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Supercritical Fluid Chromatography in Natural Product Analysis – An Update

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ABSTRACT

The wide chemical diversity of natural products has challenged analysts all over the world and has been a driving force for the development of innovative technologies since decades. In the last years, supercritical fluid chromatography (SFC) has finally emerged from the shadow of liquid chromatography (LC) and gas chromatography (GC) and has become a powerful tool in modern natural product analysis. Whereas in the past the technique had mainly been restricted to a small group of nonpolar compounds, it has largely expanded its suitability in the last years and has demonstrated possibilities without boundaries. This mini-review, focused on the latest applications, provides a brief update on the current status of SFC in natural product analysis with the aim to demonstrate its applicability for both polar and nonpolar plant constituents. The approaches cover the whole range of polarity, including carotenoids, flavonoids, water-unstable ginkgolides, and even highly polar triterpene saponins with several sugar residues.

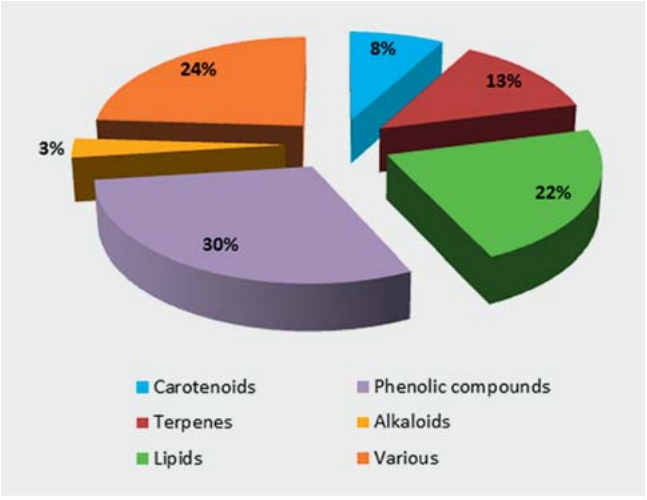
ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
BPR	backpressure regulator
DAD	diode-array detector
DEA	diethylamine
ESI	electrospray ionization
LOQ	limit of quantitation
RP-HPLC	reversed-phase HPLC
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
UHPLC	ultra-high performance liquid chromatography
UHPSFC	ultra-high performance supercritical chromatography

Introduction

Already developed more than 50 years ago, SFC was overshadowed by LC and GC for a long time [1]. It was mainly criticized for its weak UV sensitivity, poor quantitative performance, and limited reliability. In the last decade, the introduction of a new generation of commercially manufactured instruments tackled most of these limitations, and SFC has developed to a powerful analytical tool combining advantages of short analysis times and unique selectivity with low operating costs and environmental friendliness [2, 3].

The wide chemical diversity of natural products has always challenged analysts. Therefore, the availability of such highly efficient analytical technologies is of tremendous interest [4]. Initially, the application field of SFC in natural product analysis was relatively modest, focusing mainly on nonpolar compounds [5, 6]. However, the technique has largely expanded its suitability and has become an accepted analytical alternative. As shown in



▶ **Fig. 1** Range of SFC applications in natural product analysis categorized by substance classes from 2012 to 2016. Scifinder. Date of information gathering: May 2017.

▶ **Fig. 1**, the range of applications is broadly diversified and extends from the separation of nonpolar lipids to the analysis of highly polar triterpene saponins with several sugar residues [3, 4, 7, 8].

The number of publications dealing with the application of SFC for natural product analysis is increasing steadily (▶ **Fig. 2**), demonstrating its potential as complementary alternative to other well-established techniques as (U)HPLC or GC. These remarkable advances since the last review [7], enlightening the role of SFC in plant analysis, motivated us to present a brief update focusing on applications of the last two years.

Theoretical Background and Instrumentation

SFC operation is based on the use of a supercritical fluid as mobile phase. The supercritical condition is obtained whenever pressure and temperature of a gas or a liquid exceed their critical values [9]. In this state, features of both, the liquid and the gaseous state, are connected in a unique way: high dissolving capabilities and densities like a liquid are paired with low viscosity and high diffusivity of a gas [10, 11].

Nowadays, supercritical CO_2 is most widely used, because its critical values (31 °C and 74 bar) are easily attainable and it is inert, nontoxic, readily available, and cheap. Additionally, it is also an environmental friendly alternative to the standard organic solvents [10, 12]. Supercritical CO_2 is a highly lipophilic solvent with a polarity similar to hydrocarbons [13]. Therefore, SFC is often incorrectly considered as a normal phase system [14]. Analysis of more polar solutes requires the addition of an organic modifier (primarily an alcohol) [15, 16]. This modification causes an increase of the critical point with the consequence that most separations do not occur under supercritical but rather under so-called subcritical conditions. Due to the fact that both states have comparable characteristics, it is not of particular importance for the operator [17, 18].

