of gas and liquid. However, in the original design the CO_2 was liquefied in the cooling head but then allowed to heat up again before entering the check valve. To compensate for this the connecting tubing was shortened. In addition a wider bore tubing was used so that the pump head was not starved of liquid CO_2 at higher flow rates. The insulation around the pump head was also extended to cover the connecting tubing, so maintaining liquid conditions.

{ii} Cross contamination.

Cross contamination could occur between extracts if due care and attention were not taken. It was found that the narrow bore connecting tubing for the detector cell was rapidly blocked during several extractions. It became necessary to replace all the tubing after the extraction vessel (including the BPR) with wider bore tubing to prevent this problem. However, this did cause the base line stability to be reduced a little, but this was not a problem for SFE. It would appear that once the tubing from the extraction vessel had left the oven, it rapidly cooled down due to the large heat sink in the UV flow cell. This reduction in temperature may reduce the solubility of the waxes, lipids and pigments in the now possibly subcritical CO_2 [229], so explaining why greasy green deposits were found in the tubing. To avoid this it may be possible to heat the flow cell to the same temperature as the extraction, thus maintaining the solubility.

Further problems were encountered with precipitation/deposition occurring in the exit of the Jasco BPR. Just after the valve seat where the CO_2 vaporises, condensation of the extract can occur on the wall of the nut as shown in Figure 8.9. This can cause contamination between samples and requires careful cleaning between extractions, which is time consuming. This problem could possibly be reduced by using a shorter nut which would fit flush to the base of the BPR and would not have the same tendency to collect the sample. It would also require a change in the heating block so that the collection vessel could be fitted directly to the bottom of the BPR block.

FIGURE 8.9. Schematic of the Jasco 880-81 BPR.

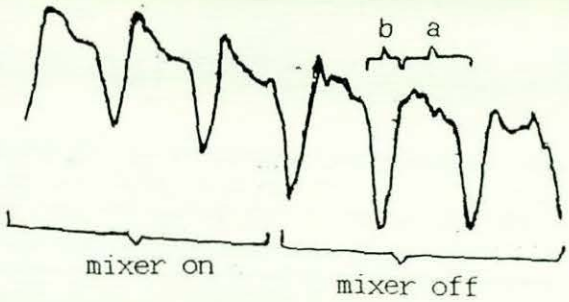


{iii} Introduction of the modifier.

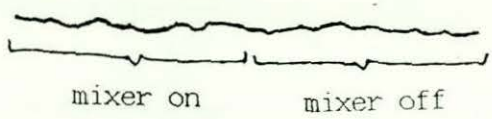
The problem of adding modifiers to give an even flow and stable base line proved to be very complicated. This phenomenon of a pulsating flow and degrading flow rate has been related to such factors as the compressibility of the fluid, the seal material and the dead volume and plunger volume of the pump [244]. The Jasco 880-PU HPLC pumps are claimed to have overcome the biggest problem of the compressibility of the eluent, by the use of an "intelligent cascade" delivery system [244]. This is achieved by the first pump ("compression pump") compressing the eluent up to the pressure required, the eluent is then passed to the second pump ("metering pump") which delivers the compressed eluent continuously at a precise rate. This system appears to work when the modifier is delivering 100 μl or more of organic eluent into the pressurised CO_2. However, when the system delivers between 50-10 μl (which is at the very bottom of its delivery range) an undulating base line is produced (Figure 8.10 {i}). Different compressibility settings have been tried for both the CO_2 and modifier pumps, but pulsation still occurred. The compressibility value of the modifier pump was adjusted to 155 (water has a value of 70 to 80). The CO_2 pump had this value adjusted to the highest possible setting of 256, as it has been shown that liquid CO_2 has a compressibility value which is three times higher than that of the polar modifier ethanol [244].

FIGURE 8.10. Base line noise with and without modifier under several SFC conditions. Spherisorb 5 μm (4.6 x 250 mm) column, CO_2 at 250 bar, 65°C and 2.5 ml min^{-1}. UV detection at 210nm, 0.08 AUFS. a = metering pump on the in stroke, b = compression pump on in stroke.

{i} Modifier pump:-
Jasco PU 880.
20 μl min^{-1} MeOH



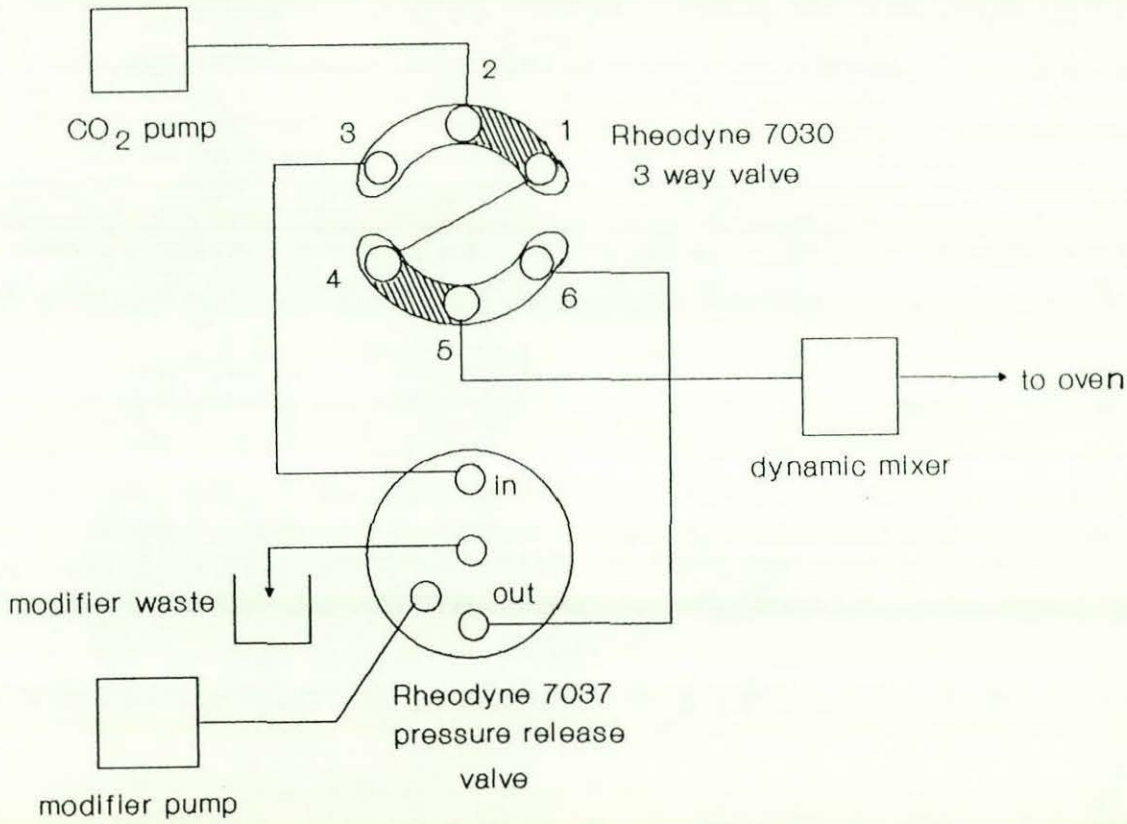
{ii} Modifier pump:-
Philips PU 4100
20 μl min^{-1} MeOH



In an attempt to remove this problem of inefficient delivery of modifier into the pressurised system by the Jasco pump, a dynamic mixer was used to try and produce a more homogeneous eluent (Figure 8.10 {i}). A limited improvement was obtained with the smaller undulating peaks being removed, however the underlining trend still remained. An alternative dual reciprocating pump (Philips PU 4010) was used instead of the Jasco pump, this also could delivered down to 10 μl, but the same problems were incurred. A completely different approach was then undertaken by using a semi-micro delivery HPLC pump (Philips PU 4100) which had a maximum delivery of 5 ml min^{-1}. This pump with its short rapid pump strokes alleviated the problem (Figure 8.10 {ii}). This suggested the "10 ml" pump heads on the Jasco pump were too large for the very low flow rates. Consequently there was a lag in delivery.

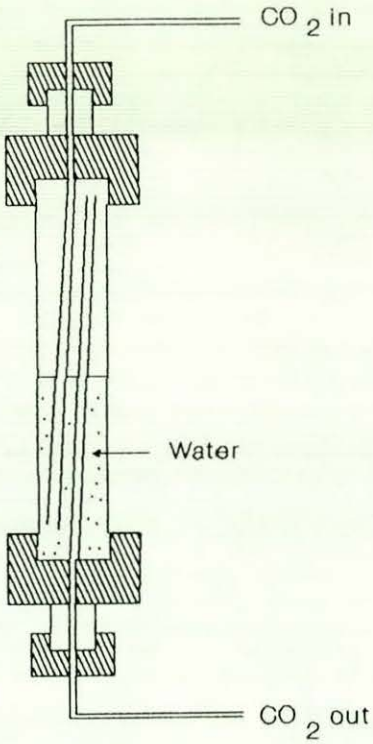
Another problem was the large pressures the modifier pump experienced when the modifier was initially introduced into the system. The use of a Rheodyne BPR (7037) prevented the back flow of modifier and enabled the modifier pump to be pressurised prior to being exposed to the CO_2 eluent (Figure 8.11). This reduced the equilibrium time for obtaining a modified eluent.

FIGURE 8.11. Modifier pump with Rheodyne 7037 pressure regulator to produce a "ready pressurised" modifier system. Note the pressure regulator (7037) should be set to a pressure of at least a 100 kg cm^2 higher than the system working pressure.



In some extractions water was used as a modifier, however, since its solubility in CO_2 is very low (≈ 0.1% [245]) it was not possible to introduce it into the system by the conventional means of a HPLC pump. It was feared that even at the lowest flow rate of 10μl min^{-1}, a two phase system would be formed with water saturated CO_2 and a separate water layer. Instead, an empty HPLC column half filled with water was incorporated into the design, so that CO_2 bubbled through the water and became a single CO_2/water saturated phase (Figure 8.12) [245].

FIGURE 8.12. Device for introducing water into the CO_2 solvent.



{iv} Collection vessel efficiency.

It was found that the sample recovery of essential oils from a cellulose matrix (Chapter 9.) using the standard Jasco (10ml) collection vessel with the Jasco BPR, was very poor, with the odour of essential oils being detected during the extraction process. The recovery was particularly bad for monoterpenes which in some cases were not collected at all. It has been suggested that during the depressurisation process the analyte molecules may nucleate and become entrained in the expanding gas, so that an aerosol is formed [178]. Experiments have suggested that the solute particles in the 0.01-0.02μm range can be formed by the rapid expansion of SCF solutions through pressure restrictors [246]. As a consequence, because of the low volume of the collection trap, most of the sample is blown out of the system as an aerosol, before it has had a chance to condense on the sides of the flask. If very low flow rates were used the results improved but the extraction times became unacceptably long.

A number of alternative designs for the trap were examined. Some success was achieved by using preparative GC traps with convoluted gas paths or increased wall areas. However, the same problems of aerosol formation

occurred, so that the higher flow rates generated in SFE (litres per minute instead of ≈ 200 cm^3 min^{-1} in prep-GC) produced low recovery yields. Traps packed with glass beads or helices were considered but rejected as they would hamper sample collection.

Methanol was investigated, to attempt to trap the analytes in a liquid. However, bubbling the extract through methanol in a glass vial (5 ml) at room temperature using a fine bore HPLC tube (0.01" i.d.), proved impractical. The flow rate through the methanol was too rapid, so that the methanol and analytes were aspirated into the atmosphere and lost. Furthermore, the tip of the crimped tube would frequently freeze up and block, causing an erratic flow through the solvent. A sintered block was placed over the end of the crimped tube in an attempt to disperse the CO_2 more evenly in the solvent, however this appeared to have little effect on the recovery yield. Hawthorne [247] suggests that the problem lies with using narrow bore HPLC tubing, a more practical alternative may be the use of a fused silica capillary tube (10 cm x 25 μm i.d.).

Temperature effects were investigated (Table 8.4). The prep. GC traps were cooled in ice but this had little effect. Since the volatility of the essential oils at 0°C was very low, the most likely mechanism for loss was still through solute aerosol formation. Liquid nitrogen could not be used with these traps as they filled up with solid CO_2 within 5 minutes whereas the extractions took 15-20 minutes pumping CO_2 at 1 ml min^{-1}. To compensate for this the traps were extended, but within ten minutes the narrow inlet and outlet tubes had blocked. Eventually the use of a simpler, larger flask (100 ml) was tried at different temperatures (Table 8.4 and Figure 8.13). Cooling in liquid nitrogen was found to give the best results. This collection vessel had a sufficiently large volume so that all the CO_2 could condense as a solid. The vessel was then subsequently allowed to evaporate in a freezer at -10°C. Any loss due to aerosol formation was now in theory completely eliminated. However, the recovery of limonene was still very low (Table 8.4). This is thought to be due to the very volatile nature of the monoterpene, which probably leads to it being lost during the preparation and storage of the test matrix. Conventional extraction using the solvent, dichloromethane, also produced low yields of limonene (Table 8.4), though the yield was higher than the SFE method. This suggested that some sample loss was still occurring for the very volatile analytes during the collection process. To keep sample variation and loss to a minimum, 25 g batches of spiked cellulose were prepared and kept at -10°C in a freezer.

This matrix could be kept up to 1 month without any significant deterioration.

The 100 ml collection vessel (Figure 8.13) can be used for up to 30 minutes at 2 ml min^{-1}. With shorter extractions smaller collection vessels (50 ml) can be used, and for timed extracts a simple test tube with a side arm is sufficient. The side arm is used to monitor the effluent and prevent liquid nitrogen entering the flask when topping up the dewar. In each case the position of the liquid nitrogen level is important. If too much liquid nitrogen is added so that the neck of the collection vessel is covered, then the CO_2 condenses on the walls of the neck and the flow is blocked very rapidly. The level should ideally be maintained at about point A in Figure 8.13.

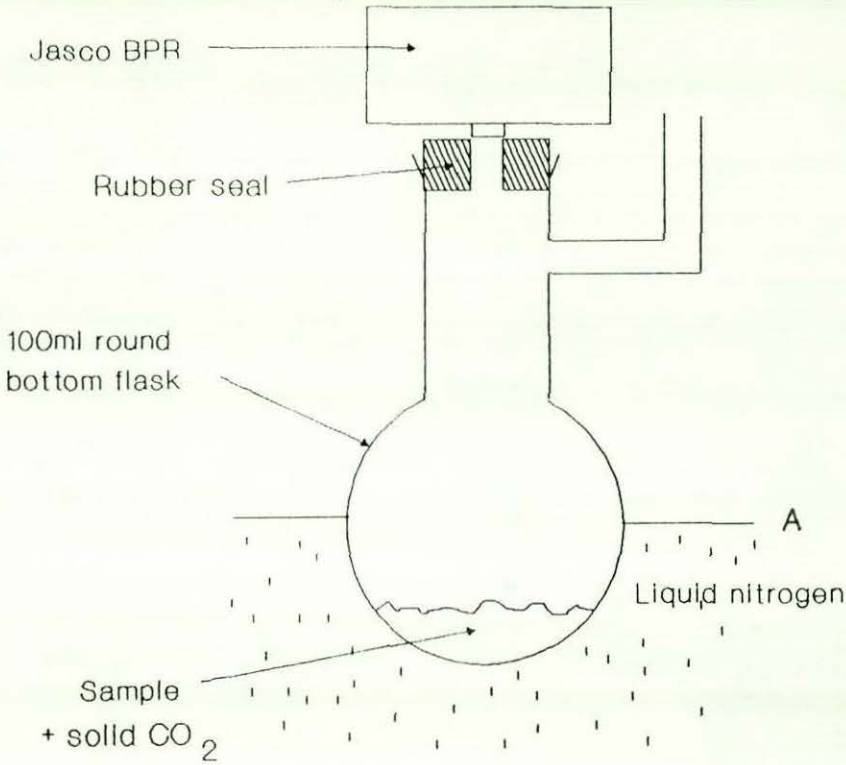
It has been suggested that the use of modifiers with a high critical temperature, such as methanol, in CO_2 can also substantially decrease aerosol formation as reflected by much higher extraction recoveries [178]. this may be due to the formation of sizable liquid-methanol droplets during depressurisation, which likely contain or scavenge many of the analyte molecules and have a larger deposition efficiencies due to their size and liquid character.

TABLE 8.4. Collection efficiency of various collection vessel designs. a = solvent extraction, b - g = SFE extraction at 250 bar, 40°C at 0.8 ml/min CO_2.

Collection vessel	% recovery					
	Limonene	Carvone	Eugenol	Caryophyllene	Santonin	Total oil
a	46	88	81	95	98	82
b	0	18	13	20	21	18
c	14	44	32	67	52	42
d	12	27	21	32	35	25
e	2	10	8	16	15	10
f	9	28	34	32	62	33
g	23	86	81	97	93	76

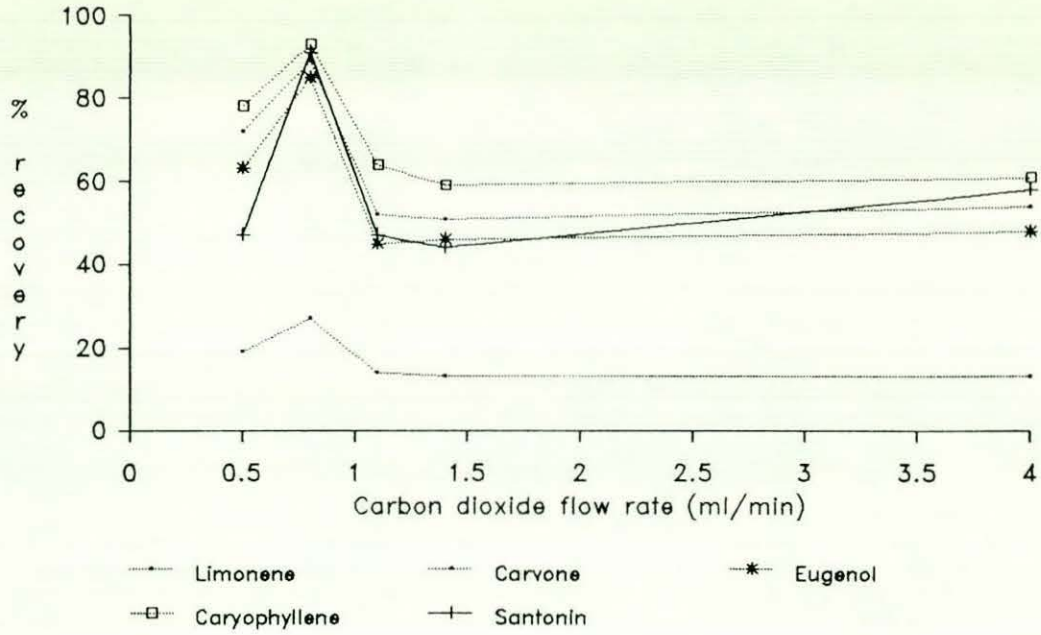
a = Organic solvent extraction with dichloromethane.
b = Standard Jasco collection vessel (10 ml) at room temperature.
c = Prep-GC collection vessel in liquid nitrogen (≈ -170°C)
d = Bubbled extract through methanol at room temperature using a crimped fine bore HPLC tube.
e = Collection vessel (Figure 8.13) in MeOH/ice (≈ -15°C).
f = Collection vessel (Figure 8.13) in acetone/dry ice (≈ -60°C).
g = Collection vessel (Figure 8.13) in liquid nitrogen (≈ -170°C).

FIGURE 8.13. Collection vessel.



The flow of the CO_2 is also important (Figure 8.14). The optimum flow for the 100 ml collection vessel was about 0.8 ml min^{-1}. If lower flow rates were used liquid oxygen from the air condensed in the vessel and at higher flow rates (1.5 ml min^{-1}) losses occurred with essential oils escaping through the exhaust arm (Figure 8.13). It is interesting to note the % recovery of santonin increased at the very high flow rates (4 ml min^{-1}), suggesting that at the lower flow rates the extractions were not being collected for a long enough period of time. However, at such low flow rates the extraction time to get a 100% santonin would become unrealistic.

FIGURE 8.14. Effect of flow rate on collection of essential oils extracted by SFE from a cellulose matrix.



8.5 Conclusions

The conventional extraction methods had varying degrees of success in distinguishing feverfew from its adulterants. The headspace analysis proved inconclusive and was impractical due to the amount of sample required. Steam distillation looked promising but relied on minor components to distinguish the plants, many of which could be artifacts. The solvent extractions were able to remove a number of terpenes including the polar sesquiterpenes, from which a distinction could be made. However, a lot of extraneous material was also removed, these non-volatile materials causing problems in the GC analysis. However, there was sufficient variation in the essential oil content in the plants to enable a classification to be obtained.

TLC possessed adequate resolution to identify the plants, based on the presence of parthenolide. But, this traditional method of screening plant materials may not be suitable when analysing the reported tansy variety which contain parthenolide. In such instances capillary GC may be more appropriate, as a greater number of components are resolved on which to base a distinction. The French Pharmacopoeia method used HPLC to determining the sesquiterpene lactones but this proved ambiguous, due to the limits of resolution in the system. The SFE method managed to remove the widest range

of essential oils but apparently without any of the artifacts incurred by the conventional extractions. It was this supercritical technique which was deemed most suitable for further investigation.

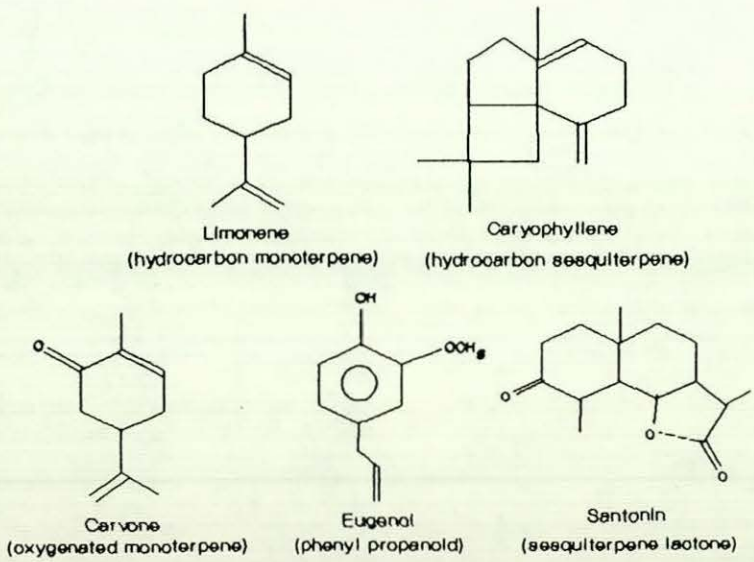
Chapter 9

Supercritical fluid extraction of essential oils

9.0 Introduction

Following the initial survey using conventional extraction techniques to isolate the essential oils from feverfew and its reported adulterants [3] (Chapter 8), an alternative method, SFE, was investigated. Conventional extraction techniques had proved cumbersome and sometimes undesirable due to the unstable nature of the essential oils. SFE was therefore deemed a more gentle and appropriate method for the isolation of such thermally liable compounds. Initially an attempt was made to assess the selectivity of SFE using feverfew plant material, however, this proved too complex to monitor. It was therefore decided to use a model plant matrix of cellulose, spiked with examples of five classes of essential oil (Figure 9.1). This matrix was extracted by SFE, using a range of temperatures, pressures and modifiers to ascertain the optimum extraction and recovery conditions. The accuracy of the model was then assessed by analysing feverfew.

FIGURE 9.1. The five test compounds spiked onto the model plant matrix.



9.1. Terpene test mixture

The model plant sample was designed to be as representative of feverfew as possible, with a set of test compounds selected to reflect those found in the herb. Limonene and caryophyllene are non-polar hydrocarbons, carvone is a polar monoterpene, eugenol a phenolic and santonin a sesquiterpene lactone which was chosen to mimic the reported active ingredient, parthenolide. The test compounds were spiked onto the matrices as a dichloromethane solution, at concentration levels (Table 9.1) comparable to those reported for similar compounds in the plant [49,234]. In this study a 2 ml extraction vessel was used which could contain about 0.5 g of spiked cellulose matrix. This corresponds to a sample size of about 1 mg of each test compound (with the exception of santonin at 2.5 mg), being exposed to sub- and supercritical CO_2.

TABLE 9.1. Concentration of test compounds in model matrix.

Compound	Amount of test compound spiked onto 1 g of matrix
Limonene	2.4 mg
Caryophyllene	2.4 mg
Carvone	2.4 mg
Eugenol	2.4 mg
Santonin	5.0 mg

In the subsequent extractions, the recovery of limonene was always very low regardless of conditions or matrices used. This low yield may be due to the loss of this very volatile monoterpene during matrix preparation when the solvent was being evaporated and during the collection process. This would suggest that the low limonene recoveries were probably an artifact of the experimental method. The recovery yields of the test compounds from the matrix were determined by GC analysis. The usual method protocol of internal and external standards were used to quantify the extracts (Chapter 7).

9.2 Selecting plant model matrix

A simple plant model was required, so that trends in SFE due to the polarity, volatility or molecular size of the solute could be ascertained. Spiked matrixes, such as silanised glass beads [178] and glass wool [227], have been used previously to investigate the solubility of analytes in

supercritical fluids (SCF). In this study, trial extractions with silanised solid glass beads (500 μm) were investigated as a possible plant model matrix but proved a poor substitute for the plant material, the analytes being rapidly washed off within 1 minute, even in mild extraction conditions (Table 9.2).

A Hypersil silica (12 μm) matrix was also investigated but this proved too retentive (Table 9.2). The unmodified silica surface is presumed to be covered with polar silanols [248] which appear to strongly retain the polar test compounds (i.e. the sesquiterpene lactone), so that a modified SCF was required to obtain an exhaustive extraction of the compounds from the matrix.

The most realistic plant model matrix proved to be α-cellulose (Sigma, non-assayed). This matrix has many similarities to the plant material, having originated from a plant, and required comparable extraction conditions to those required to remove the essential oil from feverfew (Table 9.2). Due to the porous nature of the cellulose, the analytes may be distributed throughout the matrix and adsorbed onto it. Therefore cellulose was chosen as the model matrix, because it appeared to reflect the adsorption and diffusion parameters present in the plant material.

TABLE 9.2. Extraction conditions required to isolate test compounds from the matrix, compared to the extraction of feverfew.

Matrix	SFE conditions for an exhaustive extraction.
Glass beads	130 bar, 45°C, 1 ml/min CO_2
Cellulose	250 bar, 45°C, 1 ml/min CO_2
Silica	250 bar, 45°C, 1 ml/min CO_2, 5% MeOH
Feverfew	200 bar, 45°C, 1 ml/min CO_2

9.3 Effect of temperature and pressure on extraction

The spiked cellulose matrix was exposed to various temperatures and pressures to determine optimum selectivity and extraction yields. Both sub- and supercritical CO_2 were investigated (Figure 9.2 {i-iv}). Each extract was carried to completion by UV monitoring and was irrespective of time, so that the extraction was only terminated after the UV absorbance reading had gone back to the original absorbance value prior to the extraction. Extraction with liquid CO_2 (-10°C) at various pressures demonstrated that the extraction yield for the majority of the test compounds was independent

of pressure (Figure 9.2 {i}). This is to be expected as the density of CO_2 varies very little with pressure at this temperature (Figure 9.3). In fact, the recovery yield appears to emulate the density profile, demonstrating the importance of density in the extraction process. Furthermore, at these conditions the volatility of the solutes may be small, so that its influence on solubility would be minimal. However, there was an anomaly in this pattern as the polar lactone, santonin, increased in solubility with pressure and the recovery yields for limonene were very low. It would appear that for the majority of test compounds there was no advantage in working at the higher pressures and thus most extractions with liquid CO_2 take place at 55 bar (cylinder pressure) [152].

On using liquid CO_2 at room temperature (Figure 9.2 {ii}), a similar extract to that of cold liquid CO_2 (Figure 9.2 {i}) was obtained. However, the recovery yields were lower, presumably because the density of the solvent is now lower (Figure 9.3). The influence of volatility on extraction yields appeared to have little effect as the corresponding increase in solvent temperature from −10°C to 20°C did not enhance solubility. An advantage of using liquid CO_2, is that the cost of the equipment is greatly reduced compared to SFE, due to the readily accessible temperatures and pressures, cylinder pressure being sufficient in many cases. For example, the British patented method for hop extraction is by the use of liquid CO_2 from −5 to +15°C [152].

Extracting the model matrix with a supercritical fluid gave a completely different extraction profile (Figure 9.2 {iii}) to that of liquid CO_2. The recoveries were higher than those obtained with the subcritical fluid at 120 and 250 bar (Figure 9.2 {ii}). This is interesting, because this supercritical fluid had a lower density (Figure 9.3) and dielectric constant than the subcritical solvents and would therefore be expected to possess a lower solubility power, especially for the polar lactone, santonin. The increase in recovery is probably due to the supercritical fluid possessing a higher diffusivity than the liquid phase, thus aiding the penetration of the solvent into the matrix and enhancing the mass transfer rate of the test compounds out of it. The increase may also be due to the formation of solvent clusters which are thought to be prevalent near the critical point in the supercritical phase [193]. Therefore in the supercritical region at 120 bar, the local solvent density about the solute may be higher than the bulk density shown in Figure 9.3 [183].

At low pressures (50 bar) the solvent is a dense gas with a corresponding

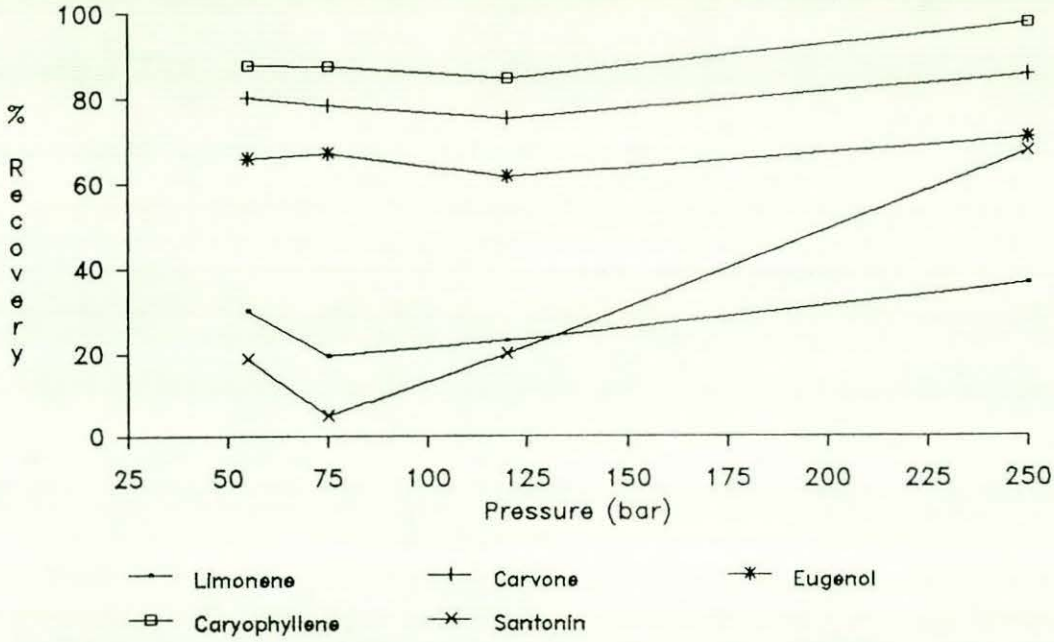
low density (Figure 9.3), with low recoveries being obtained for all the test compounds (Figure 9.2 {iii}). Above the critical pressure (75 bar) the recovery increases slightly in accordance with the slight increase in density (Figure 9.3). This observation would appear to contradict the notion that a supercritical fluid might be intrinsically different as a solvent from the liquid phase, as no significant change in the solubility occurred between the phases. These observations are similar to those found in the literature [177,178]. However, the recovery did increase dramatically as the pressure was increased from 75 to a 120 bar, as this corresponded to a large increase in density. To efficiently extract the polar lactone, santonin, a higher pressure of 250 bar was required. Therefore using mild extraction conditions of 120 bar at 40°C, it may be possible to obtain a partial fractionation of an essential oil, as the majority of the less polar compounds should be efficiently extracted, compared to only a partial extraction of the polar sesquiterpene lactone. It would appear that the components which are not very polar, have smaller molar masses and larger vapour pressures, are more soluble in the SCF than the polar ones. The recovery yield profile obtained from the model matrix (Figure 9.2{iii}) is comparable to the solubility isotherms obtained for similar components by Stahl *et al* [227], though in this case the oils had been coated onto silanised glass beads (Section 6.6, Figure 6.11).

On further increasing the temperature of the supercritical fluid a drastic change took place in the profile of the recovery yields (Figure 9.2 {iv}). Near the critical point (75 bar) an increase from 40 to 80°C caused an increase in the recovery yield even though there was a corresponding small drop in density. This was related to the vapour pressure of the analytes, which for the majority of test components (the exception being santonin) increase by about 10 times over this temperature range [227]. Therefore in this isobaric region the analyte volatility dominates the density effect. At higher pressures (120 bar) an increase from 40 to 80°C was accompanied by a very large drop in density (Figure 9.3) so that the density effect dominates and there was a corresponding drop in the recovery (Figure 9.2 {iv}). These two competing effects of solvent density and essential oil volatility have also been demonstrated by Stahl *et al.* [227] (Section 6.6, Figure 6.12).

At a higher temperature and pressure of 250 bar and 80°C, the density had increased sufficiently so that the majority of the test compounds, with the exception of santonin, could be efficiently extracted (Figure 9.2 {iv}).

FIGURE 9.2. Recovery yields of essential oils from a spiked cellulose matrix.

{i} SFE conditions, -10°C, 0.8 ml/min CO_2.



{ii} SFE conditions, 20°C, 0.8 ml/min CO_2.

