Supercritical Fluid Extraction of Terpenoids and Steroids from Plants
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Introduction

Terpenoids and steroids constitute the largest known group of plant secondary metabolites. The different groups of terpenes are formed from isoprene \( (C_5) \) units in enzymatic reactions. Head-to-tail condensation of isoprene units results in the formation of monoterpenes \( (C_{10}) \), sesquiterpenes \( (C_{15}) \), diterpenes \( (C_{20}) \), sesterpenes \( (C_{25}) \) and polyterpenes. Tail-to-tail coupling of \( C_{15} \) and \( C_{20} \) units leads to the precursors of triterpenes \( (C_{30}) \) and carotenes \( (C_{40}) \) [1].

Supercritical fluid extraction (SFE) processes have gained increasing interest in production of high-value plant extracts for pharmaceutical, cosmetic and food industries in recent years. A large number of books and review papers have been published [2-5]. There are several reviews of systems, for which high-pressure phase-equilibrium data have been published [6-8]. The comprehensive book of Stahl and coworkers [2] contains a systematic review about the SFE of terpenes.

This chapter describes a recent period of supercritical fluid extraction of botanical feedstocks. The natural terpenoids are discussed subsequently. It is demonstrated that most plants contain a wide spectrum of biologically active compounds which can be dissolved or precipitated selectively. Some of the most important trends affecting the uses of SFE extracts are identified.

Monoterpenes and Sesquiterpenes

Mono- and sesquiterpenes and their oxygen containing derivatives are the major constituents of essential oils. Essential oils are defined as products obtained from a plant starting material, either by steam distillation, or by mechanical pressing (e.g. citrus oils). The essential oil is subsequently separated from the aqueous phase by physical methods. Various non-terpene components such as phenylpropanoids \( (C_6-C_9) \), aliphatic hydrocarbons, acids, alcohols, esters, nitrogen- or sulfur-containing compounds are also present in essential oils. As low molecular lipophilic substances with high vapour pressure, the essential oil constituents are well soluble in liquid or supercritical carbon dioxide [9,10]. Hence, essential oil components have been extracted from a wide range of plant materials, and have found applications in many product areas [11]. A comprehensive review of published literature on the yields, major components and major applications for 42 botanical extracts was published by Moyler [12]. Supercritical fluid extraction and fractionation of essential oils were reviewed by Reverchon, recently. The literature in this field covering the period from 1991 to 1995, was also summarized in this paper [5]. Recent publications on SFE of plants containing essential oils, starting from 1996, are summarized in Table 1.

Comparison of SFE extracts with distilled essential oils results that the composition of the products is determined by the isolation methods. Normal steam distillation usually results in chemical changes, loss of the lightest components and loss of certain water-soluble components. Carbon dioxide extracts are closer in composition to the natural essential oils present in the plant materials. Alteration of volatile components during distillation can be recognized by comparing the oils obtained by steam distillation and SFE. The hydrolysis of esters to the corresponding alcohols was observed in lavandin and clary sage oils. The hydrolysis of thymol bound in glycosides resulted higher thymol concentration in thyme essential oils. During the distillation of the oil of chamomile flowers the blue chamazulene is formed from the colourless matricine.

Extraction of essential oil components can be carried out with liquid \( \text{CO}_2 \) (60-70 bar, 5-10\(^{\circ}\)C) or with supercritical \( \text{CO}_2 \) of low densities \( (0.4-0.5 \text{ g/cm}^3) \), e.g. at 90 bar and 40\(^{\circ}\)C). At these conditions the light fractions of waxes and resins are co-extracted with the volatile components. This is generally not a disadvantage in flavouring and perfumery applications.