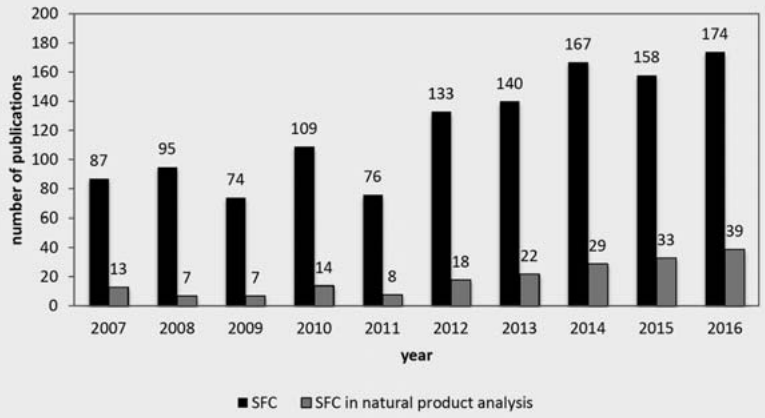
The introduction of modern state-of-the art instruments (also called UHPSFC) by several manufacturers was the major driving force for the renewed interest in SFC in recent years [2]. Although these innovative systems are largely based on UHPLC technology, the use of a supercritical fluid as mobile phase requires several important setup adaptations [18]: a BPR is required to enable accurate control of the pressure and an adapted pumping system to fulfil the mobile phase characteristics [13]. Modern instruments benefit from an optimized BPR device that not only reduces pressure variations during analysis but also ensures quicker adaptations to changes in flow rate and mobile phase composition [19]. Revised CO_2 delivery systems, guaranteeing adequate cooling of the incoming CO_2 to insure its liquid state, are another key factor for the new, reliable SFC performance [13, 18]. In addition, the new instruments include lower injection volumes, reduced void volumes to limit band broadening, and higher upper pressure limits [2, 19]. Although the pressure limits (400 to 600 bar) are still quite low compared to UHPLC systems (over 1000 bar), this is mostly not a limiting factor due to the fact that the much lower viscosity of CO_2-based mobile phases generates only low pressure drops compared to liquids used in UHPLC [13]. Nearly all stationary phases and column designs tailored for HPLC, including columns packed with sub-2-µm and core-shell particles, are suitable for SFC as well [19–23]. Recently, the ongoing interest in SFC as a potent analytical technique led to an increasing availability of stationary phases specifically designed for SFC use [22]. Modern SFC systems are compatible with a wide range of different detectors, including MS, evaporative light scattering detector, and DAD [13, 24]. The latter was often criticized for its low sensitivity, largely attributed to density and refraction index changes, caused by pressure oscillations. This pressure-induced UV noise was another factor that could be significantly improved by the introduction of the modernized BPR devices [19, 25]. Beside DAD detection, the hyphenation of SFC to MS is continuously growing in importance. While in the past APCI was considered as prevailing ionization source, ESI gained in popularity in the last years [26].

Among these technical improvements also the recent introduction of a fully automated system, combining online SFE and SFC with MS detection in a single flow path, is of great interest for natural product analysis. The addition of polar organic solvents to the supercritical CO_2 allows the extraction and simultaneous analysis of compounds with a wide range of polarities and makes it to an interesting future approach [27, 28].

More information about the theoretical background, instrumentation, practical approaches, and different applications are available in recent publications [6, 9, 13, 18, 22, 29–32].

Applications on Natural Products

For a long time, SFC analysis focused on nonpolar plant ingredients like lipids and carotenoids. In the last years, an increasing interest in the often underestimated potential of SFC for analysis of polar compounds could be observed. The following section, categorized by substance classes, gives an overview of recent SFC applications on natural product analysis with the aim to dem-



▶ **Fig. 2** Number of publications per year in the field of SFC in general and of SFC in natural product analysis over the last 10 years. Source: Scifinder. Date of information gathering: May 2017.

onstrate its wide applicability for both polar and nonpolar plant constituents. Some selected applications including detailed conditions are listed in ▶ **Table 1**.

Lipids

Since its introduction, SFC has consequently conquered lipid analysis as one of its major application fields. A vast number of studies focused on the qualitative or quantitative determination of those important food ingredients. Some recent reviews provide an excellent overview of SFC analysis in this field, from the beginnings to the current state including up-to-date applications; recent advances in lipid analysis will therefore not be further discussed [3, 33–37].

Carotenoids

The use of SFC for the separation of carotenoids was already mentioned in 1968 [38]. Since then, a large number of papers was published, which emphasizes the important role of SFC as an alternative analysis technique in this sector [36]. There are different forms of carotenoids: free carotenoids and more stable forms, esterified with fatty acids [39]. To release all esters and to simplify the analysis, most of the investigations were performed after a saponification step [40]. Two recent applications are worth mentioning as this simplification was avoided to prevent artifact formation and to preserve all information on the natural carotenoid profile.