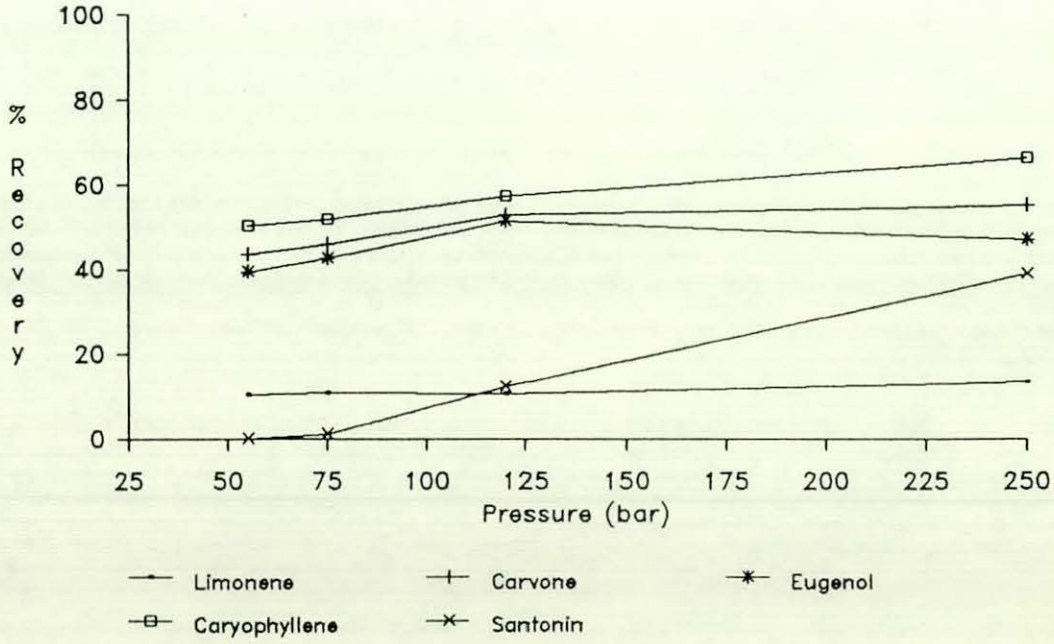
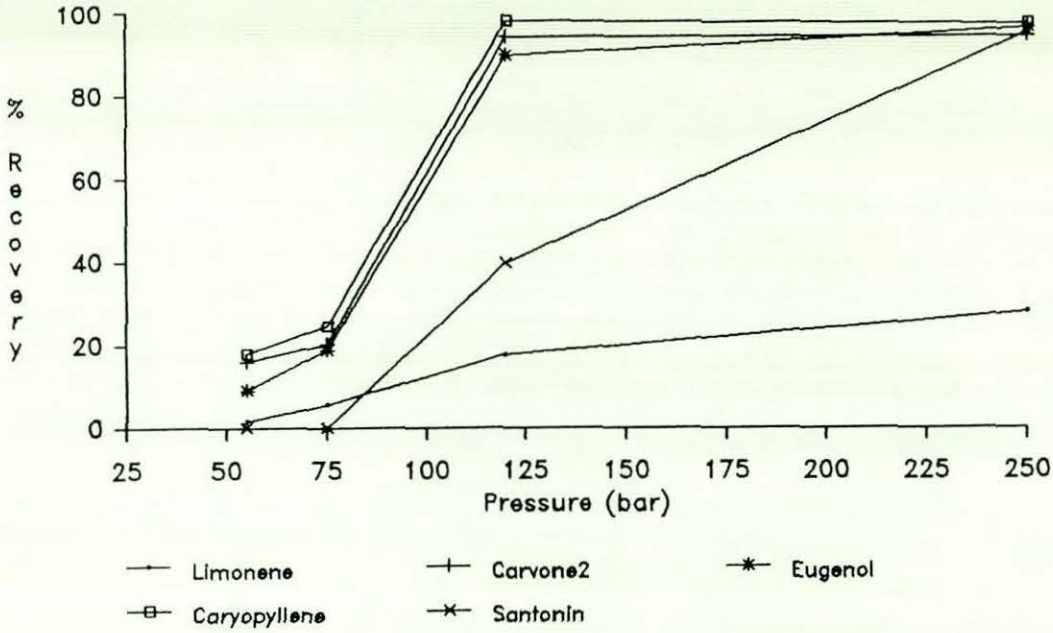


FIGURE 9.2 {iii}. SFE conditions, 40°C, 0.8 ml/min CO_2.



{iv} SFE conditions, 80°C, 0.8 ml/min CO_2.

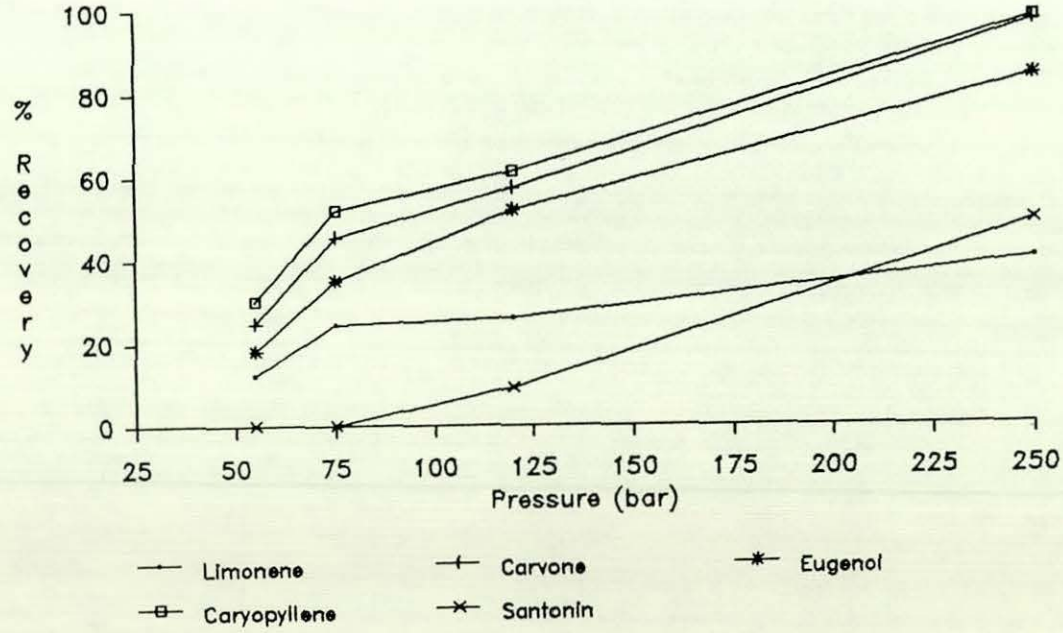
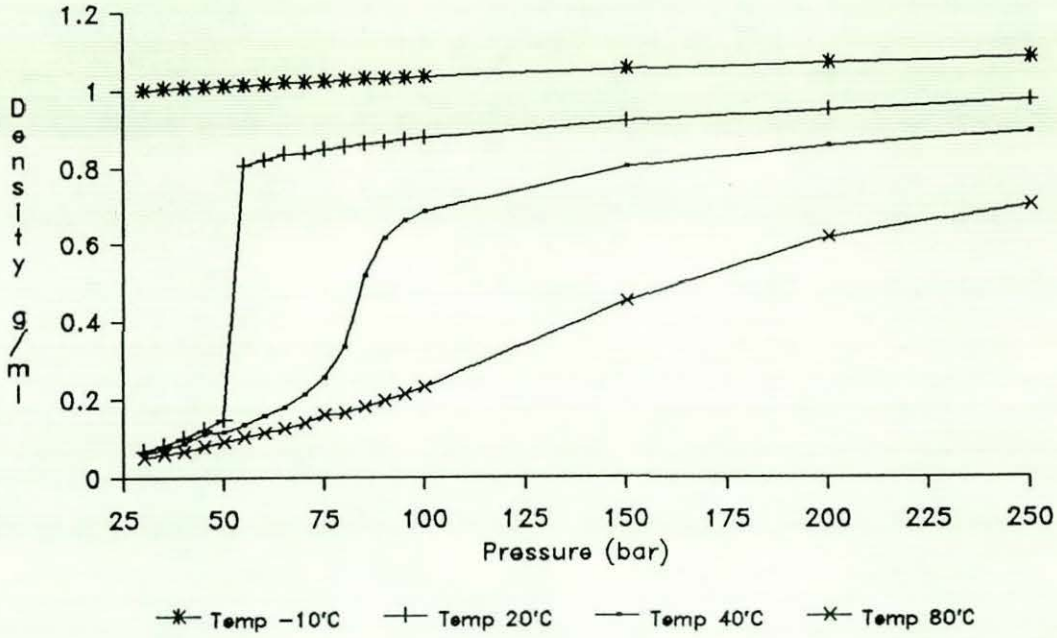


FIGURE 9.3. Density profile of CO_2.



This is important because if the matrix was extracted at 250 bar and 40°C (Figure 9.2 {iii}) then a total extraction of test compounds should be achieved. If the CO_2 extraction solution was then exposed isobarically to a temperature increase of 80°C, the santonin would probably start to precipitate out, so partially achieving selectivity. However, it was not possible to test this process experimentally.

Again, just like the subcritical solvent, the supercritical fluid recovery profile appeared to emulate the corresponding density profile, demonstrating the importance of density on the extraction efficiency.

9.4. Model for supercritical fluid extraction

Using the spiked cellulose matrix, the test components were all present in minor amounts in the extraction vessel (< 2.5 mg, Section 9.1), so that the concentration of the analyte in the SCF is probably well below the solubility limit (≈ 200 mg/NL CO_2 at 120 bar, 40°C [227]). If the test compounds are present on the cellulose matrix as an inert surface layer, so that solubility was the only limiting step in the extraction process, then according to the solubility data from Stahl *et al.* (Section 6.6, Figure 6.11) the compounds should have all been extracted in less than one minute at 120 bar and 40°C. This is assuming that the low flow rate of 0.8 ml/min

CO_2 used in this study is similar to the unspecified "low" flow rate used by Stahl *et al.*, which would enable an equilibrium to be reached between the test compounds and the SCF. (i.e. at 120 bar and 40°C the solubility of the terpenes is about 200 mg/NL CO_2 = 200 mg/1000 cm^3 CO_2, at 1 bar and 20°C = 200 mg/1.78 g of CO_2. As the pump delivers CO_2 at -10°C and 55 bar {density ≈ 1 g/ml}, within two minutes sufficient CO_2 should have been added to the matrix to extract all the components present).

However, the results from the cellulose matrix suggest an extraction time of at least 20 minutes is required (Figure 9.4). This would suggest that the solubility parameter addresses only part of the extraction process. The extraction of the test compounds may also depend on their distribution between the SCF and sorptive sites on the sample matrix. The rate of extraction may therefore be determined principally by the rate of mass transfer out of the matrix and not on the solubility of the analyte in the solvent. Furthermore, cellulose is known to contain water [250], so that besides the possible adsorption of polar solutes to the cellulose, partition of solutes between the water bound to the cellulose and the supercritical solvent may also occur.

The extractions using CO_2 have so far been discussed in terms of the final recovery yield. However, for a further insight into the extraction mechanism, a series of timed extractions for sub- and supercritical CO_2 were investigated (Figures 9.4-6). Using supercritical CO_2 at 250 bar and 40°C, timed extracts were taken to obtain an extraction profile (Figure 9.4 {i}). It would appear that the non- to medium-polar essential oils are quickly removed from the matrix but the polar analyte santonin, had a much slower extraction rate. This could be due to a number of factors such as, santonin being less soluble in the supercritical solvent, or being more strongly adsorbed to the matrix; or being solvated in the water bound to the cellulose.

The extraction rates from the present study were incorporated into the Bartle *et al.* "hot ball model" which assumes that the rate determining step in extraction is diffusion [211] (Section 6.2). A characteristic of such extractions is that the majority of the analyte is removed during a short period of time at the beginning of the extraction, but subsequently the extraction rate tails off dramatically. A physical explanation of the shape of the extraction curve is that initially the analyte present on the surface of the matrix will diffuse rapidly into the SCF. As the extraction continues this step becomes eroded, but nevertheless the concentration gradient near

the surface of the matrix is large, and diffusion, which is proportional to the concentration of the gradient continues to be at a high rate. Eventually, a smoother concentration profile is established over the whole matrix and the diffusion loss becomes a simple exponential decay. To represent this data the model expresses the amount of analyte extracted with time as $\ln(m/m_o)$ where m is the mass of analyte in the matrix at time t and m_o is the mass of analyte in the matrix at zero time (t_o). A plot of $\ln(m/m_o)$ versus time gave the characteristic profile seen in Figure 9.4 {ii}.

The curve of $\ln(m/m_o)$ obtained from the non-polar analyte caryophyllene appeared to be the closest fit to the predicted hot ball model pattern, with an initial steep fall becoming linear with time (Figure 9.4 {ii}). Shown on the time axis is the position for $t_r = 1$, i.e. the time in which the linear portion of the curve falls by an amount $\ln(m/m_o) = 1$. The steep fall occurs at a time closer to $t_r = 1$ rather than 0.5 as predicted by the ideal hot ball model. This suggests the curve is shallower than expected, probably because the initial diffusion of the analyte into the SCF is not rapid. This may be due to the caryophyllene being partially retained by the cellulose matrix, such retention mechanisms not being accounted for by the hot ball model. However, extrapolation of the linear portion of the curve to the t = 0 axis gives an intercept of approximately -0.5, which is in agreement with the ideal model [211]. Although the initial steep fall appears as a relatively small feature, it represents the loss of the majority of the material from the matrix, with 72% of caryophyllene being extracted during the initial period corresponding to $t_r = 1$. However, the time required to extract 99% of the caryophyllene corresponds to $t_r \approx 4.0$.

With the polar analytes a limited recovery was found, the more polar the analyte the shallower the curve (Figure 9.4 {ii}). This may be due to analytes being retained on the cellulose, the more polar the analyte, the greater the retention and thus the slower the extraction rate. Alternatively, this may be an artifact of the experimental procedure. It has been suggested that regions close to the surfaces of the matrix may become depleted of analytes due to evaporation, so that the initial fall would be smaller [211]. This is undoubtedly the case for limonene where the initial fall is very shallow. The effect of solubility limitation could also reduce the initial rate of fall of the curve, though in this instance this is not considered likely due to the low levels of analytes involved.

Thus, two mass transfer steps may be present, an initial non-linear

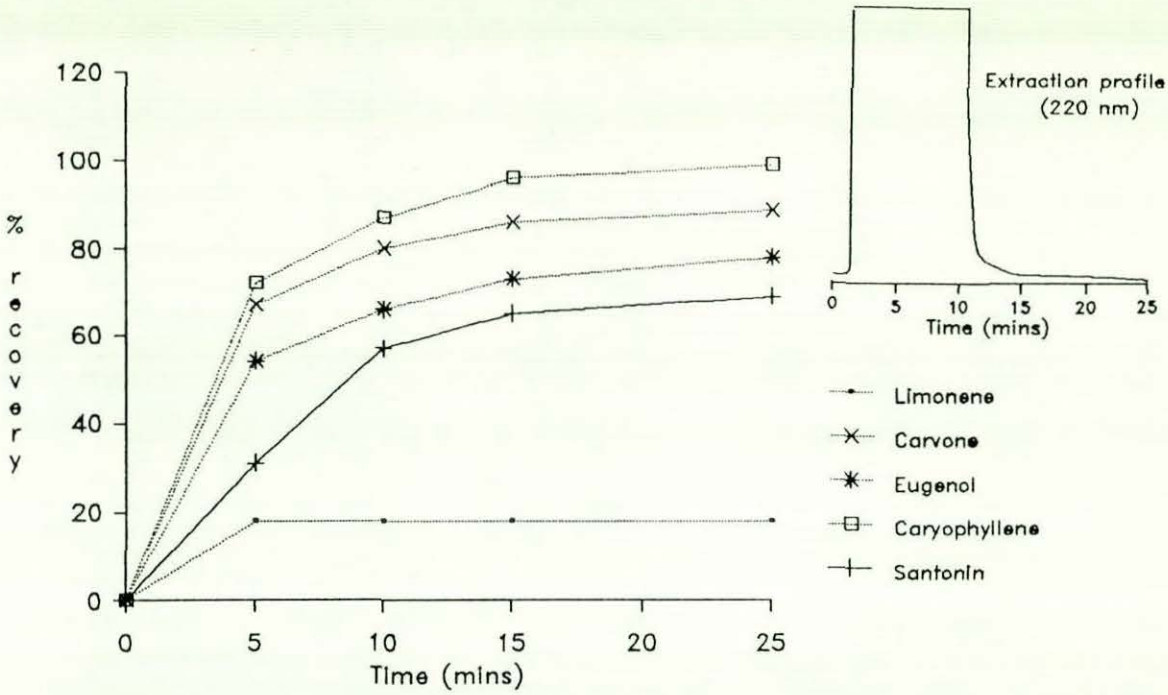
surface extraction then a slower diffusion step, the analytes being possibly bound by polar interactions to the cellulose. It has been suggested that the SCF may compete with "active sites" on the matrix, the greater the density of the SCF, the greater the intermolecular interactions and corresponding solubility [251].

Timed extractions using subcritical CO_2 were also investigated (Figure 9.5 {i}) and fitted to the "hot ball model" (Figure 9.5 {ii}). At these conditions the CO_2 is a dense liquid with a higher density than in Figure 9.4 (i.e. 1.1 g/ml instead of 0.89 g/ml). Initially the rate of extraction is comparable to the SCF (Figure 9.4), however, the recovery yield is lower as the CO_2 has a lower diffusivity and presumably can not penetrate into the cellulose matrix so easily. From the diagram (Figure 9.5 {ii}) it would appear as if only the surface oil was extracted, all the profiles being curves, reaching a final constant value very quickly, with no linear extraction region to suggest that a steady state diffusion out of the matrix may be occurring. The exception was santonin which appears to have a different rate of extraction, being initially slow, then increasing with time but eventually tailing off. The reason for the slow extraction is unclear and may be related to the lower diffusivity of the solvent or possibly due to a limited solubility of santonin in the cold liquid CO_2.

Dense CO_2 gas was also investigated as a solvent (Figure 9.6 {i}) and the results incorporated into the "hot ball model" (Figure 9.6 {ii}). At these conditions the CO_2 had very weak solvating properties and a very low density (0.13g/ml) Thus, the most polar analyte santonin, was not extracted at all and the second most polar analyte, eugenol, was only extracted slowly with time. This gives a characteristic hump on the extraction profile at about 20 minutes, when monitoring with a UV detector (Figure 9.6 {i}). An unusual plot is obtained when using the "hot ball" model (Figure 9.6 {ii}). Limonene, caryophyllene and carvone decrease as normal with time, but eugenol increases up to a final value. Presumably in this instance the solubility in the solvent may be the rate determining step. It has already been shown that eugenol has a limited solubility in CO_2 at 60 bar and 40°C of about 0.2 mg/NL CO_2 [227]. Thus even with the highly soluble caryophyllene, the weak solvent only extracted 38% of the analyte after 5 minutes compared to 72 and 75% being extracted in 5 minutes with sub- and supercritical conditions respectfully (Figure 9.4 {i} and 9.5 {i}). It would appear as if only the surface oils were extracted as most of the plots were curves, reaching a final steady state after 20-25 minutes (Figure 9.6 {ii}).

FIGURE 9.4. Extraction profile and "hot ball model" data for the supercritical CO_2 extraction of test compounds spiked onto a cellulose matrix. Extraction conditions, 250 bar, 40°C, 0.8ml/min CO_2.

{i}



{ii} Note, m = mass of analyte at time t in matrix. m_o = mass of analyte at zero time (t_o) in matrix

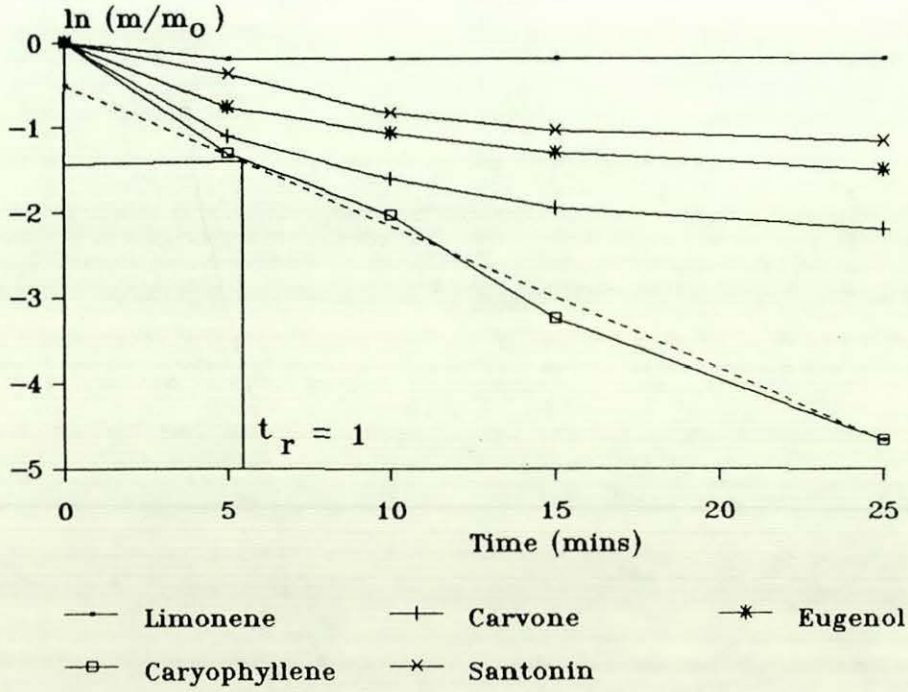
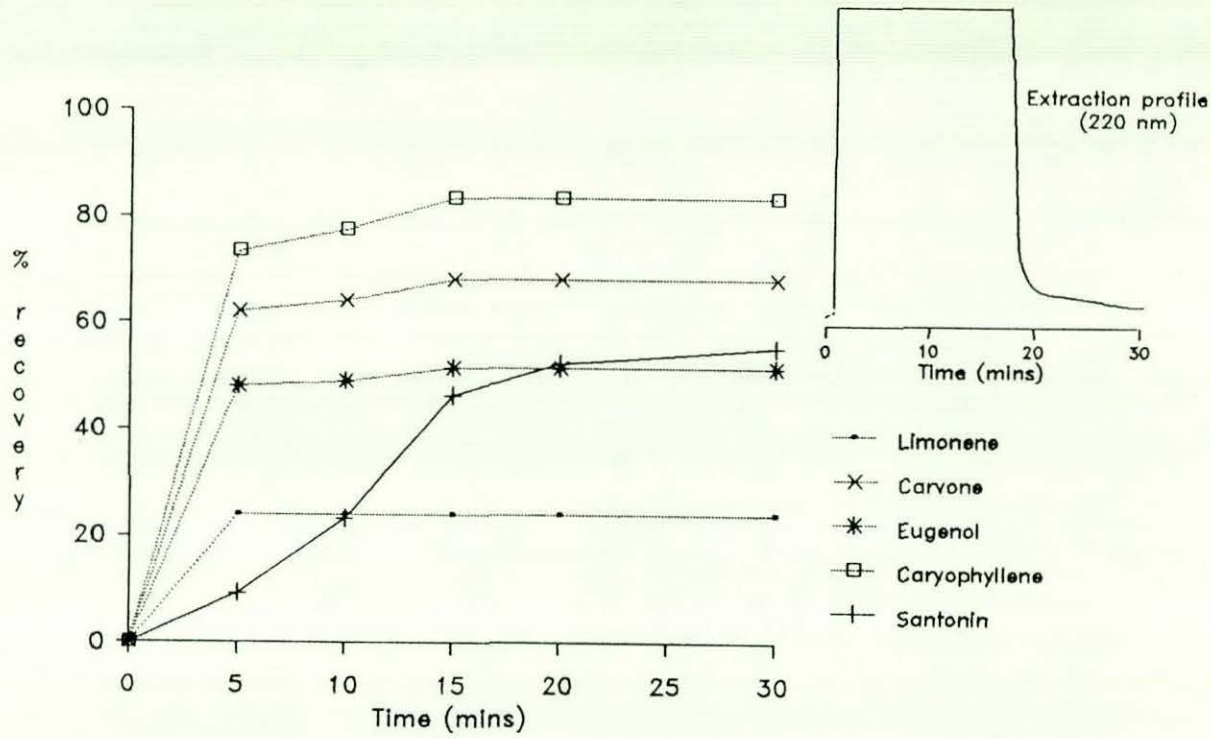


FIGURE 9.5. Extraction profile and "hot ball model" data for liquid CO_2 extraction of test compounds spiked onto a cellulose matrix. Extraction conditions, 250 bar, -10°C, 0.8 ml/min CO_2.

{i}



{ii} Note, m = mass of analyte at time t in matrix. m_o = mass of analyte at zero time (t_o) in matrix

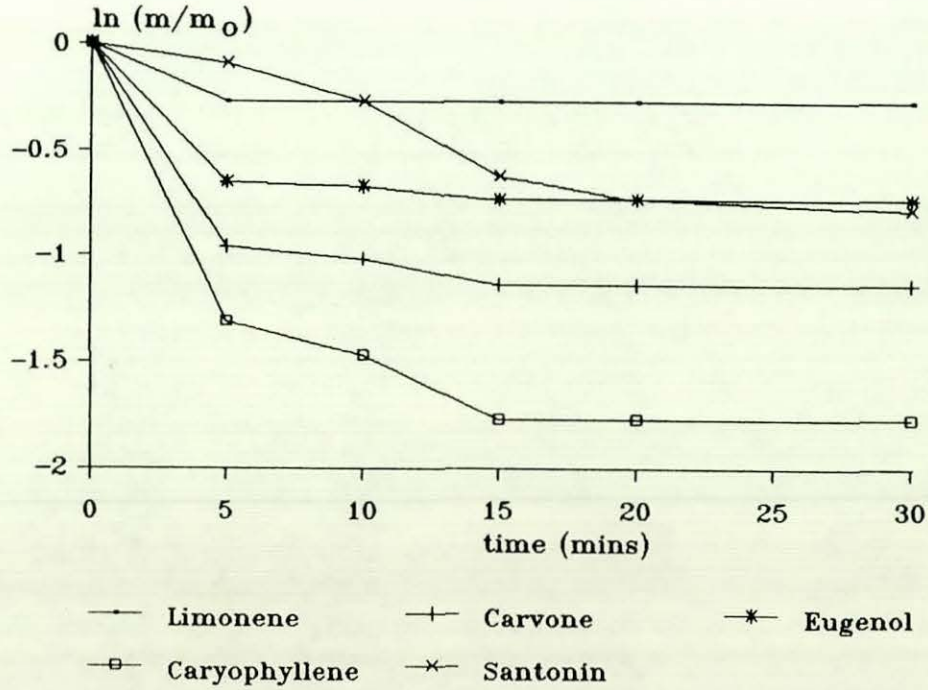
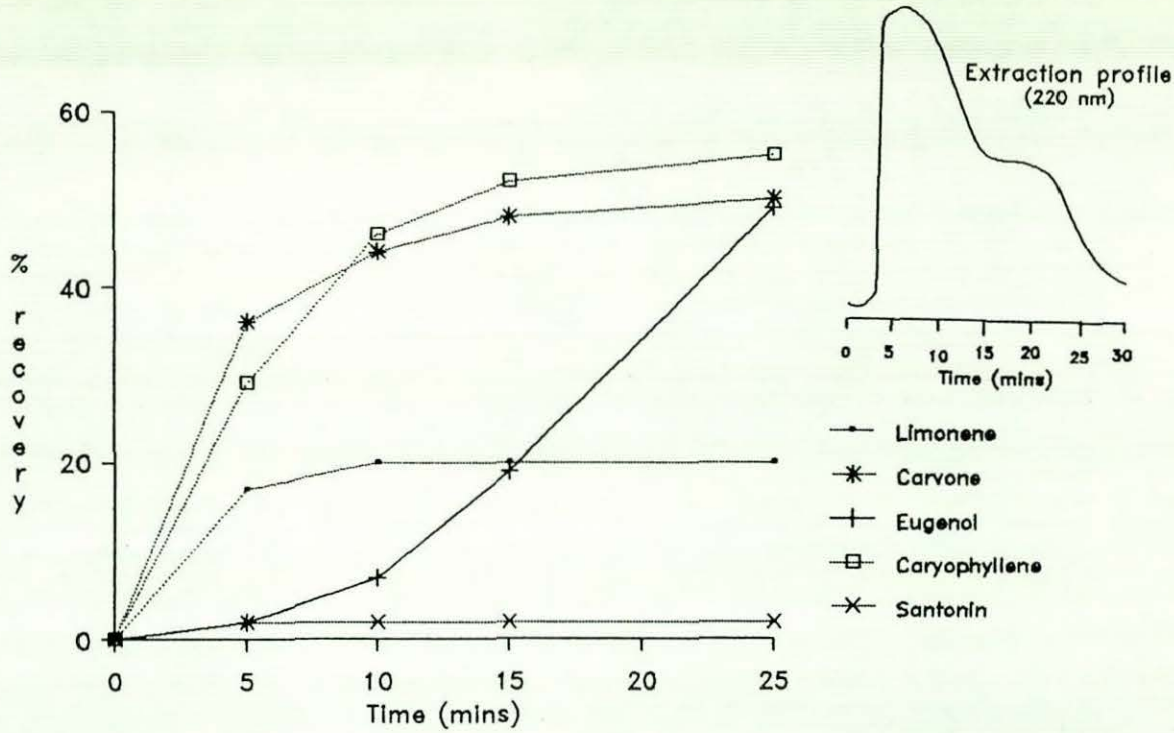
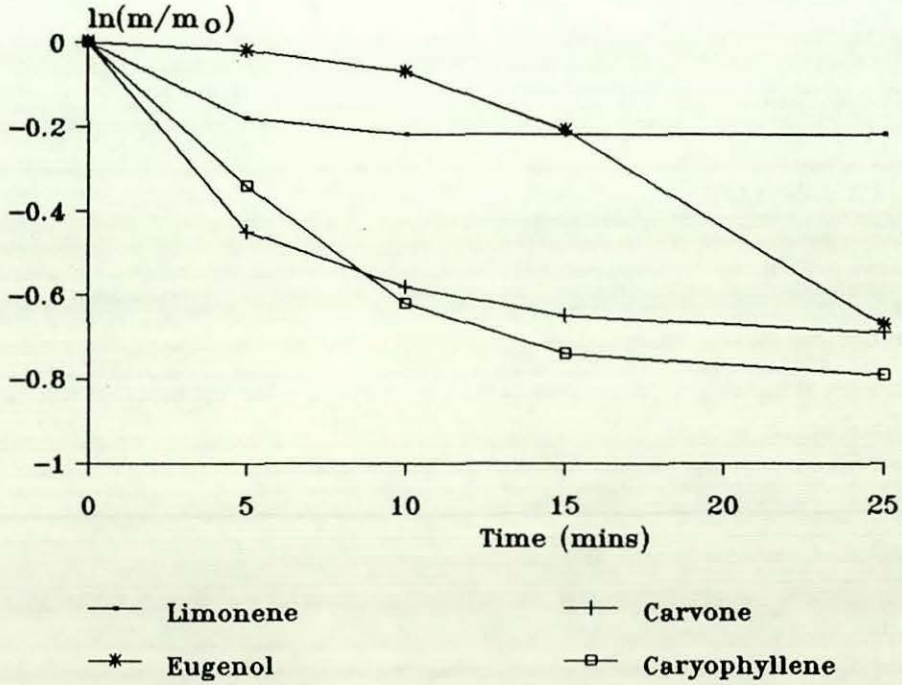


FIGURE 9.6. Extraction profile and "hot ball model" data for extraction dense CO_2 gas extraction of test compounds from a spiked cellulose matrix. Extraction conditions, 55 bar, 40°C, and 0.8 ml/min CO_2.

{i}



{ii} Note, m = mass of analyte at time t in matrix. m_o = mass of analyte at zero time (t_o) in matrix



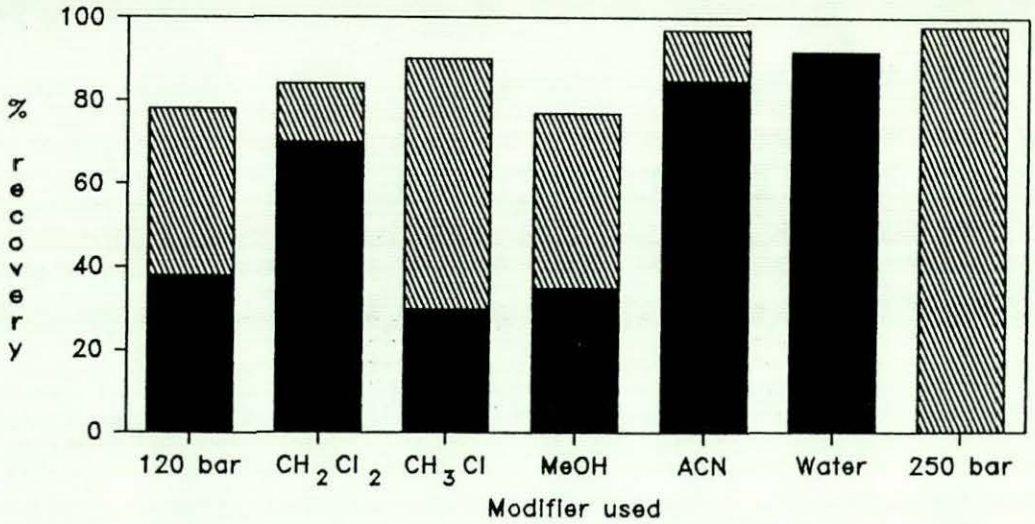
9.5 Effect of modifiers on extraction

The sesquiterpene lactone, santonin, was used to mimic the reported active ingredient, parthenolide, in feverfew. Santonin required high pressures to achieve a good recovery yield (250 bar and 40°C), so a variety of co-solvents were added to CO_2 at various percentage levels in an attempt to improve the recovery at lower pressures (120 bar and 40°C). In each case a second extraction with CO_2 alone at 250 bar, was used to determine the residue santonin in the matrix. It would appear that the polar solvents, water and acetonitrile, but surprisingly not methanol, produced an enhanced solubility, presumably because they interact more strongly with the analyte than CO_2 (Figure 9.7). Modifiers may not only induce changes in the nature of the solvent but also influence the matrix. It has been well documented in supercritical chromatographic studies [185,252] that polar modifiers may deactivate adsorption sites on polar matrices (silica based packed columns). The modifier may successfully displace or desorb the polar analytes from the cellulose matrix, so enabling the analytes to quickly diffuse into the SCF solvent. Less polar modifiers appeared to have little effect. The exception was dichloromethane, this was surprising as it is the most non-polar co-solvent investigated. There is evidence in the literature that non-polar co-solvents can be as effective as polar ones, n-octane and methanol producing similar enhanced solubilities for benzoic acid in supercritical CO_2 [199]. In that instance it was suggested that the enhanced solubility was due to the large dispersion value (δ^D = 7.6 $\{cal/cm^3\}^{1/2}$) of the non-polar modifier n-octane.

