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Review

Analysis of non-saponifiable lipids by super-/subcritical-fluid chromatography

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Abstract

Because of the particular properties of carbon dioxide or carbon dioxide/modifier mobile phases, super- or subcritical-fluid chromatography (SFC) can be an alternative to more classical chromatographic methods such as gas chromatography (GC) or high-performance liquid chromatography (HPLC) for the separation of unsaponifiable lipids. These fluids can also be helpful in the extraction and/or the concentration steps of sterols, tocopherols or carotenoids from complex samples. Supercritical extraction, off-line prefractionation or semi-preparative supercritical fluid chromatography, carried out before the analysis are described. The effects on separation of analytical parameters such as pressure, nature of and modifier percentage or stationary phase nature are also reported. The performance of capillary, packed or capillary packed columns is discussed, as well as the consequences of their use (choice of stationary phases, type of coupled detector). Numerous examples of fine separations are reported.

Keywords: Reviews; Supercritical fluid chromatography; Subcritical fluid chromatography; Lipids, non-saponifiable

Contents

1. Introduction

Due to their high molecular mass or to their chemical structure, unsaponifiable lipids are not very volatile and have a low solubility into hydro-organic solvents. Consequently, their analysis by GC requires

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either the use of a high temperature (beyond 300°C) or derivatisation, when their analysis by HPLC involves the use of organic mobile phase often containing a chlorinated solvent (methylene chloride or chloroform). In other respects, because the chemical differences between compounds can be subtle (cis/trans or positional isomerisation) in one family (tocopherols, carotenoids), high selectivities and efficiencies should be reached by chromatographic methods.

Sub- or supercritical fluids, such as carbon dioxide, have interesting physical (diffusivity, viscosity) and chemical (elution power) properties, which can successfully be applied to the separation of these compounds.

Because of the low viscosity of supercritical fluids, smaller pressure drops are observed in super-/subcritical-fluid chromatography (SFC) by using the same columns as in HPLC.

Moreover, due to the higher diffusion coefficients (D_m) of solutes into the supercritical fluids, the optimum of chromatographic efficiency is reached in SFC with greater linear speed of mobile phase than in HPLC. These two properties (low vicosities, high diffusion coefficients) allow the use of higher flow-rates, which reduces the analysis time at equivalent efficiency. With packed columns, SFC flow-rates classically range from 3 to 5 ml/min.

Solubility of compounds into carbon dioxide depends on the fluid density, which is related to the analytical pressure and temperature [1]. The density of pure carbon dioxide ranges from 0.2 to 0.8 g/ml by increasing the pressure or decreasing the temperature. Based on the solubility parameter of Hildebrand (δ), this corresponds to an eluotropic strength ranging from that of perfluoroalcane ($\delta=7$) to that of chloroform ($\delta=10$) [2].

Both capillary and packed columns are used in SFC. The two types of column offer different separation performances.

Due to the high flow-rates and the addition of modifiers to carbon dioxide, the reduced high plates are not necessarily higher with capillary columns. By using these columns, the mobile phase is generally pure carbon dioxide, which allows the use of an FID detector and easy coupling with a mass spectrometer by using a slightly adapted GC–MS interface. By keeping the temperature constant, a positive pressure gradient is often required to increase the eluotropic strength of the mobile phase and ensure good efficiency during the analysis. However, with oven temperature between 150 and 200°C, only medium densities (0.5–0.6 g/ml) are reached at the end of the gradient with high limit pressure (35 MPa). Whatever the gradient, selectivity tuning is limited to the stationary phase choice, as CO_2 density changes modify the total solubility of compounds in the mobile phase but not the specific interactions.