Removal of waxes can be solved by stagewise precipitation of products using two or more separators in series. The separators are operated at different pressures and temperatures to precipitate the resinous compounds and volatile components in different vessels. This method was suggested by Stahl and co-workers [62]. Recently, this fractional separation technique has been refined by Reverchon and co-workers [63,64] for selective precipitation of cuticular waxes and volatile oils. Fractionation of volatile and fatty oils can be also carried out by this method [29,62].
<table>
<thead>
<tr>
<th>Plant</th>
<th>Botanical name</th>
<th>Major constituents</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angelica</td>
<td>Angelica archangelica L.</td>
<td>β-phellandrene, α-pinene, sabinene</td>
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<td>Caraway</td>
<td>Carum carvi L.</td>
<td>carvone, limonene</td>
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<td>Cedarwood</td>
<td>Juniperus virginiana L.</td>
<td>cedrol, cedrene, thujopsene</td>
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<tr>
<td>Celery</td>
<td>Apium graveolens L.</td>
<td>limonene, β-selinene, 3-butylphthalide</td>
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<tr>
<td>Chamomile</td>
<td>Chamomilla (Matricaria) recutita (L.) Rauschert</td>
<td>α-bisabolol, bisabolol oxide, spiroether</td>
<td>18-21</td>
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<tr>
<td>Chervil</td>
<td>Anthriscus cerefolium Hoffm.</td>
<td>methyl chavicol, 1-allyl-2,4-dimethoxybenzene</td>
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<td>Clary sage</td>
<td>Salvia sclarea L.</td>
<td>linalyl acetate, linalool, α-pinene</td>
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<td>Coriander</td>
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<td>Cumin</td>
<td>Cuminum cyminum L.</td>
<td>cuminaldehyde, cuminy alcohol</td>
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<td>Fennel</td>
<td>Foeniculum vulgare Mill.</td>
<td>trans-anethole, fenchone</td>
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<tr>
<td>Ferulago</td>
<td>Ferulago nodosa L.</td>
<td>α-pinene, myrcene</td>
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<tr>
<td>Feverfew</td>
<td>Tanacetum parthenium (L.) Schulz</td>
<td>camphor, chrysanthyle acetate</td>
<td>31</td>
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<tr>
<td>Hieron</td>
<td>Spilantes americana Mutis</td>
<td>α- and β-bisabolones</td>
<td>32</td>
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<td>Eucalyptus</td>
<td>Eucalyptus globulus Labill.</td>
<td>1,8-cineole, p-cymene</td>
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<td>Lavender</td>
<td>Lavandula angustifolia Mill.</td>
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<td>Cymbopogon citratus Stapf.</td>
<td>geranial, nerolic acid</td>
<td>37</td>
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<tr>
<td>Lovage</td>
<td>Levisticum officinale Koch.</td>
<td>3-n-propyldene phthalide, α-terpinyl acetate</td>
<td>38</td>
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<td>Nutmeg</td>
<td>Myristica fragrans Houtt</td>
<td>myristicin, terpinen-4-ol</td>
<td>39</td>
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<td>Orange</td>
<td>Citrus aurantium L.</td>
<td>limonene, linalool</td>
<td>40-43</td>
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<td>Oregano</td>
<td>Origanum vulgare L.</td>
<td>carvacrol, thymol</td>
<td>44</td>
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<tr>
<td>Pepper</td>
<td>Piper nigrum L.</td>
<td>α- and β-pinenes, β-phellandrene</td>
<td>45</td>
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<td>Peppermint</td>
<td>Mentha piperita L.</td>
<td>menthol, menthone</td>
<td>46-48</td>
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<td>Pine</td>
<td>Pinus sylvestris L., Picea abies L.</td>
<td>1,8-cineole, borneol, camphor</td>
<td>49</td>
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<tr>
<td>Rock rose</td>
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<td></td>
<td>50</td>
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<tr>
<td>Rosemary</td>
<td>Rosmarinus officinalis L.</td>
<td>1,8-cineole, camphor, verbrenon</td>
<td>18,51</td>
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<td>Sage</td>
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<td>1,8-cineole, camphor, β-caryophyllene</td>
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<td>Santolina insularis L.</td>
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<td>Sandalwood</td>
<td>Santalum album L.</td>
<td>santalol</td>
<td>55</td>
</tr>
<tr>
<td>Savory</td>
<td>Satureja hortensis L.</td>
<td>thymol, carvacrol</td>
<td>56</td>
</tr>
<tr>
<td>Spearmint</td>
<td>Mentha spicata L.</td>
<td>pulegone, carvone</td>
<td>18,57,58</td>
</tr>
<tr>
<td>Tagetes</td>
<td>Tagetes lucida L.</td>
<td>estragole, anethole, methyl eugenol</td>
<td>20</td>
</tr>
<tr>
<td>Thyme</td>
<td>Thymus vulgaris L.</td>
<td>thymol, carvacrol, γ-terpinene, p-cimene</td>
<td>34,59-61</td>
</tr>
</tbody>
</table>

**Sesquiterpenoid Lactones**

Sesquiterpenoid lactones have a rather scattered botanical distribution. They occur in the Angiosperms (Apiaceae, Lauraceae, Menispermaceae), and chiefly in the Asteraceae. Many lactones have potential applications in phytotherapy. There are many structural variations in the group of lactones [1].

**Chamomile (Matricaria chamomilla L.), German chamomile (Matricaria recutita Rauchert)**

The chamomile is a widely applied medicinal plant. The flowerheads are used as drug. The volatile oil (Aetherolum chamomillae) is blue and the high quality drug contains 0.75-1.3% volatile oil. The chamazulene is responsible for its dark blue colour, which is a sesquiterpene with two, five and seven carbon atoms units. (The structures of the main components are shown in Figure 1.).
Figure 1. Major components of chamomile extract

The chamazulene is formed during the distillation from colourless, solid compounds the proazulenes from which the matricine, a sesquiterpene-lactone with three units is the most important. This compound posses anti-inflammatory and anti-spasmodic effects. The other anti-inflammatory compounds of the volatile oil are the bisabolol, which is a sesquiterpene alcohol (it is expected to be minimum 10% of oil) and its oxides. The third important group form the en-in-dicycloethers, which mainly posses spasmolytic activity. A good quality oil has to content minimum 10% of it.

The most important non-volatile, hidrophilic compounds are the spasmolytic flavonoids apigenin, quercetin, luteolin, patuletin and their glycosides from which some are soluble in water, others in alcohol. Coumarines (umbelliferon, herniarin) are mainly found in tubular herbal drugs, which are spasmolytic too. The chamomile flowers contain tannins, bitter agents, vegetable acids, carbohydrates, slime agents too.

The first examination has showed that the major volatile compounds of chamomile are soluble in CO$_2$ and they can be extracted at 40°C temperature and 70-100 bar pressures [65].