Bonaccorsi et al. [39] identified more than 100 different compounds belonging to chlorophylls, free carotenes, free xanthophylls, and xanthophyll mono- and diesters in sweet bell peppers through the offline coupling of SFC and LC. The first dimension was performed on a SFC system using an Acquity UPC² HSS C_{18} SB column and ethanol as modifier, while the second dimension consisted of a RP-HPLC combined with DAD and MS detection and was performed on a C30 stationary phase. The high orthogonality of SFC and HPLC clearly enhanced the separation power and facilitated the rapid quantitation as well as stability studies of carotenoids in overripe yellow and red bell peppers.

Cleavage of a fragment from the usual carotenoid structure leads to the formation of apocarotenoids. Giuffrida et al. [41] developed the first SFC/MS method for the determination of native apocarotenoids in red habanero pepper, the hottest pepper in the world. Twenty-five apocarotenoids (14 free apocarotenoids and 11 apocarotenoids fatty acid esters) were separated on a novel fused-core C30 column with methanol as modifier in less than 5 min. The compounds were detected by selective ion monitoring in the negative mode utilizing a triple quadrupole mass spectrometer and an APCI interface. Identity was further confirmed by selective reaction monitoring in positive and negative ionization mode.

Terpenes

Terpenes are a wide-spread group of plant constituents of large chemical diversity. While early investigations were mainly focused on lipophilic terpenes, lately interest in more polar compounds has increased. Particularly noteworthy in this context is a publication by Huang et al. demonstrating the potential of SFC/MS for the analysis of triterpene saponins [42]. Nine kudinosides, six stauntosides, or 11 ginsenosides could be well resolved on a ZORBAX RX-SIL column within 10 min with slightly different parameters. As shown in ▶ **Fig. 3**, addition of 5% or 10% water to methanol as modifier was mandatory in all cases to improve resolution and reduce analysis time; 0.05% formic acid was added to enhance ionization. The methods were successfully applied to the analysis of kudinosides in *Ilex latifolia*, and ginsenosides in *Panax quinquefolius* and *Panax ginseng*.

Twenty years after the first SFC study on *Ginkgo biloba*, interest has recently been rekindled [43]. Ginkgolic acids and terpene lactones were determined in extracts and dietary supplements using an Acquity UPC² BEH 2-EP column and a mixture of isopropanol/methanol (50 : 50 v/v) with 10 mmol ammonium acetate as modifier [44]. Quantitation of low concentrations of both ginkgolic acid (LOQs < 100 ng/mL) and terpene lactones (LOQs < 1 µg/mL) could be achieved by single quadrupole MS detection. The developed method might be an alternative to existing methods with

▶ **Table 1** Selected SFC applications in the analysis of natural products, categorized by substance classes.

Compounds	Plant species	Stationary phase	Analytical conditions	Detection	Quant.	Ref.
Carotenoids						
Apocarotenoids	Red habanero pepper	Ascentis Express C30 (4.6 × 150 mm, 2.7 µm)	A: CO_2, B: MeOH Gradient elution mode, 2 mL/min, 35 °C, 150 bar	MS (APCI)	✓	[41]
Carotenoids	*Chlorella* sp. *Scenedesmus* sp. Rosehip	Torus 1-AA (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: MeOH Gradient elution mode, 2 mL/min, 35 °C, 160 bar	DAD/MS (ESI)	✓	[69]
Carotenoids	Dietary supplements	Acquity UPC² HSS C18 SB (3.0 × 150 mm, 1.8 µm)	A: CO_2, B: MeOH/EtOH (1 : 2 v/v) Gradient elution mode, 1.8 mL/min, 35 °C, 152 bar	DAD	✓	[70]
Terpenes						
Terpene lactone and ginkgolic acids	*Gingko biloba*	Acquity UPC² BEH 2-EP (3.0 × 150 mm, 1.7 µm)	A : CO_2, B: 10 mmol ammonium acetate in MeOH/Isopropanol (1 : 1 v/v) Gradient elution mode, 1.4 mL/min, 30 °C, 103 bar	DAD/MS (ESI)	✓	[44]
Ginkgolides	*Gingko biloba*	(n/a)	A: CO_2, B: 5 % water and 10 mmol ammonium acetate in MeOH Gradient elution mode, 2 mL/min, 40 °C, 200 bar	MS (ESI)	✓	[68]
Kudinosides, stauntosides, and ginsenosides	*Ilex latifolia, Panax quinquefolius and Panax ginseng*	ZORBAX RX-SIL (4.6 × 150 mm, 5 µm)	A: CO_2, B: 5–10 % water and 0.05 % formic acid in MeOH Gradient elution mode, 3 mL/min, 20 °C, 160 bar	DAD/MS (ESI)	–	[42]
Ginsenoside, nucleoside, and nucleobases	*Panax ginseng*	ZORBAX RX-SIL (4.6 × 150 mm, 5 µm)	A: CO_2, B: 5 mmol ammonium acetate in MeOH Gradient elution mode, 3 mL/min, 35 °C, 160 bar	DAD/MS (ESI)	–	[71]
Diterpenoid acids (continentalic acid and kaurenoic acid)	*Aralia continentalis*	Acquity UPC² Torus 1-AA (2.1 × 150 mm, 1.7 µm)	A: CO_2, B: 0.1 % formic acid in MeOH Isocratic elution mode, 0.6 mL/min, 40 °C, 138 bar	DAD	✓	[72]
Sesquiterpenes and other constituents	*Matricaria chamomilla, Chamaemelum nobile*	Acquity UPC² BEH 2-EP (3.0 × 150 mm, 1.7 µm)	A: CO_2, B: 0.5 % formic acid in MeOH/Isopropanol (1 : 1 v/v) Gradient elution mode, 1.7 mL/min, 50 °C, 103 bar	DAD/MS (ESI)	–	[73]
Camphor	*Tanacetum parthenium*	Acquity UPC² BEH 2-EP (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: Isopropanol Gradient elution mode, 2.0 mL/min, 50 °C, 138 bar	DAD	✓	[74]
Alkaloids						
Indole and oxindole alkaloids	*Mitragyna speciosa*	Agilent RX-SIL (2.1 × 50 mm, 1.8 µm)	A: CO_2, B: 10 mmol ammonium acetate in MeOH Gradient elution mode, 0.5 mL/min, 25 °C, 180 bar	DAD	–	[46]
Spiro oxindole alkaloids	*Uncaria macrophylla*	Torus 1-AA (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: 0.1 % DEA in ACN Isocratic elution mode, 1.2 mL/min, 45 °C, 138 bar	DAD	–	[48]
		Torus Diol (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: 0.1 % ammonium hydroxide in ACN Isocratic elution mode, 1.2 mL/min, 30 °C, 124 bar			
Aconitum alkaloids	*Aconitum pendulum*	Acquity UPC² BEH 2-EP (2.1 × 150 mm, 1.7 µm)	A: CO_2, B: 10 mmol ammonium acetate in MeOH Gradient elution mode, 0.8 mL/min, 55 °C, 145 bar	DAD/MS (ESI)	✓	[49]

