The use of supercritical fluid chromatography for natural product analysis as well as underlying theoretical mechanisms and instrumental requirements are summarized in this review. A short introduction focusing on the historical development of this interesting separation technique is followed by remarks on the current instrumental design, also describing possible detection modes and useable stationary phases. The overview on relevant applications is grouped based on their basic intention, may it be (semi) preparative or purely analytical. They indicate that supercritical fluid chromatography is still primarily considered for the analysis of nonpolar analytes like carotenoids, fatty acids, or terpenes. The low polarity of supercritical carbon dioxide, which is used with modifiers almost exclusively as a mobile phase today, combined with high efficiency and fast separations might explain the popularity of supercritical fluid chromatography for the analysis of these compounds. Yet, it has been shown that more polar natural products (e.g., xanthones, flavonoids, alkaloids) are separable too, with the same (if not superior) selectivity and reproducibility than established approaches like HPLC or GC.

Abstract

The use of supercritical fluid chromatography for natural product analysis as well as underlying theoretical mechanisms and instrumental requirements are summarized in this review. A short introduction focusing on the historical development of this interesting separation technique is followed by remarks on the current instrumental design, also describing possible detection modes and useable stationary phases. The overview on relevant applications is grouped based on their basic intention, may it be (semi) preparative or purely analytical. They indicate that supercritical fluid chromatography is still primarily considered for the analysis of nonpolar analytes like carotenoids, fatty acids, or terpenes. The low polarity of supercritical carbon dioxide, which is used with modifiers almost exclusively as a mobile phase today, combined with high efficiency and fast separations might explain the popularity of supercritical fluid chromatography for the analysis of these compounds. Yet, it has been shown that more polar natural products (e.g., xanthones, flavonoids, alkaloids) are separable too, with the same (if not superior) selectivity and reproducibility than established approaches like HPLC or GC.

Abbreviations

ACN: acetonitrile
APCI: atmospheric pressure chemical ionization
CAD: charged aerosol detector
CD: circular dichroism
DAD: diode array detector
DCM: dichloromethane
DGC: dense gas chromatography
DPPH: 2,2-diphenyl-1-picrylhydrazyl
ECD: electron capture detector
ELSD: evaporative light scattering detector
ESI: electrospray ionization
FID: flame ionization detector
FPP: fully porous particles
FTIR: Fourier transform infrared spectroscopy
GC: gas chromatography
GMP: good manufacturing practices
HPGC: high-pressure gas chromatography
HPLC: high-performance liquid chromatography
HPTLC: high-performance thinlayer chromatography
IC: ion chromatography
IMS: ion mobility spectrometry
IPA: isopropylamine
IR: infrared
LOD: limit of detection
MALDI-TOF-MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MS: mass spectrometry
MTBE: methyl tert-butyl ether
NMR: nuclear magnetic resonance
ODS: octadecylsilyl
OTC: open tubular column
RP-HPLC: reversed-phase HPLC
RSD: relative standard deviation
SIC: silver ion chromatography
SFC: supercritical fluid chromatography
SFE: supercritical fluid extraction
TAGs: triacylglycerols
THF: tetrahydrofuran
UHE/LP: ultrahigh efficiency/low pressure
**Introduction**

Whenever temperature and pressure exceed a substance-specific critical value (i.e., the critical point), a supercritical fluid of a compound is obtained (Fig. 1). In this state, neither higher pressure can revert to a liquid again nor elevated temperatures to a gas. In simplified terms, supercritical fluids can be seen as highly compressed gases with liquid-like density [1]. They combine characteristics of both physical states in a unique way, such as low viscosity and high diffusivity [2]. In terms of chromatography (supercritical fluid chromatography), this results in a low pressure drop even at higher flow rates, faster separations, increased solvating power, and efficiencies, so that fluids in supercritical conditions often are considered as ideal mobile phases [2,3]. Today, carbon dioxide-based fluids are used almost exclusively because of several reasons [4]. Critical temperature (31°C) and pressure (74 bar) are easy to reach with the conventional type of HPLC instrumentation; CO2 is inert, nonflammable and cheap, it can be liquefied and stored in cylinders without problems, and it is considered environmentally friendly (“green”). The latter might not remain without opposition, but as carbon dioxide used for SFC is an industrial by-product, the overall environmental impact of using it is zero (in contrast to the production/disposal of regular solvents) [5]. The polarity of supercritical CO2 is similar to hexane, but by adding modifiers like methanol, ethanol, or acetone, solvent strength is adjustable, so that normal- or reversed-phase stationary phases can be utilized [6,7]. Carbon dioxide, due to its rather low critical temperature, is also ideal for the analysis of thermo-labile compounds and in case the isolation of constituents is attempted, no final evaporation of the solvent is required. At atmospheric pressure, CO2 converts to the gaseous state and discharges, leaving the purified fractions/compounds behind.