The low temperature (T$_{\text{c}}$<80°C) is required because of the thermal degradation of matricine. Laboratory extraction experiments has shown that the active compounds can be fractionated by choosing the proper processing parameters. As Table 2. shows the matricine was hardly extracted at 80 bar pressure while a product with high content of α-bisabolol could have been extracted [66].
The active compounds and characteristic fragrances of chamomile can be extracted by supercritical fluid extraction in significant efficiency (above 90%). Polysaccharides, acids of plant, flavonoids are not soluble and by using a well-chosen extraction technique the carotinoids and chlorophyll are not co-extracted. By using one staged supercritical fluid extraction ($P_E=120-160$ bar, $T_E=40-50^\circ\text{C}$) a yellow, water-free pleasant chamomile fragrance extract was obtained, while the residue in the extractor was completely odourless [2]. In addition other experiments has shown that the extraction with dinitrogen-monoxide in the case of similar conditions produced more extract than the carbon dioxide. But more active compounds were not indicated by using N$_2$O, just the amount of undesired compounds were increased [2]. The active compounds of chamomile are soluble in liquid CO$_2$, the supercritical state could not be absolutely necessary [67].

According to our experiments [18] the distilled oil was substantially different from the extract of supercritical fluid extraction. The concentrations of farnezene, α-bisabolol, chamazulene in the distilled oil were significantly higher and the concentration of en-in-dicycloether and herniarin were lower than in the extracts of SFE. Recently, there has been increased interest in extraction of non-volatile components. The spasmolytic activity of chamomile is mainly due to the presence of flavonoid compounds, in the first place of apigenin. It is bound in apigenin-7-O-β-glucoside, which is not soluble in CO$_2$. However, after separation of volatile compounds by SFE the free apigenin can be obtained by fermentation of the residue [21]. It was shown that the apigenin can be recovered by SFE [19].

A summary of supercritical fluid extraction of chamomile flowers is given in Table 3.

### Table 3. Supercritical fluid extraction of chamomile flowers

<table>
<thead>
<tr>
<th>Solvent Parameters</th>
<th>Subject</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$, N$_2$O 80-300 bar, 40-80°C</td>
<td>extraction of volatile components and matricine</td>
<td>2,65,66</td>
</tr>
<tr>
<td>CO$_2$ 60-400 bar, 22-29°C</td>
<td>extraction with liquid CO$_2$</td>
<td>67</td>
</tr>
<tr>
<td>CO$_2$ 200 bar, 40°C</td>
<td>comparison of distilled and extracted oils</td>
<td>68</td>
</tr>
<tr>
<td>CO$_2$ 80-120 bar, 35-50°C</td>
<td>fractionating the product in two separators in series</td>
<td>69</td>
</tr>
<tr>
<td>CO$_2$ 100-400 bar, 40-60°C</td>
<td>effects of pressure and temperature on total yield, application tests</td>
<td>18,70</td>
</tr>
<tr>
<td>CO$_2$ 80-240 bar, 40-80°C</td>
<td>solubility and extraction behaviour of selected components, modeling</td>
<td>71,72</td>
</tr>
<tr>
<td>CO$_2$, 90-400 bar, 40-50°C</td>
<td>recovery of apigenin</td>
<td>19</td>
</tr>
</tbody>
</table>

**Common wormwood (Artemisia absinthium L.), sweet wormwood (Artemisia annua L.)**

The bitter taste of this plant is due to sesquiterpene lactones. Major components are artabsin and a dimeric sesquiterpene lactone absinthin. (Structures of the mentioned sesquiterpene lactones are given in Figure 2.). By steam distillation the bitter components decompose to chamazulene and dihydrochamazulene. A disadvantage of alcoholic extraction is that the poisonous β-thujone is co-extracted with the bitter components. Fast and quantitative removal of thujone can be achieved by extraction with liquid or supercritical carbon dioxide [73]. Thujone as non polar essential oil component is very soluble in CO$_2$. At 90 bar and 40°C the solubility of thujone is about 5 wt% while that of artabsin is only 0.4 mg/g CO$_2$. The most important bitter component, the absinthin is practically insoluble in carbon dioxide at 40°C and pressures up to 200 bar. A complete removal of thujone at 100 bar and 40°C resulted a loss of about 75% of the artabsin, while absinthin is completely retained in the residue.

Artemisinin and artemisinic acid were extracted from aerial parts of sweet wormwood by SFE with CO$_2$ modified with 3 % (v/v) methanol. This method allowed quantitative recovery of active components. In all tested cases, artemisinin and artemisinic acid were extracted in a higher yield with supercritical CO$_2$ than with liquid-solid extraction [74].
Figure 2. Structures of sesquiterpene lactones mentioned in the text

**Arnika (Arnica montana L.)**

The drug consists of the dried capitulum. It contains essential oil, sesquiterpenoid lactones (helenalin, dihydrohelenalin and their esters), triterpenoids, flavonoids, carotenoids and more active components. Sesquiterpenoid lactones can induce contact dermatitis, which makes the selective removal of these compounds necessary. A German patent claims that the essential oil and the allergic lactones can be selectively extracted with supercritical CO$_2$ [75]. Extraction at 300 bar and 70°C for 6 hours resulted in almost complete removal of sesquiterpenoid lactones, while the flavonoid glycosides and other polar active components were restrained in the residue. A second step extraction with polar solvents (e.g. alcohols) can be used for isolation of these components.