continued

▶ **Table 1** *Continued*

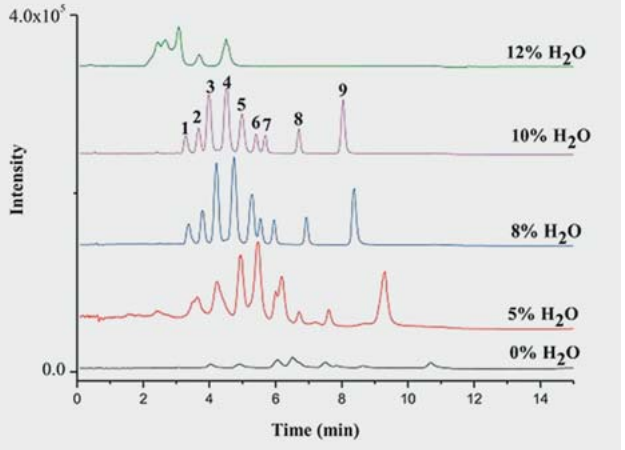
Compounds	Plant species	Stationary phase	Analytical conditions	Detection	Quant.	Ref.
Sesquiterpene pyridine alkaloids	*Tripterygium wilfordii*	Acquity UPC² BEH 2-EP (2.1 × 50 mm, 1.7 µm)	A: CO_2, B: MeOH Gradient elution mode, 1 mL/min, 45 °C, 138 bar	DAD/MS (ESI)	–	[75]
Indole alkaloids	*Alstonia scholaris*	Acquity UPC² BEH 2-EP (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: 2 mmol ammonium formate in MeOH Gradient elution mode, 1.5 mL/min, 50 °C, 138 bar	MS (ESI)	✓	[67]
Indole alkaloids	*Evodiae frucuts*	Acquity UPC² BEH (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: MeOH Gradient elution mode, 2 mL/min, 35 °C, 207 bar	DAD	–	[76]
Phenolic compounds						
Kavalactons	*Piper methysticum* (Kava-Kava)	Acquity UPC² BEH (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: 0.6 % DEA in MeOH Gradient elution mode, 1 mL/min, 70 °C, 130 bar	DAD	✓	[56]
Anthraquinones	Rhubarb	Acquity UPC² HSS C_{18} SB (3.0 × 100 mm, 1.8 µm)	A: CO_2, B: 0.05 % DEA in MeOH Gradient elution mode, 2 mL/min, 30 °C, 150 bar	DAD	✓	[57]
Coumarins	*Angelica dahurica*	Acquity UPC² CSH Fluoro-Phenyl (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: 0.1 % DEA in MeOH Gradient elution mode, 1.5 mL/min, 30 °C, 130 bar	DAD	✓	[53]
Coumarins	*Ammi visnaga fruit*	Acquity UPC² HSS C_{18} SB (3.0 × 100 mm, 1.8 µm)	A: CO_2, B: 0.1 % DEA in MeOH/ACN (1 : 1 v/v) Gradient elution mode, 1.5 mL/min, 30 °C, 140 bar	DAD	✓	[55]
Pyranocumarins	*Angelica gigas Nakai*	Acquity UPC² CSH Fluoro-Phenyl (2.1 × 150 mm, 1.7 µm)	A: CO_2, B: EtOH Isocratic elution mode, 0.6 mL/min, 35 °C, 138 bar	DAD	✓	[54]
Flavonoids	*Chrysanthemum marifolium*	ZORBAX RX-SIL (4.6 × 150 mm, 5 µm)	A: CO_2, B: 0.1 % phosphoric acid in MeOH Gradient elution mode, 3 mL/min, 40 °C, 200 bar	DAD	✓	[51]
Flavonoids	*Radix astragali*	Acquity UPC² CSH Fluoro-Phenyl (n/a)	A: CO_2, B: MeOH Gradient elution mode, 0.5 mL/min, 40 °C, 110 bar	DAD	✓	[52]
Phenolic acids	Wine	Acquity UPC² BEH 2-EP (3.0 × 150 mm, 1.7 µm)	A: CO_2, B: 0.1 % TFA in MeOH Gradient elution mode, 2 mL/min, 55 °C, 130 bar	DAD	✓	[77]
Cannabinoids	*Cannabis sativa*	Acquity UPC² BEH 2-EP (3.0 × 150 mm, 1.7 µm)	A: CO_2, B: 1 % water in Isopropanol/ACN (8 : 2 v/v) Gradient elution mode, 1.4 mL/min, 30 °C, 103 bar	DAD/MS (ESI)	✓	[58]
Miscellaneous						
Curcuminoids	Turmeric	Acquity UPC² BEH (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: 10 mmol oxalic acid in MeOH Gradient elution mode, 0.9 mL/min, 40 °C, 124 bar	DAD	–	[78]
Destruxins	*Metarhizium brunneum*	Acquity UPC² BEH 2-EP (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: 0.02 % TFA in MeOH/ACN (8 : 2 v/v) Gradient elution mode, 2 mL/min, 60 °C, 140 bar	DAD/MS (ESI)	✓	[63]
Tocopherols and tocotrienols	Soybean oil	Amine Luna NH_2 (2.0 × 150 mm, 3 µm)	A: CO_2, B: 0.1 % formic acid in EtOH Gradient elution mode, 1.5 mL/min, 30 °C, 130 bar	DAD/MS (APPI)	✓	[79]