FIGURE 9.7. Recovery of santonin using various co-solvents.

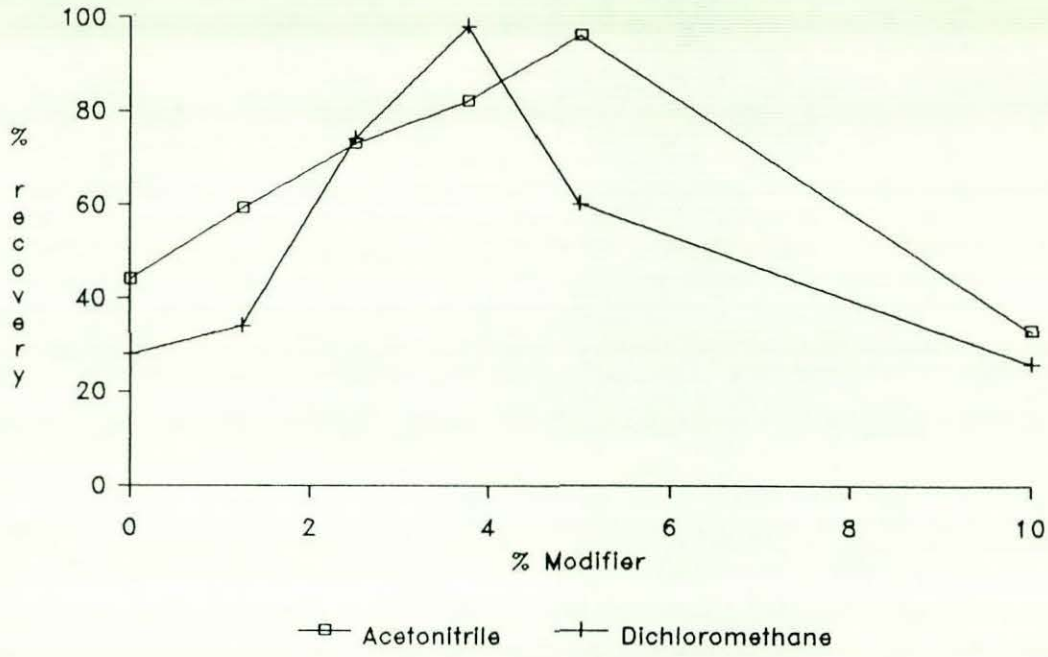
■ = % santonin removed from matrix at 120 bar, 40°C, 0.8 ml/min CO_2 and 4% organic modifier or water saturated CO_2.

▨ = % santonin that could be subsequently obtained by re-extracting matrix with 250 bar, 45°C CO_2) in the absence of a modifier.



The influence of co-solvent concentration was investigated for both acetonitrile and dichloromethane, with both co-solvents producing very similar profiles (Figure 9.8). It was found that there was an optimal concentration in the region of about 4.5% acetonitrile, with the extraction taking about a third of the time that is required when using unmodified CO_2. This is in agreement with Deye *et al.*, who found that about 5% of a polar modifier was needed before an appreciable increase in solvent strength was discernible [186]. This would initially suggest that the modifier had increased the extraction yield by enhancing the solubility of the polar lactone in the SCF. However, as has been shown in the initial work with inert glass beads (Section 9.1), solubility is presumed not to be the rate determining step for santonin at this low level. Instead, a more likely mechanism may involve santonin being adsorbed onto the cellulose. The polar acetonitrile modifier may compete with the analyte for the adsorptive sites, so "releasing" the analyte into the SCF. However, a high percentage of modifier was required (≈ 5%) suggesting a large number of adsorptive sites may be present. An interesting anomaly is that at very high concentrations of modifier (10%) the recovery drops to a yield which is comparable to just using CO_2 as a solvent (Figure 9.8). The reasons for this are unclear.

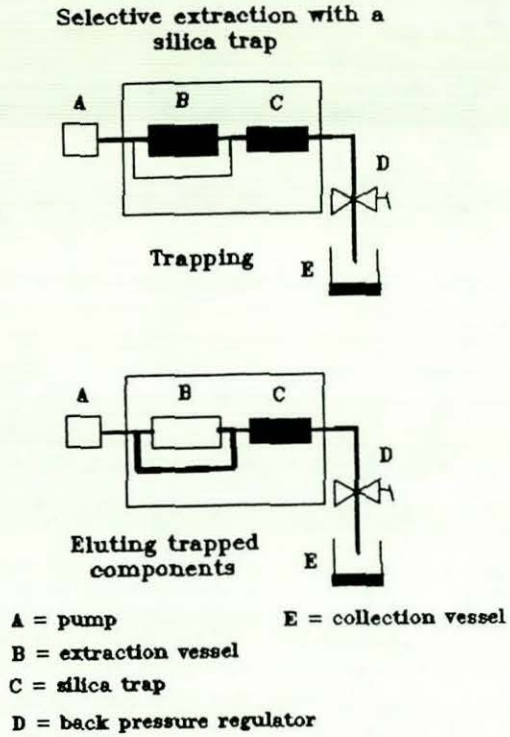
FIGURE 9.8. Effect of concentration of acetonitrile and dichloromethane modifier on % recovery of santonin. SFE conditions, 120 bar, 40°C, 0.8 ml/min CO_2.



9.6 Use of a silica "trap" to obtain selectivity

As santonin was used to represent the reported active ingredient in feverfew, different methods were investigated to determine if it could be fractionated from the other test compounds. None of the direct extraction conditions would selectively extract santonin to the exclusion of the other compounds. A SCF extraction combined with trapping was investigated, to make use of the polarity differences between santonin and the other test compounds. A short (4 x 120 mm) Hypersil 12μm silica column was placed after the extraction vessel to act as a trap (Figure 9.9). By using high pressure CO_2 (250 bar at 40°C), the majority of the test compounds were extracted from the matrix onto the on-line silica column, where, under these extraction conditions only some of the extracted components were eluted. The extraction was monitored by a UV detector at 220 nm to ascertain the elution profile from the silica column (Figure 9.10). When no more components appear to be eluting from the column, the extraction vessel was switched out off line and methanol (12%) added isobarically, to elute the analytes retained on the column.

FIGURE 9.9. Selective extraction by means of a silica trap.



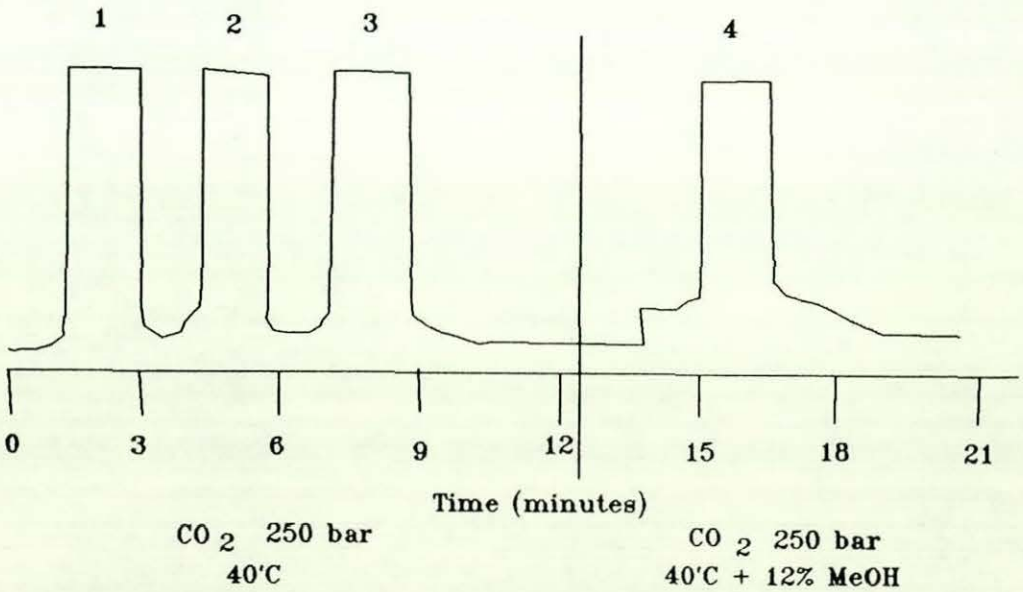
The majority of the test compounds were extracted from the cellulose matrix and eluted from the silica column at 250 bar and 40°C. The components were reasonably well resolved on the small column so that each peak could be individually collected (Figure 9.10). At this high extraction pressure the non-polar hydrocarbons, limonene and caryophyllene appeared to be virtually unretained on the silica column. This was to be expected as a later SFC study shows (Chapter 10.) that the non-polar essential oils were not highly retained on a silica column. Therefore, to obtain a fractionation of all the essential oils by SFE/SFC a density programme would have been required to enable the analytes to be fully resolved on packed column SFC. However, it was not possible to test this process experimentally.

A low recovery (44%) was obtained for santonin, suggesting that some of the lactone was still retained on the column. It was presumed that sufficient modifier (12%) was present to cover the highly adsorptive silanols sites reported on bare silica [253]. However, SFC results (Chapter 10) have shown that even when a high percentage of modifier (8%) was used with CO_2 (200 bar at 40°C), a fast flow rate of 2.5 ml/min CO_2 was required for santonin to be eluted from the column within 5 minutes. As the optimum flow rate for the collection of analytes in this SFE system is only 0.8

ml/min (Chapter 8), it is possible that the elution of santonin from the silica column was not given sufficient time to enable the majority of the analyte to be eluted. The use of higher modifier concentrations was deemed unsuitable as the eluted components would have been diluted in a large volume of co-solvent during the collection process. A more practical alternative may be to use a less retentive column for santonin such as an ODS column (Chapter 10) or to investigate a more polar modifier such as water.

One of the other disadvantages of using a trap is the potential of carry-over. If a complex matrix was to be investigated which contained a variety of components, it is feasible that some of the components may elute very slowly from the silica column, to appear in subsequent extracts. To avoid this, disposable cartridges could be used for each extraction, though this may become prohibitively expensive. However, the technique does have the potential to fractionate a range of components, with the obvious conclusion of prep SFE/SFC.

FIGURE 9.10. Fractionation of essential oils by means of an in-line silica trap. Monitored by UV detector at 220 nm (AUFS 2.56).



1 = Limonene (26% recovery) + caryophyllene (94% recovery)

2 = Carvone (75% recovery) + caryophyllene (2% recovery)

3 = Eugenol (57% recovery) + carvone (5% recovery)

4 = Santonin (44% recovery)

9.7 SFE of feverfew plant material

The optimised extraction conditions found for the cellulose model, of high pressure supercritical CO_2 (250 bar, 45°C), were applied to feverfew. The same experimental procedure used on the cellulose model, was also used on the plant material, the extracts being collected in liquid nitrogen and analysed by capillary GC. The major peaks in the feverfew extract were subsequently also identified by GC-MS (Chapter 11).

When these conditions were applied to feverfew the sesquiterpene lactone, parthenolide, was readily extracted. But, milder extraction conditions (120 bar, 45°C) also produced similar yields from the plant material. When modifier was used in conjunction with the high pressure CO_2 (250 bar, 45°C), it was discovered the amount of parthenolide extracted could be doubled (Table 9.3). This enhanced solubility was selective, with only the sesquiterpene lactone being detectably influenced by the presence of a modifier. In fact, water appeared to reduce the extraction yield of the less polar essential oils, possibly by making the SFC too polar so reducing the solubility of the oils in the solvent.

TABLE 9.3. % parthenolide extracted from feverfew under various modified SFE conditions. In all experiments CO_2 pumped at 0.8 ml/min and monitored by UV (220nm). When modifier present CO_2 is at 250 bar, 40°C. For peak identification see Section 6.3 and Figure 9.13 {ii}..

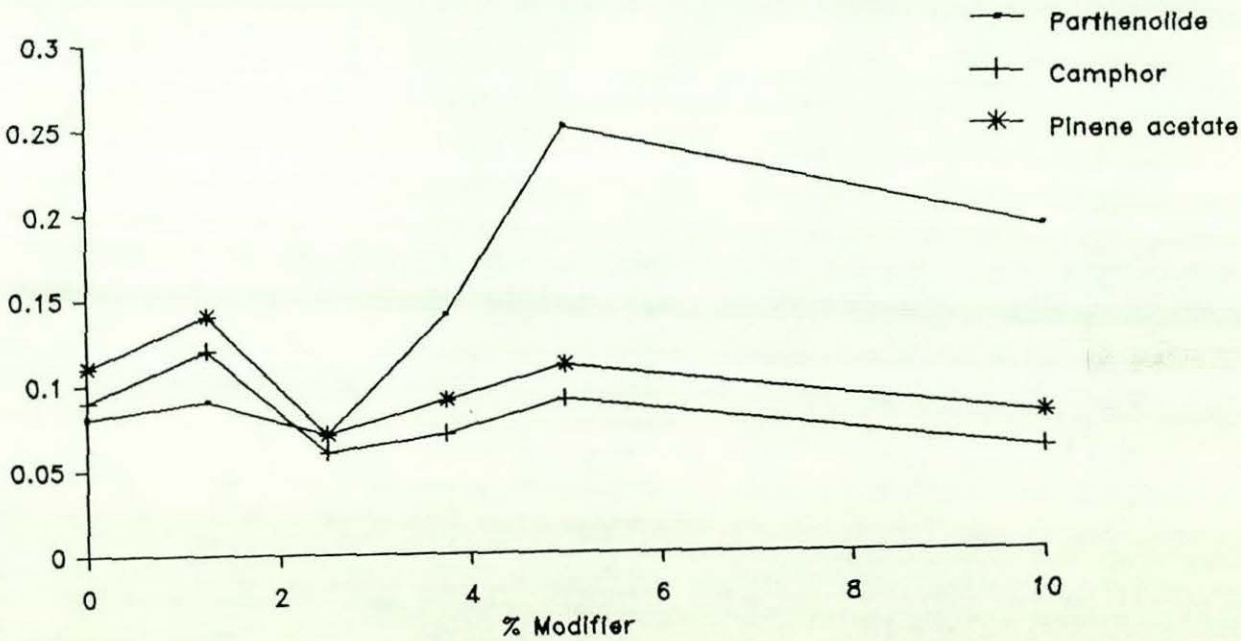
SFE conditions	% essential oil in dried plant material[b]		
	parthenolide	camphor	pinene acetate
120 bar, 40°C	0.08	0.10	0.11
250 bar, 40°C	0.07	0.08	0.10
250 bar, 40°C + MeOH (4%)	0.16	0.05	0.08
250 bar, 40°C + ACN (4%)	0.14	0.07	0.09
250 bar, 40°C + H_2O[a]	0.14	0.04	0.06

a = CO_2 saturated with water (Section 8.5.2)
b = Feverfew, homegrown from Fisons seeds, fresh plant material dried and stored for 1 month

Due to the low levels of parthenolide reported in feverfew (0.1-0.5% by dry weight of plant material) and the high solubility limit of the oxygenated sesquiterpenes in supercritical CO_2 (≈ 200 mg/NL CO_2 at 120 bar at 40°C [227]), it was presumed that solubility was not a limiting factor in the extraction process. It would therefore appear as if the parthenolide could be present in different sites on the cellulose matrix. About a third

to a half of the sesquiterpene lactone was easily obtained with mild extraction conditions, however, the rest of the lactone required a modifier to displace it, as if it had been retained by sorptive sites on the plant. As only the lactone was influenced by the presence of a modifier (Figure 9.11), it was presumed that the possible sorptive sites were polar in nature. This idea of an analyte being present as a "free" and "bound" form has been put forward by King *et al.* who investigated the extraction of rape seed with liquid CO_2 [212].

FIGURE 9.11. Effect of concentration of acetonitrile modifier on amount of parthenolide extracted from feverfew (Homegrown from Fisons seeds, fresh plant material dried and stored for 1 month). SFE conditions, 250 bar, 40°C, 0.8 ml/min CO_2.



The results suggest that the mechanism which is selectively retaining the sesquiterpene lactones on the plant material, is to be appreciably more retentive than the adsorption sites presumed present on cellulose, as feverfew required both a high pressure and a modifier to obtain the optimum yield of parthenolide (i.e. the spiked cellulose matrix required just high pressured CO_2 at 250 bar and 40°C to obtain the maximum yield of sesquiterpene lactone). However, the level of modifier required to induce enhanced solubility from feverfew was comparable to the cellulose plant model system (Figure 9.8), with an optimum concentration being in the order of 5% modifier (Figure 9.11). Again very high levels of modifier result in a reduced solubility. The percentage modifier appeared to have little

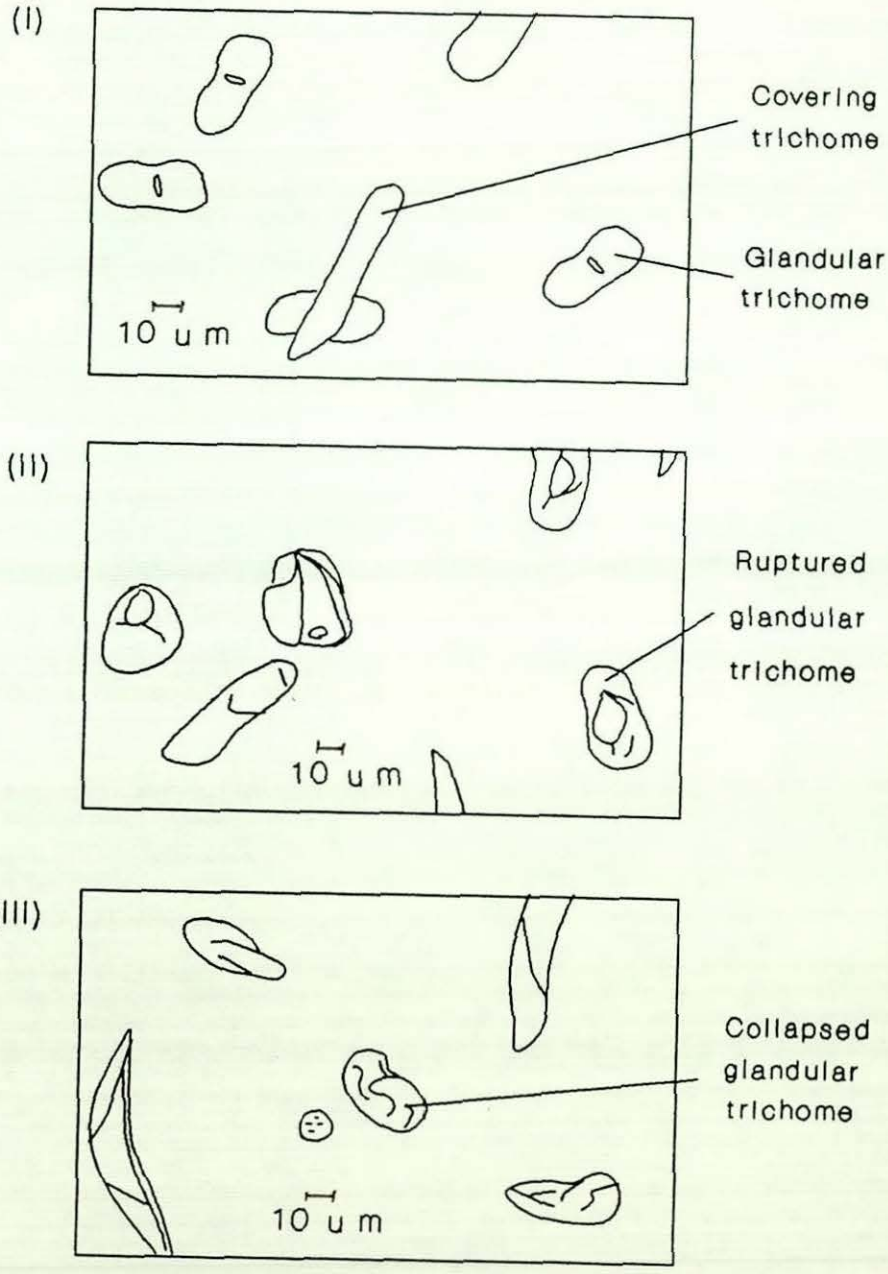
influence on the extraction yields of the easily extracted essential oils such as camphor and pinene acetate (Table 9.3). Thus, although prep-SFE/SFC (Chapter 11) produces a highly pure parthenolide extract, the yield might only be 50% of the total.

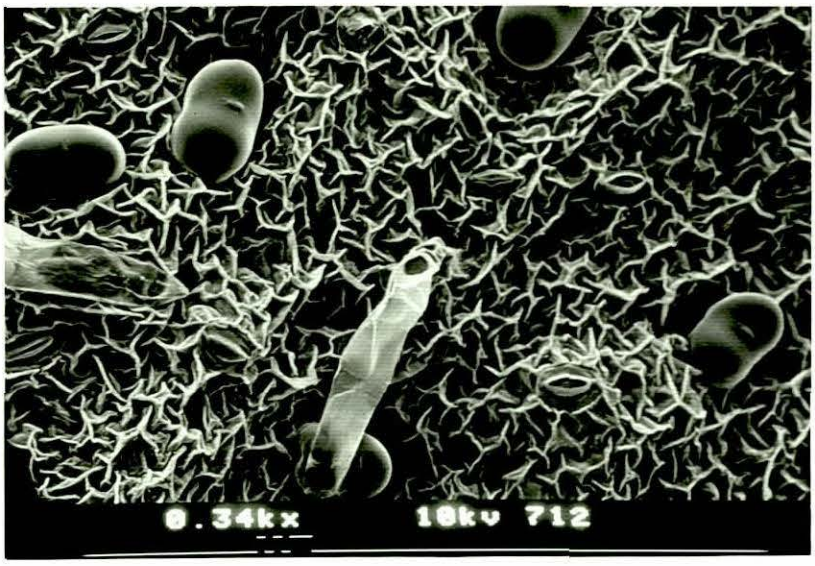
Alternatively, parthenolide may be microencapsulated physically into the plant by means of glandular trichomes reported to be present on the leaves of feverfew [34]. The effects of extraction on these trichomes has been investigated by using a scanning electron microscope (SEM) to examine air dried feverfew leaves exposed to various SFE conditions (Figure 9.12). A photograph was taken before any extractions, showing the intact glandular trichomes, with a two lobed appearance and a characteristic protuberance or ridge between the two lobes (Figure 9.12 {i}). When the glandular trichomes were exposed to supercritical CO_2 (250 bar, 40°C), the trichomes appeared to ruptured at the ridge which was presumably the weakest point on the structure (Figure 9.12 {ii}). This effect appeared universal for all the glandular trichomes, though the degree of rupturing varied, some possessed small openings while others were completely split open. The glands with the small openings were examined more closely and found to be empty inside. If the sesquiterpene lactones were present solely in these glandular trichomes, then when they ruptured the supercritical fluid should have been able to extract all the oils in them. Sugiyama *et al.* demonstrated that supercritical CO_2 could rupture similar oil containing structures present on lemon peel [156].

The feverfew samples were also exposed to modified supercritical CO_2 (250 bar, 40°C, 10% MeOH) but the glandular trichomes underwent a different physical change, "collapsing" on exposure to the solvent (Figure 9.12 {iii}). Blakeman *et al.* discovered a similar phenomenon with the feverfew glandular trichomes when using conventional liquid extraction with chloroform [34]. As the modified CO_2 was able to obtain a much higher recovery yield for the parthenolide than the unmodified CO_2, this suggests that the parthenolide may not be present solely in the glandular trichomes.

All the modifiers had the disadvantage of increasing the amount of extraneous material so that the extracts possessed a greasy consistency with a deep orange or green colour (Table 9.4). The extracts were examined by UV spectrometry and the orange colour was attributed to carotenoids (major peak at 446 nm and two minor peaks either side [107]) and the green to chlorophyll (characteristic peaks at 214, 322, 436 and 665 nm [107]). Although these materials do not effect the GC results as they are non-

FIGURE 9.12. SEM of trichomes on dehydrated surface of feverfew leaves.
(i) Intact trichomes before extraction.
(ii) Exposed to 250 bar, 40°C, 1.0 ml/min CO_2 for 20 minutes.
(iii) Exposed to 250 bar, 40°C, 1.0 ml/min CO_2 and 10% MeOH for 20 minutes.

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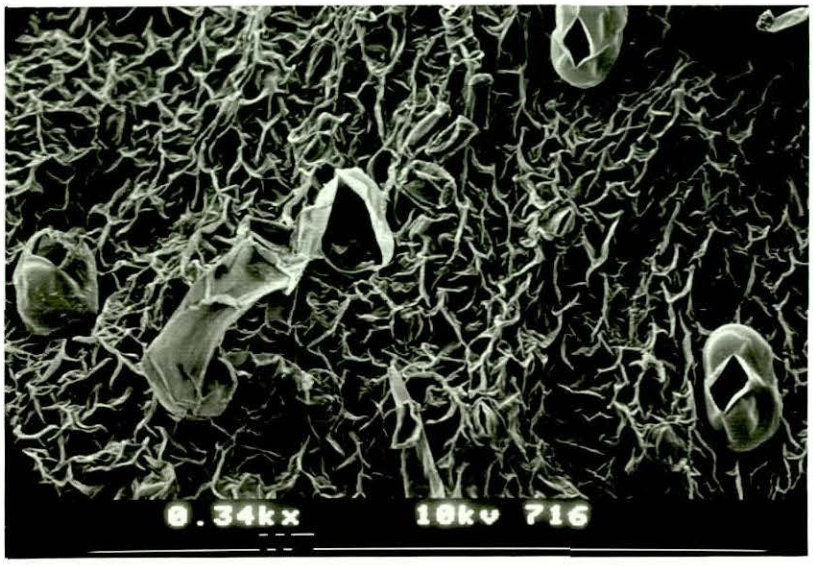
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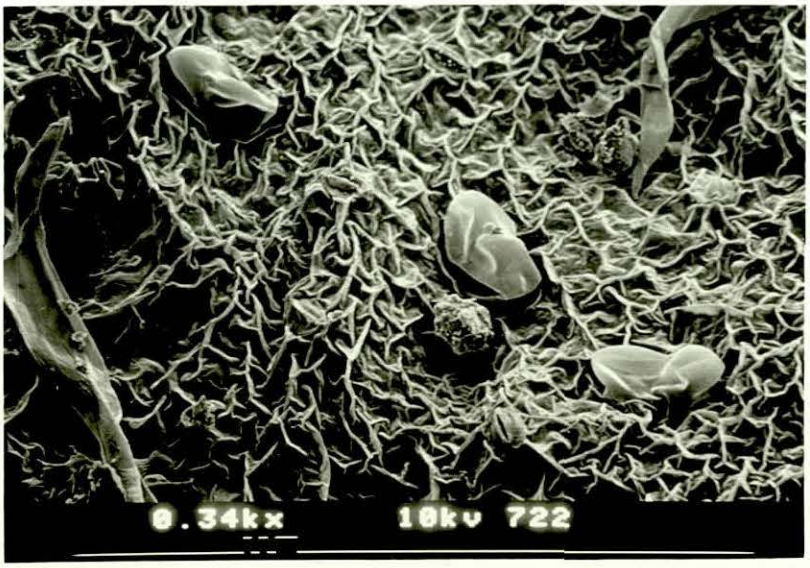

0.34kx 10kv 722

volatile, they do cause practical problems, such as blocking the syringe and shortening the life of the GC column because of contamination. The modifiers would appear to increase the extraction capability but also reduces the selectivity.

TABLE 9.4. Effect of extraction conditions on the presence of pigments. SFE conditions, 250 bar, 40°C, 0.8 ml/min CO_2.

Modifier	Colour	Inference
None	Yellow/orange	Carotenoids extracted
Water	Deep orange	Carotenoids extracted
Acetonitrile	Green	Chlorophyll extracted
Methanol	Green	Chlorophyll extracted

9.8 Timed extractions from plant material

This concept of free and bound sites may be further exemplified by examining the rate of extraction of the essential oils from the plant. Timed extracts (Figure 9.13 {ii}) were taken from the plant while simultaneously monitoring the extraction process by UV (Figure 9.13 {i}). It would appear that using just supercritical CO_2, the majority of the oils detected were extracted within 4 minutes. Between 4 and 20 minutes, very low levels of oil were extracted, presumably because the oils were having to diffuse out the plant and after 20 minutes no oils were detected. With comparable extraction conditions the oils from the cellulose matrix took 20 minutes to be exhaustively removed (Figure 9.4). This rapid extraction from the plant material would suggest that only the surface oils or unbound oils had been extracted, with no comparable extraction process seen in the cellulose plant model. There is no evidence to suggest that the polar test compound is present as free and unbound forms on the cellulose. Instead, it is envisaged that all the sesquiterpene lactone is adsorbed onto the matrix, being totally removed at high pressures or with a modified low pressure SCF.

The extraction profile obtained by UV is unique to the plant material (Figure 9.13 {i}), as there is a characteristic "hump" present which is not usually seen with the model plant matrix. Furthermore, unlike the model plant, this extraction profile does not closely correspond to the presence of essential oils. Thus, the extraction profile is probably due to extraneous material from the plant matrix being co-extracted to give the extract a yellow/orange colour (carotenoids). Therefore monitoring the extraction process was of limited value as the extraction profile would over

estimate the extraction time required to isolate the oils.

Data is available on the SCF extraction of two of the major carotenoids (carotene and lutein) which are present in the leaves of higher plants [254]. The results obtained by Favati *et al.* [254] found that at a 100 bar and 40°C only 10% of the carotene and 0.5% of the lutein was extracted. This probably corresponds to the pale yellow extracts obtained from feverfew at 120 bar and 40°C. At higher pressures of 300 bar (40°C), 96% carotene and 30% lutein were extracted which may relate to the yellow/orange extract obtained from feverfew at 250 bar and 40°C. However, Favati *et al.* state that it is only at the very high pressures of 700 bar (40°C) that green pigments (pheophytin a and b) are extracted. In this study it was not until polar organic modifiers, such as methanol, were used that such green coloured extracts were seen.

To reduce the amount of pigment extracted, a large cellulose trap (Jasco 10ml extraction vessel packed with α-cellulose) was substituted for the silica trap shown in Figure 9.9. As the essential oils were extracted at high pressures (250 bar, 40°C CO_2), the oils were virtually unretained in the cellulose, but the pigments were retarded. Thus the essential oil extracts were qualitatively indistinguishable from those obtained without a trap, however they were much paler in colour (very pale yellow). The trap could retain pigments for up to two hours before they started to appear in the extracts. At this point the trap was extracted with modified CO_2 (MeOH 5%), to reveal that less than 5% of the isolated oils had been retained on the matrix. To avoid cross contamination a disposable cellulose cartridge could prove ideal as an on-line sample clean up process. However, this trap would not work in the presence of modifiers, where the co-extraction of pigments, lipids and waxes would be a major problem.

FIGURE 9.13 Timed SFE extractions from feverfew.
(i) Extraction monitored at 220nm with a UV detector (AUFS 2.56).

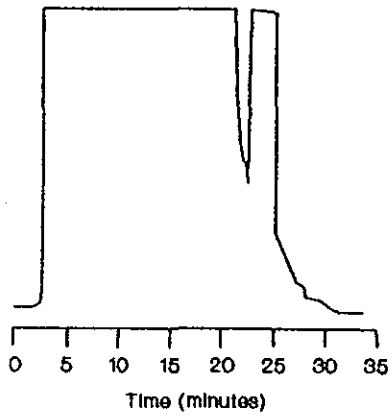
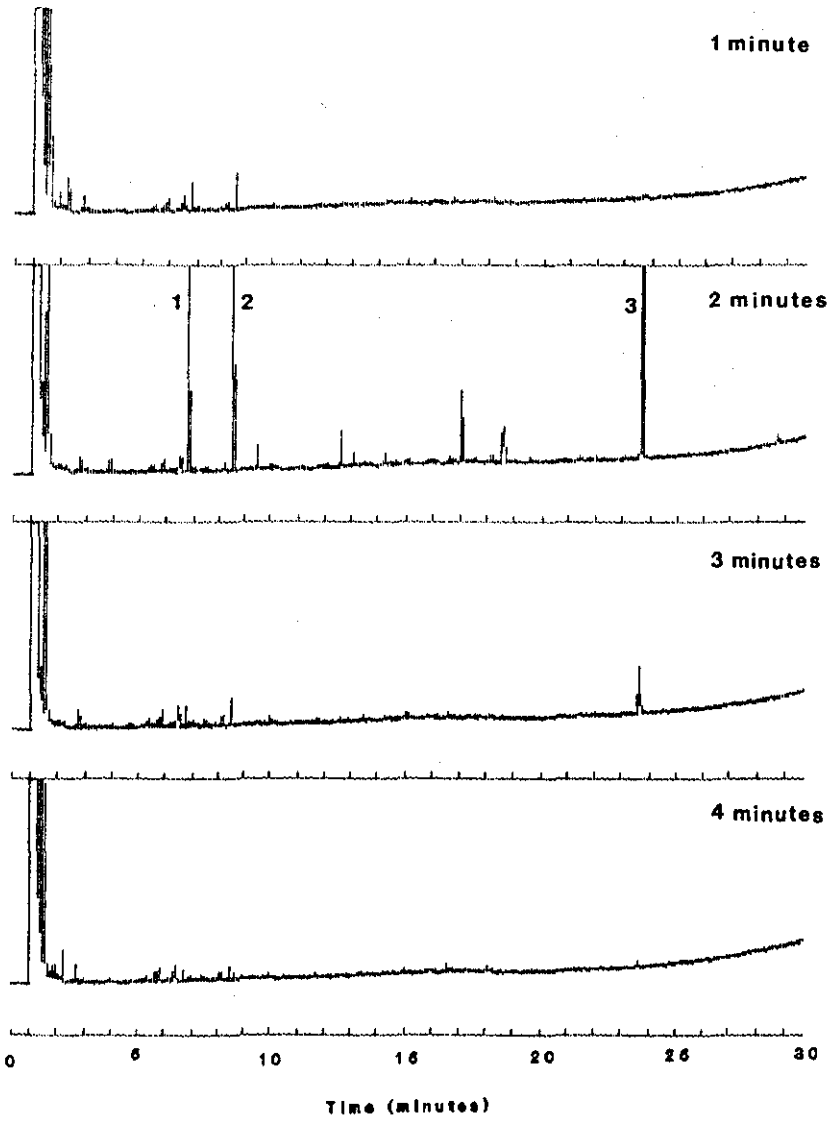


FIGURE 9.13 {ii}. Timed extraction from feverfew analysed on capillary GC (non-polar BP1 column, temperature 60 to 300°C at 8°C min^{-1}, then isothermal at 300°C for 8 minutes). 1 = camphor, 2 = pinene acetate, 3 = parthenolide.



