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# Supercritical fluid chromatography for vitamin and carotenoid analysis: an update covering 2011-2021

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ABSTRACT

As it is known, the chromatographic separations of vitamins is not a trivial issue. The subtle heterogeneity among homologs belonging to the same group makes it difficult to perform their speciation. On the other hand, differences in terms of structure complicate the simultaneous analysis of the main forms belonging to the several vitamin groups. Long times of analysis as well as the use of conventional toxic organic solvents, such as hexane and MeOH, are other downsides of conventional methods based on liquid chromatography. Over the last few years, Ultra-Performance Convergence Chromatography (UPCC or UPC$^2$) has emerged as a unique separation technique for its green character and the unparalleled ability to achieve chiral and achiral separations with an unequalled speed and reproducibility. Conceived after 2010, UPCC merges the advantages of the conventional supercritical fluid chromatography (SFC) with those of the ultra-high performance liquid chromatography (UHPLC) technology. Its application to vitamin and carotenoid analysis has shown a potential in solving the above-mentioned issues, which is still untapped. This review offers a comprehensive perspective of the SFC advancements in the last ten years and the advantages that arise from its application in vitamin and carotenoid analysis. Compared to conventional techniques, the flexibility of UPCC is unique, making it possible the simultaneous analysis of different group of achiral and chiral vitamins within the same short chromatographic run.

## 1. Introduction

Vitamins are organic substances counting a wide spectrum of molecules characterized by heterogenic chemical structures and different biological activities. These micronutrients are involved in many metabolic functions which are essential for the human organism. Depending on their solubility in water, vitamins are classified into two main families: fat-soluble vitamins (FSVs), including A, D, E, and K vitamins, and water-soluble vitamins (WSVs), encompassing all vitamins B and vitamin C. Each of these groups is composed of several structurally related bio-active forms, known as "vitamers", which differ in chemical structure, biopotency and stability. [1].

Group B vitamins ($B_1$, $B_2$, $B_3$, $B_5$, $B_6$, $B_8$, $B_9$, $B_{12}$) are required in important metabolic pathways related to the energy production and redox status reactions of the intermediary metabolism (decarboxylation, transamination, acylation, oxidation, and reduction). Additionally, they are necessary for the methyl-group transfer in fundamental biochemical reactions, such as the amino-acid conversions and the synthesis of fatty

*Abbreviations:* 1-AA, 1-aminoanthracene; 2-EP, 2-Ethylpyridine; 2-PIC, 2-picolylamine; ABPR, atmospheric back pressure regulator; ACN, acetonitrile; APCI, atmospheric pressure chemical ionization; APPI, atmospheric-pressure photoionization; BEH, ethylene bridgen hybrid; BHT, butylhydroxytoluene; BPR, backpressure regulator; CC, convergence chromatography; CSH, charged surface hybrid; D, dimension; DAD, diode array detector; DEA, diethylamine; DPH, diphenyl-; ET, extraction time; EtOH, ethanol; ESI, electrospray ionization; FSVs, fat-soluble vitamins; GC, gas-chromatography; HFo, formic acid; HPLC, high performance liquid chromatography; HILIC, hydrophilic interaction liquid chromatography; HSS, high strength silica; IMS, ion mobility mass Spectrometry; iPOH, isopropryl alcohol; LE, liquid extraction; LLE, liquid liquid extraction; LLOD, lower limit of quantitation LOD, limit of detection; LOQ, limit of quantification; NARPLC, non-aqueous reversed phase LC; MeOH, MeOH; MP, mobile phase; MS, mass spectrometry; $NH_4Fo$, ammonium formate; NPLC, normal phase LC; PDA, photodiode array detector; PFP, pentafluorophenyl; PTAD, 4-Phenyl-1,2,4-triazoline-3,5-dione; QToF, quadrupole-time-of-flight mass spectrometry; RPLC, reversed phase LC; RTF, ready-to-feed; SFC, supercritical fluid chromatography; SFE, supercritical fluid extraction; SPE, solid phase extraction; SLE, supported liquid extraction; SLE, solid liquid extraction; SRM, single reaction monitoring; T, temperature column; TBME, tert-Butyl methyl ether UPCC or UPC$^2$, ultra-performance convergence chromatography; UHPLC, ultra high performance LC; UPSFC, ultra performance SFC; WSVs, water-soluble vitamins.

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acids, cholesterol, steroids, glucose and DNA [1]. Vitamin C (ascorbic acid) is an effective antioxidant, which plays a role as a co-substrate in oxygen-requiring hydroxylation reactions in several biological functions. Moreover, it is implicated in the metabolism of collagen, lipids and some essential amino acids, as well as in the iron absorption and in the release of corticosteroids and neurotransmitters [2]. Vitamin A-active compounds embrace retinoids, such as retinol, retinaldehyde, retinoic acid, and their precursors (provitamin A carotenoids). Vitamin A plays an important role in the cellular growth, differentiation and reproduction, as well as in the immune system maintenance. In addition, rod and cone cells, which are indispensable for vision processes in the retina, are mainly composed of vitamin A [3]. Vitamin D is represented by two main forms denominated ergocalciferol ($D_2$) and cholecalciferol ($D_3$), but more than 50 metabolites with different biological activity have been identified so far. From a chemical point of view, vitamin D homologs are sterol-derivate compounds that have a central role in the absorption and transport of calcium and phosphorus [1]. Vitamin E is the generic term for referring to structurally analogous compounds, known as tocopherols ($\alpha$-, $\beta$-, $\gamma$-, $\delta$-T) and tocotrienols ($\alpha$-, $\beta$-, $\gamma$-, $\delta$-T3). Vitamers E have an antioxidant function in cell membranes against free radicals, preventing the peroxidation of unsaturated fatty acids. Vitamin K comprises phylloquinone ($K_1$) and a group of 9 menaquinones ($K_2$). They have a common naphthoquinone nucleus and a side chain that differs in length and number of double bonds. The main function of vitamin K is connected to the blood coagulation, but menaquinones also take part in bone-modelling processes [4,5].

Due to the relevance of vitamins in human physiology and health, the requirement of accurate information on their form, concentration and distribution in both food and biological matrices has become an important issue in several scientific fields. The determination of FSVs and WSVs in biological samples allows one to establish their bioavailability in the organism, as well as the clinical status of a patient. From an analytical perspective, the effective detection and quantification of these compounds, which are often present at trace levels in complex matrices, require highly sensitive and selective methodologies [6,7].

In this context, high performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) have been the gold standard in the analysis of vitamins for years [6,7]. The reasons can be found in the variety of chromatographic modes (normal phase LC (NPLC), hydrophilic interaction liquid chromatography (HILIC), reversed phase LC (RPLC), non-aqueous reversed phase LC (NARPLC)) and in the large assortment of stationary phases and particle types (2-5 μm porous particles, sub-2-μm particles, core-shell particles, and monoliths) [8]. Conventional chromatography has successfully been applied to several problems connected to the resolution and the individual quantification of structurally related vitamin forms. Some examples are: the separation of the eight E vitamers, in particular of $\beta$- and $\gamma$- isomers [9]; the separation of vitamins K from isobaric interferences co-extracted from complex matrices such as milk or dairy products [5,10]; and the differentiation of vitamins $D_2$ and $D_3$ [11]. Outstanding achievements have been obtained in the separation of WSVs by RPLC [12,13] and HILIC [14]. Nevertheless, in many instances, the results obtained by LC in terms of efficiency, selectivity and peak shape, as well as in terms of homologue resolution, required drastic or expensive chromatographic conditions. In fact, sub-ambient temperatures (5 - 10°C) and long run times (up to 1h or longer) are sometimes necessary to ensure the expected results. As a consequence, especially for the analysis of FSVs, high volumes of toxic organic solvents, like dichloromethane or hexane-based mixtures, are necessary [6].