continued

▶ **Table 1** *Continued*

Compounds	Plant species	Stationary phase	Analytical conditions	Detection	Quant.	Ref.
Vitamine E isomers	*Moringa oleifera* leafs	Acquity UPC² BEH 2-EP (3.0 × 100 mm, 1.7 µm)	A: CO_2, B: MeOH/Isopropanol (1 : 1 v/v) Gradient elution mode, 1.5 mL/min, 50 °C, 124 bar	DAD	✓	[80]
Goitrin and epigoitrin	*Isatis indigotica*	(S,S)-Whelk-O 1 (4.6 × 250 mm, 10 µm)	A: CO_2, B: MeOH Gradient elution mode, 3 mL/min, 40 °C, 120 bar	DAD/MS (APCI)	✓	[81]
Aflatoxins	Edible oil	Acquity UPC² BEH 2-EP (2.1 × 100 mm, 1.8 µm)	A: CO_2, B: MeOH Gradient elution mode, 1.0 mL/min, 50 °C	MS (ESI)	✓	[82]

Quant: quantitation; Ref: reference; A and B: mobile phase components; APPI: atmospheric pressure photoionization; TFA: trifluoroacetic acid



▶ **Fig. 3** Effect of water content in the mobile phase on the separation of nine kudinosides from *Ilex latifolia* leaves. Peak assignment: 1. Kudinoside F, 2. Kudinoside A, 3. Ilekudinoside G, 4. Kudinoside E, 5. Kudinoside C, 6. Kudinoside G, 7. Latifoloside Q, 8. Latifoloside H, 9. Kudinoside O. Reproduced with permission from [42] [rerif].

the advantage to avoid hydrolysis of ginkgolides that occur during RP-HPLC with aqueous eluents and without the need for derivatization of the ginkgolic acids as it is necessary prior to GC analysis.

Only recently, Zhu et al. [45] compared the separation of 20 different spirostanol saponins by UHPSFC and UHPLC underlining the complementarity of both techniques. While UHPSFC showed to be advantageous for the separation of spirostanol saponins with the same aglycone and a different sugar residue, UHPLC was preferable for the resolution of saponins with the same sugar residue and different aglycones. Up to now, no application to real samples was demonstrated.