1 minute = colourless, odourless extract

2 minutes = bright yellow extract with a fresh cut grass odour.

3 minutes = yellow, odourless extract

4 minutes = yellow/orange odour less extract

20 minutes = orange, odour less extract (same as 4 minute chromatogram)

9.9 Use of silica trap on plant material

A silica trap was investigated to enhance the selectivity of the extraction. The results obtained from the cellulose plant model, suggested that selectivity could be achieved by such means (Section 9.5). The experimental procedure used for the cellulose matrix was also applied to feverfew (Figure 9.9). Supercritical CO_2 (250 bar, 40°C) was passed through the plant material and onto the short on-line silica column. The extracted plant analytes eluted from the silica column were monitored by a UV detector to produce the elution profile seen in Figure 9.14 (peaks A-F). Once the extraction appears to be completed with no further analytes eluting from the column, then the extraction vessel containing the plant material was switched out off line. The silica column was then isobarically exposed to a modified CO_2 solvent (10% methanol) which eluted a number of previously retained analytes (Figure 9.14, peak G). Unfortunately, parthenolide could only be qualitatively extracted from feverfew, with the recovery yield being low as only supercritical CO_2 was used as the solvent during the extraction process. If modifier was used then a higher yield of parthenolide was obtained but the sesquiterpene lactone was no longer selectively retained. Due to the speed at which the essential oils were extracted, they were effectively injected within a short time onto the silica trap so that the elution profile (Figure 9.14 {i}) was comparable to a very crude SFE/SFC result. The initial components isolated appeared to be related to the odour of the plant, the larger, less volatile components were often coloured and isolated later and the very polar components were retained.
A similar procedure was used to successfully isolate the polar essential oils in tansy (Section 11.6).

FIGURE 9.14. Fractionation of essential oils from feverfew by means of an on-line silica trap.
{i} Extraction profile with a silica trap in-line. SFE conditions, 250 bar, 40°C, 0.8 ml/min CO_2. At point (x) plant material is switched out off line and 10% methanol introduced. Monitored by UV detector (AUFS 2.56).

A = Colourless, sweet pine odour extract.
B = Colourless odourless extract.
C = Pale yellow, faint sweet odour extract.
D = Yellow, camphor odour extract.
E = Yellow, faint sweet smell extract.
F = Pale yellow, odourless extract.
G = Yellow/orange extract, faint cut grass smell extract.

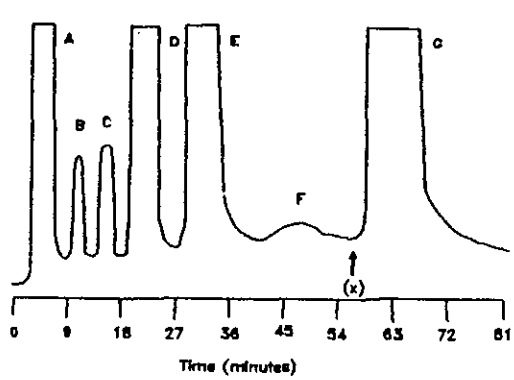
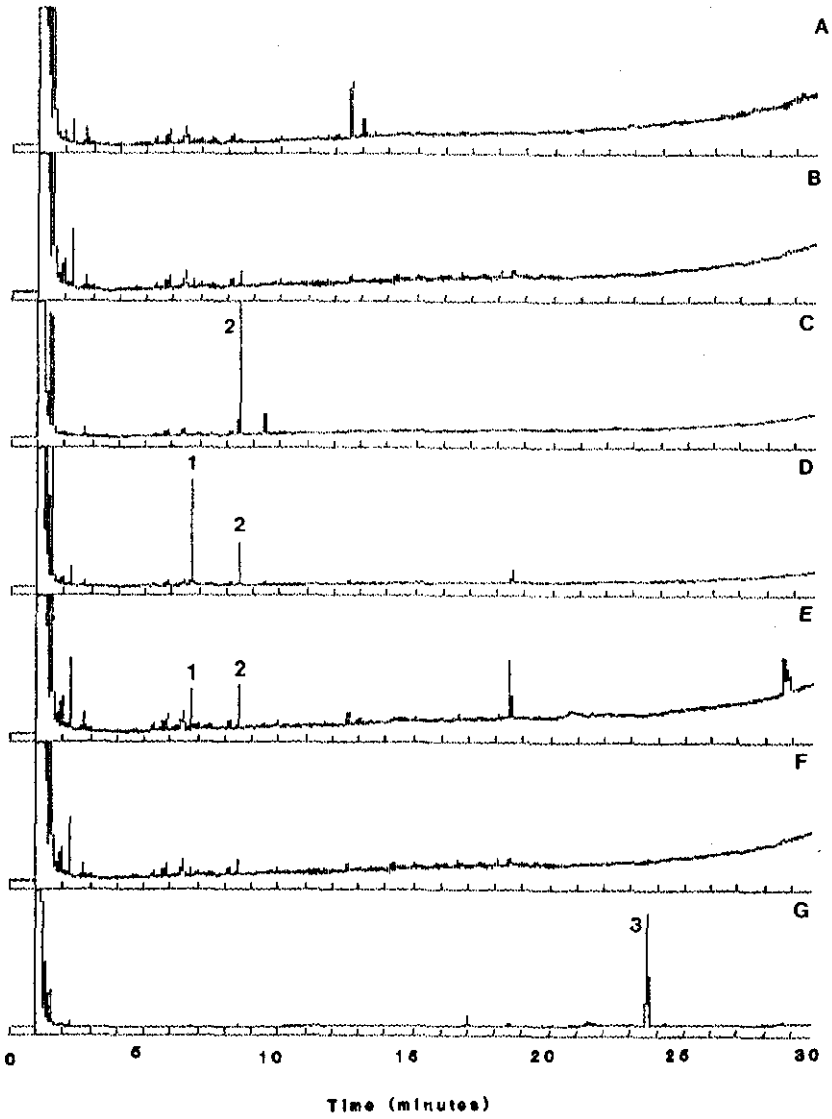


FIGURE 9.14 {ii}. Timed extracts from feverfew with a silica trap in-line. 1 = camphor, 2 = pinene acetate and 3 = parthenolide. Description of each sample is given in Figure 9.14 {i}. Capillary GC conditions given in Figure 9.13 {ii}.



9.10 Sample preparation

As nearly all the commercial products of feverfew consist of the plant being in a dried powder form, the effects of drying the plant material were investigated (Figure 9.15). Drying caused about an 80% loss in plant weight but the process appeared to have little effect on the % parthenolide extracted (based on a dry weight basis). This is to be expected as parthenolide is not particularly volatile. However, variations in extraction

yields can occur, depending on the extent of drying. If the plant material was only dried for 4 hours (50°C) after being harvested, then high yields of parthenolide could be achieved (≈ 0.16%) as if traces of water were still present. This yield would drop over the weeks as presumably the last traces of water evaporated away (≈ 0.09%) or due to the possible degradation of parthenolide on drying. On drying the plant for 24 hours (50°C) a much lower but consistent yield could be obtained (≈ 0.08%).

Drying does have an effect on the more volatile oils such as camphor and pinene acetate, which can decrease by 5-25%. Moreover, a number of minor components were lost as they were only found in the extracted whole fresh plant material or in a steam distillate (Figure 9.16 {i}). If fresh plant was ground-up to a green paste then the yield of essential oils was greatly diminished (Figure 9.16 {ii}). It is thought that the water from the ruptured cells in the plant, may act as a barrier to the supercritical CO_2, as the SCF has a very low solubility in water and the paste matrix would resist diffusion by CO_2. This same idea may apply to the low recovery rate of parthenolide from the rehydrated feverfew material which also forms a dark green paste (Figure 9.15). Therefore, in terms of the parthenolide content, drying appears an acceptable way of storing the plant material.

FIGURE 9.15. Effects of sample preparation on extraction efficiency.

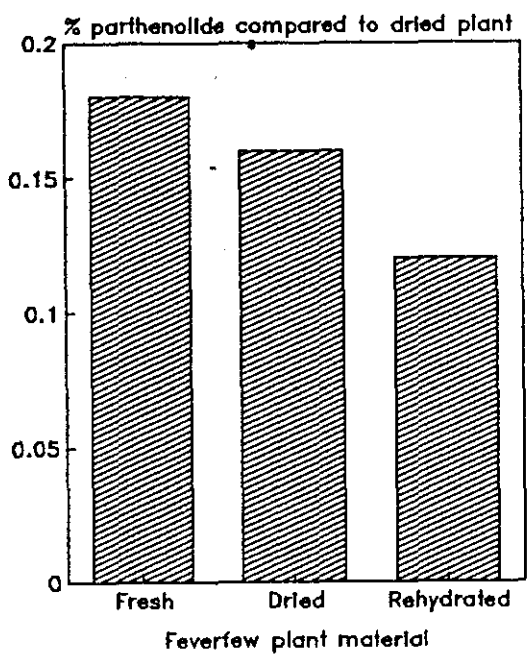
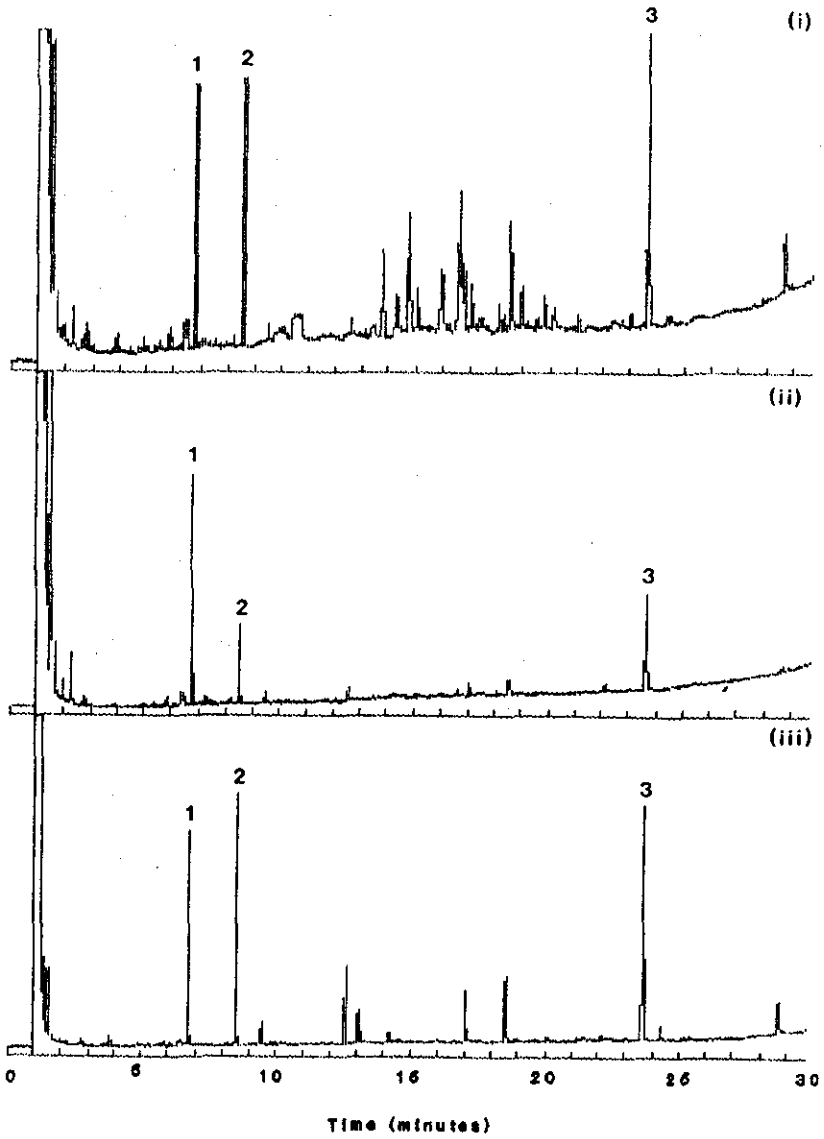


FIGURE 9.16. Effects of sample preparation on the essential oil content of feverfew (homegrown from Fisons seeds). {i} Whole fresh plant material, {ii} Fresh plant material ground up to form a green paste, {iii} Fresh plant material dried for 4 hours at 50°C. SFE conditions, 250 bar, 40°C, 0.8 ml/min CO_2. Capillary GC conditions given in Figure 9.13 {ii}.
1 = camphor, 2 = pinene acetate and 3 = parthenolide



9.11 Conclusions

The use of a plant model matrix proved very helpful in the selection of extraction conditions and suggested an insight into the extraction mechanisms. It demonstrated the importance of the diffusion parameter and its consequences in recovery yields. The model was reasonably accurate in predicting the extraction conditions for the majority of analytes in the plant material, with the overall trends being similar. However it did not represent some of the more complex processes such as microencapsulation,

which may be occurring with the polar sesquiterpene lactones.

The results appear to indicate that the sesquiterpene lactones may be present as "free" and "bound" forms. The modifier may replace the analyte molecule bound on the adsorption site, so that a more efficient extraction can take place. It would appear that cellulose may best represent the unbound analytes on plant material, which incurs the problem of having to diffuse out of the matrix and silica may best represent the bound analytes on the plant material, which requires a modifier to free them from the matrix (See Table 9.1).

Chapter 10

SFC separations of essential oils

10.0 Introduction

Packed column supercritical chromatography (SFC) was investigated as a possible model for the SCF extraction of essential oils and plant phenolics from a plant matrix. A direct comparison between the SFC conditions required to elute these components from a column was to be made with the conditions required to extract the essential oils from a plant matrix. A variety of stationary phases, temperatures, pressures and modifiers were investigated to ascertain which conditions would best emulate the SFE process, so hopefully avoiding the arbitrary "hit and miss" technique so often used to determine optimum extraction conditions.

A similar idea was investigated by McNally *et al.* [255] in which chromatographic capacity factors from SFC, were related to SFE parameters, so that a prediction of the extraction efficiency could be made. The predictions shortened the method development time in SFE but were still unable to ascertain the optimum extraction conditions. A more theoretical approach was undertaken by Bartle *et al.* in which a simple relationship between the solubility and capacity factor of an analyte was established [256]. An assumption was made that the degree of retention of a solute in SFC, as measured by the capacity factor k', is qualitatively inversely related to the solvating power of the mobile phase for that solute. The more soluble it is in the mobile phase, the less it will be retained. The theory of the relationship has been discussed [257,258] which in its simplest form is:-

$$S = C/k'$$

where S is the solubility (per unit volume), C is a constant for a particular column, solute and temperature and k' is the chromatographic capacity factor which is expressed as:-

$$k' = (V_R - V_M)/V_M$$

where V_R is the volume of CO_2 required to elute the solute and V_M is the volume of the mobile phase in the column which corresponds to the void volume marker. However, to obtain a value for C the solubility data or the vapour pressure of the solute must be known. In the case of parthenolide, the active ingredient in feverfew, such data is not available and thus the technique is of limited value. Therefore, the approach taken by McNally *et al.* was deemed more applicable to this study.

10.1 Chromatographic analysis of the essential oils

Gas chromatography (GC) is by far the most widely used technique to analyse essential oils, as they have boiling points varying from 150°C to over 350°C and all but a few have ideal vapour pressures for GC [225]. The early results obtained in GC analysis of terpenes have been thoroughly discussed and reviewed in the literature [259-261], with a recent up-date [225] showing the general refinement and diversification of the technique. Using capillary columns in conjunction with temperature programming, a very large number of compounds in a complex mixture such as an essential oil can be resolved. However, the relatively high temperatures required may cause problems for the analysis of thermally unstable constituents. High injection temperatures usually employed in GC (above 120 - 150°C) may dehydrate compounds like linalool [262], camphene hydrate [263] or α-terpineol [264].

As a consequence, alternatives have been investigated such as high performance liquid chromatography (HPLC). However, this technique is restricted in its use due to the limited resolution compared with capillary GC and to a lesser extent by the lack of a sensitive universal detector, as many essential oils possess a poor chromophore [265]. Nevertheless, HPLC has been used with some success in the selective determination of certain compounds in essential oils such as eugenol [266] and the analysis of essential oils from lemon, bergamot and orange using microbore columns and a UV detector [267]. HPLC has been particularly useful in the analysis of sesquiterpene lactones, where it is claimed that GC is of limited use because the compounds are not always sufficiently volatile and may need derivatisation [233,268].

Another more recent alternative is SFC, though few applications were found in the literature for essential oil analysis. A limited survey was carried out at Loughborough University [269], using a Spherisorb ODS and a PS-DVB polymer column with a FID detector. Both columns produced reasonably

good peak shapes for the non-polar analytes with CO_2 as the mobile phase. However, tailing peaks were observed on the ODS column for the polar analytes containing hydroxyl groups and modifiers were not investigated as these would interfere with detection. Morin *et al.* have separated and identified sesquiterpene hydrocarbon mixtures on a bare Spherisorb silica column with on-line FT-IR detection [270]. These non-polar but extremely thermosensitive organic compounds required low-temperature rather than low-density experimental conditions. Manninen *et al* applied capillary SFC to the analysis of some plant volatile oils, the results being comparable with those obtained by capillary GC [271]. For a complex mixture of peppermint oil or basil oil, SFC was claimed to be more quantitative than capillary GC, especially for the oxygenated compounds. However, the separation efficiency of capillary GC for the monoterpene hydrocarbons was better than that of SFC.

10.2 Retention of essential oils on various columns

Examples of four typical groups of essential oils, limonene (a hydrocarbon monoterpene), carvone (an oxygenated monoterpene), caryophyllene (a hydrocarbon sesquiterpene) and santonin (an oxygenated sesquiterpene) were investigated using supercritical CO_2 and packed column SFC. Quercetin, a plant phenolic was also analysed, to represent the extraneous material that can be co-extracted with the essential oils by SFE. All the analytes were injected at about a 2 mg/ml concentration onto the column. Chromatographic experiments utilising different stationary phases and conditions showed the ability to vary the capacity factor, k' (Table 10.1 and 10.2).

From the results a general pattern is discernable. The silica column appears to be acting in the normal phase mode with the most non-polar analytes eluting first and the most polar last. This is comparable to the elution order found in the normal phase HPLC system using a hexane eluent (Table 10.1 and 10.2). Several researchers have compared the selectivity and solubility properties of supercritical CO_2 to hexane. Phillips *et al.* found that CO_2 gave a similar elution order to hexane when using a silica column [189], with the solvent strength of CO_2 being seen as comparable to hexane [185]. It has been suggested that the polarity of the functional groups on the solute is probably more influential than size in determining the elution order [272].

TABLE 10.1. Elution order of essential oils and plant phenolic from different stationary phases. See Table 10.2 for chromatrographic conditions.

	SFC		HPLC	GC
Polymer[a]	ODS[b]	Silica[c]	Silica[d]	BP-1[e]
limonene	limonene	limonene	limonene	limonene
carvone	caryophyllene	caryophyllene	caryophyllene	carvone
caryophyllene	carvone	carvone	carvone (+ MeOH[f])	caryophyllene
santonin	santonin (+ MeOH[f])	santonin (+MeOH[f])	santonin (+ MeOH[f])	santonin
quercetin	quercetin (+ MeOH[f])	quercetin (+ MeOH[f])	quercetin (+ MeOH[f])	quercetin

a = Polymer (PLRP-S 5 μm 150 x 4.6 mm) stationary phase.
b = ODS (Spherisorb 5 μm 250 x 4.6 mm) stationary phase
c = Silica (Spherisorb 5 μm 250 x 4.6 mm) stationary phase.
d = Silica (Spherisorb 5 μm 250 x 4.6 mm) stationary phase and hexane as a mobile phase.
e = BP-1 (5 μm film of dimethyl polysiloxane 12m x 0.33mm i.d.) stationary phase.
f = Addition of methanol modifier to mobile phase.

TABLE 10.2. Elution conditions of essential oils and plant phenolic from different stationary phases. a – e as in Table 10.1.

	SFC			HPLC	GC
Solute	Polymer[a]	ODS[b]	Silica[c]	Silica[d]	BP-1[e]
Limonene	100 bar, 1.5ml/min	100 bar, 1.5ml/min	100 bar, 1.5ml/min	0.5ml/min	116°C
Caryophy.	100 bar, 1.5ml/min	100 bar, 1.5ml/min	100 bar, 1.5ml/min	0.5ml/min	146°C
Carvone	100 bar, 1.5ml/min	300 bar, 2.5ml/min	300 bar, 2.5ml/min	2.5ml/min + 1.6% MeOH	200°C
Santonin	250 bar, 2.5ml/min	250 bar, 2.5ml/min + 2% MeOH	200 bar, 2.5ml/min + 8% MeOH	2.5ml/min + 3.2% MeOH	250°C
Quercetin	would not elute	would not elute	300 bar, 2.5ml/min + 20% MeOH	wouldn't elute	not volatile

Note:– All the SFC and HPLC work was carried out at 40°C and all the analytes were injected individually, eluting off the column within fourteen minutes.

TABLE 10.3. Corresponding capacity factors (minutes) of essential oils and plant phenolic, from chromatographic conditions shown in Table 10.2. Note for GC the values are retention times in minutes. a – f as in Table 10.1.

	SFC			HPLC	GC
Solute	Polymer[a] (k')	ODS[b] (k')	Silica[c] (k')	Silica[d] (k')	BP-1[e] (R_t)
Limonene	1.72	8.68	0.11	5.20	4.04
Caryophyllene	7.70	30.47	0.95	5.95	7.52
Carvone	4.68	1.87	13.00	1.33[f]	12.21
Santonin	7.04	10.46[f]	4.42[f]	12.96[f]	22.76
Quercetin	-	-	2.93[f]	-	-

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

TOTAL AREA= 2.4412E+02
MUL FACTOR= 1.0290E+00

SFE extract

The polar plant phenolic, quercetin, required a higher percentage of modifier with the supercritical CO_2 to elute it from the column. This is to be expected as flavonoids are highly retained on silica in HPLC, some being irreversibly adsorbed [109]. Pietrogrande *et al.* investigated the use of various solvents to elute flavonoids from a silica column [139]. The solvents dichloromethane, chloroform, ethyl acetate and 2-propanol were assessed, but only the last produced measurable retention values. Moreover, most of the compounds examined gave rise to large, tailed peaks, revealing the existence of slow irreversible adsorption equilibria on energetically heterogeneous adsorption sites on the silica surface [273,274]. A similar peak shape was obtained on the silica column in SFC (Figure 10.1).

The unmodified silica surface is thought to have two major types of silica groups present, namely silanols and siloxane bridges, but many complex interrelationships can occur between them [248,275-276]. It is assumed that the main retention mechanism for polar analytes is via hydrogen-bonding with the silanols [277]. However, a retention mechanism is still present for the non-polar analytes as they do not elute with the solvent front. This could be due to steric hindrance or the presence of siloxane bridges on the silica surface. These groups are seen as hydrophobic and are capable of π-π interactions and will thus retain aromatic compounds such as limonene and caryophyllene [248].

The ODS column possessed the same elution order as the silica column, suggesting that both stationary phases are acting in the normal phase mode in SFC (Table 10.1 and 10.3). This would appear anomalous as the ODS stationary phase is non-polar, so that a reverse phase elution order was expected. Therefore an alternative to the conventional reverse phase mechanisms would appear to exist. Schoenmakers *et al.* suggested that a mixed retention mechanism exists in SFC [278]. It has been shown that bonded stationary phases such as ODS, have at best only about half of the adsorption sites on the silica surface coated with this organic rich layer [253], so that a large unreacted silica surface is present to potentially contribute to the retention mechanism. The surface activity associated with these adsorptive sites may be a serious limitation for packed column SFC when mobile phases of low polarity, such as CO_2, are called upon to elute polar analytes. In LC this conventional bonded phase is thought to be not nearly as much of a problem, since the mobile phase is usually more polar (e.g., methanol-water in reverse phase) and consequently deactivates the exposed silica sites on the stationary phase [277]. Thus, in SFC the bonded

phase appears to possess two retention mechanisms, one attributed to the bonded alkyl substituents, so that the stationary phase may have a greater affinity for the non-polar analytes, and the other, often referred to as the secondary retention mechanism, is due to the remaining accessible silica surface, which adsorbs the polar analytes such as santonin and carvone.

With the polymer PLRP-S column, the presence of adsorptive silanol groups are eliminated and compounds are no longer retained via this adsorption mechanism. It would appear as if the analytes were eluting in order of volatility, possessing the same elution order as the capillary GC (Table 10.1). Although tailing is significantly reduced on the polymer column (Section 10.3.), species capable of hydrogen bonding on the silica-based columns (i.e. santonin and carvone) are also retained on the PLRP-S. This has been explained by the π electrons in the extensive aromatic ring structure of PLRP-S [279]. These electrons are easily polarizable causing induced dipoles and π-π electron interactions with the solute electron orbital. As these interactions are not as strong as the adsorption interactions present on the silica based columns, these polar analytes are correspondingly less retained, requiring much milder elution conditions.

The plant phenolic, quercetin failed to elute of either of the non-polar columns (ODS or PLRP-S). This was a little unexpected as most HPLC studies use reverse phase C_{18} columns to analyse flavonoids [109]. Previous studies have shown that using the same ODS column in an isocratic LC mode ($MeOH/H_2O$ 30:70, Section 5.1), caused quercetin to be highly retained (R_t = 29.7 minutes). It may be that inadequate or inappropriate modifier was used in the SFC mode, as only up to 14% was investigated. However, unlike the other analytes, the flavonoids are not particularly soluble in CO_2. Stahl *et al.* demonstrated that some plant phenolics such as the hydroxycoumarin aesculetin, appeared not to be extractable in supercritical CO_2 [182]. Therefore, the problem may not lie solely with the stationary phase but also with a solubility limitation which is only overcome with a large amount of modifier.

The essential oils were also investigated by capillary GC, the analytes eluted in order of their boiling point. Thus the most volatile eluted first (limonene) and the least volatile, last (santonin). The plant phenolic, quercetin would require derivatisation as it lacked sufficient volatility for GC analysis [107].

10.3. Peak shape

On the silica-based columns good peak shapes were obtained for the non-polar analytes, whereas the polar analytes tailed quite badly. This tailing was most pronounced on the silica column using unmodified supercritical CO_2 as an eluent (Figure 10.1). The polymer column proved ideal for the essential oil analysis, all the oils were separated with Gaussian peaks and none required modifier.

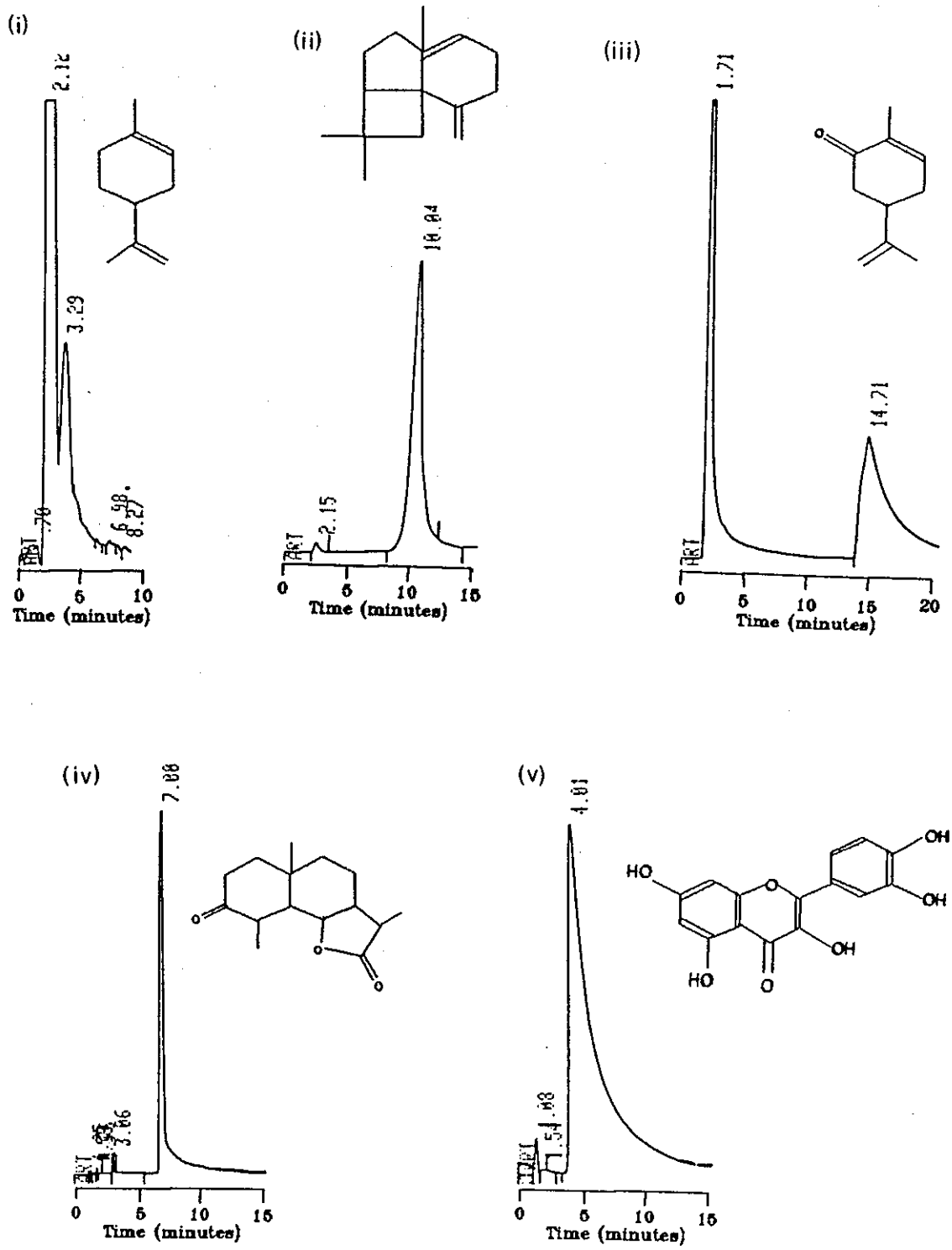
This problem of poor peak shapes for polar analytes on silica based packed column in SFC, has been reported by others [278,280] and has been explained in terms of adsorption of polar analytes to the accessible silanol groups [278,281]. This view is confirmed by the results from the polymer column, as this stationary phase possesses no such silanol groups and so gives good peak shapes for all the essential oils regardless of polarity.

10.4. Effect of modifier

The elution of polar compounds in SFC often requires the addition of polar solvents to the mobile phase. The effects of such modifiers on retention and selectivity in packed-column SFC have been the subject of a number of investigations [185,282-284]. In these reports, the addition of a small amount of modifier (<1%), was shown to drastically reduce the retention of the polar solutes.

The addition of a modifier to the mobile phase can induce changes in the nature of the mobile and stationary phase. Modifier effects include coverage of the "active sites" (i.e, silanols) [280], swelling or modifying the stationary phase [285], increasing mobile phase density [286], and/or increasing the solvent strength of the mobile phase [287]. It has been suggested that the major role of the modifiers on a bonded silica stationary phase (ODS) is the deactivation of the adsorptive sites on the column wall or on the packing material [185,283]. The modifier molecules will compete with the solute molecules for the interaction with the surface of the packing material. Under suitable conditions, they will shield the silanols. Hence the number of silanols contributing to the retention will decrease and consequently, the major retention mechanism will be the interaction of the solute with the chemically bonded phase. The result is a decrease in retention time and an improvement in peak shape.

FIGURE 10.1. Peak shapes of essential oils analysed on a silica column (Spherisorb, 5μm 4.6 x 250mm) in SFC. UV detection at 220 nm (AUFS 0.64). Chromatographic conditions: {i} Limonene 120 bar, 1.1 ml/min at 40°C; {ii} Caryophyllene 120 bar, 1.1 ml/min at 40°C; {iii} Carvone 300 bar, 2.5 ml/min at 40°C; {iv} Santonin 200 bar, 2.5 ml/min, 8% MeOH at 80°C; and {v} Quercetin 300 bar, 2.5 ml/min, 20% MeOH at 40°C.



It has been demonstrated that only polar modifiers showed a measurable influence on retention of polar solutes on an ODS, Chromspher, column [288]. This suggested that the interactions between the surface and the modifiers was primarily due to hydrogen-bonding (proton accepting) interactions. For example, benzene, the molecular size of which is similar to that of THF, shows no measurable adsorption compared to THF [288]. This is in agreement with Boudreau *et al.*, who classified silica as a proton-donating surface, which could consequently show interaction particularly with proton-accepting modifiers or solutes [289].

From adsorption isotherm data the concentration of modifier that was required to obtain almost complete surface deactivation (≈90–95%) has been estimated for methanol on an ODS, Chromspher, column [288]. At 1.1% (v/v) methanol in supercritical CO_2 the surface coverage and, hence, the surface deactivation, is close to its maximum. The main effect of increasing the methanol concentration above 1.1%, will be a modification of the mobile phase, because the stationary phase coverage remains almost constant. However, the influence of mobile phase modifications were found to be small in comparison with the initial effects caused by the stationary-phase deactivation.