**Technical Aspects**

**Historical development and current state of instrumentation**

It is maybe a surprising fact that the first report on SFC, at this time the technique was called HPGC or DGC, occurred in the year 1962 already, slightly before the evolution of HPLC started. Klesper et al. used a mobile phase of supercritical fluoromethane derivatives to successfully separate a mixture of porphyrins on a polyethylene glycol stationary phase [8]. A few years later, Sie and coworkers described CO2 as a suitable eluent and UV as a possible detection mode [9]. In the 1970 s, SFC instruments with pressure programming were constructed [10], the first preparative applications reported [11], and an SFC-MS interface described [12]. The decade thereafter brought mainly improvements in column technology, leading to two different strategies. OTCs developed by Novotny and coworkers resembled the GC columns used today, i.e., the inner surface of a silica capillary with a small internal diameter (typically 50 µm) is coated with a thin film of stationary phase, e.g., a polysiloxane [13]. These materials showed several disadvantages, such as that the mobile phase pressure could not be changed independently of the flow velocity, or the commonly used type of detector (FID) was incompatible with organic modifiers. This explains why the interest in this SFC variant faded rather quickly [14]. The second strategy, which was developed at the same time and is standard nowadays, is the use of packed columns as employed for HPLC [15]; for further details on stationary phases and SFC, see the respective chapter in this review. The design of a packed column SFC system is schematically shown in Fig. 2. As can be seen, it resembles the common HPLC setup to a large extent. In addition to pumps for the mobile phase (CO2 and modifier), an injection and detection device, it requires two additional components: an oven to facilitate supercritical/subcritical conditions and a backpressure regulator, commonly positioned after the detector. Its function is to adjust the fluid pressure above the critical pressure independent of the mobile phase flow rate or composition. Thus, this electronic device originally developed by Saito et al. is most important to maintain SFC conditions throughout the entire separation system [1].

**Stationary phases**

Pure, liquefied carbon dioxide is a solvent with nonpolar character, and SFC was often considered an alternative for normal-phase chromatography only. In this context, polar stationary phases like silica gel, cyano- or aminopropyl-bonded silica are suitable options [16]. But as the eluotropic strength of CO2 can be modified by polar additives, reversed-phase type materials (e.g., alkyl-bonded silica) are useable as well. Thus, in respect to column technology, SFC, at least as it is most commonly used today, resembles “standard” liquid chromatography to a large extent. The same stationary phases (including aromatic, halogenated, or porous graphitic carbon and polymeric phases) and column dimensions as in HPLC can be used regardless if the separations are performed in the subcritical or supercritical stage [17,18]. For a differentiation of the latter two, see the comments below (Theoretical Considerations). This indicates that SFC is rather versatile in respect to applicable stationary and mobile phases, and both need to be considered in order to improve chromatographic performance and selectivity during method development.

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**Fig. 1** Phase diagram of a compound indicating the supercritical fluid stage above critical pressure (Pc) and temperature (Tc).

**Fig. 2** As can be seen, it resembles the common HPLC setup to a large extent. In addition to pumps for the mobile phase (CO2 and modifier), an injection and detection device, it requires two additional components: an oven to facilitate supercritical/subcritical conditions and a backpressure regulator, commonly positioned after the detector. Its function is to adjust the fluid pressure above the critical pressure independent of the mobile phase flow rate or composition. Thus, this electronic device originally developed by Saito et al. is most important to maintain SFC conditions throughout the entire separation system [1].
Theoretical Considerations

By definition, SFC-based separations should be performed under supercritical conditions, which only occur above critical pressure and temperature. These values vary depending on the composition of the mobile phase, and they generally increase by adding modifiers, e.g., with 5% of methanol in CO₂, the critical temperature rises to 51°C and the critical pressure to 105 bar [1]. But there is a continuous transition between the supercritical and the liquid (subcritical) state, and the operator cannot really tell which fluidal stage is actually reached. Diffusion coefficients depend on fluid density so that separation efficiency and fluid compressibility are reduced in the subcritical stage, but otherwise supercritical and subcritical fluids have similar characteristics [2, 16]. Therefore, even “SFC” separations are often performed in the subcritical region at a temperature or pressure below the critical value, sub- and supercritical fluid chromatography are not differentiated most of the time.

When working with SFC, one has to be aware of some phenomena not occurring in regular HPLC. They are based on fluid compressibility and absorbance of the mobile phase on the stationary phase, and primarily occur on flow rate changes and modifier addition [18]. Varying the flow rate naturally changes the internal pressure in the column. Yet, in SFC, this also modifies the mobile phase density and, consequently, its solvent strength and retention factor [23]. Modifiers are usually advantageous to improve compound solubility in the mobile phase, modify solute-mobile-phase interactions, and improve peak resolution [24]. However, in SFC, the proportion of the adsorbed modifier onto the stationary phase is strongly controlled by the mobile phase composition. This explains a different polarity of the stationary phase depending on the percentage and type of modifier added. Consequently, these changes also have an effect on compound retention.

More details on the underlying mechanisms, models for prediction, and the separation kinetics of SFC can be found in excellent reviews by Lesellier [16], Poole [17], and Medina [25]. They indicate that kinetic optimization and retention mechanisms are more complex and interdependent on system conditions than in conventional HPLC.

Detection

As for stationary phases, SFC is quite flexible in respect to the type of detector used. If the mobile phase consists of pure CO₂, IR or FID can be utilized; when a modifier is added DAD, ELSD, MS, CD, or CAD are options [16, 19–21]. Depending on the type of detector, the backpressure regulator of the instrument is positioned. For example, with DAD and CD, it needs to be after the detector to maintain the pressure (required for the liquid state of the mobile phase) in the detector, and accordingly, detector cells have to be pressure resistant up to the maximum value reached during separation. For ELSD or MS, the mobile phase needs to be evaporated before detection/ionization anyway, so that the evaporation of CO₂ under ambient conditions is an advantage. In this case, the backpressure regulator is placed before the actual detection device, and the gaseous effluent can directly be introduced into the detector. The most popular SFC-MS interfaces are APCI and ESI. For ion generation, the addition of proton-donating organic modifiers like methanol is usually advantageous [22].