**Feverfew (Tanacetum parthenium L. Schultz Bip.)**

Feverfew is an aromatic herbaceous perennial, native in Europe. Current interest in this plant is mainly focused on migraine headache treatment. The compounds responsible for this activity are sesquiterpene lactones. Parthenolide is the predominant molecule.

Dried powdered feverfew was extracted with CO$_2$ at 250 bar and 45°C. The major constituents in the extract were identified as camphor, chrysanthenyl acetate, and parthenolide. The extract was compared with the essential oil obtained from the same batch of raw material. A number of additional peaks in GC chromatogram were found but no parthenolide was present in distilled oil. If methanol was added to the CO$_2$ as co-solvent the yield of parthenolide was increased [76,77].

In our laboratory a 3$^2$ full factorial design with three repeated experiments in the center of the design was performed to map quantitatively the effects of pressure and temperature of the extraction on the total yield and recovery of parthenolide [31]. The yield expressed in terms of extract mass per 100 g dried plant material (Y in wt%) was used as dependent variable. The three-dimensional response surface plot fitted on the experimental results showing the maximum of the yield can be seen in Figure 3a. The proposed extraction parameters are the highest pressure and temperature investigated. The parthenolide yields (YP) are expressed in terms of parthenolide extracted by SFE per mass of dried raw material. The influence of the extraction parameters (pressure, temperature) on the parthenolide yield as dependent variable was also investigated. The three-dimensional response surface plot fitted to the analytical results (Figure 3b.) shows the optimal extraction parameters at which the maximum of parthenolide yield is obtained, and this plot is very similar to that belonging to extraction yields. It shows that highest amount of extract containing parthenolide in considerable concentration can be produced with the optimum extraction parameters (400 bar and 60 °C).
Figure 3. Three-dimensional response surface plot: effects of pressure and temperature a: on the yield b: on parthenolide yield (YP, mg parthenolide/100 g dried material)

The yield obtained by SFE was compared with those resulting from steam distillation and conventional Soxhlet extraction with n-hexane and ethyl alcohol. Among these methods the Soxhlet extraction with ethyl alcohol was the most effective one to obtain the maximum yield (26.46 %) of plant extract. The yield obtained by SFE was similar to that resulting from hexane extraction (5.4 % and 4.92 %, respectively). The yield of essential oil obtained by steam distillation was only about one tenth of the yield obtained by SFE or hexane extraction.

Considering the results on the composition of different extraction methods SFE has been successful for obtaining products rich both in parthenolide/other sesquiterpene-γ-lactones and essential oil. Conventional solid-liquid extractions (Soxhlet-extraction) with n-hexane resulted extracts containing considerable concentration of parthenolide, camphor, and chrysanthenyl acetate, though the extraction was not quantitative for parthenolide and more polaric sesquiterpene-γ-lactones. The chrysanthenyl acetate concentration was lower in comparison with SFE products. Soxhlet extraction with ethyl alcohol resulted a different composition of extract both qualitatively and quantitatively. The main difference reflects the less selective character of alcohol as solvent, though parthenolide was present almost in the expected amount. The presence of various phenolic substances (flavonoids, etc.) was also proved.

Holy (blessed) thistle (Cnicus benedictus L.)

In herbal medicine small amounts of it are used to treat digestive disorders, lack of appetite and promote to flow of gastric secretions and bile. Blessed thistle is also used to make liqueurs and was once used in beer making. Cnicin, the bitter sesquiterpenoid lactone is an antibacterial agent too. The herb also contains traces of an essential oil, tannins, mucilage and minerals.

Supercritical fluid extraction with carbon dioxide by using factor levels of similar extraction tasks was unsuccessful for obtaining cnicin as major and some minor sesquiterpene lactones characteristic for blessed thistle. The extract contained only some volatile compounds, waxes and phytosterols [78]. Repeating the experiments using CO\textsubscript{2} mixed with 4 % ethanol, cnicin was recovered quantitatively. No more sesquiterpene lactones were detected in the SFE extracted plant material. A comparison of the cnicin contents of the samples is given in Figure 4. When the extraction was carried out in two steps using pure CO\textsubscript{2} (step 1.) and CO\textsubscript{2} mixed with co-solvent (step 2.) subsequently, the second extract was enriched with sesquiterpenic lactones and cnicin, which concentration was six times higher than in the corresponding alcoholic extract.
Figure 4. IR spectra of extracts from *Cnicus benedictus*: 1. Alcoholic extract; 2. SFE extract using CO$_2$; 3. Alcoholic extract of the plant material after CO$_2$ extraction; 4. SFE extract using CO$_2$ mixed with co-solvent; (presence of sesquiterpene lactones: distinct absorption bond at 1745-1780 cm$^{-1}$).