Over the past decade, the attention of the scientific community has increasingly been focused on a sustainable chemistry, with the aim of reducing the use of toxic organic solvents and making analytical methods greener. In this perspective, the supercritical fluid chromatography (SFC) is a separation technique which has gained great success in various analytical fields, including vitamin analysis, because of its excellent selectivity for the separation of compounds covering a wide range of polarities [15]. In modern SFC, which combines the features of both gas chromatography (GC) and LC, typical UHPLC silica-based columns are used, while the mobile phase consists of a fluid which is heated and pressurised beyond its critical point (gas and liquid states are indistinguishable). In this supercritical state, the mobile phase displays the diffusivity and viscosity of a gas as well as the density and solvating power of a liquid. Compared with other compounds, $CO_2$ is advantageous for its inertia, cheapness, greener and safer nature, weak UV absorbance at low wavelength, low critical point (74 bar and 31°C), and miscibility with a wide range of organic solvents (from MeOH to heptane). Since all compounds with log P values ranging between -2 and 10 are suitable for SFC, this technique has the untapped potential to realize the separation of a wide spectrum of compounds in a single run with high resolution and speed [16]. Like NPLC, SFC exhibits high selectivity for the separation of molecules with structural similarity (enantiomers, positional isomers, geometric isomers, homologues). Although its use is still growing, SFC has proved to be a competitive technique in several fields and in particular in vitamin analysis. A clear advantage of SFC is its ability to separate mixtures of analytes in significantly shorter times than the traditional LC. Due to the low viscosity of the compressed $CO_2$, modern SFC can employ UHPLC-like columns (1.7 μm, 3 mm ID, 100 mm) with a pressure drop lower than 250 bar, even at the flow rate of 5 mL/min. In other words, analysis times in SFC, like in UHPLC, are up to 10 times faster than in HPLC, but SFC has a lower consumption of toxic solvents than UHPLC [16]. An additional benefit, which is relevant for FSV analysis, is the compatibility of the $CO_2$-based mobile phase with non-polar solvents (e.g. hexane) commonly used in sample preparation procedures. In fact, while RPLC needs to dry organic extracts and to reconstitute them in a solvent mixture compatible with the mobile phase, SFC enables the direct injection of non-polar solvents into the system: this allows one to simplify the sample preparation step, to reduce wastes, to save time and to increase the productivity.

This review presents the advancements of SFC in vitamin analysis by remarking how novel methodologies based on SFC may be applied to solve the most relevant problems in vitamin determination. The basic principles which regulate SFC will be briefly explained, and an overview of the applications covering the last ten years (2011-2021) will also be provided, with the aim of underlining advantages and disadvantages compared to the conventional LC methods.

## 2. The evolution of SFC

The study of supercritical fluids began in 1822, when the French physicist Charles Cagniard de la Tour discovered the critical point of a substance by carrying out experiments involving sound discontinuities in the Papin digester (a prototype of a pressure cooker, filled with a liquid and containing a flint ball) [17]. By moving the device it was possible to hear the sound of the ball as it penetrated the liquid-vapor interface. When the device was heated beyond the boiling point of the liquid, the splashing sound stopped. Thus, Cagniard de la Tour was able to identify the temperature at which the boundary between the liquid and the gaseous phases disappeared. These studies were resumed during the 1860s by the Irish chemist Thomas Andrews, who coined the term supercritical fluid [18].The first attempt to introduce a supercritical fluid into a chromatographic system dates back to the 1960s by Klesper et al. [19]. Until the early 1980s, all the SFC instruments were home-made by using hardware components of other chromatographic equipment. From the beginning, there has been a real difficulty in finding a short and simple sentence to represent the very essence of the technique. The term SFC was coined in 1967 [20], but previously other tentative names had been: "*turbulent flow chromatography*", "*dense gas chromatography*" and "*ultra-high pressure gas chromatography*". Nevertheless, there is a clear difference between SFC and GC. The mobile phase in GC slightly interacts with analytes and it works as a carrier gas, while in SFC it has an active role in the compound elution. It was

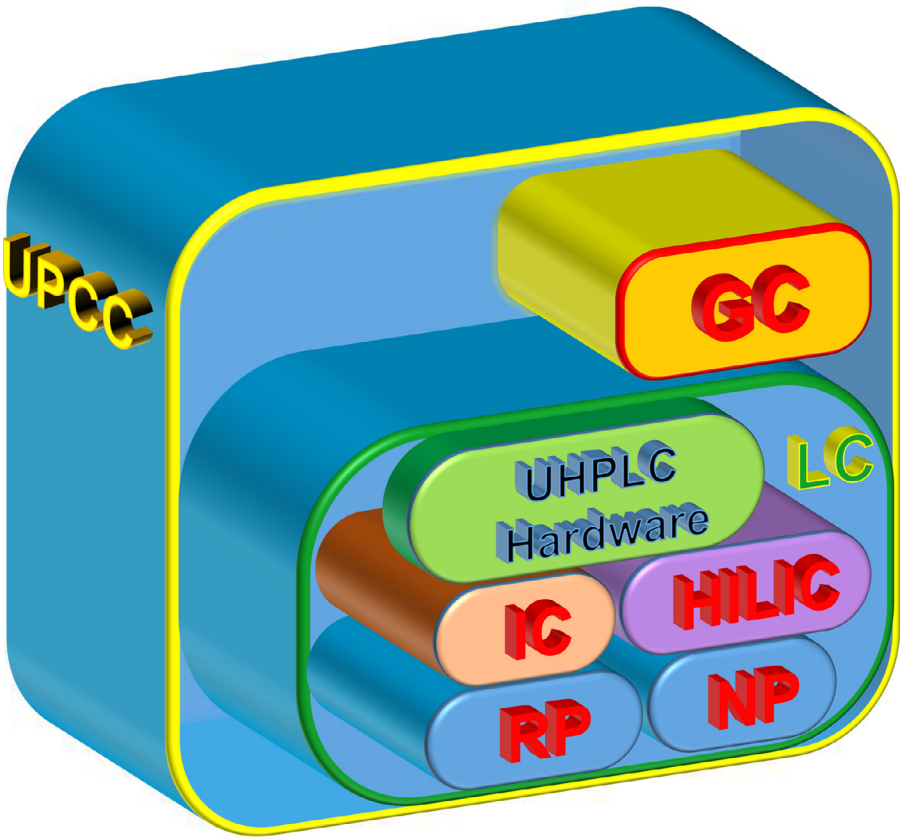


**Fig. 1.** Schematic description of ultra-performance convergence chromatography (UPCC) as a synthesis of different chromatographic techniques and technologies.