With packed columns the increase of the column length, allowed by the low fluid viscosity, can raise the efficiency up to 200 000 plates (100 000 plates by meter) [3,4]. By working at constant pressure and temperature, the addition to the carbon dioxide of an organic solvent, called a modifier, improves the compound solubility. This addition increases the eluotropic strength which can be then compared to that of pure methanol or acetonitrile [5,6]. It also changes the specific interactions between the solutes and the modified mobile phase which allows the tuning of the selectivity. Moreover, the modifier strongly reduces the active silanol sites which favors the column efficiency [7]. Depending on the compound chemical structure, UV or evaporative light-scattering detectors (ELSD) are set up in the chromatographic system. Coupling with mass spectrometer requires an API interface.

2. Extraction or purification of unsaponifiable compounds

Among the different samples containing unsaponifiable lipids, vegetable oils have often been studied. They are mainly constituted of triacylglycerols (95–98%) and unsaponifiable compounds are the minor constituents [8]. From these oils, direct analysis of sterols, tocopherols, tocotrienols and carotenoids without saponification is hindered by their low concentration, and is often difficult due to the broad peak of triacylglycerols [9].

Elimination of triacylglycerols can be carried out either during the extraction of lipids from the seeds by supercritical extraction, or by fractionation of an extracted oil using semi-preparative supercritical fluid chromatography. The supercritical fluids are especially well suited to these methods because their

eluent strength can be modified by backpressure changes, and because after the concentration step they are easily eliminated by simple depressurisation at atmospheric pressure.

2.1. Supercritical fluid extraction

Direct extraction was carried out from seeds [10] and plants [11], either with pure CO_2 or with ethanol/CO_2 (95:5, v/v). Extraction conditions are generally kept constant to extract all lipids contained into the sample. The volume of extraction cells (Fig. 1, 2) can reach 400 ml, and extraction time can take several hours, depending on the sample and on the extraction fluid nature. The elimination of triacylglycerols after extraction can be achieved by using two separators in which the extraction fluid is depressurised in two steps (Fig. 1). The pressure maintained in the first step (Fig. 1, 3) (10 MPa) was lower than the extraction pressure (35 MPa) that reduced the triacylglycerol and waxes' solubility allowing the accumulation of the high molecular mass compounds in this separator. Then, the tocopherols were collected into the second separator (Fig. 1, 4) with a lower pressure (1 MPa). However, the co-extraction of squalene in the tocopherol fraction is noticed [11].

Dynamic SFE–SFC coupling has been used by just connecting a restrictor between the extraction cell and the analytical column (Fig. 2) [12,13]. This restrictor controls the extraction backpressure. With this method of coupling, the extraction cell volume is reduced (from 50 to 300 μl), which is convenient for small samples (<1 mg), and allows fast extraction (up to 90 s). The pure CO_2 is depressurised into this restrictor in order to reduce the temperature which enables cryofocusing of the extracted compounds either in the restrictor [12] or directly in the column stationary phase [13]. With an open capillary analytical column an external cryofocusing is often needed to achieve SFE–SFC [13], which is not required with capillary packed columns, probably because of their greater retention capacity.

Fractionation of lipids can be carried out by mixing a solid sorbent with the oil samples in the extraction cell. With a NH_2-bonded sorbent, Snyder et al. have removed 95% of triacylglycerols in the first extraction step with pure CO_2 (30 ml) at 80°C [14]. Sterols were then extracted by adding methyl-ter-butyl ether (MTBE) (10%) to the CO_2. MTBE was more efficient in extracting the sterols from the