Supercritical Fluid Chromatography – Applications on Natural Products

Only a few reviews on the use of SFC for natural product analysis or isolation have been published to date, and they primarily focus on nonpolar analytes like fat-soluble vitamins [26], fatty acids [27], or essential oils [28, 29]. Some of them are more than ten years old already, so they possibly do not reflect the current state of research anymore. Thus, the following chapters are intended to provide an updated survey of the versatile use of SFC in this area of research, arranged by the type of application, which is either preparative (prep-SFC) or analytical.

Preparative/semipreparative

Supercritical fluids are utilized for preparative purposes in two techniques, SFE and SFC. In the first case, supercritical CO₂, most of the time with modifiers, is used for extraction, a procedure that has been described for many matrices and compounds. Preparative applications on food, by-products of industrial processes, environmental samples, pharmaceuticals, and natural products are summarized in a review by Herrero et al. [30]. Con-
Cerning natural products, the applications are manifold, ranging from essential oils in ginger [31], chamomile [32], or *Salvia officinalis* [33] or carotenoids in algae [34] to more polar constituents like valereneic acid in *Valeriana officinalis* [35] or xanthones in *Garcinia mangostana* [36]. More recent SFE papers even describe the extraction of prebiotic carbohydrates (lactose, lactulose, galactose, and tagatose) from solid mixtures [37], or free amino acids from broccoli leaves [38]. In the latter publication, the optimum conditions were found to be 250 bar at 70°C, using supercritical CO₂ with 35% methanol as the solvent; the overall extraction time was 35 min. The obtained yields were comparable to a conventional solvent extraction with methanol/water (7/3).

As a second preparative approach, SFC can be used for the isolation/purification of individual compounds either in the preparative or semipreparative scale. The technique is especially popular for the enantioseparation of pharmaceuticals, because it allows faster separations than conventional HPLC, is convenient to use for the enantioseparation of pharmaceuticals, because it allows faster separations than conventional HPLC, and demands no final evaporation of the solvent is required), and a large number of chiral stationary phases with divergent properties is available.

Several reviews provide a good overview on this topic [2, 7, 39, 40]. The use of SFC for the isolation of natural compounds is less common, even if some respective reports can be found in the literature (Table 1).

### Table 1: Examples of natural products isolated or purified by prep-SFC.

<table>
<thead>
<tr>
<th>Biological material</th>
<th>Compounds</th>
<th>Stationary phase</th>
<th>Analytical conditions</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pachastrissa</em> sp.</td>
<td>Jaypine B, ent-jaypine B</td>
<td>Chiralpak IC</td>
<td>CO₂ + 25% DCM/MeOH (8/2), 100 bar/35°C</td>
<td>[41]</td>
</tr>
<tr>
<td><em>Trigoneofoenum-graecaem</em></td>
<td>Spirostanol saponins</td>
<td>Chiralpak IC</td>
<td>CO₂ + MeOH (33%), 120 bar/40°C</td>
<td>[42]</td>
</tr>
<tr>
<td><em>Citrus sinensis</em></td>
<td>Polymericlaxoflavones</td>
<td>Daicel AD</td>
<td>CO₂ + DEA (0.25%) + MeOH (45%), 98 bar/30°C</td>
<td>[43]</td>
</tr>
<tr>
<td>Tuna (Thunnus thynnus) oil</td>
<td>Fatty acid esters</td>
<td>Kromasil 10–3C18</td>
<td>CO₂, 145 bar/65°C</td>
<td>[44]</td>
</tr>
<tr>
<td>Fish oil</td>
<td>Fatty acid esters</td>
<td>Silia gel</td>
<td>CO₂, 200 bar/80°C</td>
<td>[45]</td>
</tr>
<tr>
<td>Corn bran oil</td>
<td>Phytosterol esters</td>
<td>Amine-propyl silica</td>
<td>CO₂ + EOH (2–10%), 690 bar/80°C, 345 bar/40°C</td>
<td>[46]</td>
</tr>
<tr>
<td>Palm oil</td>
<td>Tocopherol, tocotrienols</td>
<td>Nucleosil diol</td>
<td>CO₂ + MTBE (6%), 190 bar/40°C</td>
<td>[47]</td>
</tr>
<tr>
<td>Rosmarinus officinalis</td>
<td>Antioxidants</td>
<td>Supelco SIL diol</td>
<td>CO₂ + EOH (10%), 130 bar/80°C</td>
<td>[48]</td>
</tr>
<tr>
<td>Rosmarinus officinalis</td>
<td>Carcsonic acid</td>
<td>Virdis SCF</td>
<td>CO₂ + EOH (5–20%), 100 bar/40°C</td>
<td>[49]</td>
</tr>
<tr>
<td>Thymus vulgaris</td>
<td>Thymol</td>
<td>Kromasil 60–5SIL</td>
<td>CO₂ + EOH (5%), 150 bar/50°C</td>
<td>[50]</td>
</tr>
<tr>
<td>Piper methysticum</td>
<td>Kava lactones</td>
<td>C4 Protein</td>
<td>CO₂ + IPA + MeOH (2–10%), 125 bar/80°C</td>
<td>[51]</td>
</tr>
</tbody>
</table>