necessary to wait until 1983 to have the first SFC commercial system: it was marketed by Hewlett Packard and it took inspiration from LC setups. Despite the technological innovations introduced over the following years, the SFC instruments of 1st and 2nd generation were unable to control key parameters such as temperature, pressure, and density of a supercritical fluid in a reproducible way [21]. The difficulties with back-pressure regulation, consistent flow rates, inadequacy of chromatographic column technology, and high baseline noise with UV detectors were the technical hitches that blocked the SFC evolution up to 2010. Until this date the technique was limited to niche applications. Between 2010 and 2011, Agilent Technologies, Inc. and Waters Corporation, independently of each other, developed the so-called instruments of 3rd generation, by taking advantage of UHPLC hardware and technological solutions, such as electronically controlled back pressure regulators (BPRs), cooled pump heads for a better control of the flow rate and mobile phase composition, and UV cells modified to compensate the differences in refractive index between $CO_2$ and co-solvents (resulting in a significant reduction of the baseline noise) [22]. To stress the transformation of a modern SFC equipment from those of the previous generations and to indicate the broad applicability of the technology, the term convergence chromatography (CC) or ultraperformance convergence chromatography (UPCC or UPC$^2$) was introduced [16]. As simply depicted in **Fig. 1**, UPCC is the junction point of different techniques (GC and LC) and technologies (UHPLC hardware), whose combination has resulted in holistically or hybrid designed instruments, bracketing all the areas amenable to both NPLC and RPLC. UPCC also includes parts of ion chromatography and HILIC and shows full compatibility with the most modern stationary phases. Basically, the modern SFC opens new frontiers in the separation science, with potentialities which are still unexpressed and worthy to be explored. A great potential is expected in vitamin separations, especially those addressed to define their natural distribution in real complex samples, such as food and biological samples.

## 3. SFC mobile phase suitable for vitamin analysis

Although other compounds such as ammonia, nitrous oxide and light hydrocarbons have been used in SFC [23], $CO_2$ has become the best choice for its definite advantages: i) it can reach supercritical conditions with a moderate energy consumption; ii) since it is not-flammable, not-corrosive and low-toxic, $CO_2$ is considered a safe fluid for operators; iii) as a side-product from several industrial processes, it is inexpensive and environmentally friendly; iv) from an analytical point of view, it shows weak UV absorbance at low wavelength, and it is miscible with most organic solvents and a wide-polarity range of analytes.

Despite what the name suggests, the mobile phase used in SFC is not necessarily in the supercritical state. During a chromatographic run, it can change from supercritical to subcritical state by finely adjusting its temperature and pressure. Thus, the retention of solutes is settled by performing temperature and pressure gradients. [24]. In other words, the density of the mobile phase remarkably affects the analyte solubility and, thus, it is the main indicator of the retention behaviour. In the supercritical region, at a constant temperature, the $CO_2$ density can be continuously adjusted by modifying the pressure. An increase in pressure leads to a higher density, which favours the analyte solubility and the reduction of its retention factor. On the other hand, the effect of the temperature is correlated to the operative pressure. At lower pressures (density < 0.5 g/mL), the decrease in temperature reduces retention factors. In this region, the mobile phase behaviour is close to the gaseous state in which the density is inversely proportional to the temperature. On the other hand, at high pressure (density > 0.5 g/mL), the decrease in temperature increases the retention factors [24,25]. The peculiar characteristics of SFC mobile phase make it possible the separation of a wide spectrum of medium-polar and non-polar substances, including both FSVs and WSVs [26] by performing a density gradient of $CO_2$.

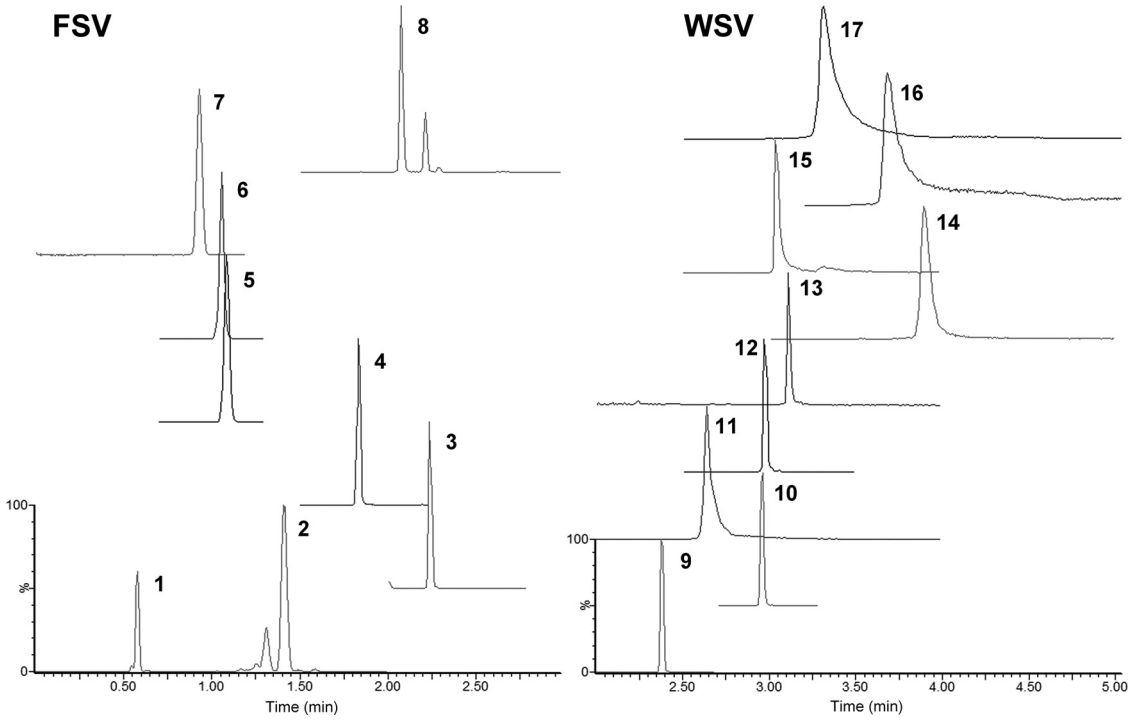


**Fig. 2.** UPCC-MRM chromatograms of 17 vitamins. (1) retinol acetate, (2) retinol palmitate, (3) $D_2$, (4) $\alpha$-tocopherol, (5) $K_2$, (6) K1, (7) $\alpha$-tocopherol acetate, (8) $\beta$-carotene, (9) nicotinamide, (10) nicotinic acid, (11) pyridoxine, (12) d-pantothenic acid, (13) biotin, (14) thiamine, (15) riboflavin, (16) $B_{12}$, (17) ascorbic acid. Method conditions as follows; column: $C_{18}$SB (50 × 3.0 mm i.d., sub 2 $\mu$m; modifier: MeOH/water (95/5, v/v) with 0.2% $NH_4$Fo; gradient condition: 2% (0.5 min), 2–30% (2.0 min), 30–85% (0.8 min), 85% (2.7 min), 85–100% (0.2 min), 100% (1.3 min), 100–2% (1 min), 2% (1.5 min); flow rate: 1.2 mL/min at a column temperature of 40°C; back pressure: 15.2 MPa (6.0 min), 15.2–10.3 MPa (0.2 min), 10.3 MPa (1.6 min), 10.3–15.2 MPa (0.5 min), 15.2 MPa (1.7 min). Reprinted from [31] with permission from Elsevier.

The supercritical $CO_2$, whose polarity is similar to that of hexane/heptane, is mainly suitable for the separation of FSVs and carotenoids. However, compressed $CO_2$ can be mixed with MeOH, ethanol, isopropanol, and acetonitrile, obtaining a high-versatile mobile phase, also suitable for WSVs. The selectivity for the more polar vitamers can be enhanced by varying the organic modifier percentage during the chromatographic run.