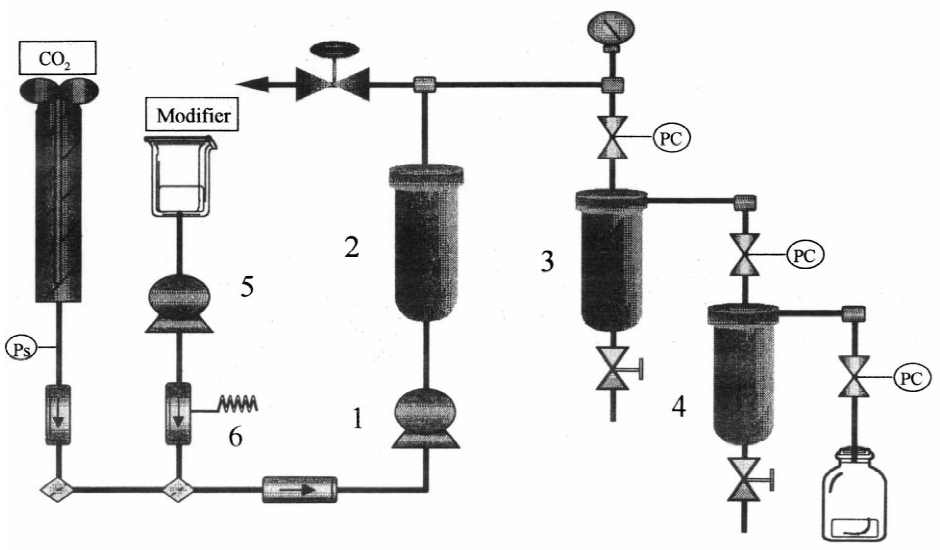

Fig. 1. Supercritical fluid extraction (SFE) pilot plant scale: (1,5) pumps; (2) extraction cell; (3) first separator; and (4) second separator (reprinted with permission from Ref. [11]).

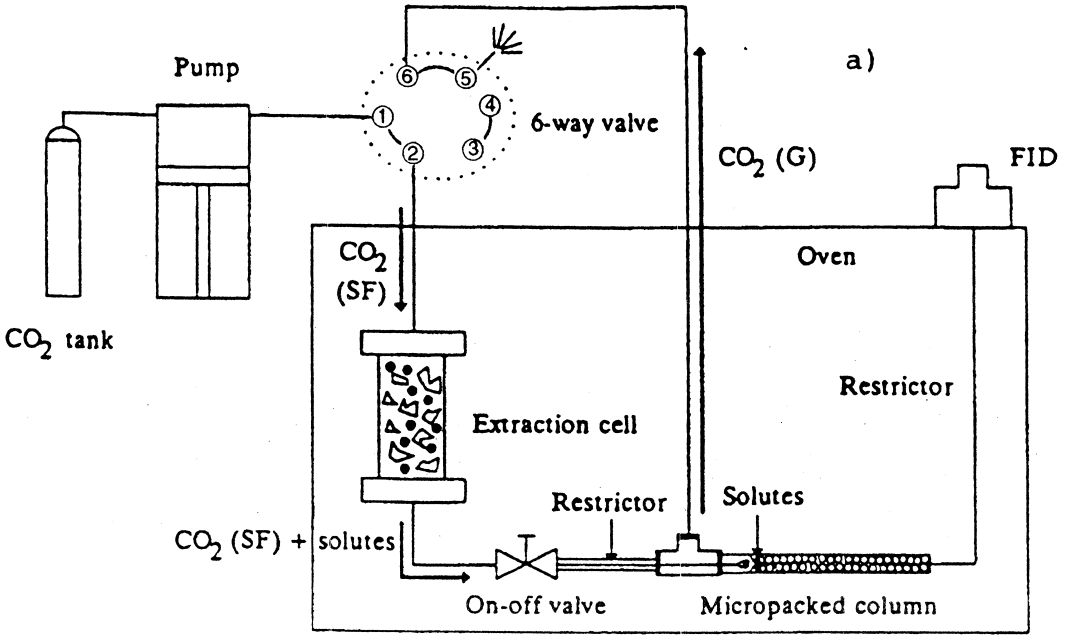

Fig. 2. Schematic diagram of the SFE–SFC device designed for on-line coupling with micropacked columns (reprinted with permission from Ref. [13]).

NH_2-bonded sorbent than methanol or methanol/chloroform mixture. The concentration of sterols was increased from 0.2% in the initial oil to 40% in the MTBE fractions.