A slightly larger number of papers report on the isolation or fractionation of natural products from crude herbal material using non-chiral stationary phases. As can be seen from Table 1, mostly nonpolar compounds like fatty acid esters, phytosterols, and tocotrienols from different kind of oils (tuna, fish, corn or palm oil) were purified [44–47]. For these matrices, reversed-phase (C18) or normal-phase (silica, amino-propyl silica, or diol) materials were suitable, using pure carbon dioxide or mixtures with modifiers (e.g., ethanol, MTBE) as the eluent. Referring only to one respective example in detail, Han et al. purified eight tocopherols (α-T, β-T, ω-T, and γ-T) and tocotrienols (α-T₃, β-T₃, ω-T₃, and γ-T₃) from palm oil [47]. In their study, they compared two types of normal-phase material, silica, and diol, and found the latter to be advantageous as Lichrosorb silica did not enable the separation of constituents like β-T and β-T₃. Experimental conditions were optimized in an analytical scale using standard compounds; the actual sample (5 g of crude palm oil) was first saponified with KOH and extracted with hexane. The unsaponifiable matter was dissolved in DCM and this solution was finally separated by SFC. Actual isolation yields were not presented in detail, but quantitative results for one sample were given.

Two manuscripts emphasize the enrichment of antioxidant and antimicrobial compounds from rosemary (*Rosmarinus officinalis*) leaves [48] and the isolation of carsonic acid from the same material [49]. The applied separation conditions were comparable, except in the latter study, the use of an SFC-specific stationary phase (Virdis SFC 2-ethylpyridine from Waters) resulted in a much higher purity of carsonic acid (92%) and a better separation from carnosol. All preparative experiments were conducted with supercritical rosemary extracts, which were dissolved in ethanol (5 mg/mL) and then injected (100 µL per analysis). Under optimized conditions, the target analyte could be concentrated 4.5 times compared to the starting material. Additionally, the content of carsonic acid clearly correlated with the antioxidant properties of rosemary extracts as determined in the DPPH assay. Oth-
er studies reported on the enrichment of thymol from thyme (Thymus vulgaris) leaves by SFC [50] and the semipreparative separation of kava lactones [51]; in both, the respective supercritical fluid extracts were used as starting material. The analytical conditions do not differ much from those already described, except that Ashraf-Khorassani et al. used a rather uncommon stationary phase (C4 protein from Vydac) and two 250 × 4.6 mm columns in tandem to resolve seven lactones in Piper methysticum.

**Analytical**

**Vitamins:** First attempts of carotenoid analysis by SFC were carried out on capillary columns, primarily focusing on the separation of α- and β-carotene isomers. Respective studies with tomato and carrot extracts showed the successful separation of xanthophyll, lycopene, and α- as well as β-carotene by using different SB–cyanopropyl–polymethylsiloxane columns from Lee-Scientific [52]. However, subsequent publications described the use of packed columns only (Table 2). Some methodological studies investigated the influence of parameters like temperature, organic modifiers, and stationary phases on the separation of carotenoids [53–55]. Lesselier et al. [54] tested the efficiency of 22 different stationary phases, and in another publication by the same group, the impact of different modifiers on the chromatographic resolution of the same compounds at a raised temperature, so that the optimum separation was described to be at 22–25 °C, which is in the subcritical region of carbon dioxide already. Increasing the CO2 pressure led to decreased capacity factors; however, selectivity was independent from temperature and pressure, and was only influenced by modifiers. Pure solvents as well as binary and ternary mixtures were screened, but most of them resulted in a concentration-dependent decrease of the capacity factors of carotenoids. On the other hand, a methanol-acetonitrile mixture (7:3) resulted in an improved separation of the respective trans/cis isomers. Matsubara et al. [56] developed the first comprehensive profiling method for carotenoids and their structural isomers by SFC with MS detection. The method allowed for the separation of β-carotene, lycopene, zeaxanthin, lutein, antheraxanthin, neoxanthin, and violaxanthin in a green algae (Clamydomonas reinhardtii) within 15 min on a monomeric ODS column from Merck (Purospher Star RP-18e, 250 × 4.6 mm), at an outlet pressure of 100 bar and a column temperature of 35 °C. The flow rate was set to 3 mL/min using 10–30% methanol with 0.1% (w/w) ammonium formate as the modifier. Under the same conditions, a monolithic silica column (Chromolith Performance RP-18e) permitted an even faster separation of the seven carotenoids. A few years later the same group used similar conditions to investigate epoxy carotenoids, which are degradation products of carotenoid oxidation [57]. Five major carotenoids and six epoxy isomers were successfully identified in human serum using a Purosphere RP 18e phase. In 2012, Abrahamsson et al. [58] described the first validated SFC method for the analysis of major carotenoids in Scenedesmus sp., a microalga (Fig. 3). Optimum results were obtained with two columns in series, a SunFire C-18 (4.6 × 250 mm; 5 µm) and a silica-bonded 2-ethylpyridine column (4.6 × 250 mm; 5 µm), both with 100 Å pore size and produced by Waters. CO2 and methanol (in gradient mode) were employed as the mobile phase, at a flow rate of 5 mL/min and 32 °C column temperature. Method validation revealed similar results compared to conventional HPLC. For example, the LOD for the different carotenoids was between 0.02 and 0.05 mg/L, and recovery rates ranged from 97 to 103%; repeatability was found to be be-