Depending on its polarity, an organic modifier can be used till 30-40 %. Obviously, as the concentration of the modifier increases, the operating parameters are often below the critical point and the fluid mixture turns into a liquid state (subcritical state); however, this event is unimportant for analytical practical purposes [27]. Under these conditions, the mobile phase is compatible with both polar and non-polar stationary phases and the range of vitamins that can be analysed with SFC is dramatically extended [28]. Due to these benefits, most SFC applications addressed to vitamns are conducted using $CO_2$ opportunely mixed with modifiers and additives. These last ones can significantly improve the peak shapes and the analyte retention. Additives act as the third component of the mobile phase, being used at low concentrations (usually 0.01 – 1%). In general, salts, acids, and bases are added to the mobile phase to adjust polarity, suppress ionization, form an ion-pair with the charged analytes, and/or remodel the properties of the stationary phase surface by interacting with its active sites [27,29,30]. Different classes of WSVs and FSVs can be separated by adding calibrated volumes of water and/or additives, such as small organic acids (e.g. trifluoroacetic acid) and bases (e.g. ammonia) or salts (e.g. ammonium formate or acetate). Usually, the addition of water to the SFC mobile phase is not practised because of the low miscibility with $CO_2$ and the dramatic pressure leaps. Recently, however, the use of water-rich modifiers is becoming more frequent. Percentages of water from 1% up to 8% mixed with MeOH have been used in UPCC methods for the analysis of WSVs. For example, Pyo [31] obtained excellent results in the separation of nicotinic acid, nicotinamide and ascorbic acid, vitamin K, vitamin D and vitamin E, by using a water-modified supercritical $CO_2$ mobile phase. In 2014, Taguchi et al. [28] applied the UPCC for the simultaneous separation of WSVs and FSVs, by using a water-rich modifier (MeOH/water (95/5, v/v) with 0.2% $NH_4$Fo) with a gradient profile up to 100% in the organic modifier. As a result, 17 vitamins were separated in 4 min as shown in **Fig. 2**.

Regarding the effect of organic cosolvents on the separation of carotenoids [32], it has been observed that, using C18 columns, solvents with a dielectric constant ($\varepsilon$) >30, like MeOH and ACN, describe a U-curve; ethanol, acetone, and 1-propanol, having $20<\varepsilon<30$, regularly decrease retention when solvent proportion increases; finally, solvents with $\varepsilon<10$, like heptane, tetrahydrofuran, and chloroform, have the same effect as the previous solvents but with a higher slope.

## 4. Chromatographic columns for the SFC of vitamins

The modern SFC usually works with packed columns, taking advantage of the already known stationary phases for LC. However, in the last decade, the growing demand for this technique has stimulated the development of novel stationary phases specifically designed for SFC. Thanks to the versatility of $CO_2$-based mobile phases, a full compatibility of SFC with most stationary phases is guaranteed. In fact, SFC can be used with several types of stationary phases, such as the polar bare-silica, silica bonded with polar ligands (aminopropyl, cyanopropyl, etc.), mixed-polarity bonded silica (aliphatic ligands with polar end-capping or polar embedded groups and aromatic ligands such as pentafluorophenylpropyl), till to the least polar $C_{18}$- and $C_{30}$-bonded silica. Lesellier et al. [16] have well highlighted how SFC shows different retention mechanisms based on the nature of stationary phases. More specifically, SFC exhibits retention patterns correlated to the density of the mobile phase and comparable to RP (non-polar stationary phase) and NP chromatog-

raphy (polar stationary phases). Furthermore, in presence of mixed polarity columns, the retention patterns are not ascribable to those of LC, but they are peculiar of SFC.

In this scenario, chromatographic columns of bare silica with 3.5 - 5 μm and sub-2 μm particles diameter have often been used to separate FSVs [33]. For example, in a typical quality control of vitamin D and its impurities in pharmaceutical raw material, the application of SFC turns out to be very promising for its speediness [34,35]. Even though encouraging results are obtained in the resolution of *cis*- and *trans*- isomers of phylloquinone ($K_1$) [36] as well as for the separation of vitamers E [37-39], the best condition for the use of bare silica is when few vitamins are simultaneously analysed. On the other hand, SFC methods based on $C_{18}$ stationary phases, including those packed with sub-2 μm particles, appear to be more suitable for the multi-residual analysis of vitamins of different classes [28,40-45], carotenoids included [46]. Among all, HSS $C_{18}$ SB by Waters has proved to be very effective: composed of high-strength silica (HSS), it has shown high selectivity thanks to the high silanol activity and a medium carbon load (8.5%) [40,41]. Recently, the retention behavior of carotenoids in SFC conditions on $C_{18}$-bonded stationary phases has also been reviewed by Lesellier and West [32], considering the effect of several cosolvents with different dielectric costants ($\varepsilon$).

In the next section, devoted to illustrating some selected applications related to vitamin analysis by modern SFC, it is also discussed the performance of several types of $C_{18}$ columns, as well as that of other stationary phases specifically designed for SFC separations.

## 5. Selected SFC applications for vitamin analysis

Although LC is the gold standard for vitamin analysis, modern SFC has all potentialities to overcome some drawbacks connected to the chromatographic separation of WSVs, FSVs and carotenoids, as pointed up below.

Petruzziello et al could analyse several carotenoids and 14 vitamers of the A, D, E, and K groups in human plasma [47] on a HSS $C_{18}$ using a UPCC instrument. The separation was performed in a gradient mode, keeping the column at 40°C, with $CO_2$ and a mixture of MeOH + 20 mM $NH_4Fo$ + 2% $H_2O$ (v/v) as a modifier. Since the detection was performed by a triple quadrupole equipped with an electrospray (ESI) source, a mixture of isopropanol/heptane (3:7, v/v) at 100 μL/min was used as the MS make-up solvent. The method guaranteed the separation of structurally similar compounds, such as the isomers lutein and zeaxanthin and the family of tocopherols, in 8 min by using a relatively small volume of organic solvent. Compared to the other analytes, an anomalous high limit of detection (LOD) was observed for α-tocopherol probably due to the MS detector saturation, despite the selection of a little intense ion current related to an isotopologue.

Tyśkiewicz et al. [42] developed a UPCC-diode array detection (DAD) method for the simultaneous determination of FSVs (*cis*- and *trans*-retinyl palmitate, *cis*- and *trans*-retinyl acetate, retinol, α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, ergocalciferol, cholecalciferol, *cis*- and *trans*-phylloquinone and menaquinone-4) in the waste fish oil after saponification. All forms, geometric isomers included, were efficiently separated on a HSS $C_{18}$ SB column (100 mm × 3.0 mm I.D., 1.8 μm), kept at 35°C, within 13 min using $CO_2$ and MeOH as the mobile phase. When the proposed method was applied to real samples, α-tocopherol, γ-tocopherol, δ-tocopherol and retinol were identified.