2.2. Semi-preparative supercritical fluid chromatography

With oils, it is also possible to use semi-preparative supercritical fluid chromatography with classical packed columns to separate and collect either sterols or tocopherols [9–16]. Medvedovici et al. have separated six lipidic fractions in less than 8 min by using an aminopropyl silica gel (20×0.46 cm) with 8 or 10% methanol as modifier (Fig. 3). The free sterols are eluted between the diglycerides and the monoglycerides. An additional solvent was added during the CO_2 depressurisation to prevent deposition of analytes in the tubing before the collection vials [15]. A total of ten successive collections were done before analysis of the sterolic fraction, allowing the quantification of 20 ppm of stigmasterol in olive oil by GC–MS.

Saito and Yamauchi [9] have proposed a semi-preparative SFC system with a CO_2/ethanol mobile phase (96:4, v/v) by recycling the minor tocopherol fraction up to a complete separation from the triacylglycerol fraction (Fig. 4). Two silica columns (see part 10 and 10′ in Fig. 4) (25×1 cm) were connected by a switching valve. From the three fractions separated by the first column, only the second one, containing α- and β-tocopherols, was switched on the second column, allowing the elimination of the first (triacylglycerols) and the third fraction. β-tocopherol was fully purified after two cycles and α-tocopherol after three cycles. A 0.2-g sample of tocopherols was collected from 1.8 g of wheat germ oil.

Also with a CO_2/ethanol mobile phase, Choo et al. have compared octadecylsilane (ODS) and silica stationary phases [16]. ODS phase was selected (10×1 cm) and allowed the separation of four fractions from 500 mg of crude palm oil. The second fraction contained only α-tocopherols and tocotrienols, the third one was composed of triacylglycerols, and the fourth was carotenoids. Partial coelution between carotenoids and triacylglycerols was noticed in the fourth fraction.

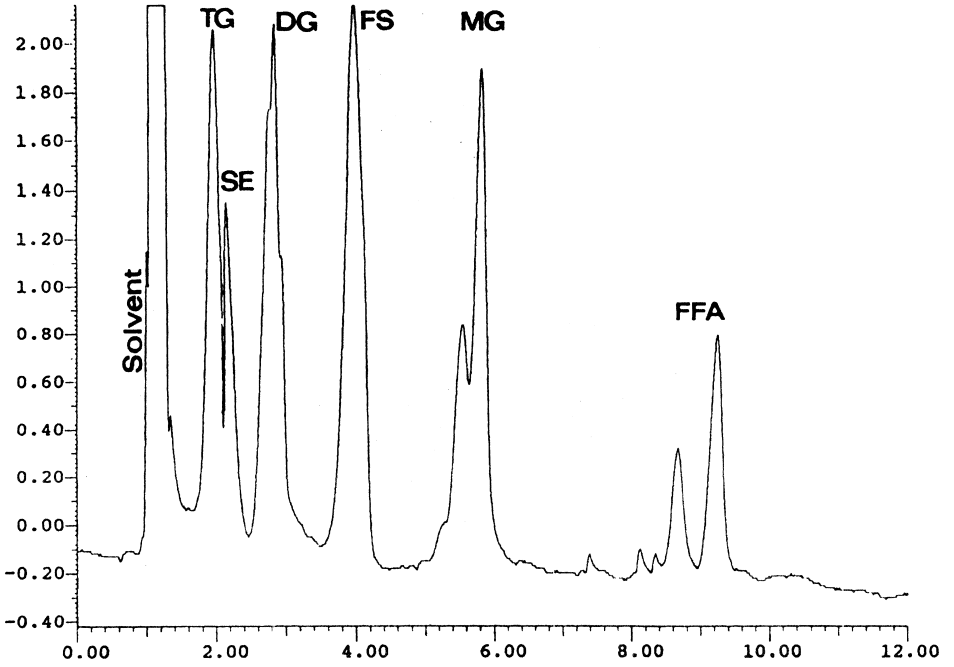

Fig. 3. Separation of different classes of lipids by SFC. Column: aminopropyl silica gel (20×0.46 I.D.; 5 μm); mobile phase: CO_2/methanol (92:8, v/v); $P=15$ MPa; $T=70$°C; $\lambda=210$ nm (reprinted with permission from Ref. [15]). DG, diglycerides; FFA, free fatty acids; FS, free sterols; MG, monoglycerides; SE, sterol esters; TG, triglycerides.