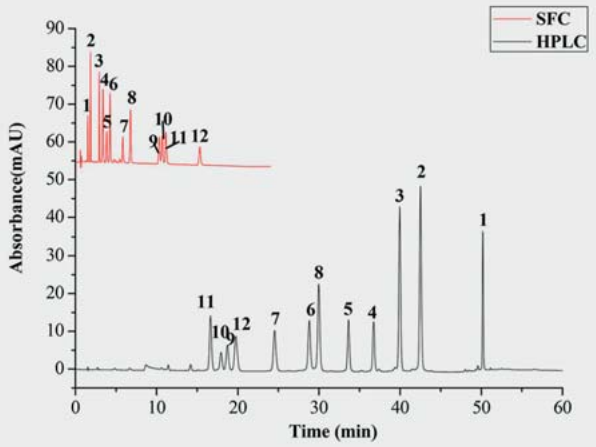
Alkaloids

Few applications focused on analysis of alkaloids in the very early years of SFC; recently reawakened interest in this topic can be observed.

The psychoactive plant *Mitragyna specicosa* and kratom, a product obtained thereof, are widely used as pain suppressor and low cost substitute for opioids [46]. Due to the addiction potential and toxicity in multiple organ system the U. S. Food and Drug Administration (FDA) has called for detention of all related products [47]. Although mitragynine and 7-hydroxymitragynine are the main psychotropic constituents, other epimeric indole (speciogynine and speciociliatine, which are diastereomeres of mitragynine, paynantheine and 3-isopaynantheine) and oxindole alkaloids (corynoxine A and corynoxine B) are present as well. Wang et al. [46] succeeded in the simultaneous separation of all eight compounds in 7 min using an Agilent RX-SIL column and a mixture of CO_2 and methanol containing 10 mmol ammonium acetate as mobile phase. SFC method provided faster separation and superior resolution compared to both UHPLC and GC method. Similar alkaloids were also in the focus of another study: Yang et al. [48] resolved two pairs of 7-epimeric oxindole alkaloids (rhynchophylline and isorhynchophylline, corynoxine A and corynoxine B) from *Uncaria macrophylla* on a Torus 1-AA column as well as on a Torus Diol column. Acetonitrile (containing 0.1% DEA or 0.1% ammonium hydroxide) was chosen as modifier, because of its ability to suppress the epimeric interconversion of these analytes longer than other modifiers. Scaled up to preparative SFC, all four alkaloids were isolated with purities higher than 95%.

Due to the extraordinary toxicity of *Aconitum* alkaloids, highly sensitive and reliable analytical methods are mandatory for an adequate risk assessment. In a recent publication, separation of five alkaloids in *Aconitum pendulum* extracts was obtained on an Acquity UPC² BEH 2-EP column [49]. Short runtime (3 min), excellent validation results (recovery rates from 92.3 to 101.2%), and LOQ values between 0.03 and 0.08 ng/mL obtained with an MS detector in the positive ESI mode, indicated that SFC systems can easily keep up with other techniques.

The separation of the amine alkaloids in *Piper longum* was reached through the offline coupling of SFC and UHPLC [50]. The first dimension was performed on a SFC system using a XAmide column and methanol as modifier. The manually collected, dried fractions were re-dissolved and subsequently analyzed on a UHPLC system and an HSS T3 column (second dimension). Due to the high orthogonality of both systems, not only separation



▶ **Fig. 4** Separation of the 12 flavonoids in both SFC and HPLC modes. Peak assignment: 1. Kaempferide, 2. Baicalein, 3. Kaempferol, 4. Luteolin, 5. Quercetin, 6. Morin, 7. Myricetin, 8. Baicalin, 9. Hyperoside, 10. Luteoloside, 11. Myricitrine, 12. Buddleoside. Reproduced with permission from [51] [rerif].

power was increased, but also the detection of low contained compounds, which were overshadowed in the one-dimensional separation, was achieved.

Phenolic compounds

The frequent occurrence and biological activity render phenolic compounds to one of the most interesting ingredients in plant kingdom. In the last few years an increasing number of analytical applications were investigated focusing on flavonoids [51, 52], coumarins [53–55], kavalactones [56], anthraquinones [57], and cannabinoids [58].

Huang et al. [51] presented very recently a first SFC-DAD-UV method for the analysis of 12 flavonoids, among them flavones, flavanols, as well as mono- und diglycosides. Baseline separation was achieved using a ZORBAX RX-SIL column (4.6 × 150 mm, 5 µm) and 0.1% phosphoric acid in methanol as modifier at a column temperature of 40 °C and an outlet pressure of 200 bar. The authors compared their SFC method with HPLC analysis of the standard compounds utilizing a ZORBAX SB-C18 column with the same dimensions and particle size. Whereas the SFC analysis took 18 min, 55 min were necessary to obtain baseline separation by HPLC (▶ **Fig. 4**). The SFC method was subsequently validated for five representative congeners (limits of quantitation from 2.19 to 5.86 µg/mL, recoveries between 100.2% and 104.1%, precision better than 2.4% for aglyca and 4.6% for glycosides) and applied to the quantitative analysis of hydroethanolic extracts of *Chrysanthemum marifolium*.