### Table 2: Examples of vitamin analysis by supercritical fluid chromatography.

<table>
<thead>
<tr>
<th>Biological material</th>
<th>Compounds</th>
<th>Stationary phase</th>
<th>Analytical conditions</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrots, tomatoes</td>
<td>Lycopene, α-carotene, β-carotene + isomers</td>
<td>SB-phenyl-S5</td>
<td>CO2 + EtOH (1%), isobaric, 45–50 °C</td>
<td>[52]</td>
</tr>
<tr>
<td>Carrots</td>
<td>All-trans α- and β-carotenes</td>
<td>LiChrospher 100 RP 18, Spherisorb ODS</td>
<td>CO2 + MeOH/ACN (15%), 150 bar/22 °C</td>
<td>[53]</td>
</tr>
<tr>
<td>Scenedesmus sp.</td>
<td>Canthaxanthin, astaxanthin, lutein, chinenone, violaxanthin, zeaxanthin, neoxanthin, β-carotene</td>
<td>SunFire C18, Viridis SFC silica 2-ethylpyridine</td>
<td>CO2 + MeOH (10–25%), 120 bar/32 °C</td>
<td>[58]</td>
</tr>
<tr>
<td>Allium cepa (onion)</td>
<td>Linoleic acid, δ-sitosterol, palmitic acid, α-tocopherol</td>
<td>SB-methyl-100</td>
<td>CO2, 100–320 bar (pressure programming), 280 °C</td>
<td>[59]</td>
</tr>
<tr>
<td>Vegetable oils</td>
<td>α-, β-, γ-, δ-tocopherols</td>
<td>ODS-silica gel</td>
<td>CO2 + MeOH (0.5%), 150 bar/32 °C</td>
<td>[60]</td>
</tr>
<tr>
<td>–</td>
<td>vitamin A acetate isomers</td>
<td>Vydac peptide and protein C18</td>
<td>CO2, 130 bar/60 °C</td>
<td>[64]</td>
</tr>
<tr>
<td>–</td>
<td>vitamin K1 isomers</td>
<td>RegisPak C18</td>
<td>CO2 + MeOH (5%), 150 bar/30 °C</td>
<td>[66]</td>
</tr>
<tr>
<td>Palm oil</td>
<td>Coenzym Q 10</td>
<td>Metaphase RP 18</td>
<td>CO2 + MeOH (6.6%), 180 bar, 50 °C</td>
<td>[67]</td>
</tr>
</tbody>
</table>

**Fig. 3** Supercritical fluid chromatography separation of carotenoids [(2) astaxanthin, (5) lutein, (6) zeaxanthin, (7) β-carotene, and (8) neoxanthin)] in a Scenedesmus sp. extract; compounds A–F are unidentified chlorophylls. Reproduced with permission from [58].
tween 0.9% and 2.4% RSD. Accordingly, the authors considered their procedure advantageous because it is the first validated method that is rapid as well as environmentally sustainable (range of methanol as the modifier during analysis: 9–25%).

Several SFC methods have been published for the analysis of tocopherols in plant seeds [59], vegetable oils [60], wheat germ [61], and microalgae [62]. Separations were described on capillary as well as packed columns; in the first case, using pure CO₂ and adjusting the desired selectivity by the choice of stationary phase [59,61]. With packed columns, the use of modifiers was mandatory. In terms of technical advancement, Ibanez et al. [63] described the optimization of a dynamic online SFE-SFC coupling method for tocopherol analysis. They investigated the performance of capillary columns of varying dimensions (180 and 500 µm i.d.) filled with porous silica particles (10 mm, 60 Å, Hi-chrom), which were coated with different stationary phases (polar: 3–20% Carbowax 20 M; nonpolar: 10% SE-54 (95% methyl- and 5% phenyl-silicone; Supelco)). In spite of generally higher separation efficiencies with smaller inner diameter capillaries, the larger diameter was chosen with regards to the requirements for an online coupling to SFE (i.e., higher sample loading volumes and capacity). In respect to the coating material, only the polar type was able to separate all four analytes (α-, β-, γ-, δ-tocopherol) in 30 min. Coating the particles with higher percentages of Carbowax 20 M (3–10%) resulted in a better resolution, but also increased the retention times.

An interesting study reported on the hyphenation of SFC to ¹H NMR for the analysis of vitamin A acetate isomers [64]. By using a Vydac peptide and protein C18 column, five respective cis/trans isomers could be separated and structurally identified in less than 25 min. Isomerized vitamin A acetate was dissolved in DCM and separated by SFC first, and then was subjected to NMR analysis (Bruker ARX 400) using a specially designed pressure-proof probe head with a volume of 120 µL. The NMR spectra of several phthalates could be recorded even in the continuous flow mode. To date, there are only two reports describing the separation of vitamin K isomers by SFC, and both were carried out by Berger et al. In an application note, the cis- and trans-isomers of vitamin K₁ could be resolved in 2 min on an Agilent RX-Sil column (3 × 100 mm, 1.8 µm), which is 3 times faster than conventional HPLC [65]. A recent publication describes the separation of seven enantiomers of vitamin K₁ on a chiral stationary phase (Regis-Pack CLA-1, 250 × 4.6 mm, 5 µm) using 5% methanol in CO₂ as the mobile phase at 2 mL/min, 30 °C, and 150 bar, respectively. However, a baseline separation could not be obtained for all of the compounds [66]. Coenzyme Q₁₀, which is structurally related to vitamins K and E, shows promising health benefits for the treatment of cardiovascular diseases and is a by-product in the extraction of vitamin E from palm oil. The compound could easily be resolved from other constituents in the ethanolic fraction of the unsaponifiable matter of palm oil and palm fiber oil by SFC on RP18 material, as described in the manuscript by Han et al. [67].