3. Separation of unsaponifiable compounds

3.1. Capillary columns

As discussed previously, screening of both unsaponifiable compounds and triacylglycerols can be difficult because the low initial concentration of these minor constituents requires higher loading of the samples in the column, which strongly reduces the chromatographic separation [17]. However, by using non-polar capillary columns, several minor compounds were separated from different oils [10,17,18]. The elution order (squalene, α-tocopherol, cholesterol or γ-sitosterol and triacylglycerols) is related to the molecular mass. Temperature is always kept constant (from 140 to 170°C), when linear or multi-step density gradients are carried out to increase the eluent strength of the mobile phase.

Staby et al. reported the separation of numerous compounds (free fatty acids, squalene, α-tocopherol, cholesterol, cholesteryl esters, triacylglycerols and diacylglycerides) on a DB-5 column with a thick film (0.4 μm) [19].

Quantification of squalene by cSFC carried out with a DB-5 column (5% phenyl–95% dimethypolysloxane) has showed an almost linear correlation between the squalene content found by SFC and the iodine determination. The squalene percentage in several shark liver oils reach more than 50% of the liver weight. Mono-alkyl glycerol esters obtained by saponification of diacylglycerol ethers have been separated on a polar DB-225 phase (50% cyanopropylphenyl–50% methylpolysiloxane) (Fig. 5). Monopalmityl, monostearyl and monooleyl glycerol ether were the main ethers contained in the shark liver oils (*E. princeps*) [20]. However, due to the high squalene content, a previous separation of squalene by TCL is required to avoid the column overloading during the monoglycerol ethers analysis.

Among different stationary phases (SB-methyl, SB-octyl-50, SB-phenyl-5 and Carbowax), Snyder et al. have reported that the best resolution between β- and γ-tocopherols (positional isomers) was obtained