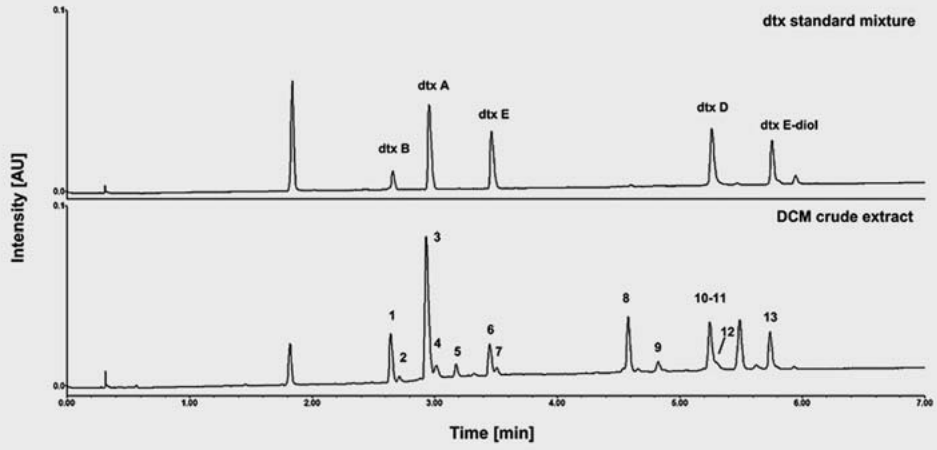
A few SFC methods have been published up to now for the determination of coumarins. Pfeifer et al. [53] presented a validated SFC-DAD-UV method for the determination of eight congeners in *Angelica dahurica* roots, Kim et al. [54] resolved two pyranocumarins in *Angelica gigas* roots, and Winderl et al. [55] succeeded in the first complete separation of all coumarins in *Ammi visnaga* fruit.

The first report on the successful separation of anthraquinones has been published as well [57]. Five anthraquinones (chrysophanol, physcion, emodin, aloeemodin, and rhein) could be resolved in less than 5 min on an Acquity UPC^2 HSS C_{18} SB column using methanol with 0.05% DEA as modifier and DAD detection. The method was validated (LOQs < 1.34 µg/mL, recovery rates between 95.4% and 103.1%, precision better than 6.92%) and successfully applied to the analysis of rhubarb extracts.

Cannabis has been used for centuries due to its manifold medicinal properties but was banned as one of the most popular illegal recreational drugs worldwide [59]. Whereas analysis of its constituents was mainly of interest in body fluids and hair samples to prove drug abuse for many years, the situation has changed drastically as marijuana–and not only Δ9-THC–has recently been brought into medicinal use in several countries. Due to this changed situation, reliable standardization and quality control is badly needed [60]. Wang et al. [58] presented very recently a promising SFC-DAD/UV-MS method for the quantitative determination of nine of the most abundant cannabinoids. Separation was achieved within 11 min using isopropanol/acetonitrile (80 : 20 v/v) with 1% water as modifier and a BEH 2-EP column with sub-2-µm particles. Specificity was proved by MS detection, LOQs were reported as 5 µg/mL for acidic and 10 µg/mL for neutral cannabinoids, recoveries ranged from 96.1 to 107.6%, and the overall precision was better than 7.6%. The method was applied to the analysis of 30 cannabis and hashish samples (acetonitrile/methanol extracts [80 : 20 v/v]). The results were in good agreement with a standard UHPLC method (variations ± 13.0%). The SFC-DAD/UV-MS method might be an alternative to existing methods with the advantage of orthogonality to UHPLC, increasing the power of identification of congeners in complex matrices, and without the need for decarboxylation or derivatization as it is necessary prior to GC analysis.

Murauer et al. [56] developed a fast and validated method for the determination of all major lactones in *Piper methysticum*, a plant that was long considered as an herbal alternative to synthetic anxiolytics but banned from the market due to assumed hepatotoxicity in 2002. Baseline separation was obtained in less than 4 min using an Acquity UPC^2 BEH column and a mixture of CO_2 and methanol with DEA as mobile phase. With 70 °C a rather high column temperature, already 10 °C above the recommended maximum by the column manufacturer was selected, because only under these conditions baseline separation of kavain and yangonin was possible.

Recently, a supercritical based protocol for the extraction, analysis, and isolation of six polar compounds (o-vanillin, styracin, vanillin, trans-cinnamic acid, vanillic acid, and shikimic acid) from *Styrax*, an exudate from various Liquidambar trees, has been published [61]. A mixture of supercritical CO_2 and ethanol (1 : 1) was used for the extraction. The generated extracts were resolved on an Acquity UPC^2 BEH 2-EP column using 0.1% phosphoric acid in methanol as modifier. Scaled up to preparative SFC, styracin and trans-cinnamic acid were isolated on a Viridis BEH 2-EP column in only 7 min. Compared to conventional workflows, the author described supercritical based methods as a cheap, time-saving, and environmentally friendly alternative that will gain in value in the future.



▶ **Fig. 5** Representative UHPSFC-PDA chromatogram of a *Metarhizium brunneum* DCM crude extract and a destruxine standard mixture containing dtx A, B, D, E, and E-diol. Reproduced with permission from [63].