Fatty acids and acylglycerols: Fatty acids and acylglycerols, often termed “lipids”, are the best-studied compounds in SFC analysis. Several reviews have summarized respective applications mainly focusing on food-related matrices [68–70].

The standard method for fatty acid analysis is definitely gas chromatography. However, most derivatives show low volatility and moderate polarity (e.g., hydroxy fatty acids), so that derivatization is commonly required for their analysis by GC. For SFC, these properties are no limitation, and, accordingly, the separation of fatty acids has been carried out on OTC as well as on packed columns. The advantages and limitations of individual stationary phases are summarized in a paper by Senorans and Ibanez [27]. They conclude that OTCs show high efficiencies, but in terms of loadability, sample capacity, and short analysis times, packed columns are a much better choice. Hirota and Sogabe were the first to describe two-dimensional supercritical fluid chromatography as it is already established for GC [71]. Their system comprised a silica gel column (SuperCrestSIL, 50 × 4.6 mm; 5 µm) in the first dimension, coupled to an ODS-column (L-column ODS, 50 × 4.6 mm; 5 µm) in the second dimension. Both were maintained under 130 bar pressure, but at a different temperature (first column: 20 °C, second column: 50 °C); pure CO₂ served as the mobile phase, with a flow rate of 2 and 3 mL/min, respectively. After the first column, the effluent was trapped in a tube (1 m × 0.25 mm i.d., 100% methyl silicone, 0.25 µm film thickness) and then flushed on the second-dimension column. Fatty acid methyl esters (FAMEs) in various oils (e.g., fish or soybean) were qualitatively analyzed. First-dimension separations were primarily based on the number of double bonds, while the second-dimension separations depended on the chain length of the analytes. Thus, two orthogonal separation modes could be combined in a unique way. In a subsequent publication of the same group, they modified their setup by also incorporating SFE, so that sample cleanup, extraction, and analysis became feasible in one step [72]. Practical applicability was confirmed for styrene oligomers in polystyrene; natural products were not analyzed.

Two just recently published reviews summarize the publications on the SFC analysis of triglycerides. Bamba et al. concluded that monolithic columns connected in series are preferable for profiling TAGs due to high-resolution, high-throughput, and low-back pressure [69]. When SFC-MS was used, TAGs were usually detected as ammonium adducts [M + NH₄]⁺, and a cone volume of 35 V₆₃ was shown to be ideal for the fragmentation of most compounds. The review of Bernal et al. excellently summarized the different stationary phases already described for triglyceride analysis and discussed their impact on retention [68]. The authors observed that the most important factor for separation on capillary columns is the number of acyl-carbons in the respective molecules. On these stationary phases, coelutions occur frequently, so that a combination with MS might be required. Reversed-phase materials are a more selective option when using packed columns, either with UV-, ELSD, or MS detection [73–75]; the described separation conditions were typical for SFC. Lesselier et al. investigated the influence of different polar modifiers and obtained the best results with acetonitrile. An increase in selectivity was noticed when decreasing the temperature from 30 to 5 °C. On the other hand, pressure changes had no big impact on selectivity [73]. A less common approach is the use of SIC in combination with SFC [76]. SIC is based on a cation exchange mechanism, which facilitates the separation of compounds like fatty acids and acylglycerols because of the number, position, and configuration of double bonds. A silica-based strong cation exchanger column (Nucleosil 100–5 SA) was first loaded with silver ions, and their excess was removed by flushing with 1% ammonium acetate solution. After this step, “conventional” SFC separations were performed with acetonitrile and isopropanol (in the gradient mode) as modifiers (Table 3). Just recently, Lesselier et al. published an innovative method for triglyceride separation [77]. They developed an UHPL/SFC using FFP. With such core-shell materials, high theoretical plate numbers above 100 000 were observed, and as these phases generate a low-back.
pressure, several columns could be coupled without the need of an ultrahigh-pressure chromatographic system. Five columns (Kinetex C18, 150 x 4.6 mm, 2.6 µm) in tandem at a low temperature (17°C) and with ACN as a modifier significantly improved the separation of TAGs in vegetable oils compared to previous publications (Fig. 4).

Steroids: SFC offers a new perspective for sterol analysis because of its mainly hydrophobic character, the possibility of direct coupling to SFE, and the advantage to renounce from derivatization reactions. Yet, the drawbacks of early publications were long analysis times and missing precision data [78, 79]. Huber et al. presented a rapid and precise method for cholesterol determination in milk fat by SFC [80]. Compared to conventional GC analysis, they used an SB Phenyl 5 (20 m x 0.5 µm) capillary column for separation with CO2 as the mobile phase and FID detection. Carbon dioxide density was changed during analysis from 0.012 to 0.45 (g/mL)/min in the gradient mode. The authors considered their approach more accurate, but GC analysis was faster and cheaper. Baiocchi et al. investigated the performance of different capillary columns with either FID or ECD detection [81]. With stationary phases of medium polarity like polyethylene glycol (Carbowax) and polydimethylsilicone, good resolution and reasonable analysis times were reached for several testosterone and progesterone derivatives. For later eluting compounds (trenbolone, corticosterone, cortisone-21-acetate, and prednisolone-21-acetate), ECD was advantageous due to a higher detection sensitivity. However, a significantly reduced flow rate and CO2 pressure (9 bar) were necessary to achieve a low background noise and to avoid baseline drifts. The SFC analysis of sterols with different polarity and structural features (e.g., androstenone, testosterone, cortisone, or estradiol) on packed columns is described as well, also studying the influence of column materials and modifiers on retention. If silica-based materials are used, nonpolar components like androstenone can be eluted using pure CO2; low percentages of methanol are required when separating steroids present, such as enanthates, valerates, or capronates. More polar steroids (e.g., keto and hydroxy steroids) might require derivatization prior to SFC analysis [82, 83]. One of the few medicinal plant-related applications is the already mentioned study by Zhao et al., who successfully isolated stereoisomeric spirostanol saponins in T. foenum-graecum by SFC-ELSD on Chiralpak IC material [42].