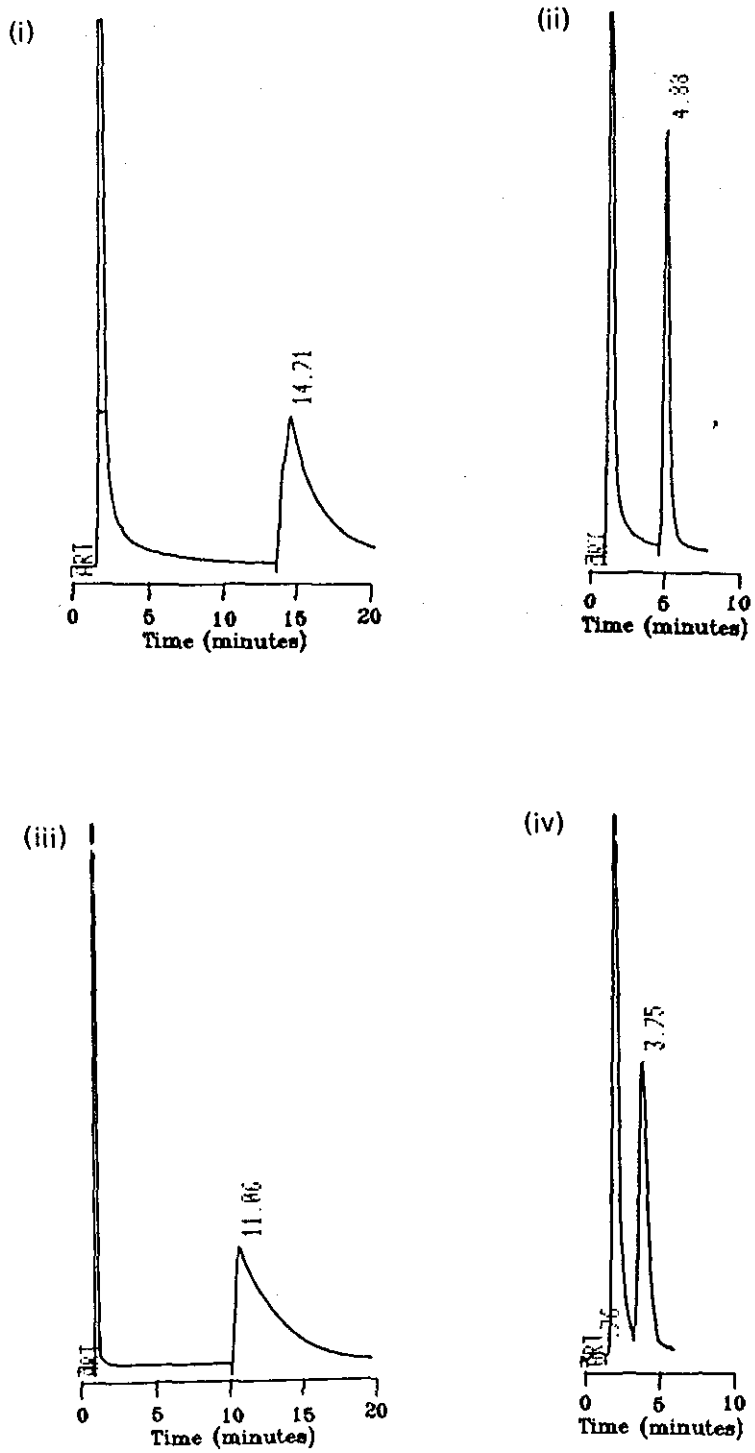
Mobile phase properties liable to change upon the addition of modifiers to the supercritical fluid, include both density, and the nature and extent of physiochemical interactions between the solute and the mixed mobile phase. The addition of modifier may result in a substantial increase in the mobile phase density. The effect of this density increase on retention is claimed to be comparable to the effect of a pressure induced density increase with neat CO_2 [288]. The assumption of a changing solvating power on the addition of a modifier to CO_2 is supported by spectroscopic measurements of solvatochromic shifts [185,290]. At low concentration of methanol in supercritical CO_2 (≈1%) no solvatochromic shift is seen (22), it appears it is not until about 4% methanol is present that an appreciable increase in solvent strength is discernible [186]. Phillips *et al.* claims

that at 3% methanol concentration in supercritical CO_2, the modifier may start to interact with the solute as either a Lewis acid or base with hydrogen bonding capabilities [189]. The presence of clusters [193] has also been suggested as has the idea that the modifier may act as a surfactant causing a decrease in interfacial tension between the mobile and stationary phase [189].

Berger *et al.* suggest that the role of density changes and active sites have been overemphasised while other factors, particularly mobile phase solvent strength, have been underemphasised [291]. The data generated by Berger *et al.* on silica based packed columns in SFC [291], indicated that the primary mode of action of the modifier was neither covering the active sites or in increasing density but in producing significant changes in the mobile phase solvent strength. The linear relationship between retention and mobile phase solvent strength suggested that coverage of active sites played no more than a minor role in retention shifts. This confliction of views demonstrates that there is at present no one agreed concept of retention mechanisms in packed column SFC.

The results in this study concur with many of the views found in the literature. The polar essential oils tailed badly on the silica based columns, with the very polar santonin only eluting in the presence of modifier. The improvement in peak shape with the introduction of modifier for the medium polar solute, carvone can be seen in Figure 10.2. It would appear that the solute is more highly retained on the silica column due to silanols being the major retention mechanism, with the modifier greatly reducing the retention time and improving the peak shape. Conversely, with the ODS column, the silanols are seen as the secondary retention mechanism which is responsible for tailing peaks. Therefore, the modifiers main influence was seen with the dramatic change in peak shape (though if very low pressures of 100 bar were used, then a larger influence on retention could be seen). If the columns are used under the same conditions (100 bar, 60°C, 1.5 ml/min CO_2, 2% methanol), with sufficient methanol modifier to cover the silanol groups on the ODS packing material, it was found that carvone was more highly retained on the silica column (R_t = 14.96 mins) than on the ODS packing material (R_t = 8.69). This suggested that more than 2% modifier would be required to cover the silanols present on the silica packing material. The presence of modifier (methanol) also improved the peak shape of polar solutes on the silica column in normal phase HPLC, with a hexane eluent. In the absence of silanols, the polymer column separates all

FIGURE 10.2. SFC conditions for carvone :-
Silica (Spherisorb 5 μm 4.6 x 250mm) column. UV detection 220 nm (AUFS 0.64)
{i} 300 bar, 40°C, 2.5 ml/min CO_2.
{ii} 100 bar, 40°C, 1.5 ml/min CO_2, 0.05 ml/min MeOH (3.3%).
ODS (Spherisorb 5 μm 4.6 x 250 mm) column. UV detection 220 nm (AUFS 0.64)
{iii} 150 bar, 60°C, 1.3 ml/min CO_2.
{iv} 150 bar, 60°C, 1.3 ml/min CO_2, 0.01 ml/min MeOH (0.77%) at 220nm.

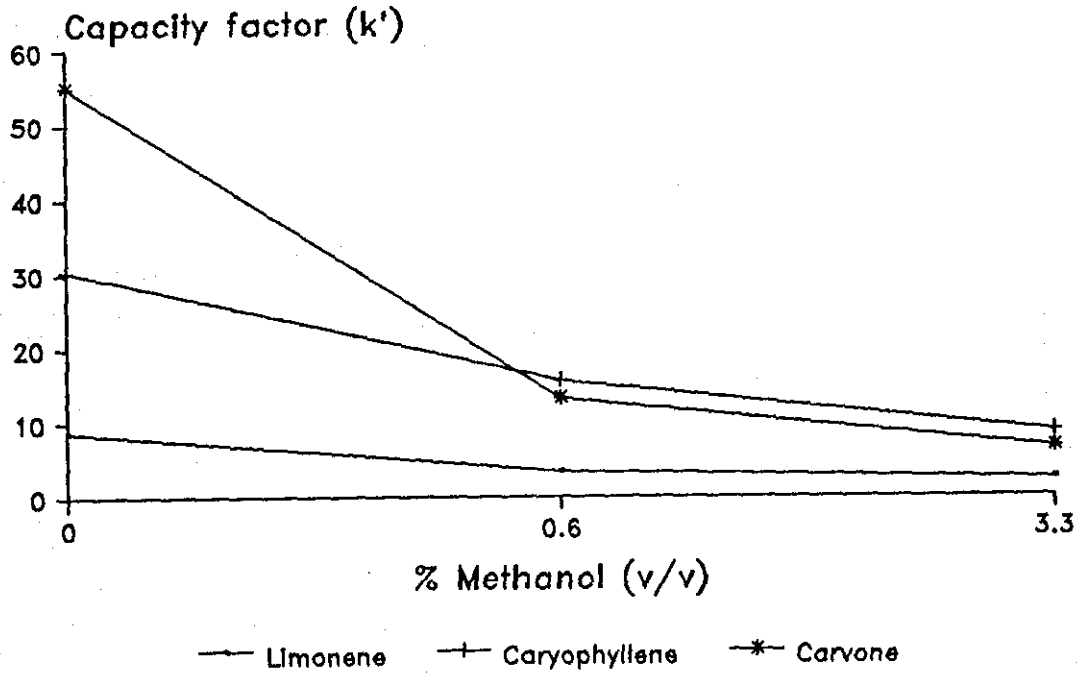


the oils as Gaussian peaks in supercritical CO_2, with no modifier required.

Besides the modifier affecting the peak shape and retention time of solutes on an ODS column, it may also change the elution order (Figure 10.3). In the absence of modifier, the highly retentive silanols present on the bonded stationary phase, may selectively adsorb the polar analytes so that carvone is seen to elute after the non-polar essential oils limonene and caryophyllene. With the initial introduction of modifier (0.6%), the retention of carvone is greatly reduced compared to the non-polar analytes. The result is a change in the elution order with carvone now eluting before caryophyllene. Assuming the modifier shields the silanol groups at this low concentration of 0.6%, then the main retention mechanism on the column will be with the interaction of solutes with the bonded alkyl substituents. Under these circumstance the non-polar caryophyllene will be more strongly retained than the polar carvone. At the higher modifier concentration (3.3%) where all the silanols are presumed to be adsorbed with methanol, a modifier-solute interaction becomes more prominent which may affect both polar and non-polar solutes. However, under these chromatographic conditions solubility is not considered a limiting factor [227], thus the reduction in retention times at these high modifier levels will be minimal compared to the results achieved with the initial introduction of modifier. Even at these high levels of modifier (3.3%) where the silanols are presumed to contribute little to the retention mechanism, the polar analytes such as carvone and santonin (Table 10.2 and 10.3) do not elute at the solvent front. This suggests there is an additional retention mechanism for the polar analytes, possibly due to simple steric hindrance or reverse phase interactions with the chemically bonded alkyl substituent.

This theory may also explain why adjusting the modifier concentration from 3.3% to 0.6% only takes in the region of 20 minutes to equilibrate, presumably as the modifier is mainly solvated in the mobile phase. Conversely, to reduce the modifier concentration from 0.6% to 0.0% may take up to 5 hours as the methanol is presumed to be absorbed onto the free silanol groups present.

FIGURE 10.3. Influence of modifier on retention of essential oils on an ODS (Spherisorb 5 µm 4.6 x 250 mm) column. SFC conditions 100 bar, 60°C, 1.5 ml/min CO_2 detected at 220 nm (AUFS 0.64).



10.5. Reproducibility

Problems were encountered using silica based columns, as repeated injections of tailing polar solutes gave decreasing retention times until a reproducible time was attainable. Once this was achieved, reproducibility could be maintained if the conditions were unaltered. However, when the conditions were made more rigorous, such as using higher pressures, then the whole process of achieving reproducibility would have to be repeated. This phenomenon was most evident for the very polar solute with hydroxyl groups present (quercetin) as can be seen in Table 10.4 and Figure 10.4. Taylor *et al.* found a similar problem with the injection of pyridine onto a ODS Nucleosil column [277].

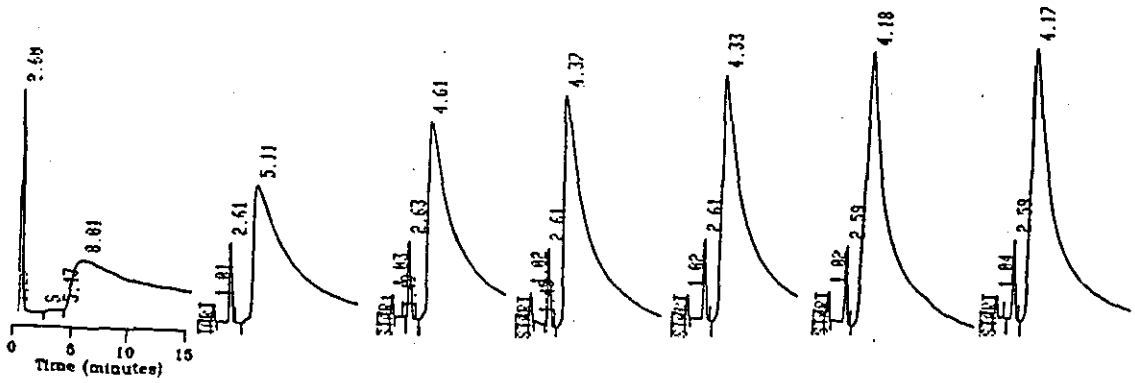
The reproducibility achieved after successive injections of polar solutes onto the silica based columns may be attributed to the adsorption of solutes onto the silanols, thus deactivating them. This is not an irreversible adsorption as the solutes can be deadsorbed at a higher densities. However, for analytes such as quercetin a polar modifier is already present (MeOH 20% v/v) to elute the compound from the silica column. At this concentration the majority of the silanols are thought to be deactivated [253]. But as the

results show (Figure 10.4.) the solute is undoubtly interacting with the stationary phase to cause a gradual decrease in retention time, as if further sorptive sites are present for the solute to adhere too.

TABLE 10.4. *Reproducibility of analytes on a silica* (Spherisorb 5 μm 4.6 x 250 mm) column. SFC conditions:- Limonene 80 bar, 40°C, 1.5 ml/min CO_2; Caryophyllene 100 bar, 60°C, 1.5 ml/min CO_2; Carvone 300 bar, 40°C, 2.5 ml/min CO_2; Santonin 300 bar, 40°C, 2.5 ml/min CO_2, 8% MeOH; Quercetin 300 bar, 60°C, 2.5 ml/min CO_2, 20% MeOH.

Solute	Capacity factor (k')					
	1st	2nd	3rd	4th	5th	6th
Limonene	1.12	1.16	1.17	1.14	1.13	1.14
Caryophyllene	1.83	1.81	1.81	1.81	1.81	1.80
Carvone	12.70	12.60	12.53	12.54	12.54	12.51
Santonin	4.88	4.72	4.67	4.58	4.50	4.42
Quercetin	7.00	4.10	3.58	3.35	3.31	3.16

FIGURE 10.4. Consecutive injections of quercetin onto a silica (Spherisorb 5 μm 4.6 x 250 mm) column. SFC conditions 300 bar, 60°C, 2.5 ml/min CO_2, 0.05 ml/min MeOH (≈3.3%) at 254 nm. 20 μl 9injection (≈ 150 μg quercetin). UV detection (AUFS 0.64)



For the medium polar analytes such as carvone, the presence of a modifier causes a reduction in retention time and improves the reproducibility of the system, presumably as the modifier deactivates the adsorptive sites which interact with this solute (Table 10.5). With the ODS packing material, the problem of reproducibility was a lot less evident, requiring only one or two injections of a polar solute before reproducibility was achieved. This may be related to the fewer number of accessible silanol groups present on the bonded stationary phase. The idea that the problem of reproducibility is related to accessible silanol groups would appear to be confirmed as reproducability was easily attainable on the polymer column which does not possess such adsorption sites.

TABLE 10.5. Affects of modifier on reproducibility of carvone on a silica (spherisorb 5 µm 4.6 x 250 mm) column. SFC conditions:– a = 300 bar, 2.5 ml/min CO_2, 40°C at 254 nm, b = 100 bar, 1.5 ml/min CO_2, 40°C, 3.3% MeOH at 254 nm.

Solute	Capacity factor (k')					
	1st	2nd	3rd	4th	5th	6th
Carvone/no MeOH[a]	12.70	12.60	12.53	12.54	12.54	12.51
Carvone/MeOH[b]	1.28	1.30	1.30	–	–	–

10.6 Affect of temperature on retention of solutes

To assess the effects of temperature on the retention of solutes, each analyte was investigated under its appropriate chromatographic condition, at three different temperatures (Table 10.6). It can be seen from the results that there may be two competing factors in SFC, namely solute volatility and solvent density. This was demonstrated by the most volatile analyte, limonene. As the temperature was increased in SFC, the retention of limonene first increased then decreased. Thus, with the initial increase in temperature an increasing retention was caused by a decrease in the solubility of the solute in the mobile phase which corresponds to a decrease in CO_2 density (Figure 10.5). Beyond the retention maximum temperature, while solubility continued to decrease, the increasing temperature caused an increase in volatility. As in GC, an increasingly volatilised solute is less strongly retained. This characteristic increase then decrease in retention time with temperature for limonene, has also been obtained for n-alkanes in SFC [292].

Conversely, the retention profile for caryophyllene (Table 10.6) was distinctly different from that obtained for limonene, even though the oils were eluted under similar chromatographic conditions. With caryophyllene the retention continually increased with increasing temperature, density being the dominate factor throughout the temperature range investigated (Figure 10.5). The reason for this may lie with the volatility of the oils, because at 80°C limonene has a much higher vapour pressure (≈0.045 bar) than caryophyllene (≈0.002 bar) [175,293]. Thus, under these circumstances volatility may be the dominate factor for limonene, but for caryophyllene with its small vapour pressure, it will be the density of the mobile phase which is of most importance.

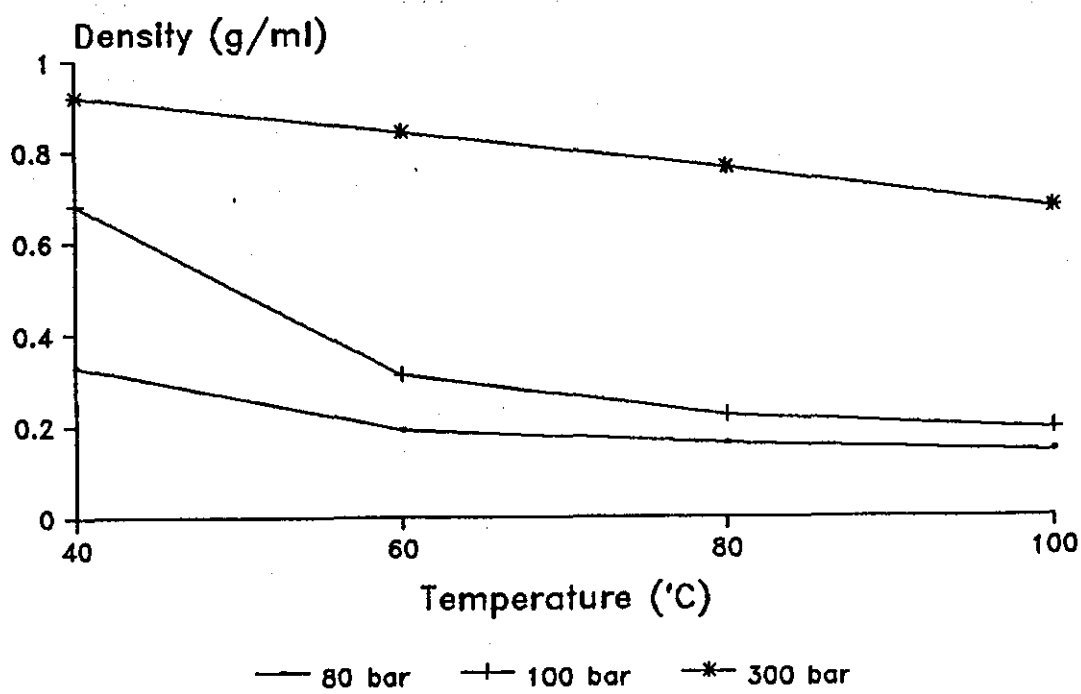
For the more polar essential oils, carvone and santonin, temperature appears to have little effect on the retention of the solutes. This may be because at the very high pressures required to elute the solutes, the effect

on density with temperature is minimal (Figure 10.5). Furthermore, the densities at these high pressures are so high that any slight decrease will have little effect on the solubility of the oils, which have been shown to be very soluble in CO_2 at these densities [227].

TABLE 10.6. Variation in capacity factors with on a silica (Spherisorb 5 µm 4.6 x 250 mm) column. SFC conditions:- Limonene 80 bar, 1.5 ml/min CO_2; Caryophyllene 100 bar, 1.5 ml/min CO_2; Carvone 300 bar, 2.5 ml/min CO_2; Santonin 300 bar, 2.5 ml/min CO_2, 8% MeOH; Quercetin 300 bar, 2.5 ml/min CO_2 20% MeOH.

Solute	Capacity factor (k')			
	40°C	60°C	80°C	100°C
Limonene	1.14	4.87	4.21	3.10
Caryophyllene	0.78	1.81	9.3	-
Carvone	13.00	13.41	14.78	-
Santonin	4.85	5.01	5.75	-
Quercetin	2.86	3.13	12.65	-

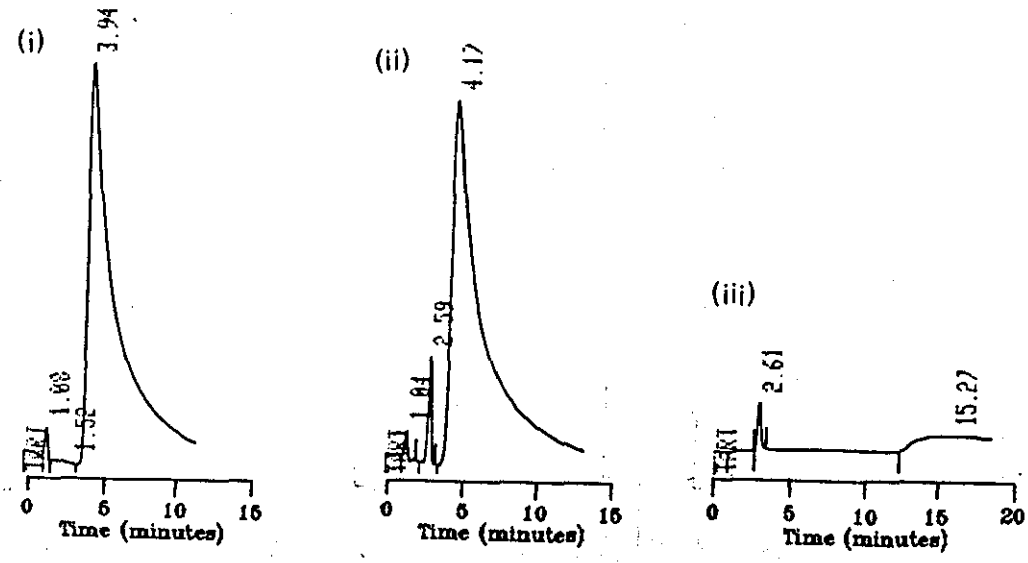
FIGURE 10.5. Density profile of CO_2 at the SFC conditions used in Figure 10.6.



For the most polar analyte, quercetin, temperature appeared to have a major influence on the retention of the solute (Table 10.6). Between 40 and 60°C there was little variation with retention time. However, at 80°C the peak shape of the solute drastically changed with a corresponding increase in retention time (Figure 10.6). Initially, as temperature has a minimal

effect on density at these SFC conditions (Figure 10.5), it would appear as if the analyte had undergone some sort of degradation process. But quercetin is an aglycone flavonol which is thermally stable and very unlikely to degrade under these circumstances. Thus at present the exact causes are still unclear.

FIGURE 10.6. Peak shape of quercetin at different temperatures, {i} 40°C, {ii} 60°C and {iii} 80°C. SFC conditions given in Table 10.6. UV detection at 220 nm (AUFS 1.28)



10.7. Evaluating SFC in predicting SFE conditions

To assess the ability of chromatographic data to predict the extraction conditions, the optimum SFE conditions for the major types of essential oils identified by GC-MS in feverfew are compared with the SFC conditions required for the solutes on the various stationary phases (Table 10.2 and 10.7).

TABLE 10.7. Optimum extraction conditions for the major groups of essential oils in feverfew. i = predicted from cellulose model as no sesquiterpene hydrocarbons were identified by GC-MS in feverfew.

Essential oil	Optimum extraction conditions
Hydrocarbon monoterpene (camphene)	120 bar, 40°C, 0.8 ml/min CO_2
Oxygenated monoterpene (camphor)	120 bar, 40°C, 0.8 ml/min CO_2
Hydrocarbon sesquiterpene[1]	120 bar, 40°C, 0.8 ml/min CO_2
Oxygenated sesquiterpene (parthenolide)	250 bar, 40°C, 0.8 ml/min CO_2 + 6% acetonitrile

By examining the SFC data general trends may be ascertained. The polymer PLRP-S stationary phase appeared to retain the non to medium-polar analytes (limonene, caryophyllene and carvone) to a similar degree to those present in the plant matrix. The ODS column was generally more retentive and the silica column less retentive than the plant matrix, though both could still be used as a rough indication of potential SFE conditions. The sesquiterpene lactone proved much more difficult to predict as it is thought to possibly exist in two "forms" in the feverfew plant material (Chapter 9). About 50% of the major sesquiterpene (parthenolide) present in feverfew could be removed at low pressures (120 bar) with supercritical CO_2. To remove the remaining "bound" parthenolide a modifier was required (250 bar, 6% acetonitrile). The silica column appeared to closely represent the possible "bound" parthenolide in feverfew. However, in the plant matrix methanol failed to increase the extraction yield, with the more polar acetonitrile modifier being required. None of the stationary phases could represent the easily extracted parthenolide found present in feverfew. It would therefore appear as if SFC could be used as a rough guide to ascertaining SFE conditions, the silica based columns giving the best overall representation of the plant material.

Additional similarities between SFC and SFE were also established. Mobile phase modifiers are well known as a major influence in chromatographic retention. An increase in modifier concentration decreased the k' values of the sesquiterpenes lactones on the silica based columns. This supports the increase in extraction efficiency obtained with increased modifier in the supercritical CO_2 solvent used on feverfew (Chapter 9.). Furthermore, the presence of a polar modifier had a limited influence on the k' values of the non-polar analytes, a similar trend being seen in SFE with modifiers having little effect on increasing the extraction yields of the non-polar analytes from the plant material (Chapter 9).

10.8 Conclusions

The separation of essential oils and plant phenolics in SFC proved to be useful in correlating some of the underlining trends in SFE. The non-polar analytes could be quickly and efficiently analysed on a wide range of stationary phases in supercritical CO_2. However, the polar analytes were characterised by poor peak shapes and poor reproducibility on the silica based columns. The use of a polar modifier greatly improves the peak shape,

analysis time and reproducibility of the analytes. Temperature also had a dramatic effect on the retention of the analytes, in which either solubility or volatility dominated the retention mechanism.

In terms of chromatography the polymer column proved to be the most applicable in analysing essential oils in SFC, though packed column SFC lacked the resolution and robustness of the capillary GC method. For predicting the overall extraction conditions, the silica based columns appeared to most closely represent the plant matrix. However, the non-polar ODS stationary phase tended to over estimate the extraction conditions for the non-polar analytes, and conversely the polar silica column would underestimate the extraction conditions. Most of the stationary phases predicted the need of a modifier to extract the polar analytes, with the non-polar ODS stationary phase underestimating the percentage modifier required and the polar silica stationary phase overestimating the percentage. Thus none of the stationary phases were able to realistically represent the plant material, however, the results did give a rough indication of the densities required and whether a modifier would be suitable. It has been suggested that packed column SFC could be useful for semi-preparative fractionation for "group type" separation of the essential oils [269].

Therefore, if no prior knowledge of the analytes was available then SFC could quickly give a rough indication of the SFE conditions required. There are limitations to this as SFC may underestimate some of the more complex processes that can exist in the plant matrix, for example, the technique can not take into account the analyte that may be present as salts, sugars or even microencapsulated. However, the technique is of value due to the speed at which an analysis can be carried out, as SFC has a much shorter method development time than SFE.

CHAPTER 11

Identification of essential oil in Feverfew and its adulterants

11.0 Introduction

A study was carried out to identify the major terpenes and essential oils in feverfew and its reported adulterants. The results have been compared with previous reports for feverfew analysis by TLC [43], UV [27,57,239], IR [45,49,57,239], NMR [27,49,57] and GC-MS [27,49,57], to give a positive identification of the reported major active ingredient, parthenolide and some of the other constituents.

11.1 Prep-SFE of essential oil

Traditional methods for the isolation of essential oils from plant material are time consuming, require large amounts of solvent and extract a lot of extraneous material. Therefore prep-SFE was investigated as an alternative. Using a 40ml extraction vessel, authenticated feverfew material (10 g) from Chelsea Physic Gardens was extracted with supercritical CO_2 at 250 bar, 45°C and 0.85mls min^{-1}, to yield a clear yellow extract, which when analysed on capillary GC was shown to be rich in essential oils (Fig. 11.1).

Prep-TLC was used to obtain the parthenolide fraction (R_f = 0.41) which contained one major component (95%) that was tentatively identified as parthenolide by its GC retention time (See Figure 11.2). This identification was confirmed by spectroscopic studies (Section 11.2). By using a solution of the parthenolide standard to construct a calibration graph, it was estimated that the extract contained 0.15% of parthenolide by dry weight of the plant. This is 68% of the amount present in the original SFE extract (Figure 11.1), so that sample loss had occurred. By using conventional solvent multi-step extraction and liquid chromatography, yields of 0.40% [27], 0.25% [34] and 0.06% [239] by dry weight of the plant, have been obtained. However the SFE method is much quicker, taking only 45 minutes instead of 3 to 4 hours.

The extraction method has been improved by using an on line SFE/silica trap (Section 9.9). This is by far the quickest extraction method and has the lowest sample loss, with 0.18% parthenolide by weight of the plant being isolated. However, the extract only contains 80% parthenolide (Figure 11.3), this is too low to be used in spectroscopic studies.

FIGURE 11.1. Temperature-programmed GC separation of prep-SFE extract of feverfew on a non polar (BP1) column. Temperature 60 to 300°C at 8°C min^{-1}, then isothermal at 300°C for 8 minutes. See Table 11.4 for peak identification (Parthenolide = peak 14).

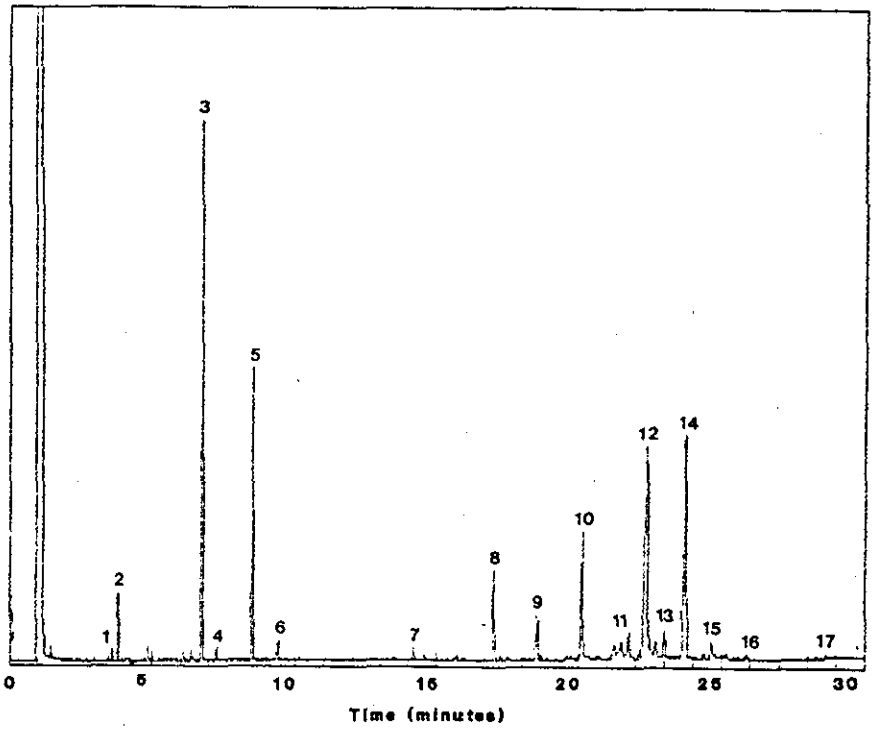


FIGURE 11.2. Temperature-programme GC separation of prep-SFE, prep-TLC parthenolide fraction from feverfew (column conditions Figure 11.1). Parthenolide = 14.