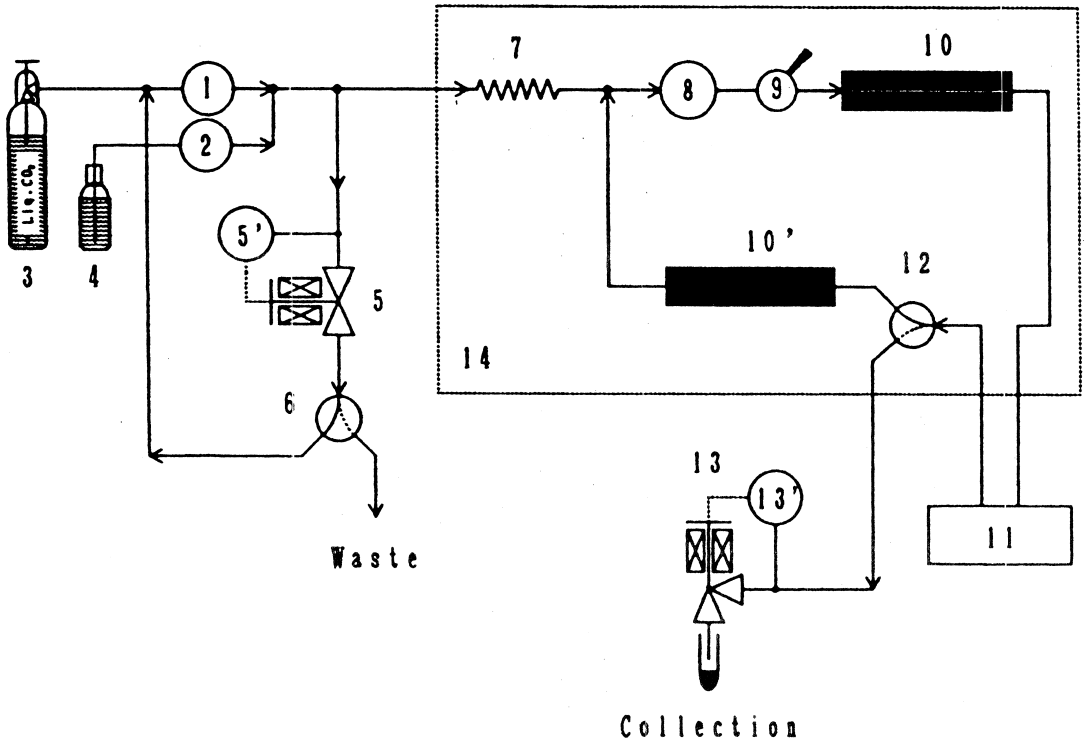

Fig. 4. Diagram of recycle preparative SFC (reprinted with permission from Ref. [9]).

on the SB-octyl-50 column [21]. Once again, the retention order follows the molecular mass: δ- (MW: 402.6), β-, γ- (MW: 416.6) and α-tocopherol (Fig. 6) (MW: 430.7). β- and γ-tocopherols, having the same molecular mass but differing in the position of one methyl group, were also resolved. The resolution between tocopherols was improved by the superimposition of a temperature gradient with a pressure gradient. On polar stationary phase (Carbowax), the tocopherol elution order is reversed: α, γ, δ. This retention order is governed by the ability of the tocopherol polar part to interact with the stationary phase. The relative retention of α-tocopherol is reduced more strongly because of the steric hindrance of two methyl groups which inhibits the interaction between the α-tocopherol hydroxyl group and the polar sites on Carbowax phase. No indication was given on the β-/γ-tocopherol separation with this polar phase.

The retention order between phytosterols and tocopherols was also inverted, but surprisingly phytosterols elute after tocopherols on SB-octyl-50 columns, and before on polar Carbowax. Four phytosterols were separated on SB-octyl-50: brassicasterol, stigmasterol, campesterol and β-sitosterol (Fig. 6) [14,21].

Whatever the retention behavior, the comparison of tocopherol content from different samples between GC and SFC shows higher values obtained by SFC. Such a trend could be due to a partial loss of analyte during the required derivatisation of tocopherols prior to GC.

With capillary columns, cholesterol was analysed from milk fat [22], woolgrease [23] and human serum [24]. Temperatures required in SFC are lower (from 65 to 130°C) than in CPG (250°C).

Few separations of carotenoids by cSFC have been reported. Because of the labile nature of carotenoids, low temperature was used (50°C). α- and β-carotene were resolved by using a SB-phenyl-50 column, and a mobile phase containing 1% ethanol [25]. Unfortunately, lycopene was eluted just between the two previous carotenes, which reduced the total separation. Separation of all trans α-carotene and