**Diterpenes**

Diterpenes constitute a vast group of C$_{20}$ compounds which are widespread in plants. The structure of diterpenes is highly variable (components mentioned in the text are shown in Figure 5.), and strictly dependent on their biogenesis. They have limited therapeutic applications [1]. Hence the interest of isolation is reasonably low.
Figure 5. Structures of the diterpenes investigated by SFE

**Clary sage (Salvia sclarea L.)**

Clary sage contains in its stems, leaves and flowering parts substantial amounts of the compound sclareol. This compound and its derivatives have been used extensively as perfume component, as a wine and foodstuffs flavourant, and as a cigarette flavourant. It has therefore been of interest to develop methods of producing high-purity sclareol. The sub and supercritical fluid extraction of clary sage pellets free from essential oil was performed with carbon dioxide and CO$_2$+propane mixture [79]. Freshly harvested and dried flowering tops of clary sage collected from the same field were extracted with carbon dioxide [23,80]. The SFE extract was compared to essential oil obtained by hydrodistillation. Only the SFE extracts contained sclareol.

**Yews (Taxus brevifolia Nutt., Taxus cuspidata Capita)**

The diterpenoid natural ingredients (e.g. taxol, paclitaxel, baccatin III) have become important anticancer agents. The solubilities of pure taxol [81] in CO$_2$ and paclitaxel [82] in CO$_2$ and N$_2$O were measured at pressures from
210 to 490 bar and temperatures 35°C to 45°C. It was shown that paclitaxel can be extracted from the barks of *Taxus brevifolia* [83]. Recent studies show the ability to isolate paclitaxel and baccatin III from needles of *Taxus cuspidata* using CO₂ and CO₂ mixed with co-solvents [84]. The needles were treated with hexane before the SFE in order to remove waxes and other non-polar materials. The highest yield of active components was achieved at 300 bar and 50°C using pure CO₂. Among the co-solvents (ethyl acetate, methanol, dichloro-methane, ethyl ether mixed with CO₂ from 0.7 to 3 %) dichloro-methane increased the recovery of paclitaxel by a factor of four. The other co-solvents had negligible effect. The highest yield of baccatin III was obtained with CO₂ and 0.7 % ethyl ether mixture. A comparison with conventional solvent extraction (using methanol and dichloro-methane in 1:1 mixture) resulted that the recovery of the polar diterpenes by SFE was not very effective. However, the concentrations of paclitaxel and baccatin III in the SFE extracts by comparison to solvent extracts were 15 and 20 times greater, respectively.

**Diterpene antioxidants**

Much work has recently studied the antioxidant activity of different plants and plant extracts. Rosemary and sage (leaves and extracts) were most effectively investigated [85,86]. Phenolic diterpenes such as rosmanol and carnosol and their derivatives are potent antioxidants. However, the antioxidant pattern is rather complex and synergistic effects of different components can be observed.

An effective supercritical fluid extraction method of the antioxidant components was developed by Gerard and co-workers [87]. Primary batch CO₂ extraction was carried out at 500 bar and 60°C. Yields and composition of extracts are given in Table 4.

<table>
<thead>
<tr>
<th></th>
<th>Rosemary</th>
<th>Sage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total yield</td>
<td>5.4</td>
<td>7.6</td>
</tr>
<tr>
<td>Volatile oil</td>
<td>1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>2.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Other compound</td>
<td>1.4</td>
<td>1.7</td>
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</table>

Table 4. Total yield and composition of rosemary and sage extracts obtained by SFE (wt% of the plant) [87]

From a practical point of view it is important that increasing of plant extract concentration in foods gives an unpleasant aroma. SFE process allows elimination of volatile components from the extracts. Deodourization can be done effectively by counter-current extraction of the primary extract with carbon dioxide in a high pressure column. The extracts presented in Table 4. were processed by counter-current extraction. The concentration of phenolic diterpenes in the rosemary extract increased from 44 % to 63 % and in the sage extract from 46 % nearly to 68 % [87]. Comparison of antioxidative activity of eight sage and twenty-four rosemary extracts obtained by different extraction methods revealed that there is no clear solvent effect on activity among hexane, ethanol and CO₂ extracts [88].

**Triterpenes and Steroids**

Triterpenes are C₃₀ compounds arising from the cyclization of 3S-2,3-epoxy-2,3-squalene or from squalene itself. Basic skeletons of same important molecules of this group are shown in Figure 6. In view of their therapeutic and industrial applications, triterpenes and steroids constitute a group of secondary metabolites of the utmost importance [1].

Sterols form a major portion of the unsaponifiable matter of vegetable oils. They exists mainly as free sterols and as sterol esters of fatty acids, although sterol glucosides and acylated sterol glucosides are also present. The first investigations of the solubility behaviour of selected steroids was completed by Stahl and co-workers. As Stahl and Glatz [89] concluded, solubility of steroids in CO₂ depends on the number of polar substituents. Steroids with one hydroxyl group (i.e. many sterols) can be dissolved at 40°C from 80 bar upwards, those with two hydroxyl groups from 120 bar, and those with three hydroxyl groups above 150 bar. All compounds with more than three functional groups are practically insoluble in pure CO₂. Saturating the carbon dioxide with water lowered the solubility of steroids. Addition of entrainers, e.g. methanol, ethanol, acetone in concentration of less than 5% increased the solubility of sterols (e.g. sitosterol, stigmasterol, cholesterol). Several experimental solubility data of selected steroids are presented in Table 5.