Although the most used LC stationary phase for the analysis of carotenoids is the $C_{30}$, which has proven to be compatible with supercritical mobile phase, there are not many reports that describe its use in SFC for this purpose [48-51]. Giuffrida et al [49] succeed in the identification of 25 apocarotenoids in habanero peppers using a 2.7 μm-fused-core particle $C_{30}$. The particles consisted of a silica nucleus encircled by a thin (0.5 μm) porous shell of stationary phase that provided a great efficiency. All the separations occurred in less than 5 min. In a further investigation, Giuffrida and co-workers determined the occurrence of carotenoids and apocarotenoids in human blood samples using similar conditions [51]. They also reported that the separation of 31 carotenoids with a sub-2 μm $C_{30}$ column was more efficiently than a conventional $C_{30}$ column; the method applied to real samples was able to identify 3 antheraxanthin monoesters and 9 apocarotenoids in yellow tamarillo for the first time [50].

Among columns designed for LC and transferred to SFC, pentafluorophenyl-(PFP), diphenyl- (DPH), and cyanopropyl-bonded silica stationary phases can play a significant role in vitamin analysis. Although PFP stationary phase provides mixed retention mechanisms mainly related to π-π, hydrophobic, and hydrogen bonding interactions, some applications did not notch up the expected success. For example, Pilařová et al showed how a PFP stationary phase was not suitable for the separation of two critical pairs, i.e. β- and γ-tocopherol and β- and γ-tocotrienol [52], and for the retention of vitamin K [42]. However, in those works, the experimental set-up (such as mobile phase composition, column temperature, etc.) was fixed and not optimized for PFP. It is important to mention that PFP column has produced good results in the separation of vitamin D [53-55]. For example, Oberson et al. [53] developed a novel SFC-MS method to quantitfy vitamin D and its main metabolites (vitamin D3, vitamin D2, and their 25-hydroxy metabolites) in breast milk after ethanolic protein precipitation and liquid-liquid extraction; saponification was avoided due to the partial degradation of some metabolites. Final extracts were derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione and the vitamin D derivatives were detected by a triple quadrupole with atmospheric pressure chemical ionization (APCI). All of the analytes were separated in 9 min on a PFP column (3.0 × 100 mm, 1.7 μm) using a gradient of MeOH-$NH_4Fo$ on $CO_2$. Make-up solvent was MeOH containing $NH_4Fo$. In the final conditions matrix effect was lower than 20% for $D_2$ and $D_3$ and lower than 5% for the metabolites. The limit of quantification (LOQ) reached levels as low as 50 pmol/L, with intra- and inter-day relative standard deviations lower than 15% and 20% for all analytes.

Méjean et al. [56] managed to separate six retinoids (all-trans-retinal, all-trans-retinol, all-trans-retinoic acid, retinyl propionate, retinyl acetate, and all-trans-retinyl palmitate) on a DPH stationary phase in about 5 min. An elution gradient separation was achieved using $CO_2$ (solvent A) and EtOH with 0.1 % HFo (solvent B). This kind of column exhibits an exclusive selectivity due to its hydrophobicity, π-π interactions, and steric hindrance. The UV detection permitted limits of detection (LODs) down to 1 pmol of injection.

Taguchi et al. [57] investigated the SFC separation of hydrophilic WSVs such as niacin and its metabolites (niacin, nicotinamide, nicotinamide *N*-oxide, and nicotinuric acid) using a HSS Cyano column (3.0 × 50 mm, 1.8 μm) and a mobile phase composed as follows: $CO_2$ (phase A) and MeOH/$H_2O$ (95/5, v/v) with 0.1% (w/v) $NH_4Fo$ and 0.15% (v/v) HFo (phase B). The SFC-MS/MS method proved to be effective also for the analysis of very polar vitamers having negative log P values.

Donato et al. [58] developed a bidimensional strategy to define the carotenoid fingerprint in red chilli pepper. SFC was used in the first dimension (1D), while RP-UHPLC was the second dimension (2D). An on-line SFC × RP-UHPLC system operated in an automated fashion, using six-port switching valves which were equipped with $C_{18}$ cartridges for trapping and focusing the analytes eluting from 1D. In order to efficiently focus carotenoids on the sorbent material and to reduce interferences of the expanded $CO_2$ on the 2D separation, a make-up flow of water was added to the SFC effluent. Besides DAD and quadrupole-time-of-flight mass spectrometry (QToF), ion mobility was the fourth separation dimension being able to discriminate analytes based on mass, shape and size.

The growing demand in SFC applications has led to the introduction of novel stationary phases tailored for this technique. In the **Fig. 3**, the chemical structures of the most relevant stationary phases used for vitamin analysis are illustrated.

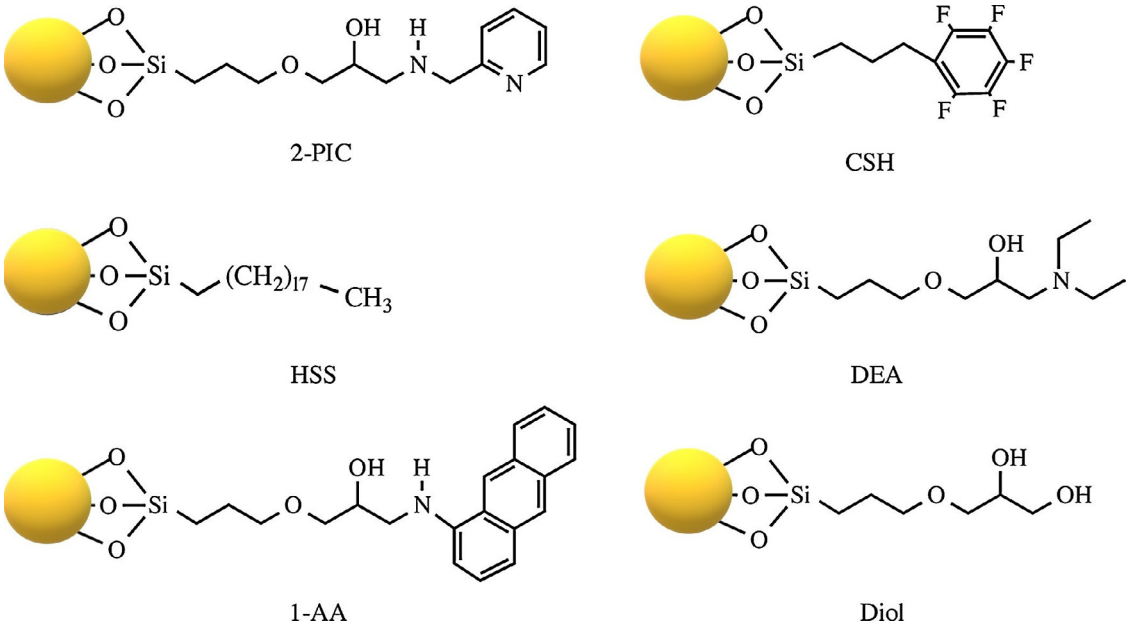


**Fig. 3.** Chemical structure of frequently tested stationary phases for vitamin analysis: 2-PIC: 2-picolylamine; CSH: PFP; HSS:$C_{18}$; Torus DEA: diethylamine; 1-AA: 1-aminoanthracene; Diol.