Terpenes and terpenoids: As can be seen in Table 3, the major terpenes in the essential oil of hops could be separated by SFC-Fourier-transform infrared spectroscopy (SFC-FTIR) [84]. Optimum SFC separations were achieved on an OTC column (SB-Phenyl-5) in 60 min, at a constant temperature of 80°C, and a flow rate of 3 mL/min CO2. Infrared spectra of the major constituents were taken as films deposited on AgCl discs and compared with those obtained after SFC separation in an IR flow cell (Perkin-Elmer 1760 x) within a scan range of 3500–800 cm⁻¹. The FTIR spectra indicated differences depending on the hop variety and can be a useful tool to determine the origin of different hop oils. Suto et al. described an online-coupled SFE-SFC method for the

Table 3 Examples for the analysis of triglycerides and terpenes by supercritical fluid chromatography.

<table>
<thead>
<tr>
<th>Biological material</th>
<th>Compounds</th>
<th>Stationary phase</th>
<th>Analytical conditions</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetable oil (soybean, sunflower)</td>
<td>Triglycerides</td>
<td>Nucleosil 100–5 S A</td>
<td>CO2 + ACN/Isooctane (1.2–12.2%), 150–300 bar/65°C</td>
<td>[76]</td>
</tr>
<tr>
<td>Vegetable oil (argan, rapeseed)</td>
<td>Triglycerides</td>
<td>Kinetex C-18</td>
<td>CO2 + MeOH/ACN (12%), 100 bar/17°C</td>
<td>[77]</td>
</tr>
<tr>
<td>Humulus lupulus</td>
<td>Myrcene, humulene, caryophyllene</td>
<td>SB-phenyl-5</td>
<td>CO2, 80–350 bar/80–140°C, 80–140°C</td>
<td>[84]</td>
</tr>
<tr>
<td>Magnoliaceae cortex</td>
<td>Magnolol, honokiol</td>
<td>Prep Pak NH2</td>
<td>CO2 + MeOH (5%), 200 bar/45°C</td>
<td>[85]</td>
</tr>
<tr>
<td>Artemisia annua</td>
<td>Artemisinin, artemisinic acid</td>
<td>Nucleosil NH2</td>
<td>CO2 + MeOH (3%), 250 bar/40°C</td>
<td>[86]</td>
</tr>
<tr>
<td>Apple pomace (Royal Gala)</td>
<td>Betulonic acid, botulin, lupenol, β-amyrin, uvaol, ursolic acid, erythrodiol, oleanolic acid</td>
<td>Synergi Polar-RP, Viridis ethylpyridine</td>
<td>CO2 + MeOH (3–10%), 120–180 bar/15–25°C</td>
<td>[87]</td>
</tr>
<tr>
<td>Antrodia camphorata</td>
<td>Ergostane triterpenoids</td>
<td>Chiralcel OJ-H</td>
<td>CO2 + MeOH (10%), 100 bar/40°C</td>
<td>[89]</td>
</tr>
<tr>
<td>Bacopa monnieri</td>
<td>Bacoside A3, bacopaside II</td>
<td>Finepak SIl-5, C-18</td>
<td>CO2 + MeOH (6%), 180–300 bar, 27–45°C</td>
<td>[90]</td>
</tr>
</tbody>
</table>

Fig. 4 Separation of rapeseed oil by supercritical fluid chromatography using a superficially porous stationary phase (Kinetex C18) and ELS-detection; influence of modifiers: 10% ACN (full black line), 10% ACN/MeOH 90:10 (red, coarse dashed line), and 12% ACN/MeOH 90:10 (blue, fine dashed line). Reproduced with permission from [77]. (Color figure available online only.)
determination of honokiol and magnolol in magnolia bark extracts [85]. SFE was carried out for 1 min at 200 bar pressure and a temperature of 45 °C using supercritical CO₂ with 5% methanol. The extract was then preconcentrated by trapping on an amino column (Capcell Pak NH₂). For SFC analysis, the extraction vessel was bypassed, and the methanol concentration increased to 15% so that the trapped analytes eluted from the amino column. With this setup separations required only 3 min. For the analysis of diterpenes in rosemary extracts see chapter “Preparative/semipreparative applications”.