Many triterpene compounds are present in various plants.
Figure 6. Structures of some triterpenes

Table 5. Solubility of steroids in supercritical fluids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>P(bar)</th>
<th>T(°C)</th>
<th>$y_{max} \times 10^5$</th>
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<td>35-100</td>
<td>0.09</td>
<td>94</td>
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</table>

* graphical presentation
**Mulberry (Morus alba L.)**

Supercritical fluid extraction screening test revealed that mulberry bark contains various extractable pharmaceutical agents (e.g. triterpenes, steroids, amines). Among them α-amyrin acetate was identified in the SFE extract. 1 mg α-amyrin acetate was isolated from 1 g mulberry bark with CO$_2$ at 250 bar and 40°C [95].

Another investigation of mulberry root bark resulted a maximum yield of 3.68 mg α-amyrin acetate from 1 g plant material at 200 bar and 60°C [96].

**Neem (Azadirachta indica A. Juss)**

Azadirachtin is a highly oxidised triterpenoid which can be used as natural pesticide. It is present only in the neem tree. Other biologically active compounds (tetranortriterpenoids e.g. nimbin, salannin) are also present in the plant.

Neem seeds were extracted with CO$_2$ to obtain the active components. Azadirachtin was extracted at high solvent density (0.9 g/cm$^3$) only. A combination of a rapid decompression after 10 min equilibrium period with two sequential extractions increased the recovery of the active components. Increasing the polarity of the solvent by addition of methanol to CO$_2$ increased the yield of the azadirachtin (about 1.5 mg/g seeds was obtained) [97]. Investigation of the selective extraction of triterpenoids from neem seeds resulted that pure CO$_2$ extracted all the nimbin and salannin, whilst some azadirachtin was left in the residue. Using CO$_2$ and methanol, the highest pressure (344 bar) and percentage of methanol (20%) resulted the highest yield of azadirachtin. An optimum was observed for extracting nimbin and salannin at 206 bar and 6% methanol [98].

Similar extraction process described in [97] was applied to recovery of the active compounds from the plant tissue culture. The cell culture were cryogenic milled with liquid nitrogen before extraction which increased the yield substantially. In the process where the sequential extraction was preceded by a rapid decompression azadirachtin was extracted completely (compared with classical solvent extraction) [99].

**Various Plants**

SFE of the bark of Cedrela toona was performed at 390 bar and 40°C. Two phytosterols, stigmasterol and 22, 23-dihydroderivative of stigmasterol were identified [100].

Recovery of stigmasterol by SFE from the Spirodela polyrhiza was less effective than by Soxhlet extraction using n-hexane. The highest yield obtained by SFE was 65 µg/g plant material which is only 56% of the active component extracted with hexane [96].

**Marigold (Calendula officinalis L.)**

The flowers contain flavonoids, carotenoids and triterpenoid compounds. The mixture of faradiol esters is quantitatively the most relevant part of the active fraction, since it represents about 75 % of the triterpene alcohols, and it shows also the highest antiinflammatory activity.

The first extraction tests of Calendulae flos revealed that at 150-300 bar pressures the non-polar active components are soluble in CO$_2$ [101].

The effects of the extraction parameters (pressure, temperature) on the yield and recovery of the active compounds were determined by 3$^2$ full factorial designed experiments in our laboratory [102]. The three-dimensional response surface plot shows that both pressure and temperature have significant effect on the yield (see Figure 7a.). The maximum yield was obtained at $P_E$ = 450 bar and $T_E$ = 60°C for marigold. The non-volatile terpenes and sterols were analysed by direct chromatography of the extracts and/or after acid methanolyses and saponification using the standard techniques. Fractions after the reactions were studied by thin-layer chromatography and TLC combined with densitometry. SFE extracts of marigold contained the characteristic triterpenoids of the plant. Major constituents were the faradiol monoester, the monool taraxasterol, lupeol and β-amyrin. The amounts of the high value faradiol components in the SFE extracts obtained at different conditions were only slightly different. Hence the maximum recovery of faradiol and its esters was obtained also at 450 bar and 60°C. The total faradiol and faradiol ester contents of SFE extracts were between 16-20 g/100 g extract. While the concentration of these principle components was very low in alcoholic extract (0.2-2.0 g/100 g).
The effects of extraction pressure and temperature on the total yield and recovery of the active components were investigated in our laboratory [103]. The three-dimensional response surface plotted to the experimental results graphically is shown in Figure 7b. The conclusion is obvious: by increasing the pressure the yield will be higher, while at this highest pressure (450 bar) the temperature has slight effect on the yield.

The triterpenoid and phytosterol contents in chaste tree extracts (calculated as β-amyrin and β-sitosterol, respectively) were compared according to the designed experiments. The maximum yields (61.4 mg β-amyrin/100 g dried seeds and 108.4 mg β-sitosterol/100 g dried seeds) were obtained at optimum extraction parameters.

**Dandelion (Taraxacum officinale Web.)**

Supercritical fluid extraction of dandelion parts was examined in our laboratory [103]. The products contained the characteristic triterpenoids and phytosterols of *Taraxacum officinale* root and herb, respectively. The concentrations of these active components (calculated in β-amyrin and β-sitosterol, respectively) are shown in Table 6.