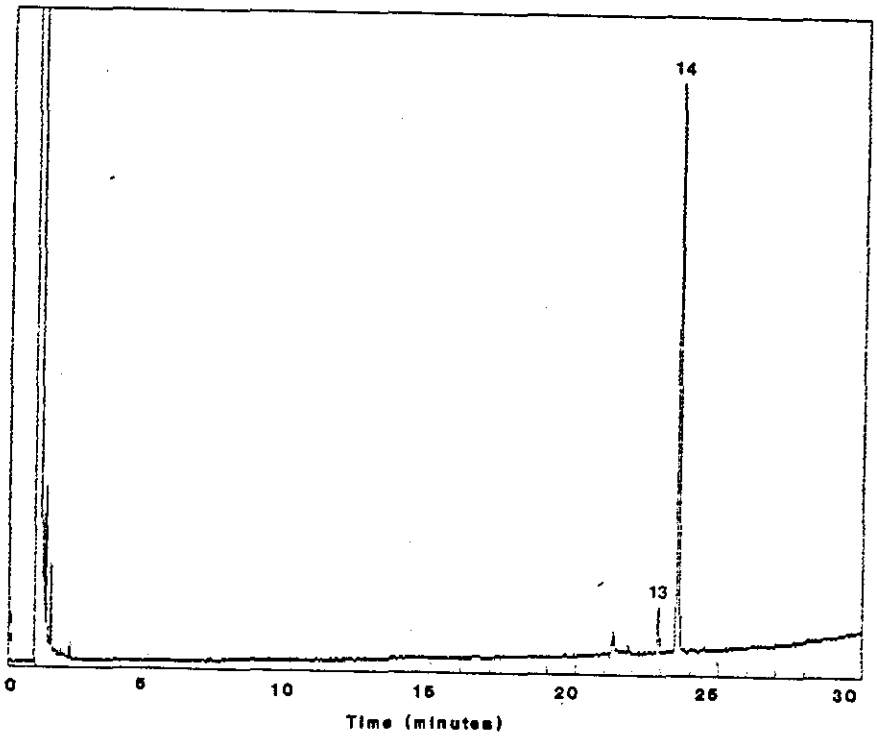
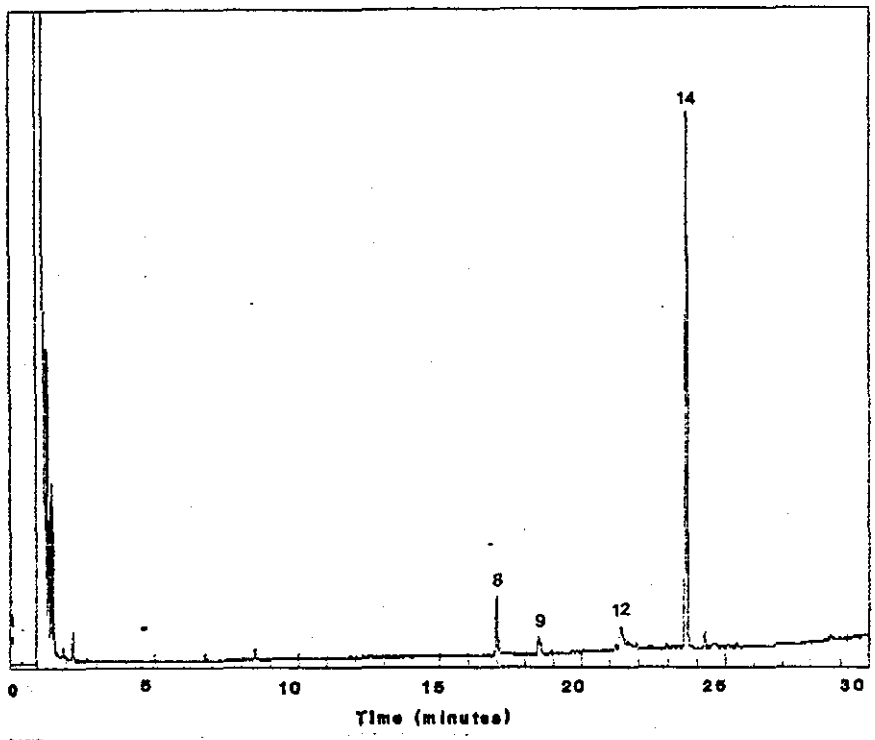


FIGURE 11.3. Temperature-programme GC separation of on line prep-SFE/silica trap parthenolide fraction from feverfew (column conditions Figure 11.1). Parthenolide = 14.



11.2 Identification of parthenolide

11.2.1 Ultraviolet analysis

The ultraviolet (UV) absorption spectra of plant constituents can aid in class identification. The majority of sesquiterpene lactones have a weak chromophore, xanthanolides and pseudoguaianolides absorbing at 215nm [233] and 220nm [268] respectively.

Using HPLC-diode array detector, the parthenolide standard was seen to possessed a λ_{max} at 218 nm (log ϵ 2.76). An identical spectrum was found for the parthenolide sample extracted from feverfew. These values are in close agreement with those cited in the literature for parthenolide at 214

nm (log ∈ 4.22) [57] or 225 nm (log ∈ 3.53) [239], although the molar absorptivity values are lower. This is possibly due to the HPLC-diode array system being able to separate the parthenolide from any trace impurities of plant pigments.

11.2.2 Infrared analysis

The infrared (IR) spectra are often used to assign a plant compound to a chemical class, and even to contribute to structural elucidation. The region in the IR spectrum above 1200 cm^{-1} contains spectral bands due to the vibrations of individual bonds or functional groups. Below 1200 cm^{-1}, most bands are due to the vibration of the whole molecule and because of its complexity, it is known as the "fingerprint" region. This region is frequently used in phytochemical studies for comparing an experimental with a standard sample [107].

The infrared spectra of sesquiterpene lactones are characterised by sharp, very distinctive absorption bands at 1770-1750 cm^{-1} (>C=O γ-lactone) [45,57,239], easily distinguishable from the background of the spectrum, even in crude mixtures [45]. The IR spectrum of parthenolide has been reported to have absorption bands at 1754 and 1650 cm^{-1} (conjugated γ-lactone) [57], 1250 cm^{-1} (-C-O-C-) [27], 1144 cm^{-1} (->C-O- of γ-lactone) [239] and 880 cm^{-1} (exocyclic $=CH_2$) with no adsorption in the region about 3500 cm^{-1} [57], indicating the absence of a hydroxyl function group. A comprehensive list of the absorption bands in the parthenolide IR spectrum was given by Govindachari *et al.* [57].

The standard parthenolide and the extracted sample (Figure 11.4), possessed IR spectrum that were indistinguishable and corresponded very closely to the reported spectra. This would suggest that the component isolated from feverfew is parthenolide.

FIGURE 11.4. Infrared spectrum of the extracted parthenolide in a KBr disc. Numerical values for absorption bands given in Table 11.1.

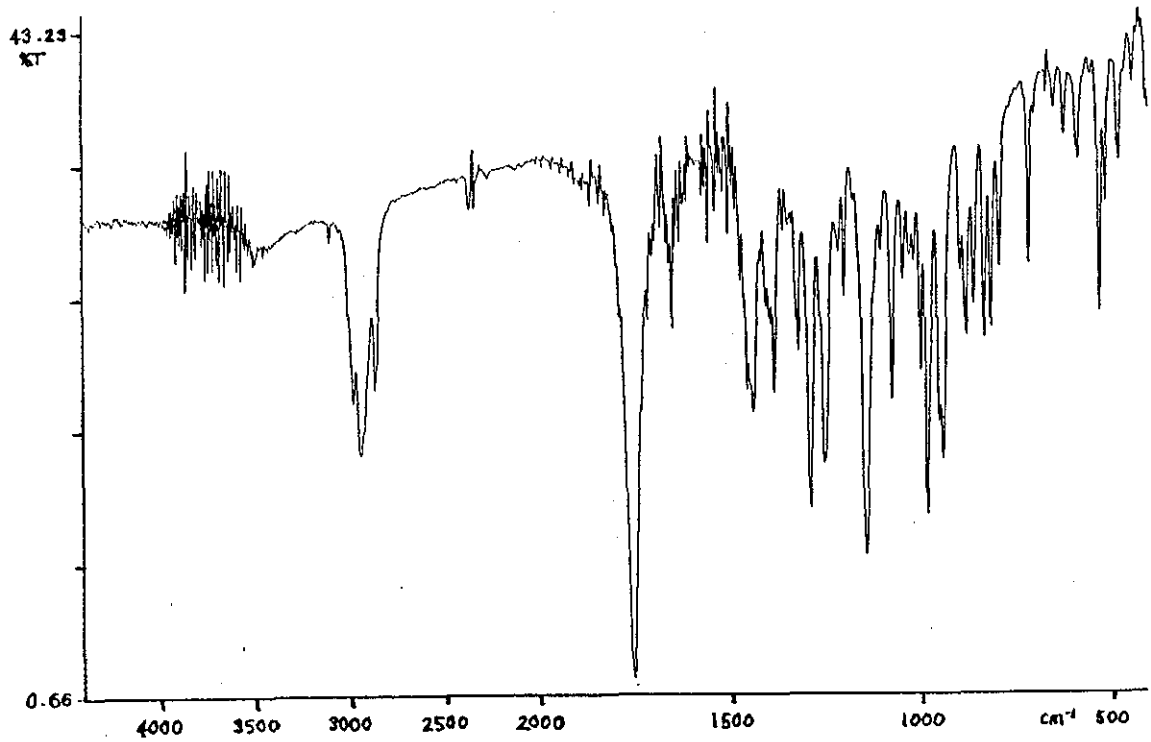


TABLE 11.1. IR spectrum of extracted parthenolide in a KBr disc.

IR Absorption (cm^{-1}), Transmittance (%)

cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%
3904	28	3854	25	3840	28	3821	28
3807	28	3751	26	3736	27	3712	27
3690	28	3676	26	3650	25	3630	27
3588	27	3568	27	2934	16	2862	20
1754	1	1685	30	1654	23	1636	29
1560	28	1542	31	1508	29	1441	18
1387	19	1322	22	1290	12	1255	15
1201	26	1144	9	1076	19	1047	27
1000	21	983	12	940	15	880	23
860	25	833	23	815	23	793	27
716	27	586	34	529	24	514	32
79	34						

11.2.3 Nuclear magnetic resonance spectroscopy

The common structural groups in typical sesquiterpene lactones, give rise to characteristic nuclear magnetic resonance (NMR) spectral features [294-297]. The most distinctive is the presence of two sets of doublets (2H) between δ5.0 and δ6.5, indicative of an exocyclic methylene group. Allylic coupling occurs between the two C-13 methylene hydrogens and the C-7 hydrogen (Figure 11.5 {i} and {ii}), for all sesquiterpenes containing either a C-6 or C-8 α,β-unsaturated γ-lactone [27].

The proton NMR of parthenolide has been reported by a number of workers [27,49,57]. The most extensive given in Jessup's PhD thesis [27]. Bohlmann *et al.* investigated a range of sesquiterpene lactones in feverfew, but only quoted the spectrum for 3β-hydroxyparthenolide. They claimed the spectrum of parthenolide and its hydroxyl derivative were very similar, with the obvious exception of the C-3 proton. The spectra obtained for the standard and extracted parthenolide are indistinguishable (Table 11.2 and Figure 11.5 {i}) and compare closely with those in the literature.

Table 11.2. The spectra of the extracted parthenolide (Figure 11.5) compared to the standard sample and the literature values (δ, $CDCl_3$).

Hydrogen on carbon no.	Chemical shifts (ppm) Standard parthenolide	Extracted parthenolide[a]	Jessup [27]	Bohlmann* *et al.*
1	5.22	5.19	5.23	5.11
2	2.37	2.37	N/A	2.42
3	2.19	2.19	N/A	-
5	2.80	2.78	2.81	2.79
6	3.86	3.86	3.87	3.91
7	2.76	2.76	2.81	2.73
8	1.56	1.58	N/A	1.63
9	2.19	2.19	N/A	2.13
13a	5.62	5.62	5.64	5.49
13b	6.34	6.34	6.35	6.24
14	1.71	1.71	1.73	1.71
15	1.30	1.30	1.32	1.29

* Note the Bohlmann *et al.* [49] spectrum data is for 3β-hydroxyparthenolide. N/A = no assignment given.

a = Figure 11.5 {i}.

FIGURE 11.5 (i). Proton NMR spectrum of extracted parthenolide.

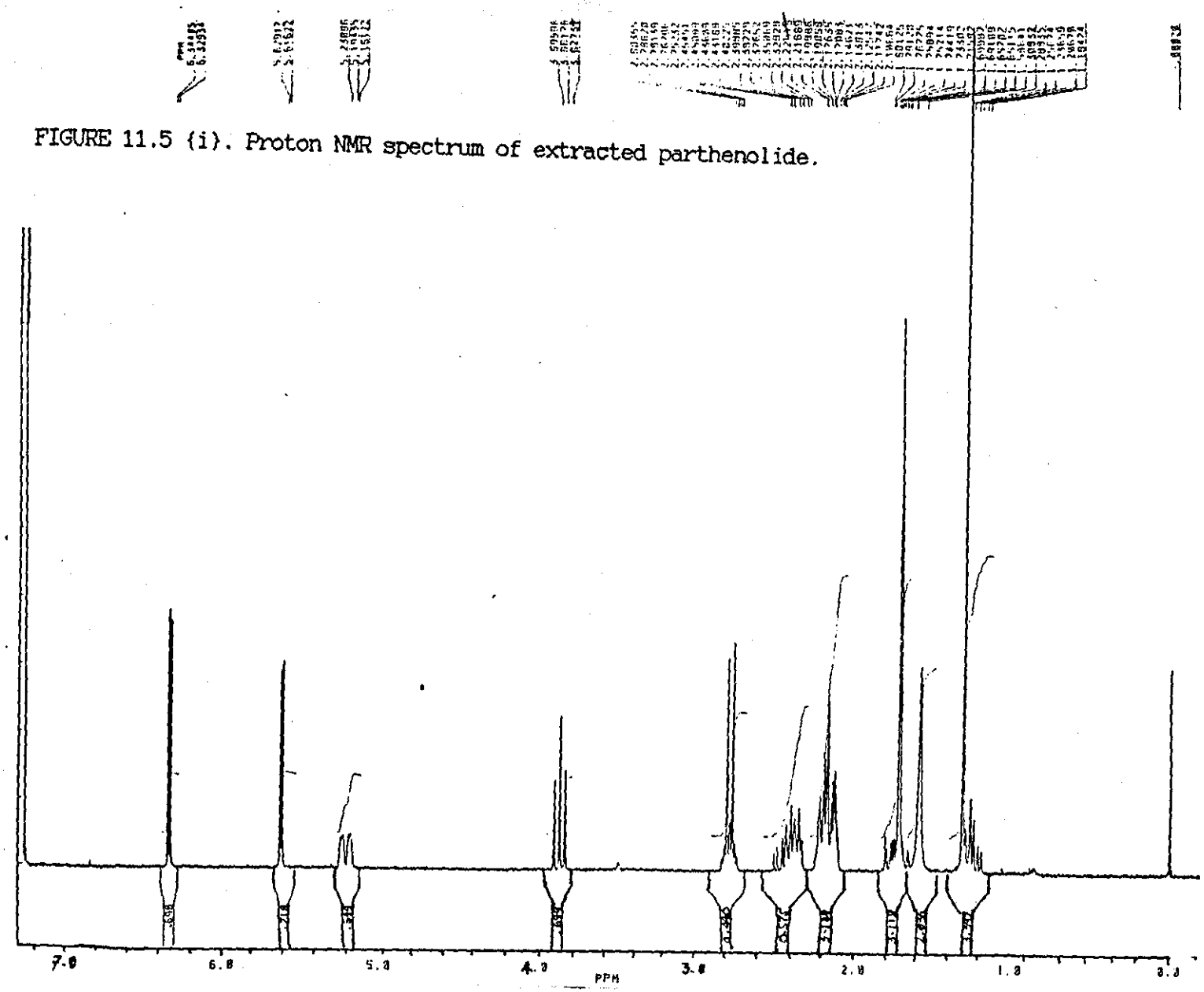
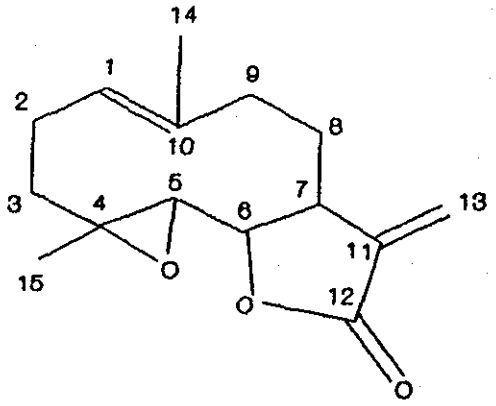


FIGURE 11.5 {ii}. Structure of parthenolide.



^{13}C NMR spectroscopy is now also routinely used to elucidate structural information. Accordingly the ^{13}C NMR spectra of the parthenolide samples were obtained and compared with that in the literature [27,52]. The decoupled ^{13}C NMR spectrum of the extracted parthenolide in $CDCL_3$ solution showed fifteen clear signals. The signals were assigned with reference to Jessup's findings [27] (Table 11.3). The ^{13}C NMR spectrum of parthenolide is seen in Figure 11.6. The results demonstrate that all four spectra are indistinguishable.

TABLE 11.3. ^{13}C NMR data for parthenolide (δ, $CDCl_3$)

		Chemical shift			
Carbon no. [27]	Standard parthenolide	Extracted parthenolide[a]	Ref. [27]	Carbon no. [52]	Ref. [52]
14 or 15	16.97	16.98	16.97	15	17.0
15 or 14	17.29	17.31	17.24	14	17.3
8 or 3	24.16	24.16	24.11	2	24.2
3 or 8	30.68	30.71	30.64	8	30.2
9 or 2	36.38	36.42	36.35	3	36.5
2 or 9	41.24	41.27	41.19	9	41.2
7	47.71	47.74	47.65	7	47.7
5	61.52	61.53	61.45	4	61.5
4	66.42	66.44	66.36	5	66.4
6	82.45	82.47	82.38	6	82.5
1	121.23	121.21	121.08	13	21.0
13	125.31	125.34	125.24	1	125.3
10	134.59	134.62	134.51	10	134.7
11	139.26	139.30	139.21	11	139.3
12	169.25	169.24	–	12	169.3

a = Figure 11.6

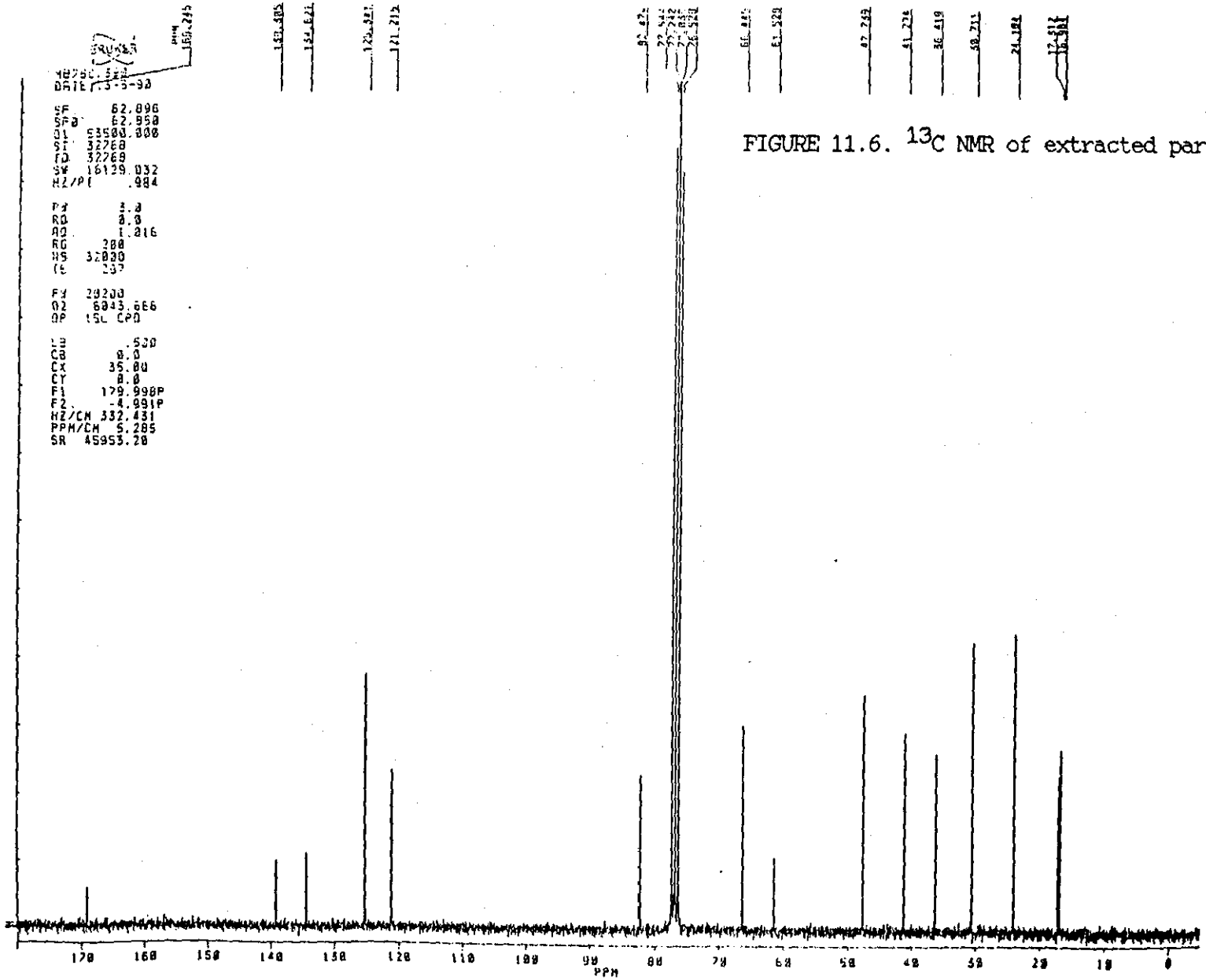


FIGURE 11.6. ^{13}C NMR of extracted parthenolide.

11.3 Gas chromatography - mass spectrometry analysis

Gas chromatography - mass spectrometry (GC-MS) is a very powerful technique in the identification of terpenes [298-304]. Unlike UV, IR and NMR techniques, GC-MS requires only microgram amounts of sample and is readily amenable to a mixture of components. However, the high injection temperatures usually employed in GC (above 120-150 °C), may dehydrate compounds like linalool [262], camphene hydrate [263], or α-terpineol [264]. All the major components in feverfew have been assigned a mass spectrum and where possible an identification has been given. Compound identification in MS is either by interpretation from the theory and from the rules for the fragmentation of organic compounds, or by empirical spectrum matching. The former technique is slow and tedious and generally requires considerable experience, the latter involves searching manually or via a computerised data base through a limited number of terpene standards.

A major problem in terpene analysis relates to the very large number of possible isomers within each group of mono- and sesquiterpenes. This makes it difficult to distinguish between the similar spectrum obtained [305]. Compounds sharing the same skeletal structure and differing only in the position of a double bond often display entirely different spectra, such as γ- and δ-cadinene [306]. Because of the limited number of reference standards and reference mass spectra, considerable difficulty was encountered in trying to identify the higher molecular weight terpenes, particularly the sesquiterpenes and oxygenated sesquiterpenes. Therefore the total number of identified components is less than those reported in the literature [27,49,57].

11.3.1 GC-MS separation of the terpenes in Feverfew

Attempts were made to identify as many constituents as possible in feverfew, by mass spectral analysis. The GS-MS analysis was carried out under comparable conditions to the GC study carried out in Section 11.2. The total ion scan of a GC-MS separation showed good resolution and matched very closely with the chromatogram obtained from a TPGC analysis in Figure 11.1.

In low molecular weight compounds (MW below about 154), a parent ion is usually observed in the EI spectra. This enables the molecular weight of the individual compounds to be determined. Terpenes can be grouped according to their respective molecular weights, for example an observed parent ion of m/z

136, corresponds to a molecular formula of $C_{10}H_{16}$. In higher molecular weight compounds (MW above about 154) the parent peak becomes very weak or absent and additional information is required via CI data. However, even this has its limitations and not all the components could be assigned a molecular weight.

To obtain a useful identification, extra information was required besides the molecular weight. The information sought came from authenticated mass spectra, fragmentation patterns, retention times of authenticated standards and the retention data of the GC-MS peaks. Several compounds have been successfully identified by comparing the GC-MS spectra with available reference mass spectra [299,305-308]. Five of the components had indistinguishable mass spectra to those found in tansy. Dihydroparthenolide has been identified from its fragmentation pattern (Table 11.4). This compound possesses a mass spectrum which is closely related to that of the parthenolide (Figure 11.7 {i} and {ii}). Most of the major fragmentation peaks have m/z values +2 higher than those found in the parthenolide mass spectrum. Dihydroparthenolide is known [57,239], but has only been obtained from feverfew by the catalytic hydrogenation of parthenolide. However, it has been reported as a constituent of *Michelia compressa* [55]. It was also found at 3% in the standard parthenolide sample.

It was noticed that differences sometimes occurred in mass spectral fragmentation pattern of a compound depending on the operating conditions. This was observed among some of the available reference mass spectra taken from different literature sources. Nonetheless, the mass spectrometric method has proved to be a valuable assistant in the identification of the components. The assignments given to the GC-MS peaks are given in Table 11.4 and the mass spectra of the extracted parthenolide and dihydroparthenolide are given in Figure 11.7 {i} and {ii} and the corresponding Table 11.5 {i} and {ii}.

TABLE 11.4. The identities of the major peaks in the TPGC separation of the SFE extract of feverfew. Peak number corresponds to peaks in TPGC traces (Figure 11.1).

Peak number	% Relative abundance	Compound	Reference mass spectra
1	0.4	α-pinene*	a,b,c
2	2.1	campene (T)	c
3	20.9	camphor* (T)	a,b,c
4	0.4	borneol (T)	a,b,c
5	10.4	pinene acetate (T)	d
6	0.8	bornyl acetate	a
7	0.4	$C_{10}H_{16}$	–
8	3.9	M.W. 144	–
9	3.1	M.W. 200	–
10	0.8	M.W. 256 (T)	–
11	6.5	M.W. 278	–
12	16.8	dihydroparthenolide	e
13	1.0	M.W. 259	–
14	17.9	parthenolide	d,f
15	0.3	M.W. 284	–
16	0.8	tetracontane 3,5,24-methyl. $C_{43}H_{88}$	d
17	0.2	pentatriacontane	d

Mass spectra references:
a = Ref. [299].
b = Ref. [307].
c = Ref. [308].
d = National Bureau of Standards (NBS) library/computer search.
e = Fragmentation pattern.
f = Ref. [27].

(T) = component also found in Tansy
* = confirmed by comparison with retention time of standard.

TABLE 11.5 {i}. CI mass spectra data from dihydroparthenolide (peak 12) and parthenolide (peak 14). The results are expressed as the ten largest peaks in each mass spectrum.

Peak no.12: m/z (% rel. int.) 72 (100), 98 (92), 84 (88), 96 (84), 70 (77), 86 (76), 97 (76), 233 (66), 112 (62), 268 (59).
Peak no. 14 m/z (% rel. int.) 231 (100), 233 (75), 266 (68), 268 (62), 95 (32), 249 (28), 81 (25), 235 (24), 84 (24), 251 (24).

TABLE 11.5 {ii}. Mass spectra data from the major components in feverfew. Results expressed as ten largest peaks in each mass spectrum.

Peak no. 1: m/z (% rel. int.), 93 (100), 91 (43), 92 (39), 77 (26), 41 (22), 79 (18), 43 (12), 121 (12),105 (9), 136 (8).

Peak no. 2: m/z (% rel. int.) 93 (100), 121 (70), 41 (37), 79 (36), 107 (28), 91 (28), 39 (28), 67 (26), 77 (20), 136 (18).

Peak no. 3: m/z (% rel. int.) 95 (100), 41 (67), 81 (65), 108 (45), 69 (34), 152 (33), 55 (32), 109 (30), 40 (30), 83 (28).

Peak no. 4: m/z (% rel. int.) 95 (100), 41 (23), 110 (22), 43 (15), 93 (12), 55 (11), 39 (11). A weak spectrum.

Peak no. 5: m/z (% rel. int.) 43 (100), 119 (71), 134 (25), 109 (22), 91 (20), 81 (16), 121 (15), 93 (13), 80 (11), 107 (10).

Peak no. 6: m/z (% rel. int.) 95 (100), 43 (91), 93 (49), 41 (49), 121 (45), 136 (32), 108 (25), 55 (22), 109 (22), 69 (19).

Peak no. 7 m/z (% rel. int.) 83 (100), 55 (43), 109 (28), 41 (22), 93 (13), 136 (13), 43 (11), 95 (10), 69 (10), 108 (9).

Peak no. 8: m/z (% rel. int.) 43 (100), 108 (41), 41 (30), 95 (27), 93 (25), 126 (19), 55 (15), 111 (15), 71 (13), 79 (13).

Peak no. 9: m/z (% rel. int.) 200 (100), 115 (62), 128 (47), 50 (41), 55 (36), 76 (31), 157 (31), 102 (30), 129 (30), 170 (25).

Peak no. 10: m/z (% rel. int.) 43 (100), 41 (96), 73 (63), 149 (60), 55 (54), 57 (50), 60 (50), 129 (29), 71 (23), 69 (22).

Peak no. 11: m/z (% rel. int.) 41 (100), 79 (72), 43 (71), 55 (61), 67 (58), 95 (50), 81 (47), 93 (39), 80 (35), 108 (32).

Peak no. 12: m/z (% rel. int.) 43 (100), 41 (63), 55 (48), 95 (32), 119 (28), 81 (27), 107 (22), 67 (22), 93 (20), 133 (18).

Peak no. 13: m/z (% rel. int.) 43 (100), 41 (86), 57 (56), 185 (56), 129 (50), 112 (40), 157 (36), 55 (26), 259 (20), 139 (16).

Peak no. 14: m/z (% rel. int.) 43 (100), 41 (64), 53 (53), 55 (32), 95 (28), 81 (27), 91 (23), 67 (22), 93 (21), 105 (19).

Peak no. 15: m/z (% rel int.) 43 (100), 41 (33), 111 (18), 55 (17), 95 (16), 53 (15), 81 (12), 109 (10), 67 (8), 91 (7), 79 (7).

Peak no. 16: m/z (% rel. int.) 57 (100), 43 (92), 71 (63), 85 (41), 55 (35), 69 (22), 83 (21), 97 (19), 56 (15), 70 (12).

Peak no. 17: m/z (% rel. int.) 57 (100), 43 (83), 71 (68), 85 (46), 55 (28), 41 (25), 69 (17), 83 (15), 56 (14), 44 (14).

FIGURE 11.7 {i}. EI mass spectrum of extracted parthenolide.

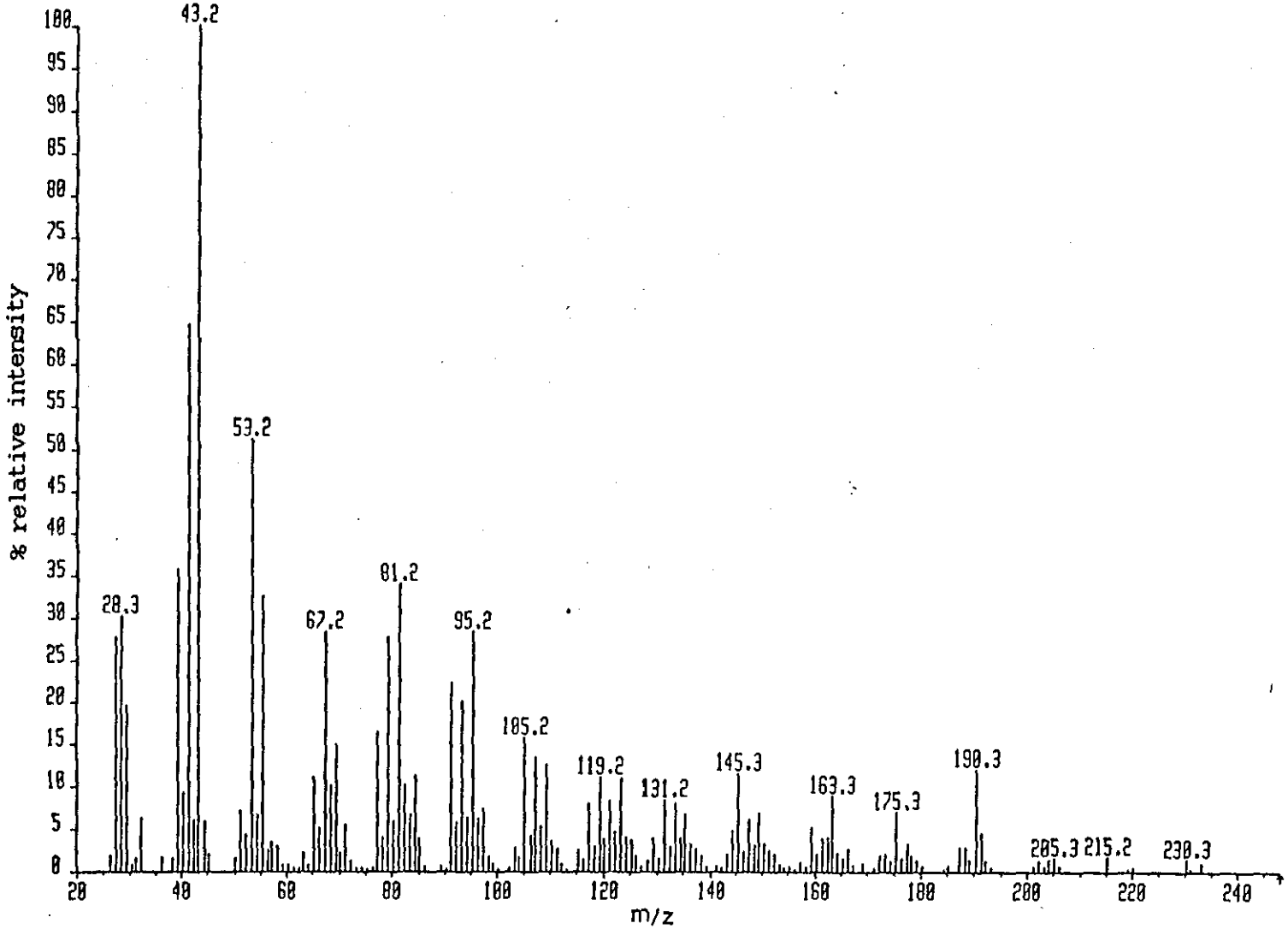
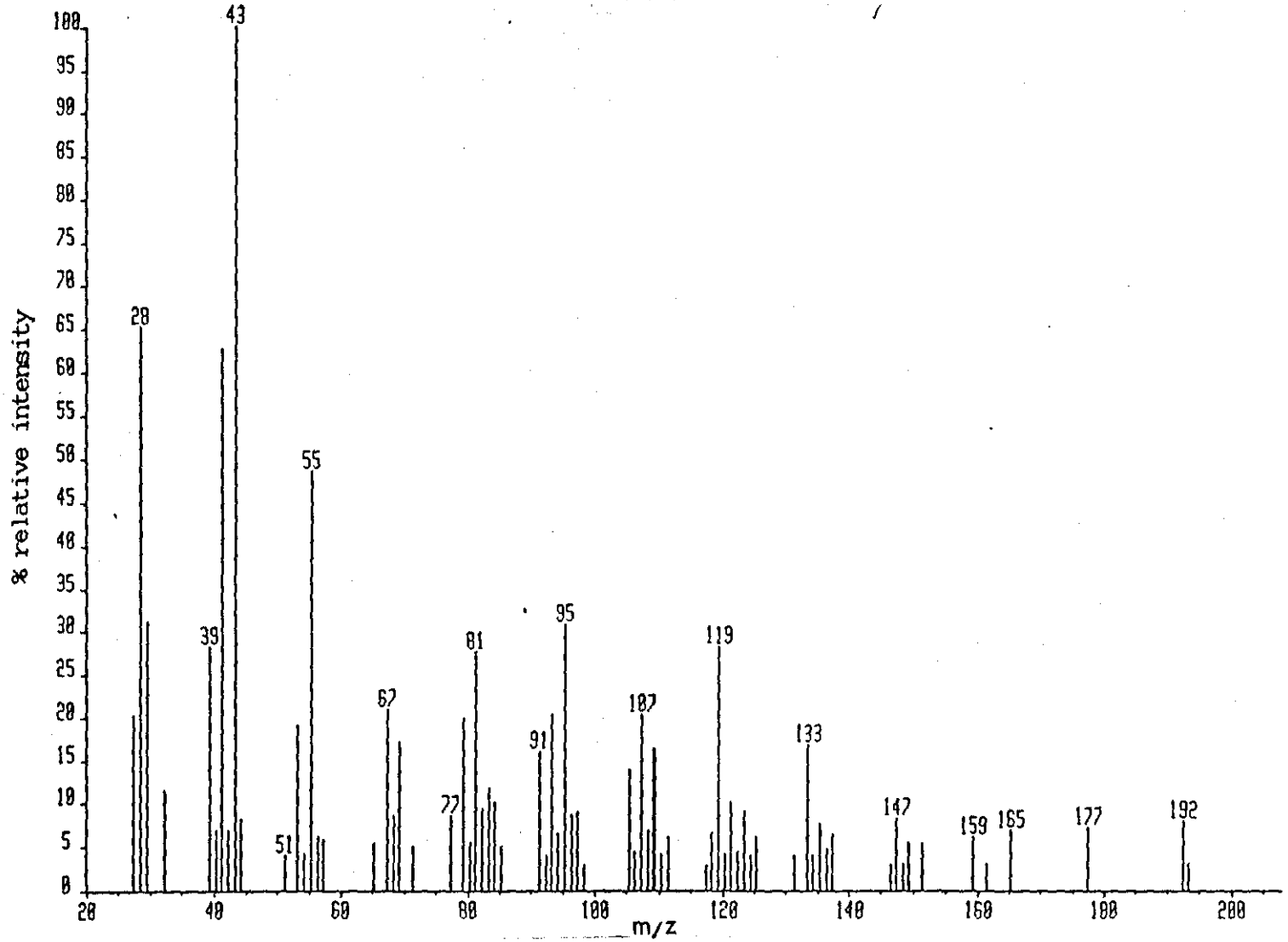


FIGURE 11.7 (ii) EI mass spectrum of extracted dihydroparthenolide.