The SFC separation of two sesquiterpene lactones in Artemisia annua, artemisinin and artemisinic acid, was described by Kohler et al. [86]. They compared two different approaches, either using a packed aminopropyl silica column (Nucleosil NH₂) together with ELSD, or a DB-WAX capillary column and an FID. Both methods were validated and analytical results were comparable (e.g., LOD for artemisinin: 0.01 µg/mL by SFC-ELSD, 0.03 µg/mL by SFC-FID), but the method using a packed column was much faster. It allowed the separation of both analytes in less than 8 min compared to 25 min by capillary SFC. As for sesquiterpenes, the number of SFC papers describing the analysis of triterpenoids is very small. Among the few is one describing the assessment of pentacyclic triterpenes in an apple pomace extract [87]. The overall best results were achieved on a Synergi polar-RP-column, while Viridis ethylpyridine material seemed to be preferable for mono-ol and di-ol acidic triterpenoids (© Fig. 5). Additionally, it could be used for semipreparative approaches. A mobile phase typical for SFC was selected (CO₂ with 3% methanol, isocratic elution) at a flow rate of 3 mL/min and a temperature of 20 °C. As the compounds of interest showed rather weak UV absorbance, an ELSD (Sedex 85) was employed for detection. Tavares et al. published the separation of four triterpene acids by capillary SFC, but utilized these compounds as standards only without showing a practically relevant application [88]. Only recently the separation of ergostane triterpenoids in the medicinal mushroom Antrodia camphorata has been reported [89]. The (R)- and (S)-forms for each of the seven enantiomers could be well resolved on a Chir-alcil OJ-H column (4.6 × 250 mm, 5 µm), eluted with 10% MeOH in CO₂ at 2 mL/min with a back pressure of 120 bar and a column temperature of 40 °C. The fact that low-polarity epimers like antcin A and antcin B could be separated was most favorable, because respective compounds are difficult to resolve by conventional RP-HPLC. Last but not least, bacoside A, bacoside B, and bacoside C could be well resolved on a Synergi polar-RP column (4.6 × 250 mm, 5 µm) at a flow rate of 3 mL/min and a temperature of 45 °C using supercritical CO₂ with 5% methanol. This setup separations required only 3 min. For the analysis of diterpenes in rosemary extracts see chapter “Preparative/semipreparative applications”.

Fig. 5 SFC separation of eight triterpenoid standards on two different stationary phases, (a) Viridis ethylpyridine and (b) Synergi polar-RP. Reproduced with permission from [87]. (Color figure available online only.)
bile phase was required due to the compounds polar character. Methanol did not have sufficient solvent strength to elute this compound from the column used (Spherisorb CN), but by adding 0.5% diethylamine, a satisfactory separation of atropine and the internal standard terbutalin could be achieved in 10 min. Peak-shape could further be improved by also adding 0.5% trifluoroacetic acid to methanol [96].

Flavonoids: As for alkaloids, the number of analytical papers on flavonoid analysis by SFC is small. In 2006, Li et al. used this technique to determine nobiletin, a polymethoxylated flavone found in sweet orange (C. sinensis) peel, and its metabolites in mouse urine [100]. SFC on a chiral column (Chiral-Pak AD-H; 250 × 4.6 mm; 5 µm) was helpful to separate two derivatives (3′- and 4′-demethylnobiletin) that could not be resolved by HPLC; all other metabolites were monitored by LC-MS. In a subsequent publication, Wang et al. compared different chiral and non-chiral column materials for the separation of the same constituents by SFC and normal-phase chromatography [101]. Because of higher resolution efficiencies, achiral columns showed advantages over chiral materials in SFC, and in terms of selectivity they were comparable. However, compared to normal-phase chromatography, huge differences were observed. For example, by SFC, the two before-mentioned nobiletin derivatives could be separated on a Chiralpak AD column with a retention time difference of 10 min. By normal-phase chromatography, they eluted (on the same phase) as barely resolved, broad signals. An extensive study investigating the effect of mobile-phase composition on retention and selectivity in achiral supercritical fluid chromatography was published 2013; for this purpose, several flavones were used as model compounds [102]. The authors stated that in the achiral SFC mobile phase, composition is merely a fine optimization parameter, which should be varied only after already having selected the most appropriate stationary phase. This stands in contrast to chiral SFC, for which the mobile-phase composition is usually most important.

Just recently, Ganzera reported on the separation of several isoflavones, representing major constituents in soy (Glycine max), red glover (Trifolium pratense), and kudzu (Pueraria lobata), by SFC [103]. Within 8 min, nine compounds could be baseline resolved on an Acquity UPC2 BEH column (1.7 µm particle size) from Waters; the mobile phase comprised CO2 and methanol with 0.05% phosphoric acid. Other applications that describe the use of SFC for the analysis of phenolic compounds are not related to natural products, but they focus on environmental issues instead [104,105].

Conclusions

Trying to summarize the current standing of SFC in analytical sciences, especially focusing on natural products related applications, results in the following observations. The technique, even if it is comparatively old and shows significant advantages over established procedures (e.g., faster, more efficient, environmental friendly), still has to be termed as an “exotic” analytical alternative. This is possibly due to the fact that commercially available, robust instruments are only sold for a comparatively short time. Many previous studies were conducted on laboratory-made equipment, which is interesting in respect to technical advancement but not suitable for routine use in industry where, for example, GMP guidelines need to be followed. Furthermore, many respective reports are rather old (≥ 10 years) and describe the application of today barely used OTC materials. All this could be misinterpreted in a sense that research has slowed down because...
of lacking interest, potential, or relevance. The opposite is the case. From more recent reports it is clearly evident that SFC is versatile in respect to technical modifications (e.g., mode of detection, selection of stationary phase) and analytes. When it comes to economic aspects, a state-of-the-art SFC system is definitely more expensive than a comparable conventional HPLC. Yet, its operation is cheaper because inexpensive CO₂ is primarily used, low amounts of solvent are consumed, and nearly no waste is generated. It could be shown that the theoretical advantages of SFC can be transferred to relevant separation problems and complex matrices like herbal extracts. Excellent separations of compounds have been described that are not possible with conventional approaches. Together with current developments on the instrumental sector, it is therefore only a matter of time till SFC finally reaches its desired place in analytical sciences.

**Conflict of Interest**

The authors declare no conflict of interest.

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