**Table 6. Isolation of β-amyrin and β-sitosterol from dandelion**

<table>
<thead>
<tr>
<th>Parts of plant</th>
<th>β-amyrin g/100 g extract</th>
<th>β-amyrin g/100 g dry plant</th>
<th>β-sitosterol g/100g extract</th>
<th>β-sitosterol g/100 g dry plant</th>
</tr>
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<tbody>
<tr>
<td>Roots</td>
<td>24.54</td>
<td>0.424</td>
<td>1.24</td>
<td>0.021</td>
</tr>
<tr>
<td>Leaves</td>
<td>11.17</td>
<td>0.446</td>
<td>3.09</td>
<td>0.123</td>
</tr>
</tbody>
</table>

Extraction with ethyl alcohol resulted higher total yield and recovery of the active compounds. However, alcohol extracted unwanted compounds too, hence the concentrations of the triterpenes in the extract were only 1/5 - 1/10 of those in SFE extracts.

**Tall pitch**

Tall products accumulate as residues from the manufacture of paper by the sulphate-pulp process. The non-distillable residue of the tall soup is the tall pitch which contains phytosterols up to 15%. Stahl and Glatz [2] studied the extraction of tall products with supercritical fluids. Dinitrogen monoxide and ethane showed much better dissolving powers than the carbon dioxide at the same conditions. A continuous extraction of tall pitch combined with stagewise precipitation using three separators in series resulted a product containing ca. 50% free sterols. The overall yield was 85%.
Carotenoids

The carotenoid group includes more than 400 tetraterpenoid compounds consisting of a sequence of eight isoprene units. Carotenoid hydrocarbons are called carotenes. Derivatives that contain oxygen functions (e.g. hydroxy, keto, epoxy, methoxy and carboxylic groups) are called xanthophylls. Some carotenoids are acyclic (e.g. lycopene) but more common are those that contain one six-membered (or occasionally five-membered) ring at one end or two rings at both ends of the molecule. The structure, and some important molecules are illustrated in Figure 8.

Due to their chromophore conjugated double bonds the carotenoids are very widespread natural pigments in plants. They provide the natural yellow, orange or red colours. In plants, the carotenoids occur universally in the chloroplasts of green tissues, but their colour is masked by the chlorophylls. Carotenoids also accumulate in flowers (e.g. marigold), fruits (e.g. tomato, paprika) and roots (e.g. carrot).

The solubility of β-carotene in supercritical fluids have been studied extensively [104-108]. The measured mole fraction was $2.54 \times 10^{-6}$ at 70°C and 429 bar. Addition of cosolvents (1 wt.% ethanol, methanol or methylene chloride in CO$_2$) increased the solubility in every case [104].
Nitrous oxide ($N_2O$) was found a better solvent for β-carotene than CO$_2$. The measured mole fractions were $1.7\times10^{-6}$ and $9.14\times10^{-6}$ at the same conditions (260 bar 67°C) in CO$_2$ and in $N_2O$, respectively [108]. The extraction of carotenoids from a wide varieties of plants has been described.

**Paprika (Capsicum annuum L.)**

Paprika is one of the most important sources of natural carotenoids. Coenen et al. [109,110] proposed a two-step process for the separation of pungent compounds and carotenoids by SFE. Aroma and pungent components were recovered at 120 bar and 40°C, and the paprika residue was re-extracted at 320 bar and 40°C to recover carotenoids. The solvent can be supercritical fluid carbon dioxide, ethane, ethylene, or a mixture of the last two. Ethanol, acetone, water and mixtures of these were proposed as modifiers.

SFE (at 350 bar and 50°C) and hexane extracts from the same raw material were compared. The carotenoid contents in the hexane extracts were significantly higher than in SFE extracts. Also the portion of yellow pigments, especially the esterified pigments was higher in the SFE extracts than in the hexane extracts [111].

The optional operating pressure for the extraction of aromatic components from paprika at 40°C was 150 bar and for the separation of pigments the pressure was 400 bar in the second step [112]. By extraction of different raw material pressures between 400 and 700 bar gave the same colour value, but the extraction time and CO$_2$ consumption was reduced remarkably when extraction pressure was increased. The recovery was about 90% from the good quality raw material [113].

**Alfalfa (Medicago sativa L.)**

Leaf protein concentrates prepared from alfalfa were extracted with CO$_2$ using pressures of 100-700 bar at 40°C. Over 90% of carotene contained in the raw material was removed at extraction pressures in excess of 300 bar. At this pressure only 29% of lutein was recorded. When the solvent pressure was increased to 700 bar, 70% lutein recovery was obtained [114].

**Anatto (Bixa orellana L.)**

Commercial annatto colours are obtained by extraction of the pigments from the pericarp of the fruit. The major pigment component of the extracts is cis-bixin with smaller amounts of other carotenoids. Supercritical CO$_2$ was used to extract natural colours. The chosen operating pressures and temperatures for extractions were 207 bar, 50°C; 310 bar, 60°C and 345 bar, 50°C. The extraction run at 345 bar and 50°C had the highest average pigment concentration (19 mg/g extract). The recovery based on the total yield extracted with the solvent mixture of ethyl alcohol/chloroform (3:1) was 31%. 0.2-0.3 mg pigment/g of seeds was isolated [115].