Miscellaneous

Cyclic hexadepsipeptides, known as destruxins, are produced by the fungus *Metarhizium brunneum,* which is used as a pest control agent [62]. Due to concerns that this use entails risks to humans and the environment, the development of validated analysis methods is of great interest. Optimum resolution was obtained on an Acquity UPC² BEH 2-EP column with a mixture of supercritical CO_2 and methanol/acetonitrile (8 : 2 v/v) containing 0.02 % trifluoroacetic acid as the mobile phase [63]. As shown in ▶ **Fig. 5**, 17 analytes were separated within 4 min. Five of them were identified using reference material, while the other eight were identified by MS. Compared to established UHPLC method, SFC is characterized by shorter analysis time, rapid equilibration, higher throughput and low operation costs, but has the disadvantage of 4–26 times lower sensitivity. According to the authors, this may be explained by the lower injection volume on one hand and the lower sensitivity of SFC-UV compared to HPLC-UV on the other hand.

Recently, Grand-Guillaume Perrenoud et al. [64] highlighted the versatility of SFC for natural product analysis. A set of 120 highly diverse natural compounds (alkaloids, organic acids, flavonoids, cardioglycosides, etc.) were selected for a systematic column screening on 15 different stationary phases applying identical elution parameters (CO_2 and MeOH with 10 mmol ammonium formate and 2 % water). The SFC system was coupled to a Q-ToF mass spectrometer operated in both positive and negative modes with ethanol as make-up liquid. According to their results, the method is suitable for almost 90 % of the tested compounds. Three stationary phases (Diol, not end-capped C18 and 2-EP) showed to be appropriate for wide-range usage. To prove this, dichloromethane and methanol extracts from white willow and yerba mate were analyzed on these three columns under the previously mentioned conditions. The obtained metabolite profiles showed the ability of the developed method for the analysis of both complex polar and nonpolar plant extracts.

Establishing a pharmacokinetic study of a natural product is challenging. Due to the low concentration, complex matrices and wide range of active ingredients, appropriate sensitive and selective analytical methods are indispensable [65]. In comparison to LC-MS, the method of choice for most approaches, SFC is still in its infancy and the number of publications is meagre [66]. Therefore, the following two approaches are noteworthy, as they underline the auspicious potential of SFC in this sector that will surely gain in value in the future.

The four indole alkaloids scholarisine, 19-epischolarisine, vallesamine, and picrinine are described as the major bioactive compounds in *Alstonia scholaris*, a widely distributed folk medicinal plant in Asia and Africa, used for the treatment of chronic pulmonary diseases. Although commercial formulations (Dengtaiye tablets, DTY) are available, their pharmacokinetic profile is still poorly explored and *in vivo* studies are missing. Recently, Yang et al. [67] developed a SFC/MS-MS method for the simultaneous quantitation of these four compounds in rat plasma using an Acquity UPC² BEH 2-EP column with 2 mmol ammonium formate in methanol as modifier. The method was subsequently validated (LOQs 50 pg/mL, recoveries between 84.47 and 95.22 %, precision in the range from 1.42 to 12.85 %) and applied to a pharmacokinetic study in rats after oral administration of 108 mg/kg Dengtaiye tablets.

The second pharmacokinetic study focused on the simultaneous monitoring of three ginkgolides and their six hydrolyzed metabolites in rat plasma after intravenous administration of the total ginkgolide extract [68]. Methanol with 5 % water and 10 mmol ammonium acetate was used as modifier and a triple quadrupole MS for detection. The use of supercritical CO_2 is favorable as it avoids spontaneous hydrolysis of ginkgolides during analysis and allows an accurate characterization of the naturally occurring metabolites. As authentic standards for the ginkgolides metabolites were missing, diazepam and ketoprofen were chosen as internal standards for the method validation (correlation coef-

ficients > 0.992, LLOQ between 0.2 and 1.0 µg/mL, recoveries of 80.0–116.3 % with RSD less than 10.1 %).

Future Perspectives and Concluding Remarks

Whereas in the early days SFC has mainly been restricted to a small group of nonpolar compounds, over the years it has largely expanded its suitability and developed to a versatile technique with great potential for natural product analysis. As shown in the summarized approaches, ranging from nonpolar carotenoids to highly polar glycosides, SFC has finally emerged from the shadow of HPLC and GC, combining some of their best features. Consequently, remarkable short methods are providing high efficient separations at low costs and without the need of toxic solvents. Moreover, the use of supercritical CO_2 offers finally an adequate solution for thermolabile and water-unstable plant ingredients inaccessible with HPLC and GC. Often criticized limitations, like poor quantitative performance and limited reliability, could clearly be tackled according to recent publications presenting separations with low LOQ values and excellent validation criteria. The upscale from analytical to preparative SFC provides a cost-saving and rapid solution for the isolation of highly pure substances due to easy solvent removal and the possibility of high sample loading. With all these features and the continuously ongoing technical development, SFC has become a promising analytical tool with a bright future in the area of natural product analysis.

Conflict of Interest

The authors declare no conflicts of interest.

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