11.4 GC analysis of terpenes in feverfew varieties.

The essential oil content of samples of the major varieties of feverfew were investigated in a trial study. Analytical scale SFE was used to obtain the essential oils, using 0.4 grams of dried plant material (containing flowers and leaves) stored at ambient temperature in a cupboard for two years. The same extraction conditions were used as described in Section 11.1. All the varieties studied were found to contain parthenolide, though inter-variety variations existed (Table 11.6). Similar variations in the sesquiterpene lactone content between varieties of feverfew and plants harvested in different seasons, have been observed by Hylands (unpublished findings). The varieties demonstrate a quantitative rather than qualitative inter-variation, a "typical" essential oil distribution being seen in Figure 11.8 (ii). Components 3, 4, 6 or 9 are absent from a few of the varieties, this may be due to the age of the plants.

TABLE 11.6. Percentage parthenolide present in common "main-line" feverfew and the feverfew varieties. Dried plant material two years old.

Feverfew variety	% parthenolide in dried plant material
Main-line feverfew (Schultz Bip.)	0.13%
Balkan Peninsula	0.13%
Schneeball	0.12%
Golden Veis	0.05%
Boule de Neige	0.05%
Flore Pleno	0.01%

According to the literature, the sesquiterpene lactones are situated in the glandular and covering trichones on the underside of the leaves [34]. Therefore the leaves and flowering heads were analysed separately for their parthenolide content. Parthenolide was found to be present in both parts of the plant, in about equal concentrations (Table 11.7). The essential oil distribution in these components were indistinguishable, both corresponding to Figure 11.8 (i) in appearance. This would suggest that parthenolide is not present solely in the trichones and that to get a representative sample of feverfew, the percentage of leaves and flowers may not necessary need to be known.

TABLE 11.7. Percentage parthenolide present in different parts of the plant. Dried plant material two years old.

Feverfew	% parthenolide in dried plant material
Feverfew grown from Johnson seeds' (leaves only).	0.26
Feverfew grown from Johnson seeds' (flowers only)	0.21
Feverfew grown from Fison seeds' (leaves only)	0.18
Feverfew grown from Fison seeds' (flowers only)	0.16

A number of feverfew plants were cultivated from seeds supplied by Johnson, Fisons, and Chambers. These plants containing leaves and flowers, were indistinguishable from the authenticated feverfew varieties, containing the same qualitative distribution of essential oil but being in higher concentrations (Table 11.8). It was not possible from the essential oil content of the plants, to determine which variety of feverfew the seeds had originated from.

TABLE 11.8. Percentage parthenolide present in homegrown feverfew. Dried plant material two years old.

Feverfew	% parthenolide in dried plant material
Feverfew grown from Fison seeds'	0.15
Feverfew grown from Chamber seeds'	0.18
Feverfew grown from Johnson seeds'	0.21

The reproducibility of the major extracted components from replicate SFE analysis proved quite complicated. A large intra-variety variation in the parthenolide content of freshly dried feverfew plants was discovered. Three samples were taken from the same plant grown from Fison seeds' and examined by

SFE off-line and GC analysis. This gave a relative standard deviation (RSD) for parthenolide of 14%. This RSD includes the possible errors associated with weighing the sample, with collecting the extract, the GC analysis, and with chromatographic peak integration, as well as with any variation in analyte concentration resulting from the small samples used (0.4g). These results compare favourably with Hawthorne *et al.* who quoted a RSD of 6-17% for individual components in basil spice on an on-line SFE/GC system [309]. The variation between individual feverfew plants of the same variety, harvested at the same time of year can be very large, with RSDs of 60% being possible. This demonstrates the problems of obtaining credible results from natural products and stresses the need to investigate large numbers of plants to ensure a representative sample.

The distribution of the essential oils appears to be influenced by the age of the plant (see Figure 11.8 {ii}). As the plant ages the parthenolide appears to degrade and a series of degradation products 10,11 and 12 appear. These have been identified in Section 11.3.1 as components with M.W. 256, 278 and dihydroparthenolide respectively. The volatile components, such as camphor (3) and pinene acetate (5) decreased dramatically over two years of storage and disappeared alltogether after four years, when the majority of the components are degradation products (Figure 11.8 {iii}).

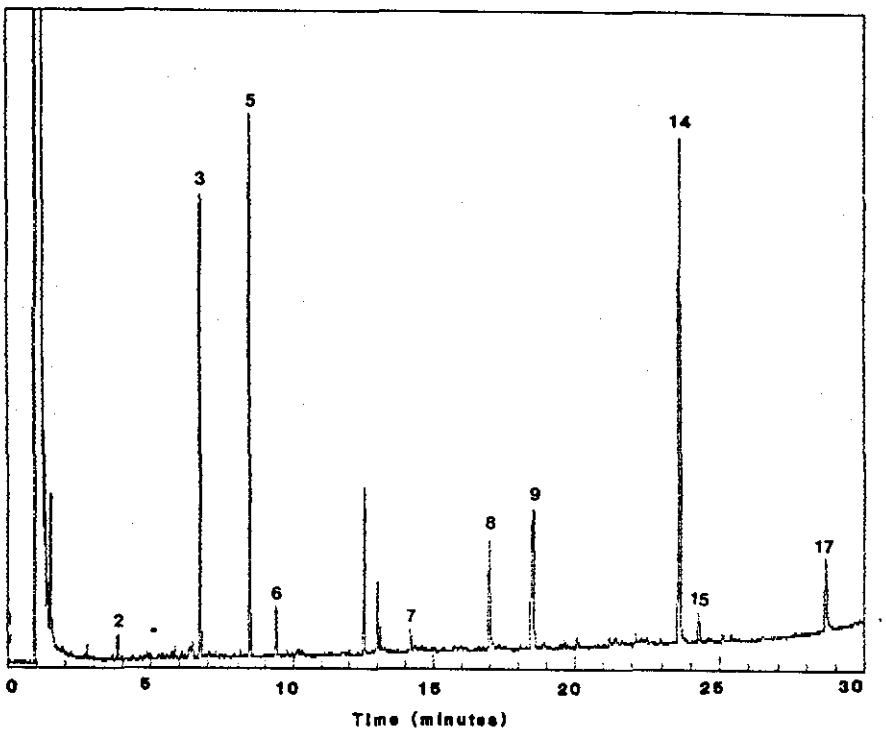
On average, between 0.1 and 0.5% parthenolide was obtained for fresh dried samples of feverfew. This value dropped to 0.04 - 0.21% after two years storage. This demonstrates one of the disadvantages of using natural products in the pharmaceutical industry, namely that they may have a short shelf life. Within two years about half of the parthenolide had degraded (see Table 11.9).

TABLE 11.9. Degradation of parthenolide in relation to the age of the plant.

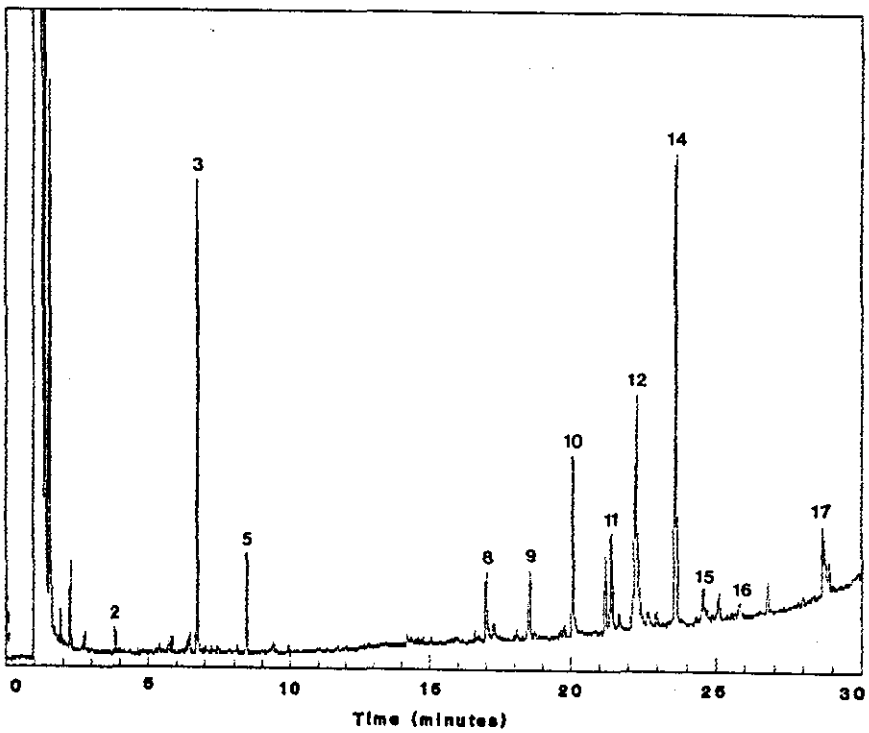
Age of dried plant	Origin of plant	% parthenolide in dried plant material[a]
One day	Fison seeds'	0.43, 0.32
One year	Fison seeds'	0.31
Two years	Fison seeds'	0.15, 0.18, 0.21
Over four years	British Analytical Control	0.002

a = SCF extractions taken from the same plant.

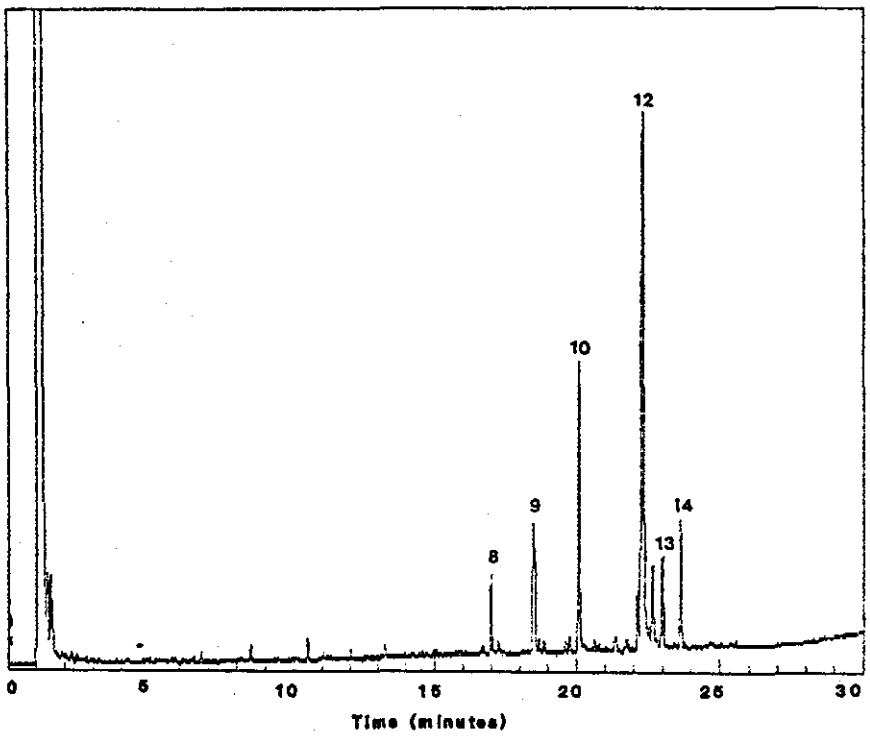
FIGURE 11.8. Changes in essential oils with the age of the plant.(i) Dried feverfew (Home grown from Fisons seeds), stored for one day (parthenolide = 14).



(ii)Dried feverfew (Home grown from Fisons seeds), stored for two years (parthenolide = 14).



(iii) Dried feverfew (British Analytical Control) stored for four years (parthenolide = 14).



Parthenolide has been shown previously to undergo oxidation to an diepoxide and to polymerise on storage [55,239]. It is also susceptible to photo-oxidation to give a peroxy terpene [52] and acid catalysed cross ring cyclisation to yield a eudesmanolide or guianolide [41] (Section 2.2.1). However, none of these reported or predicted degradation products were detected in the present feverfew studies.

11.5 GC analysis of terpenes in feverfew products

A number of feverfew products available through pharmacies and health shops, have been analysed for their parthenolide content (Table 11.10). The products were formulated as tablets, capsules or drops and could vary in the part of the plant used and the stated feverfew content. A number of the preparations did not refer to the correct botanical name of feverfew on their labels, or define the part of the plant used in their manufacture. Although it

TABLE 11.10. % parthenolide content in feverfew preparations available on the market.

Product (manufacture)	Content as stated on label	Recommended daily dose	% parthenolide based on labelled weight of plant material	Amount of parthenolide in each tablet
Feverfew capsules (Purefil)	100mg *Chrysanthemum tanacetum parthenium*	100mg	0.019	19µg
Feverfew tablets (FSC)	150mg *Chrysanthemum parthenium*	150mg	0.017	25µg
Barefoot feverfew tablets (Potter's)	200mg *Tanacetum parthenium*	200mg	0.013	26µg
Feverfew capsules (Power health)	200mg *Chrysanthemum tanacetum parthenium*	200-400mg	0.014	28µg
Feverfew tablets (Heath Heather)	25mg *Tanacetum parthenium*	25-50mg[a]	0.0	0µg
Feverfew tablets (Herbal Lab.)	125mg *Chrysanthemum (tanacetum) parthenium*	125mg[b]	0.014	18µg
Country collection feverfew capsules (Lifeplan products)	250mg *Chrysanthemum (tanacetum) parthenium*	250mg	0.0	0µg
Feverfew tablets (Seven Seas)	100mg *Tanacetum parthenium*	100mg[b]	0.08	80µg
Lomigran . Standardised feverfew capsule (R.P. SchererLtd.)	25mg *Tanacetum parthenium* containing 0.1mg active constituent	50mg[a]	0.0	0µg
Feverfew 6X homoepathic tablets (Nelson)	Feverfew	2 tablets a day	0.0	0µg
Feverfew drops (Nelson)	33% alcoholic tincture 1.5% *Pyrethrum parthenium*	5 drops twice a day	0.06mg/ml[c]	14µg[d]
Essence of feverfew (Gerard)	Tincture of feverfew 5:1	5 drops thrice a day	0.0	0µg

a = Product recommended to be taken with food.

b = Product recommended to be taken with liquid or water.

c = Value is parthenolide (mg) per ml of neat feverfew drops.

d = Amount of parthenolide in 10 drops of tincture.

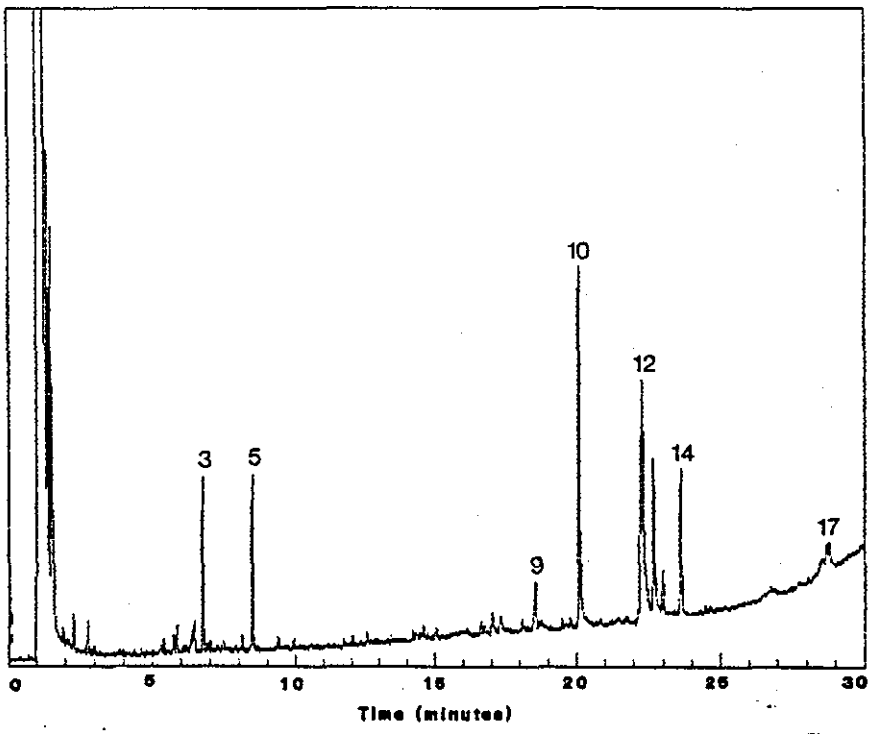
is the leaf which is recommended for the treatment of migraine, Lifeplan products state their feverfew capsules contain the whole dried herb. The daily dose recommended by the manufacturers varies from 25–400mg, the majority being above the recommended daily dose of 50mg used by Johnston *et al* [32] (Section 1.3). Only two of the products' labels suggest that the doses should be taken with food and none of the preparations contain the standard warning "Do not exceed stated dose". However, one product did display the "Keep out of the reach of children", though ironically it was the Nelson 6X homoeopathic feverfew tablets.

All the products yielded low levels or non-detectable levels of parthenolide. By studying the chromatograms obtained for the feverfew products (see Figure 11.9), it would appear that the majority of sesquiterpene lactones have degraded, the same degradation peaks (no. 5 and 7) being present as in the two year old feverfew sample (Figure 11.8 {ii}). From the low levels of parthenolide, it would appear that the majority of the products have passed their worthwhile shelf life. Based on the assumption that a minimum of 50mg of dried leaf material (about 0.15mg of active ingredient at 0.3% by dry weight) is required as a daily therapeutic dose, a 200mg feverfew tablet would roughly have a shelf life of about four years from when harvested, assuming a first order decay. Unfortunately, none of the feverfew products had an expiry date or stated their expected parthenolide content.

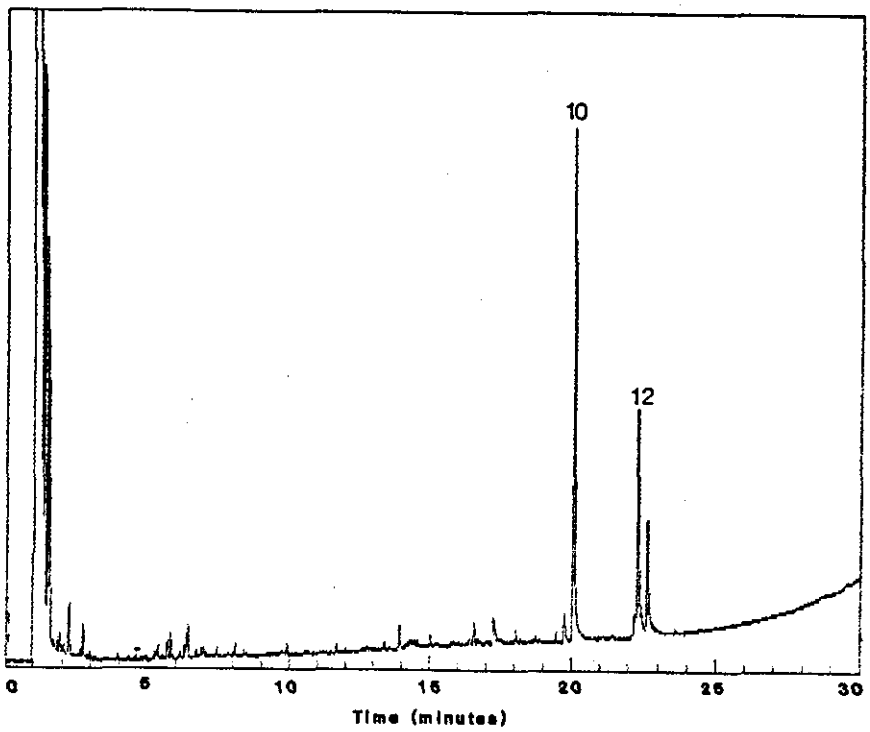
Variations in the amount of feverfew in four commercial products (based on their ability to inhibit platelet secretion) has already been reported [310]. The activity of the herbal preparations was found to be much lower than claimed. This is in agreement with the present study, dihydroparthenolide having been shown to have no cytotoxic activity [311]. Not surprisingly, the homoeopathic preparations contained no detectable activity [310], with no parthenolide found to be present in this study..

These results highlight the need for standardising commercial preparations of feverfew, as has been suggested by others [32,21]. However, this will be very hard to implement. Commercial preparations may contain the whole plant so that the active components, which is claimed to be mainly present in the leaf, will be "diluted" [34]. It is also possible that the active components are present in different amounts at different stages of plant growth. Finally there is the obvious problem of stability.

FIGURE 11.9. (i) Feverfew capsule (Purefil) contains 0.02% parthenolide (peak 14) by dry weight of plant.



(ii) Feverfew tablet (Heath and Heather) contains no detectable level of parthenolide.



11.6 GC-MS separation of the terpenes in Tansy

Two independent research groups claim that parthenolide is present in some of the tansy varieties [62,68], with one of the groups claiming that in *Tanacetum vulgaris* var. *crispum*, parthenolide is the main sesquiterpene lactone [68]. As parthenolide was initially thought to be unique to feverfew and hence a characteristic chemotaxonomic marker, this claim warranted an investigation of tansy by GC-MS.

Tansy was obtained from a variety of sources namely commercial herb suppliers and home grown. The plant was extracted and analysed under the same conditions as feverfew. There have been many publications on identifying the components in tansy by GC-MS [66,68,70-73,77]. It has therefore been possible to positively assign the major components within the plant extract. A list of the identifications is given in Table 11.11 and 11.12, with the corresponding chromatogram given in Figure 11.10 {i}.

Two chemotypes, thujone and camphor, were found in tansy. Tansy obtained from two commercial herb suppliers were of the thujone chemotype (thujone concentration 41-47% of oil extracted), while the home grown tansy was a camphor chemotype (48% of oil extracted). See figure 11.10 {i-iii}. These chemotypes can be described as "well defined" as the concentration of the major component lies within the range of 40-99% [67]. It is interesting to note that Finnish tansy are of a camphor chemotype, while plants from Central Europe and Canada are of a thujone type [12]. Tansy supplied by Cathy of Bournemouth Ltd originated from France, unfortunately the other samples were of unknown origin. The variation between the plants appears to be both quantitative and qualitative, with components such as peak numbers 8 and 9 being absent in home grown tansy (Figure 11.10 {iii}). Such qualitative variations have been found in an extensive survey undertaken by Sorsa *et al* [12].

The distribution of hydrocarbon and oxygenated monoterpenes in both feverfew and tansy, seem very similar, with a third of the components in feverfew being found in tansy. The major fingerprinting region would therefore appear to be determined by the sesquiterpene and sesquiterpene lactone constituents in the plant. This corresponds to peaks 9-12 in tansy (Figure 11.10 {i}). Sometimes peaks 10 and 11 dominated the essential oil distribution (Figure 11.10 {ii}). This gives a fingerprint similar to some of the feverfew products (Figure 11.9 {ii}). However, by GC-MS a distinction can be drawn with peak 11 (tansy), as well as the presence of monoterpenes such as thujone (peak

4) which are absent in feverfew preparations (see Table 11.9). It is interesting to note that Hendriks *et al.* found that tansy samples containing parthenolide had α- and β-thujone absent [62]. No evidence was found to confirm the claim that parthenolide is present in Tansy, though this could be explained by chemotaxonomic variations within the species and the fact that only a limited number of plants were investigated. In the light of this, a combination of sesquiterpenes and sesquiterpene lactones should be used to chemotaxonomically identify the plants, rather than solely relying on the presence of parthenolide as a single marker to confirm authenticity.

FIGURE 11.10 {i}. GC-MS of prep-SFE extract from Tansy (Cathy of Bournemouth Ltd). BP1 column. Temperature 60 to 300°C at 8°C min^{-1}, then isothermal at 300°C for 8 minutes. See Table 11.11 for peak identification. Thujone present as 41% of extracted essential oil.

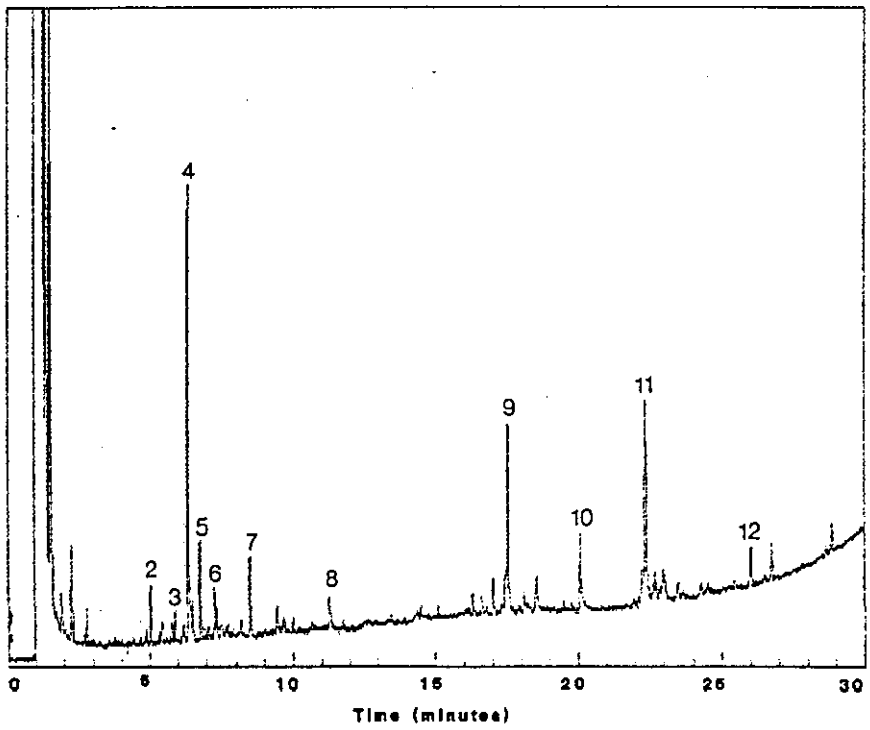


FIGURE 11.10 {ii}. Essential oil distribution in tansy obtained from Brome and Schimmer Ltd. GC conditions in Figure 11.10 {i}. See Table 11.11 for peak identification. Thujone present as 47% of extracted oil.

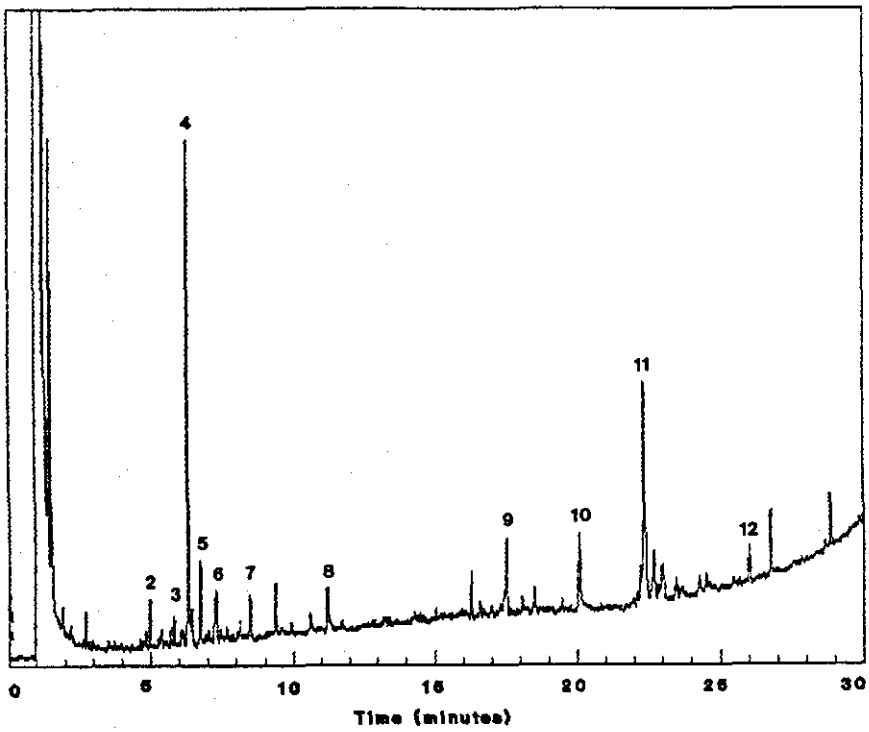


FIGURE 11.10 {iii}. Home grown tansy. GC conditions in Figure 11.10 {i}. Peak identification in Table 11.11. Camphor present at 48% of extracted oil.

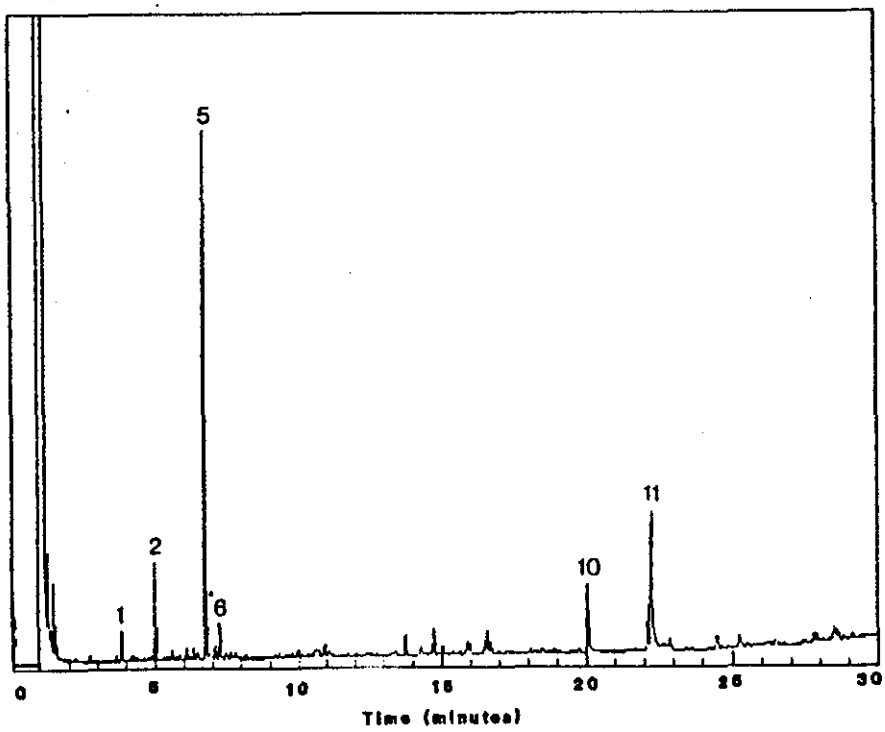


TABLE 11.11. The identities of the major peaks in the GC-MS separation of tansy. Peak number corresponds to peaks in Figure 11.10 (i).

Peak number	% Relative abundance	Compound	Reference mass spectra
1	0.4	camphene (F)	c
2	2.7	cineole	a,b,c,d
3	1.4	isothujone	a,b,c
4	27.7	thujone	a,b,c
5	6.8	camphor* (F)	a,b,c
6	3.5	borneol (F)	a,b,c,d
7	3.9	pinene acetate (F)	d
8	2.6	M.W. 184	-
9	8.9	cis-longipinane-2,7-dione	e
10	4.7	M.W.256 (F)	-
11	4.7	M.W. 230	-
12	11.9	M.W. 350	-

Mass spectra references:

a = Ref. [299]

b = Ref. [307]

c = Ref. [308]

d = NBS library

e = Ref. [72]

(F) = component also found in Feverfew.

* = confirmed by comparison with the retention time of a standard.

The individual mass spectra of these compounds is given in Table 11.12.

TABLE 11.12. Mass spectra data from the major components in tansy. Results expressed as ten largest peaks in each mass spectra.

Peak no. 1: m/z (% rel. int.) 93 (100), 121 (70), 41 (37), 79 (36), 107 (28), 91 (28), 39 (28), 67 (26), 77 (20), 136 (18).