Among the novel columns conceived for SFC separations, the 1-aminoanthracene (1-AA) stationary phase has produced appreciable results for the separation of neutral and hydrophobic molecules, including FSVs and carotenoids [59]. 1-AA can establish $\pi$-$\pi$ interactions through the aromatic rings of anthracene and supplementary interactions due to amino and hydroxyl groups. For example, Jumaah et al. [54] developed a SFC-MS method that was able to discriminate nine vitamin D metabolites ($D_3$, $D_2$, 1OH-$D_2$, 25OH-$D_3$, 1OH-$D_3$, 25OH-$D_2$, 24,25$(OH)_2$-$D_3$, 1,25$(OH)_2$-$D_2$, 1,24$(OH)_2$-$D_3$, 1$\alpha$,25$(OH)_2$-$D_3$) in about 8 min. All compounds were baseline separated on a 1-AA column. In coupling SFC with MS, the authors compared ESI and APCI, the latter providing signals six times more intense. The LODs were found to range between 0.39 and 5.98 ng/mL for 24,25$(OH)_2D_3$ and 1$OHD_2$, respectively. The method was applied to human plasma samples to verify its feasibility. Vitamin $D_3$, 25OH-$D_3$ and 24,25$(OH)_2$-$D_3$ were determined in concentrations of: 6.6 ± 3.0 ng/mL, 23.8 ± 9.2 ng/mL and 5.4 ± 2.7 ng/mL, respectively. Oberson et al [60] separated nine FSVs in only 4 min with 1-AA. Nevertheless, the stationary phase was not able to resolve *cis*- and *trans*- isomers of phylloquinone . More recently, Tyśkiewicz et al [42] optimized a SFC method for the simultaneous separation of *cis*- and *trans*-phylloquinone, *cis*-and *trans*-retinyl palmitate, *cis*- and *trans*-retinyl acetate, $\alpha$-, $\beta$-, $\gamma$- and $\delta$-tocopherol, ergocalciferol, cholecalciferol, and retinol. Several columns were tested in this study, 1-AA included, but HSS $C_{18}$ allowed the resolutiom of all the forms in 13 min only.

2-Ethylpyridine (2-EP) and 2-picolylamine (2-PIC) are stationary phases widely used in achiral separation. These columns have been manufactured for SFC systems and exhibit a broad range of retention mechanisms due to their mixed-polarity surface. Both columns were successfully applied for the separation of the 8 positional isomers of vitamin E [52,61]. Nováková et al [61] observed a complete separation of the analytes in barely 5 min with both stationary phases when 3% EtOH was used as a modifier. Moreover, the study highlighted how 2-PIC column showed the same retention pattern in less than 4 min by using 3% of MeOH, while 2-EP column was not able to resolve $\delta$-tocopherol and $\beta$-tocotrienol, under the tested conditions. This is probably due to the multiple retention mechanisms of the 2-PIC stationary phase that can work with different modifiers without losing in resolution. Amino-bonded silica was applied to the separation of tocopherols and tocotrienols, using $CO_2$ and EtOH as mobile phase [62]. Comparing atmospheric-pressure photoionization (APPI), ESI and APCI, the authors found that APPI resulted to be the most sensitive ionization technique. The chromatographic run lasted less than 5 min and was as sensitive as traditional approaches, with LODs in the tens of $\mu g$/L.

Diol-bonded silica stationary phases, with their polar surface, are widely used in SFC applications, generally providing NP behaviour. They are fairly applied to vitamin analysis even though their performance has been below expectations when multi-class separations have been tackled. Their selectivity is mainly related to the presence of hydroxyl groups which provide good peak shape and retention for the analysis of tocopherols [42].

### 5.1. SFC applied to chiral vitamin separations

Over the years, especially before its remarkable development started in 2010, SFC had essentially been applied to chiral applications. This capability has still been a strong point of the modern SFC.

It is known that a great number of chiral stationary phases are available for LC applications [63]. Although most of them have also been adopted in SFC, there are not many reports dealing with the separation of both diastereomers and enantiomers of vitamins. Dexpanthenol (pantothenyl alcohol or provitamin $B_5$) is an active ingredient of numerous vitamin B-complex supplements and cosmetic formulations. Once the skin penetration is performed, the molecule is converted into pantothenic acid which is a precursor in the biosynthesis of coenzyme A [1]. The pharmacological effect of dexpanthenol relies on the D-(R)-panthenol (eutomer), while the L-(S)-isomer (distomer) is inactive. In oder to determine the enantiomeric purity of cosmetic formulations, the enantioresolution of provitamin $B_5$ was obtained in less than 6 min, by employing a 3 $\mu$m-amylose-type immobilized polysaccharide chiral stationary phase (Chiralpak IA) and a mobile phase of $CO_2$ and 11% MeOH pumped at 2.3 mL/min, 25°C and 150 bar backpressure [64].

Vitamin $K_1$ has a double bond at the 2,3'-position on its side chain. The *trans*-isomer is the active and most abundant form, while the *cis*-isomer is inert accounting for up to 20 % of the total vitamin in both natural and synthetic products. However, besides the diastereomeric double bond, there are also 2 chiral centers at the 4' and 11' positions on the side chain [1]. Thus, there are 8 potential stereoisomers: 4 *trans*-enantiomers, and 4 *cis*-enantiomers. In nature, vitamin $K_1$ is predominantly 2',3'-trans-4*R*,11*R*-philloquinone. Most researchers measure the total vitamin $K_1$, overestimating the nutritional value of some foods,