A combined extraction method with CO$_2$ containing acetonitrile as modifier at 606 bar and 40°C afforded 2.7 mg bixin/g dry weight of annatto seeds [116].

**Sweet potato (Ipomoea batatas (L.) Poir)**

β-carotene is the predominant carotenoid of sweet potato tissue and is primarily found as all trans-β-carotene. Supercritical CO$_2$ was used to obtain a natural carotenoid extract. The most efficient conditions were at 414 bar and 48°C. Tissue pretreatment affected the efficiency of the extraction. The highest carotenoid yield, 0.23 mg/g tissue, was achieved in the extraction from freeze-dried sweet potato. Recovery approached to 80% asymptotically. Apparently, 20% of the carotenoids was inaccessible to supercritical CO$_2$ [117].

**Tomato (Solanum lycopersicon L.)**

Lycopene, which has an intense red colour, is the major pigment component in tomatoes. Dried ripe tomato skins and seeds were extracted with CO$_2$. Trace amounts of lycopene was extracted at 281.2 bar and 40°C. The concentration of lycopene increased on rising the temperature. 84% of lycopene and 93% of β-carotene were extracted compared to the product obtained by Soxhlet extraction when the SFE was performed at 80°C and 281.2 bar [118].

Dried and powdered tomato pomace was extracted with CO$_2$ at 450 bar and 60°C. 0.46 mg of lycopene and 0.04 mg of β-carotene were isolated from 1 g dried pomace [119].

By extraction of dried and powdered tomato skins at more intense conditions (400 bar and 110°C) complete recovery was achieved in 50 min. No change in the composition of lycopene isomers was observed at this high temperature. Modifiers miscible with water (acetone and methanol) gave higher recoveries than pure CO$_2$ [120].

**Carrot (Daucus carota L.)**

The two major carotenoids present in carrot (α- and β-carotenes) were extracted with supercritical CO$_2$. The highest yield was obtained at 250 bar and 57°C. 0.135 mg of α- and 0.150 mg of β-carotenes were extracted per g of freeze dried sample [121].
Extraction of carotenes from carrot tissue was performed using 5 or 10 % ethanol in CO\textsubscript{2} at 303-505 bar and 30-50\textdegree C. The optimum conditions for extraction α- and β-carotene were 303 bar and 50\textdegree C with 10 % ethanol \cite{122}. Effects of particle size, temperature and solvent (CO\textsubscript{2} or N\textsubscript{2}O) were investigated. The smallest size (d\textsubscript{p}<0.25 mm) gave higher yield. Extraction rate was increased with rising temperature when a constant density solvent was used. Extraction with N\textsubscript{2}O was faster than that performed with CO\textsubscript{2} due to the higher solubility of β-carotene in N\textsubscript{2}O \cite{123}.

**Conclusions**

Medicinal and aromatic plants, their active components – among those terpenoids, the largest known group of plant secondary metabolites – are widely used as raw materials in the production of drugs, phytopharmaceutical preparations and functional foods containing isolated natural compounds, total and selectively extracted or purified extracts. There is a growing interest in new natural products that act very specifically and topically, so as to avoid undesirable side-effects. In many cases very specific and selective recovery processes are needed.

Supercritical fluid extraction is one of the most important newer separation methods. The technology has achieved a special success in the terpenoid chemistry as extraction with supercritical fluids causes only a minimum of temperature stress for the usually sensitive molecules. Further advantages are the mild operating conditions, exclusion of air, and an inert solvent environment. These are all ideal for the preparation of medicinal plant extracts. Supercritical fluid extraction may offer possibilities to produce extracts free from non-wanted compound like the monoterpene thujone, etc. The most frequently used carbon dioxide for extraction does not add any health risk to food or pharmaceutical products, like other solvents may do.

As supercritical and near critical fluids have properties that are different to conventional solvents the extracts prepared by this method have to be considered as a new combination of constituents already known for the traditional extracts of the same plant. By selecting the fluid polarity and/or density, the solvating power of the fluid can be adjusted enabling the possibility of class-selective extraction. Therefore supercritical fluid extraction offers possibilities for increasing the phytotherapeutical potency. Besides supercritical fluid extracts contain less, almost inactive components. Therefore carbon dioxide extracts are different from the traditional preparations not only in chemical composition but in phytotherapeutical activity as well. There is an urgent need for phytochemical data of plant extracts prepared by supercritical fluid extraction. These may serve as an essential part in the pharmacological, toxicological and clinical tests in the documentation of each herbal drug.

Supercritical fluid extraction is still not yet a common technique. The broadness of applications needs for development of new applications, more detailed phytochemical analysis of extracts. Although supercritical fluid extraction does not provide the solution to every problem, but it may be an integrated part in the production of required standards of quality for phytopreparations.

**Acknowledgment**

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**Nomenclature**

\[d\] diameter of particle (mm),
\[P\] pressure (bar),
\[T\] temperature (\textdegree C),
\[y\] solubility (mole fraction),
\[Y\] total extraction yield (wt%),
\[YP\] active component yield (mg/100 g plant).

Subscripts

\[E\] extraction,
\[p\] particle,
\[S\] separation,
\[\text{max}\] maximum
\[\text{min}\] minimum.

**References**