Peak no. 2: m/z (% rel. int.) 43 (100), 81 (37), 41 (31), 71 (30), 108 (30), 84 (30), 112 (26), 55 (23), 69 (23), 154 (20).

Peak no. 3: m/z (% rel. int.) 81 (100), 110 (98), 41 (98), 68 (68), 95 (53), 67 (52), 69 (51), 109 (48), 55 (38), 43 (35).

Peak no. 4: m/z (% rel. int.) 110 (100), 41 (78), 81 (76), 95 (58), 67 (47), 69 (46), 109 (46), 68 (43), 55 (35), 43 (25).

Peak no. 5: m/z (% rel. int.) 95 (100), 41 (67), 81 (65), 108 (45), 69 (34), 152 (33), 55 (32), 109 (30), 40 (30), 83 (28).

Peak no. 6: m/z (% rel. int.) 95 (100), 41 (23), 110 (22), 43 (15), 93 (12), 55 (11), 108 (11), 96 (8), 67 (7), 139 (7).

Peak no. 7: m/z (% rel. int.) 43 (100), 119 (71), 134 (25), 109 (22), 91 (20), 81 (16), 121 (15), 93 (13), 80 (11), 107 (10).

Peak no. 8: m/z (% rel. int.) 43 (100), 94 (22), 41 (16), 95 (16), 55 (11), 68 (11), 93 (11), 136 (7), 59 (6), 79 (6).

Peak no. 9: m/z (% rel. int.) 96 (100), 82 (86), 41 (67), 125 (59), 67 (55), 97 (55), 81 (46), 55 (37), 68 (37), 110 (32).

Peak no. 10: m/z (%rel. int.) 43 (100), 149 (82), 41 (76), 55 (47), 73 (46), 57 (44), 60 (43), 129 (23), 69 (22), 71 (20).

Peak no. 11: m/z (% rel. int.) 230 (100), 41 (98), 53 (75), 105 (66), 107 (65), 43 (63), 91 (60), 119 (52), 215 (52), 55 (52).

Peak no. 12: m/z (% rel. int.) 55 (100), 230 (75), 119 (46), 83 (41), 105 (40), 41 (30), 132 (26), 215 (26), 107 (25), 53 (25).

11.7 GC separation of terpenes in German chamomile

German chamomile has also been reported as an adulterant for feverfew [3], therefore the essential oil content was determined (Figure 11.11 and 11.12). All the commercial samples of German chamomile studied consisted solely of dried flowering heads and seemed to possess few monoterpenes (Figure 11.11). Conversely, dried one year old home grown German chamomile containing the whole plant, did possess such components, though the amount present was quite small due to the age of the plant (Figure 11.12). It appears the flowering heads are rich in component (i) relative to the whole plant.

The plant is reported to be rich in sesquiterpenes such as azulenes, bisabolol and farnesene [25]. All the plants investigated in the study had similar essential oil compositions, varying in a quantitative manner. It was not possible to run a GC-MS on the plant, but none of the peaks in the home grown or commercial German chamomile corresponded to parthenolide or any of the major peaks in feverfew and tansy. Therefore, the essential oils provide an ideal means of fingerprinting German chamomile.

FIGURE 11.11. Essential oil content in German chamomile (Cathy of Bournemouth Ltd), obtained by SFE. Temperature programme 60 to 300^{0}C at 8^{0}C min^{-1}, then isothermal for 8 minutes on a non-polar (BP1) column.

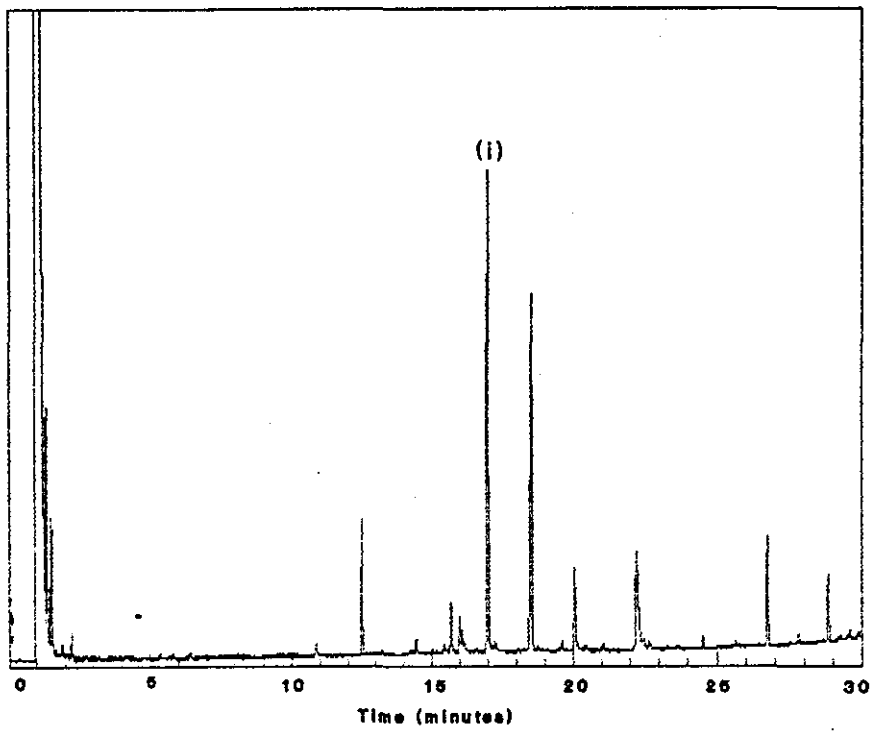
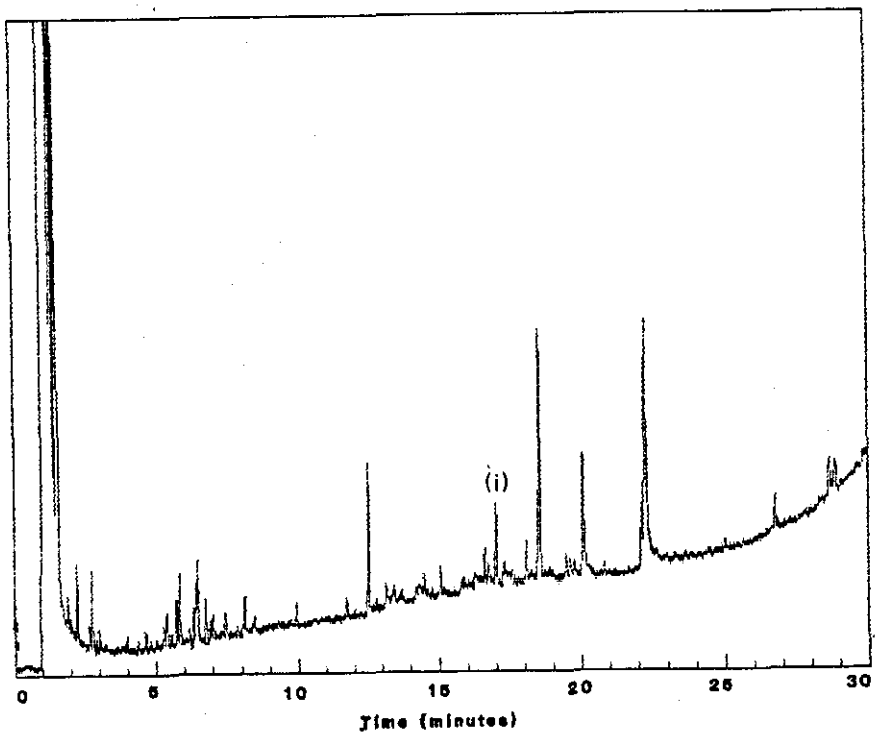


FIGURE 11.12. Home grown German chamomile (the whole plant). GC conditions as in Figure 11.11



11.8 Conclusions

The spectroscopic techniques would appear to confirm that the reported active ingredient, parthenolide [27], is present in feverfew. It is by far the major sesquiterpene lactone in the plant. Initially this was to be used as a marker to distinguish feverfew from its adulterants. However, due to the instability of parthenolide and the fact that it has been reported in tansy, it would appear to be necessary to use the full profile of terpenes as a fingerprint, in particular, the sesquiterpenes and sesquiterpene lactones. With such components a distinction between all three plants should be achievable. There appeared sufficient variation in the essential oil content of the plants, so that the identity of the constituents was not required to enable a chemotaxonomic identification. A comparison of the retention times of the terpenes on capillary GC was adequate information. The GC-MS data merely further confirmed the distinction between the plant species. It has also become apparent that the reported active ingredients in feverfew have a short shelf life, so that a knowledge of the degradation products would enable feverfew products to be positively identified.

CHAPTER 12

Conclusions

With the prospect of the Department of Health requiring evidence for the efficacy, safety and quality of herbal remedies and reports of German chamomile and tansy being incorrectly supplied as feverfew, there is a genuine need for the herbal suppliers and manufacturers to ascertain the authenticity of the feverfew plant. This study has therefore investigated a number of potential chemotaxonomic markers which could be used to distinguish the plant species.

The initial work examined the differences in the phenolic content of the plants, and from the results a distinction between the species was obtained. However, although the phenolics were easy to extract and detect, two isocratic eluents were required to resolve all the components in the complex extracts and often careful examination was needed to distinguish the subtle differences between the closely related feverfew and tansy species. These subtle differences frequently relied on obtaining the UV-visible spectrum of the individual components, which lowered the limits of detection and required a photodiode-array detector. Therefore, to fully exploit the differences in the phenolic content of these species, a gradient elution system with a photodiode-array detector may be more practical, as this system would be able to resolve and classify all the components in the extracts. However, this type of equipment involves a very large capital outlay, and may not be available to all herbal manufactures, many of which still rely on cheaper analytical techniques such as TLC, which in this instance would not be able to distinguish feverfew and tansy. Therefore a more distinctive set of chemotaxonomic markers was sought, which did not require such elaborate separation and detection methods.

The French Pharmacopoeia uses the presence of parthenolide to identify feverfew [232], although objections to this have been raised [3,139]. HPLC was the recommended technique to qualify and quantify the sesquiterpene lactone, but this study has shown that this method is ambiguous as parthenolide has been reported in tansy [62,68] and packed column LC does not have the resolution to separate parthenolide from some of the other sesquiterpene lactones. Therefore, rather than relying on one component to identify feverfew, this study used the essential oil content as a potential means of fingerprinting the plant species.

The essential oils possessed sufficient variation to enable all 3 plant species to be distinguished by a simple solvent extraction and TLC analysis. However, the distinction by TLC analysis was mainly based on the presence of a dark blue parthenolide spot which was absent in the tansy and German chamomile chromatograms. In contrast, capillary GC distinguished the plants based on a number of terpenes, in particular the sesquiterpenes and sesquiterpene lactones. As tansy may contain parthenolide, capillary GC would be the most appropriate method to fingerprint the plant species. A further investigation is required to determine the essential oil content of the tansy varieties which are claimed to contain parthenolide, so as to ascertain if these too can be distinguished from feverfew.

Conventional solvent extraction methods adequately extracted the essential oils but also removed a number of undesirable components. A more selective, "cleaner" extraction was obtained by using SFE. Initially the selectivity of this technique was assessed using the plant material, but this proved too complex, so a simpler cellulose plant model was used. The model demonstrated that by varying the temperature and pressure of the CO_2 class selective extractions could be obtained. The recovery profile appeared to emulate the corresponding density profile, demonstrating the importance of density, not pressure, on the extraction efficiency. Analyte volatility also influenced the extraction yield, with the volatility of some terpenes being the dominate effect at low densities and high temperatures. At these conditions deodourisation of the plant material could be achieved. Further selectivity was obtained by using an in-line trap, though further investigation is required into it's reproducibility and effects on the recovery yields.

The plant model also demonstrated that diffusion, not solubility was the rate determining step for the majority of the terpenes. Furthermore, the results from the plant material suggest that the very polar terpenes (i.e. sesquiterpene lactones) are present as free and bound forms. Therefore, sometimes too much emphasis can be placed on trying to increase the solubility of the analyte in the SCF, when in fact solubility may not the limiting factor. The real problem is often associated with trying to free the analyte from the matrix and transported it to the surface of the material, where in its unbound form it can be quickly removed and thus extracted. However, at present little information is available on this phenomenon and further research is required if a full understanding of SFE is to be achieved.

The plant model also highlighted the problem of collecting volatile analytes with a SFE system. This problem is often underestimated even though it can have dramatic effects on the recovery yields. To overcome the problems incurred by analyte volatility, the extract was collected in solid CO_2 which required a specially designed collection vessel. Even though the model proved helpful in the selection and extraction conditions, there were limitations, as the model was unable to simulate some of the more complex processes which can occur in the plant material. SFC was investigated as a quicker method development technique, however, none of the stationary phases were able to realistically represent the plant material, though a rough indication of the extraction densities and the need for a modifier could be predicted. As neither system could fully account for the way the analyte was situated in the plant matrix, it may be important to obtain the optimum SFE conditions by screening the plant material at various extraction conditions. To further optimise the system an on-line SFE-GCMS could be investigated, in which the whole process of extraction, separation and identification could be achieved in one step. Such a system may in the future become the routine method for screening such complex materials.

Unfortunately some of the essential oil components have the disadvantage that they are less stable than the phenolics. They appear to possess a short shelf live, degrading or evaporating to undetectable levels within several years. This problem has been particularly acute with the sesquiterpene lactones, which have degraded quite badly in some of the feverfew preparations. Therefore, problems may arise when trying to determine the amount of plant material in the product, based on the essential oil content. However, as the sesquiterpene lactones are thought to be the active ingredients in feverfew [26,27], it may be more appropriate to determine the level of lactones in the product rather than the amount of plant material. Pharmacological evidence has shown that the feverfew products investigated contain a much lower activity than expected [310], which further confirms the need to determine the amount of lactones.

By investigating a range of components in the plant species, a number of discrepancies were discovered in some of the feverfew products. One product possessed a phenolic content of unknown origin, another was mislabelled and others contained very low levels of the claimed active ingredients. This suggests that tighter controls on the contents of herbal preparations is needed, with possible guidelines being implemented stating on the product which part of the plant was used, how much is recommended for a daily dose

and an expiry date, so that feverfew products are not ingested containing degradation products of unknown toxicity and efficacy. This will obviously require further research, especially in long term studies on the stability of the sesquiterpene lactones and an elucidation of the degradation products.

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Appendix

A.1 Input data for chemometrics analysis

Data corresponds to peak areas in HPLC chromatograms of the phenolic content of feverfew, tansy and German chamomile (Section 5.6.1, Figure 5.13).

Feverfew

Peak																							
1	2	3	4	5	6	7	8a	8b	9	10	11	12	13	14	15	16a	16b	16c	17	18a	18b	19	20
18	0	28	0	0	0	1	2	0	0	1	3	2	2	3	70	0	2	0	3	4	0	2	0
6	0	49	0	6	0	3	5	0	1	1	2	6	13	0	154	0	96	0	12	2	0	2	0
23	0	30	0	3	0	4	4	0	0	0	0	0	14	8	35	0	48	0	5	2	0	1	0
47	0	0	1	1	0	1	2	0	0	0	2	5	13	5	227	0	278	0	40	6	0	15	0
38	0	40	0	2	0	1	4	0	0	1	0	2	6	4	84	0	45	0	7	2	0	0	0
13	0	22	1	6	0	4	3	0	0	0	0	1	4	0	38	0	0	39	3	1	0	0	0
30	0	3	3	2	0	2	2	0	0	0	0	0	1	4	44	0	0	57	0	5	1	0	0
30	0	0	0	0	0	2	1	0	0	0	0	2	5	2	69	0	88	0	11	3	0	2	0
37	0	13	1	1	0	0	0	0	0	0	1	4	13	0	188	0	255	0	28	5	0	1	0
98	0	18	0	1	0	1	3	0	0	1	1	3	15	1	172	0	223	0	40	5	0	0	0
122	0	3	0	4	0	6	6	0	0	0	0	0	5	0	57	0	84	0	16	2	0	0	0
55	0	11	3	3	0	8	4	0	0	0	0	2	5	0	67	0	112	0	12	2	0	1	0
30	0	10	0	0	0	1	1	0	0	0	0	2	5	0	47	0	79	0	11	3	0	3	0
48	0	3	1	1	0	2	2	0	0	0	1	2	3	3	104	0	109	0	20	2	0	2	0
10	0	10	1	3	0	3	3	0	1	2	1	3	6	0	36	0	2	0	4	2	0	0	0
2	0	9	1	1	0	6	2	0	0	0	1	3	8	1	68	0	176	0	7	2	0	0	0
13	0	11	2	3	0	3	3	0	1	3	1	4	12	1	69	0	70	0	9	4	0	1	0
20	0	15	2	4	0	3	4	0	0	0	0	4	20	11	135	0	63	0	10	4	0	2	0
6	0	5	0	4	0	1	5	0	0	1	2	1	12	0	89	0	1	0	86	34	0	0	0
8	0	29	2	2	0	8	2	0	0	0	0	1	3	0	12	0	21	0	3	3	0	1	0
2	0	83	0	0	0	1	1	0	1	2	2	6	4	0	40	0	3	0	3	3	0	0	0
10	0	15	2	3	0	4	3	0	2	1	1	1	7	0	33	0	2	0	8	2	0	0	0
15	0	10	2	1	0	3	0	0	1	1	1	3	6	3	116	0	3	0	22	9	0	0	0
16	0	35	5	4	0	16	3	0	1	1	1	2	5	2	71	0	88	0	8	3	0	0	0
14	0	6	10	2	0	4	3	0	0	0	0	2	4	1	19	0	41	0	3	3	0	0	0
44	0	18	0	0	0	1	1	0	2	0	1	5	7	2	65	0	123	0	20	1	0	1	0
32	9	0	1	2	0	2	5	2	1	1	2	1	4	1	27	0	0	2	1	1	0	0	0
18	0	15	1	4	0	7	4	0	1	2	2	1	5	2	62	0	36	0	10	1	0	0	0
7	0	9	1	2	0	4	23	0	1	2	1	0	2	12	11	0	15	0	4	0	0	0	0
11	0	8	0	0	0	2	1	0	0	0	0	1	1	2	27	0	5	0	3	1	0	0	0
80	0	30	0	0	0	1	1	0	0	0	0	1	0	2	27	0	37	0	8	1	0	0	0
60	0	30	0	0	0	20	10	0	0	0	1	2	1	4	80	0	16	0	12	3	0	0	0
40	0	61	0	1	0	2	1	0	0	0	0	3	2	5	80	0	5	0	6	1	0	0	0
16	0	36	10	6	0	9	5	0	0	1	1	5	1	8	86	0	64	0	17	5	0	0	0
20	0	62	0	1	0	2	1	0	0	0	0	5	2	5	110	0	50	0	12	6	0	1	0
19	0	30	0	0	0	1	0	0	0	0	0	3	0	4	70	0	17	0	20	4	0	0	0
36	0	30	0	0	0	2	1	0	0	0	0	1	6	12	88	0	187	0	3	19	0	0	0
50	0	30	0	1	0	1	3	0	0	0	0	6	6	7	181	0	107	0	33	8	0	0	0
56	0	60	0	1	0	18	8	0	0	1	0	3	0	8	110	0	38	0	10	3	0	0	0
23	0	60	0	0	0	6	9	0	0	1	0	5	7	7	95	0	48	0	13	2	0	0	0

Peak

1	2	3	4	5	6	7	8a	8b	9	10	11	12	13	14	15	16a	16b	16c	17	18a	18b	19	20
50	0	40	1	1	0	3	0	0	0	0	0	4	1	8	22	0	56	0	20	4	0	0	0
93	0	44	2	2	0	4	31	0	0	0	0	8	2	4	93	0	27	0	21	5	0	0	0
22	0	50	0	3	0	8	7	0	0	0	0	2	3	20	86	0	140	0	8	4	0	0	0
32	0	110	2	3	0	5	9	0	0	0	0	20	8	4	180	0	15	0	20	5	0	0	0
38	0	54	0	8	0	26	23	0	0	0	0	4	2	8	180	0	28	0	20	5	0	1	0
7	0	16	1	0	0	2	3	0	0	1	1	1	3	6	51	0	11	0	3	3	0	0	0
29	0	24	1	5	0	6	8	0	0	0	0	0	1	4	26	0	23	0	14	8	0	1	0
50	0	100	0	14	0	10	8	0	1	2	1	6	3	8	180	0	32	0	12	4	0	1	0
23	0	95	0	0	0	1	2	0	0	0	0	0	3	8	100	0	95	0	16	4	0	0	0
31	0	52	0	10	0	23	4	0	0	0	0	0	1	13	85	0	10	0	7	1	0	0	0
14	0	17	0	5	0	3	3	0	0	0	0	0	0	4	16	0	27	0	2	1	0	1	0
25	0	26	0	3	0	8	6	0	0	1	1	3	1	4	54	0	40	0	9	1	0	0	0
39	0	91	0	1	0	3	4	0	1	1	1	2	1	8	76	0	40	0	10	1	0	0	0
15	0	10	0	1	0	6	4	0	0	1	1	2	1	3	23	0	6	0	9	2	0	0	0
40	0	45	0	0	0	0	1	0	0	0	0	1	1	6	51	0	71	0	10	2	0	0	0
12	0	14	0	0	0	1	1	0	1	0	1	0	1	1	89	0	69	0	22	8	0	0	0
17	0	111	1	4	0	8	4	0	1	0	1	11	5	3	76	0	8	0	13	5	0	0	0
26	0	87	1	2	0	12	20	0	1	2	1	2	10	7	206	0	114	0	40	10	0	4	0
14	0	29	0	2	0	9	9	0	1	0	2	5	4	4	85	0	66	0	22	10	0	0	0
33	0	26	0	0	0	19	4	0	0	0	0	2	1	5	130	0	57	0	1	1	0	0	0
3	0	10	1	3	0	2	2	0	1	0	1	3	1	3	21	0	2	0	3	1	0	0	0
40	0	29	0	0	0	16	4	0	0	1	1	3	2	4	42	0	10	0	10	1	0	0	0
35	0	80	0	3	0	10	7	0	0	0	2	4	4	4	120	0	80	0	27	6	0	1	0
20	0	40	1	1	0	9	2	0	0	0	1	4	4	5	85	0	66	0	12	0	0	0	0
29	0	47	0	6	0	13	4	0	0	0	1	1	3	4	82	0	14	0	13	1	0	0	0
10	0	47	0	0	0	2	3	0	0	0	0	0	1	2	53	0	44	0	18	1	0	0	0
41	0	149	0	50	0	15	18	0	1	2	3	0	5	14	134	0	40	0	21	5	0	0	0
78	0	78	0	0	0	1	1	0	0	0	0	1	3	5	60	0	8	0	2	0	0	0	0
3	0	9	0	0	0	1	0	0	0	0	0	1	0	2	15	0	3	0	3	0	0	0	0
3	0	9	0	0	0	1	0	0	0	0	0	1	0	0	16	0	13	0	3	0	0	0	0
8	0	6	1	1	0	2	1	0	1	0	1	4	5	2	79	0	41	0	8	4	0	2	0
50	0	23	2	3	0	3	5	0	0	0	0	1	3	1	23	0	1	0	3	1	0	0	0
4	0	6	1	1	0	2	1	0	1	1	2	2	7	3	80	0	75	0	13	2	0	1	0
20	0	18	1	2	0	5	3	0	0	0	0	2	3	3	100	0	103	0	19	2	0	1	0
9	0	19	0	1	0	1	2	0	1	1	0	1	10	1	106	0	141	0	19	2	0	1	0
42	0	16	1	2	0	2	2	0	1	0	1	1	7	1	66	0	108	0	15	3	0	1	0
49	0	9	1	2	0	3	2	0	0	0	0	0	4	3	36	0	111	0	51	1	0	2	0

<u>German chamomile</u>

Peak

1	2	3	4	5	6	7	8a	8b	9	10	11	12	13	14	15	16a	16b	16c	17	18a	18b	19	20
6	6	8	0	7	2	6	0	32	7	0	0	0	0	0	5	0	0	18	0	0	0	0	1
1	4	13	0	4	2	10	0	15	3	0	0	0	0	0	1	0	0	20	0	0	0	18	0
5	2	14	0	6	6	12	0	75	5	0	0	0	0	0	8	0	0	52	0	0	3	0	0
1	0	16	0	1	0	2	0	30	1	0	0	0	0	0	4	0	0	25	0	0	2	2	1
5	5	10	0	6	2	5	0	52	5	0	0	0	0	0	5	0	0	34	0	0	1	1	1
18	16	28	0	9	10	18	0	95	14	0	0	0	0	0	8	0	0	46	0	0	4	0	0
10	2	5	0	5	0	3	0	48	4	0	0	0	0	0	12	0	0	20	0	0	4	5	2
8	6	9	0	8	2	8	0	48	6	0	0	0	0	0	6	0	0	24	0	0	2	1	0
10	2	16	0	6	1	0	0	95	0	0	0	0	1	3	11	0	0	67	0	0	2	21	15
10	9	13	0	7	2	8	0	42	10	0	0	0	0	5	11	0	0	27	0	0	0	1	0

												Peak										
1	2	3	4	5	6	7	8a	8b	9	10	11	12	13	14	15	16a	16b	16c	17	18a	18b	19	20
9	12	15	0	6	6	12	0	99	9	0	0	0	0	0	9	0	0	63	0	0	9	1	1
9	6	12	0	9	2	9	0	47	10	0	0	0	0	0	8	0	0	22	0	0	2	0	0
1	0	18	0	1	2	1	0	63	2	0	0	0	0	0	3	0	0	33	0	0	3	2	0
4	9	6	0	3	1	6	0	39	3	0	0	0	0	0	3	0	0	22	0	0	2	2	1
25	1	12	0	7	0	2	0	83	7	0	0	0	0	3	8	0	0	68	0	0	11	29	15
17	6	16	0	4	14	2	0	78	10	0	0	0	0	0	3	0	0	31	0	0	0	0	0
0	2	3	0	7	2	3	0	0	0	0	0	0	0	0	1	0	0	5	0	0	1	0	0
8	4	16	0	10	0	12	0	62	7	0	0	0	0	2	7	0	0	34	0	0	3	1	1
16	2	9	0	8	4	2	0	62	2	0	0	0	0	2	13	0	0	49	0	0	10	5	17
6	4	12	0	7	2	6	0	53	5	0	0	0	0	0	5	0	0	33	0	0	3	1	0
29	9	6	0	3	1	0	1	15	6	0	0	0	0	4	2	0	0	15	0	0	4	12	6
6	6	5	0	1	9	2	0	61	3	0	0	0	0	0	0	3	0	31	0	0	2	4	71
10	2	2	0	6	1	0	0	10	2	0	0	0	0	0	11	0	0	67	0	0	2	21	10
7	12	12	0	7	3	9	0	82	8	0	0	0	0	0	7	0	0	46	0	0	2	2	1
15	3	4	0	8	6	5	0	70	10	0	0	0	0	17	21	0	0	20	0	0	0	4	3

Tansy

												Peak										
1	2	3	4	5	6	7	8a	8b	9	10	11	12	13	14	15	16a	16b	16c	17	18a	18b	19	20
53	0	8	0	0	0	12	26	0	0	0	0	2	0	3	89	63	0	0	20	0	5	0	0
88	0	4	0	0	0	20	44	0	0	0	0	14	14	12	14	45	0	0	26	0	13	0	0
44	0	12	0	0	0	4	93	0	0	0	0	1	0	1	17	10	0	0	4	0	3	0	0

A.2. Principal component analysis

A.2.1. Percentage loading

Principal component scores		
1st	2nd	3rd
27.8%	10.0%	9.3%

A.2.2. Latent vectors (loadings)

First three loadings (latent vectors) of the peaks (variables) shown in Section 5.6.1, Figure 5.13.

Peak	Loading 1st	2nd	3rd
1	0.1726	-0.1821	-0.2599
2	-0.2934	-0.1646	0.1309
3	0.1736	-0.2804	0.2339
4	0.0929	0.0852	0.0880
5	-0.0456	-0.3658	0.3394
6	-0.2657	-0.1485	0.1280
7	0.0103	-0.4566	0.0046
8a	0.0935	-0.2902	-0.3422
8b	-0.3328	-0.1571	0.1068
9	-0.2976	-0.1936	0.1765
10	0.1651	-0.0972	0.2999
11	0.1871	-0.0773	0.3185
12	0.2015	-0.1959	-0.1208
13	0.2468	-0.0395	0.0264
14	0.1293	-0.2840	0.0055
15	0.2978	-0.1760	0.1220
16a	0.0138	-0.2580	-0.4665
16b	0.2194	0.0806	0.0455
16c	-0.2592	-0.0909	0.1001
17	0.2524	-0.1138	0.0397
18a	0.2008	-0.0388	0.1716
18b	-0.1967	-0.2834	-0.2812
19	-0.1292	-0.0047	0.0166
20	-0.1430	-0.0073	-0.0024

A.2.3. Principal component scores

First three principal component scores used in Section 5.6.1., Figure 5.14.

Feverfew

Principal component scores 1st	2nd	3rd
1.0438	0.9411	1.0641
2.5906	-0.1960	1.5076
0.6857	-1.0229	2.0335
1.0730	1.1334	-0.7130
4.1785	-0.2884	1.0062
0.7735	0.5560	0.2023
-0.5213	1.6099	-0.4984
-1.0385	1.3207	-0.7080
0.8765	1.4921	-0.5217
3.7710	0.3627	-0.4920
4.2445	-0.8349	-0.4265
0.8970	0.8925	-0.7547
0.9120	1.6411	-0.4205
0.5623	1.5086	-0.9158

Principal component scores

1st	2nd	3rd
1.1510	1.0151	0.0838
0.7088	0.9769	0.9631
1.3471	1.3951	0.2661
2.0685	0.5203	1.4001
2.1190	0.2306	-0.1557
4.3532	-0.3898	2.5425
0.1173	1.3710	0.2068
1.3497	0.5381	1.6253
0.4386	1.3079	0.5517
2.0878	-0.7051	1.1932
1.3588	1.7129	0.6865
0.1840	1.5989	-0.7662
1.4794	0.8806	-0.1684
0.3787	1.0753	0.0040
1.1109	0.3066	1.1548
0.6486	-0.0648	0.3339
-0.5755	1.8928	-0.7819
0.2825	1.1776	-1.3451
0.9640	-1.0061	-0.9842
0.7612	0.4148	-0.3627
2.2481	-0.2816	1.3145
1.3713	0.3486	-0.0622
0.5545	1.1351	-0.3934
2.3133	0.6070	0.2753
3.0258	-0.2353	-0.1635
1.4464	-1.4760	-0.5255
1.7708	-0.3788	-0.1814
1.0868	0.4089	-0.6672
2.3032	-1.1613	-1.9413
1.7534	-0.9928	-0.1235
3.6358	-2.0462	-0.2758
2.0838	-3.2585	-0.2854
0.2708	1.2452	-0.1084
0.4761	0.4959	-0.1271
2.9458	-3.0345	2.0853
1.5200	0.2819	0.2541
0.5937	-2.1966	0.1642
-0.5185	1.2473	-0.4060
0.6930	0.1745	-0.0503
1.4772	-0.3699	0.6026
0.1651	0.9452	-0.1559
0.6220	1.0314	-0.6037
0.7868	1.6151	0.0948
2.0623	-1.1723	0.5783
4.2593	-2.3063	1.8555
2.0264	-0.2996	0.6611
0.7210	-0.5024	-0.6842
-0.2512	1.4996	-0.1676
0.7394	-0.3781	-0.3429
2.5925	-1.1130	0.8577
1.1856	0.1077	-0.0647
0.7455	-0.5798	0.1050
0.2846	1.3034	-0.2780
1.4188	-0.7594	0.6885

Principal component scores		
1st	2nd	3rd
0.8286	0.3527	-0.7975
-0.76164	2.0310	-0.6788
-0.6982	2.0927	-0.6927
1.3169	0.4610	-0.2781
-0.4361	1.9021	-0.5958
1.7295	0.3898	1.1823
0.9706	1.3447	-0.1833
1.9038	0.9611	0.0495
1.2462	1.1384	-0.4049
0.9687	1.5369	-0.1800

<u>German chamomile</u>

Principal component scores		
1st	2nd	3rd
-3.2991	0.2332	0.6563
-2.977	0.5716	0.2307
-4.2629	-0.9117	0.5417
-2.0087	1.6029	-0.6703
-3.4929	0.3130	0.5486
-7.2421	-3.7659	2.0756
-3.0099	0.5506	-0.4103
-3.6310	-0.3284	0.4961
-4.2187	0.3447	0.2666
-3.9045	-0.8743	1.0705
-6.6865	-2.7519	0.6364
-3.9543	-0.7794	0.8093
-2.9922	1.0519	-0.3270
-3.4298	0.4104	0.0396
-5.5087	-1.3319	-0.6951
-5.6796	-0.7628	1.5331
-1.7527	1.4291	-0.2697
-3.5553	-1.0853	0.5484
-4.5144	-0.9098	-0.6898
-3.5769	-0.1188	0.2634
-3.5984	0.1000	-0.4306
-5.9043	0.1713	0.1740
-3.4805	1.0775	-0.0780
-5.2227	-1.1791	1.0815
-3.8072	-1.6507	1.0679

<u>Tansy</u>

Principal component scores		
1st	2nd	3rd
0.2545	-3.0564	-6.5404
1.5884	-6.3126	-7.9308
-0.0008	-1.5164	-5.1982

A.3. Presentations

1. M.D. Burford and R.M. Smith, "Chromatographic identification of herbal medicines", presented at the Research and Development Topics in Analytical Chemistry, Meeting of the Analytical Division of the Royal Society of Chemistry, Dublin, 21st-22nd March 1989.

2. R.M. Smith and M.D. Burford, "Quantitation in supercritical fluid extraction of volatile compounds from plant materials", presented at Supercritical Fluids" organised by Leeds Supercritical Fluids Group at the University of Leeds, Leeds, 25-26th July 1990.

3. R.M. Smith and M.D. Burford, "Sample preparation for chromatographic analysis using supercritical fluids", presented at the "18th International Symposium on chromatography", Amsterdam, 23-28th September 1990.

A.4. Publication

M.D. Burford and R.M. Smith, Anal. Proc., 1989, 26, 339.