**Table 1**
Selected applications (2011-2021) based on SFC for the analysis of vitamins and carotenoids.

| Analytes | matrix | Extraction conditions | Mode/detection | Column | SFC conditions | LOD, LLOQ, LOQ | Reference |
|---|---|---|---|---|---|---|---|
| $\alpha$-tocopherol, $\alpha$-tocotrienol, $\beta$-tocopherol, $\gamma$-tocopherol, $\gamma$-tocotrienol, $\delta$-tocopherol, $\delta$-tocotrienol, $K_1$, lutein, zeaxanthin, $\beta$-cryptoxanthin, 25OH-$D_2$, 25OH-$D_3$. | Human plasma. | i) Protein precipitation: IpOH;<br>ii) SPE;<br>iii) evaporation to dryeness;<br>iv) reconstitution: IpOH/heptane. | UHPSFC-ESI(+)-triple quadrupole, operated in SRM. | Viridis HSS $C_{18}$ SB (3.0 × 100 mm; 1.8 $\mu$m). | BPR: 172 bar;<br>T: 40°C;<br>MP: $NH_4Fo$ in MeOH/$H_2O$ + $CO_2$.<br>Inj. vol: 2 $\mu$L. | LODs: 0.4-0.004 ng/mL; probably for the high endogenous content of $\alpha$-T, the evaluation of LOD for this analyte does not seem realistic (3200 ng/mL). | [46] |
| *cis*-and *trans*-retinyl palmitate, *cis*- and *trans*-retinyl acetate, retinol, $\alpha$-tocopherol, $\beta$-tocopherol, $\gamma$-tocopherol, $\delta$-tocopherol, ergocalciferol, cholecalciferol, *cis*- and *trans*-phylloquinone and menaquinone-4. | Hemp Seed Oil and Waste Fish Oil. | i) Saponification: LLE (diethyl ether);<br>ii) evaporation to dryness;<br>iii) reconstitution: TBME. | UHPSFC-PDA. | HSS $C_{18}$ SB (3.0 × 100 mm; 1.8 $\mu$m). | ABPR: 12.41 MPa;<br>T: 35°C.<br>MP: MeOH + $CO_2$. | - | [41] |
| Apocarotenoids. | Chilli pepper. | i) LE: acetone;<br>ii) evaporation<br>iii) epiphase ether/hexane dried under vacuum;<br>iv) reconstitution: $CH_3OH$/MTBE. | UHPSFC-APCI-triple quadrupole. | $C_{30}$ (4.6 × 150 mm; 2.7 $\mu$m). | BPR: 150 bar;<br>T, 35°C;<br>MP, MeOH + $CO_2$. | - | [48] |
| Carotenoids and apocarotenoids. | Human blood. | direct injection without any preliminary treatment. | UHPSFC-APCI-triple quadrupole. | $C_{30}$ (4.6 × 150 mm; 2.7 $\mu$m). | BPR: 150 bar;<br>T: 35°C;<br>MP: MeOH + $CO_2$. | - | [50] |
| 25OH-$D_3$, 25OH-$D_2$, $D_3$, $D_2$, 1,25$(OH)_2$-D2, 1,25$(OH)_2$-D3, 24,25$(OH)_2$-D2, 24,25$(OH)_2$-D3. | Human milk. | Protein precipitation: EtOH;<br>ii) LLE: hexane/ethyl acetate;<br>iii) PTAD derivatization. | UHPSFC-APCI-triple quadrupole. | PFP column (3.0 × 100 mm, 1.7 $\mu$m). | ABPR:128 bar;<br>T, 45°C;<br>MP, $NH_4Fo$ in MeOH/$H_2O$ + $CO_2$. | LLOQs were as low as 2 ng/100 mL for all analytes. | [52] |
| $D_2$, $D_3$, 25-OH-$D_2$, 25OH-$D_3$, 3-epi-25OH-$D_2$, 3-epi-25OH-$D_3$, 1,25$(OH)_2$-$D_2$, 1,25$(OH)_2$-$D_3$, 24,25$(OH)_2$-$D_2$, 24,25$(OH)_2$-$D_3$. | Human serum. | i) Protein precipitation: ACN;<br>ii) extraction: ACN;<br>iii) evaporation to dryness;<br>iv) reconstitution in MeOH. | UHPSFC-ESI-triple quadrupole. | PFP column (4.6 × 250 mm, 3.5 $\mu$m). | BPR: 10.0 MPa;<br>T: 50°C;<br>MP: MeOH + $CO_2$;<br>post-column make-up solvent: $NH_4Fo$/HFo. | LOQs: from 0.071 to 0.704 ng/mL. | [54] |
| All-*trans*-retinal, all-*trans*-retinol, all-*trans*-retinoic acid, retinyl propionate, retinyl acetate, and all-*trans*-retinyl palmitate. | - | - | UHPSFC-DAD. | Diphenyl column (2.0 × 250 mm, 3 $\mu$m). | BPR:130 bar;<br>T: 55°C;<br>MP: HFo in EtOH + $CO_2$. | Instrumental LOD< 1 pmol injected. | [55] |
| Niacin, nicotinamide, nicotinamide N-oxide, and nicotinuric acid. | Rabbit plasma, human urine. | Plasma and urine:<br>i) Protein precipitation: MeOH;<br>ii) centrifugation;<br>iii) supernatant was analysed directly (plasma), diluted (urine). | UHPSFC-ESI-triple quadrupole. | HSS Cyano (3.0 × 50 mm, 1.8 $\mu$m). | ABPR: 20.68 MPa;<br>T: 40°C;<br>MP: $NH_4Fo$/HFo in MeOH + $CO_2$. | - | [56] |
| Carotenoids. | red chilli pepper (*Capsicum annuum* L.). | SPE: MeOH/ethyl acetate/petroleum ether;<br>ii) dissolution in MeOH/ MTBE);<br>iii) filtration. | 2D-UHPSFC × RP-UHPLC-PDA-Q-ToF MS-IMS. | Trapping column: Xbridge C18 (2.1 × 20 mm; 5 $\mu$m)<br>1D column: Ascentis ES Cyano (1.0 × 250 mm; 5.0 $\mu$m);<br>2D column: BEH $C_{18}$ (2 × 50 mm, 1.7 $\mu$m). | 1D<br>T =40°C (1D);<br>MP: MeOH + $CO_2$<br>2D<br>T = 60°C;<br>MP: ACN/$H_2O$ and iPOH. | - | [57] |

(*continued on next page*)

**Table 1** (*continued*)

| Analytes | matrix | Extraction conditions | Mode/detection | Column | SFC conditions | LOD, LLOQ, LOQ | Reference |
|---|---|---|---|---|---|---|---|
| $D_2$, $D_3$, 25OH-$D_3$, 25OH-$D_2$, 1-OH-$D_3$, 1-OH-$D_2$, 1,24$(OH)_2$-$D_3$, 1,25$(OH)_2$-$D_2$, 1,25$(OH)_2$-$D_3$, 24,25$(OH)_2$-$D_3$. | Plasma. | Protein precipitation: ACN; ii) centrifugation; iii) evaporation to dryness; iv) LLE: ethyl acetate/$H_2O$ v) centrifugation; vi) evaporation to dryness of organic layer: vii) dissolution in MeOH | UHPSFC-ESI-QToF. | 1-AA (3.0 × 100 mm, 1.7 $\mu$m). | ABPR: 200 bar; T:35°C; MP: MeOH + $CO_2$. | LODs: 0.39 - 5.98 ng/mL. | [53] |
| $\alpha$-carotene, $\beta$-carotene, lycopene, canthaxanthin, lutein, zeaxanthin, neoxanthin, $\beta$-cryptoxanthin, astaxanthin, and violaxanthin. | Microalgae, rosehip. | i) SFE ($CO_2$): P, 300 bar; T, 40 C; flow rate, 2 mL/min; ET, 60 min; ii) after PBR: EtOH, flow rate, 0.2 mL/min. | UHPSFC-ESI-PDA-triple quadrupole. | 1-AA (3.0 × 100 mm, 1.7 $\mu$m). | ABPR: 160 bar; T: 35°C; MP: MeOH + $CO_2$. | LODs: 2.6 - 25.2 ng/mL. | [58] |
| All-*trans*-retinol, all-*trans*-retinyl acetate, all-*trans*-retinyl palmitate, $D_2$,$D_3$, dl-$\alpha$-T, dl-$\alpha$-tocopheryl acetate, MK-4, $K_1$ | i) milk-based infant formula powders, infant cereals. ii) RTF adult nutritional products. | i) SPE or LLE: a papain solution (acetate buffer, pH 5.0/hydroquinone) at 45°C for 30 min; ii) extraction: acidified MeOH/isooctane/BHT; iii) centrifugation; iv) injection of upper layer (vitamins A, E and K) and PTAD derivatization (vitamins D). | UHPSFC-APCI-triple quadrupole. | 1-AA (3.0 × 100 mm, 1.7 $\mu$m). | ABPR: 128 bar; T: 45°C; MP: $NH_4$Fo in MeOH/$H_2O$ + $CO_2$. | LODs: 4 pg injected for vitamins A, D and K, and 40 pg injected for vitamin E. | [59] |
| $\alpha$-tocopherol, $\alpha$-tocotrienol, $\beta$-tocopherol, $\beta$-tocotrienol, $\gamma$-tocopherol, $\gamma$-tocotrienol, $\delta$-tocopherol, $\delta$-tocotrienol, tocopherol acetate. | Supplements of vitamins E (drops, capsules, tablets, and granulate). | I) Drops and capsules: i) dilution: heptane; ii) injection; II)Tablets and granulate: i) dissolved in MeOH; ii) extraction in heptane. | UHPSFC-DAD. | BEH -2-EP (3.0 × 100 mm, 1.7 $\mu$m). | BPR: 130 bar; T: 50°C; MP: MeOH + $CO_2$. | - | [60] |
| $\alpha$-tocopherol, $\alpha$-tocotrienol, $\beta$-tocopherol, $\gamma$-tocopherol, $\gamma$-tocotrienol, $\delta$-tocopherol, $\delta$-tocotrienil, tocopherol acetate. | Soybean oil. | i) Dilution with cyclohexane/BHT; ii) direct injection. | UHPSFC-DAD. | $NH_2$ column (2.0 × 150 mm, 3.0 $\mu$m). | ABPR: 130 bar; T: 30°C; MP: HFo in MeOH + $CO_2$. | LODs: 4.25-20.5 µg/L | [61] |
| *cis*-$K_1$, *trans*-$K_1$. | - | - | i) UHPSFC-DAD. | bare silica (4.6 × 150 mm, 3.5 $\mu$m). | BPR:200 bar; T:60°C; MP: ethyl acetate + $CO_2$. | - | [35] |
| 8 stereoisomers of phylloquinone: 4 *trans*-enantiomers, and 4 *cis*-enantiomers. | - | - | UHPSFC-DAD. | Chiral column: RegisPack (4.6 × 250 mm, 5 $\mu$m). | BPR set at 150 bar; T: 30°C; MP: MeOH + $CO_2$. | - | [65] |
| $D_3$,$D_2$, 25OH-$D_3$, 25OH-$D_2$, 3-epi-25OH-$D_3$, 1$\alpha$,25$(OH)_2$-$D_3$, 24R,25$(OH)_2$-$D_3$, 23R,25$(OH)_2$-$D_3$). | Human serum. | Protein precipitation: MeOH/iPOH; ii) centrifugation; iii) SLLE; iv) PTAD derivatization; v) evaporation; vi) reconstitution in MeOH; vii) injection. | UHPSFC-APCI-triple quadrupole. | Chiral column: Lux cellulose-3 chiral column (3.0 × 150 mm, 3.0 $\mu$m). | ABPR: 1750 psi; T: 20°C; MP: HFo in MeOH + $CO_2$. | - | [66] |

especially those fortified with synthetic vitamin $K_1$. The separation of vitamin $K_1$ enantiomers has been faced by HPLC [65], but SFC has proved to be more effective [36,66]. Berger et al [36] proposed a method by which was possible to obtain a baseline separation of the $K_1$ isomers in about two minutes with a totally porous bare silica column (4.6 × 150 mm, 3.5 $\mu$m) and 2.5% ethyl acetate in $CO_2$ as the mobile phase. Berger & Berger [66], with a stationary phase composed by silica modified with tris-(3,5-dimethylphenyl) carbamoyl amylose (RegisPack column) and a mobile phase of 5 % MeOH in $CO_2$, were able to separate seven out of the eight enantiomers of vitamin $K_1$ in about 20 min. A single enantiomer (probably trans-4R,11R-philloquinone) and three other ones accounted for 58 % and for 30 % of the total areas, respectively. An achiral separation of the *cis*- and *trans*-isomers indicated that the total of all *cis*-enantiomers was approximately 11.35 % of peak area.

A column packed with cellulose tris(3,5-dimethylphenylcarbamate) (150 mm, 3 mm, 3 $\mu$m) was employed for the separation of chiral metabolites of vitamin D [67]: 23,25$(OH)_2$-$D_3$, 24,25$(OH)_2$-$D_3$ and 1$\alpha$,25$(OH)_2$-$D_3$ along with the C3-epimer 3-epi-25OH-$D_3$ from 25OH-$D_2$ and 24OH-$D_2$ from 25OH-$D_2$. The mobile phase was $CO_2$ and 0.1% HFo inMeOH with a make-up solvent containing 0.1% HFo. Comparing UHPLC and UPCC, the separation by the latter was better because it was able to reduce the run time of about 2 min (6 min *vs* 8 min required by UHPLC). Such metabolites were extracted from serum samples by means of liquid-liquid extraction and, then derivatized with PTAD to improve the ESI-MS/MS detection. The employed stationary phase demonstrated its ability in resolving structurally similar metabolites, bearing hydroxyl groups, taking advantage of hydrogen bonding between the analytes and the stationary phase.

**Table 1** lists the selected applications discussed in 5 and 5.1 sections, reporting additional information related to matrix, extraction technique, experimental conditions of analysis, SFC detector, LODs and LOQs.

## 6. Comparison with liquid chromatography

The pre-eminence of SFC over LC in vitamin analysis emerges in several situations.

One case is when a large number of compounds, characterized by a subtle chemical heterogeneity, have to be separated. In fact, in many circumstances, the selectivity of LC is not enough to avoid co-elution problems, which cannot be solved even using a MS detector if isomeric molecules are involved (for instance, lutein and zeaxanthin, $\beta$- and $\gamma$-tocopherols, etc.). In such cases, NPLC and NARPLC can be effective, but they require long chromatographic runs (30-60 min) and the use of large volumes of toxic solvents [6].

Speed is another appreciable feature of SFC in vitamin analysis. A representative example is the separation of eight vitamers E, obtained in just 4 min by using a sub-2μm 2-EP column and a mobile phase composed from 98% $CO_2$ and 2% of MeOH with 10 mM $NH_4Fo$ [52]. The same mixture could be separated on a $C_{30}$ column kept at 15°C, under isocratic NARP conditions, but in 18 min [9]; moreover, despite a carefully optimization of the chromatographic conditions, the two critical pairs of $\beta$- and $\gamma$-tocotrienols, and $\beta$- and $\gamma$-tocopherols could not completely resolved at the baseline [9].

Another important aspect is the green character of SFC. Compared with NPLC and RPLC, UPCC reduces the consumption of toxic solvents and, consequently, their purchase and waste disposal costs. In particular, by using UPCC, a 30-kg $CO_2$ bottle lasts 3 weeks, while the residues of organic solvents take 1 year to fill a 10 L tank.

Last but not least, SFC allows the direct injection of organic extracts, avoiding tedious solvent exchange operations which, on the other hand, are necessary when RPLC methods are used.

## 7. Conclusions

Over the last few years, UPCC has emerged as a unique separation technique for its green character and for merging the advantages of SFC with those of the UHPLC technology. In the field of vitamin and carotenoid analysis, LC has still been considered the best option, especially in the case of carotenoids, whose separation is very effective on a HPLC $C_{30}$ column, kept at subambient temperatures, under NARP conditions. Nevertheless, as explained, long chromatographic runs and the use of toxic organic solvents make the conventional methods addressed to vitamin analysis not suitable for a sustainable chemistry. On the contrary, UPCC has all peculiarities to carry out green and cheap separations. UPCC also shows a matchless ability in performing very quick chiral and achiral separations. Thence, it can solve demanding vitamin separations, especially those of chiral forms that are still few explored. Last but not least, UPCC can be a potent system to carry out simultaneous analyses of WS and FS vitamin forms in a single run, proving a rapid comprehensive profile of food and biological samples.

## CRediT author statement

**Pierpaolo Tomai**: draft; **Chiara Dal Bosco:** Review & Editing; **Nina Felli**: Draft, Figures; **Francesca Romana Scuto***:* Review & Editing; **Giovanni D'Orazio**: Tables and references; **Alessandra Gentili**: Supervision, Conceptualization, writing- original draft preparation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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