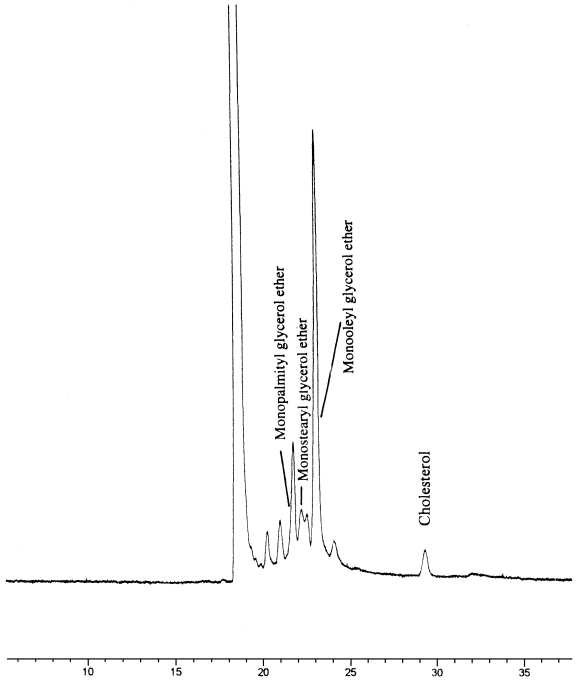

Fig. 5. Capillary supercritical fluid chromatography of the unsaponifiable matter from the liver oil of *Etmopterus princeps*. Column: DB-5 (20 m×100 μm); mobile phase: neat CO_2; T= 170°C; multistep gradient from 20.3 to 36.2 MPa (reprinted with permission from Ref. [18]).

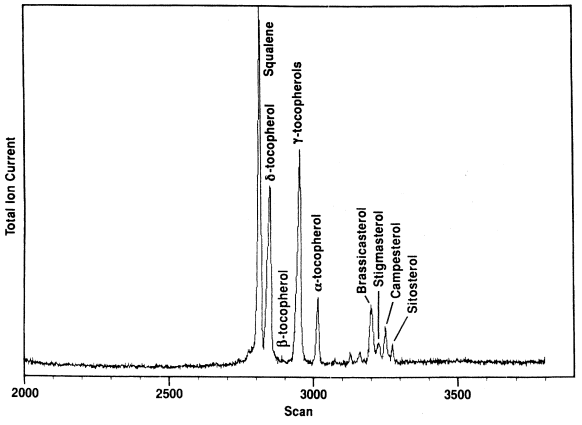

Fig. 6. Supercritical fluid chromatography–mass spectrometry analysis of a commercial antioxidant sample. Column: SB-octyl-50 (10 m×100 μm); mobile phase: neat CO_2; pressure gradient from 10 to 35 MPa; temperature gradient from 100 to 180°C (reprinted with permission from Ref. [14]).

various cis isomers was reached on two coupled SB-cyanopropyl-50 columns, the cis isomers being eluted before the trans isomer. This retention order is observed in normal-phase liquid chromatography.

Finally, cSFC can easily be interfaced with a quadrupole mass spectrometer, either in electronic impact (EI) [21] or in chemical ionisation (CI) modes [12]. As it classically occurs in GC–MS, the ammonia adduct $[M+18]^+$ is the greatest peak observed in CI, when the molecular peak is low in EI. With underivatised tocopherols, the fragmentation patterns in SFC–EI-MS are close to those observed in GC–EI-MS.

3.2. Packed capillary columns

The advantages of packed capillary SFC (pcSFC) are well reported by Shen and Lee [26]. Because of the small ratio column/particle diameter, packed capillary columns have good permeability and long columns can be used (up to 70 cm) improving the chromatographic efficiency. A reduced plate height of ~2.2 can be reached with 5-μm diameter particles, but 10-μm diameter particles are currently used.

Due to the lower mobile phase flow-rates, less dilution occurs during analysis and therefore the higher concentration of resolved compounds allows lower detection limits to be reached. However, the smaller loadability of these columns involves the injection of small sample volumes (0.25 μl).

On the other hand, the small amounts of stationary and mobile phases favor rapid temperature equilibria during temperature gradient. As in open capillary SFC, pressure gradients are always necessary in packed capillary SFC for increasing fluid density.

The internal diameter of these packed capillary columns ranges from 180 to 500 μm [26–29]. Smaller diameters offer higher efficiency but the loadability of greater diameter columns allows the separation of larger samples. For that reason, columns of 500 μm were often selected with the aim of direct SFE–SFC coupling [29]. Effectively, resolution losses were not observed on soluble vitamin separation carried out on-line after an SFE extraction.

Since they are not soluble in pure CO_2, some polymers, generally used in GC as stationary phases, are coated on the silica particles in pcSFC. Encapsu-

lated particles present both a reduced surface activity and porosity, which improve the peak shape and reduce the retention of compounds because of the lower specific surface by filling the pores. However, because no commercial encapsulated particles are available, home made coating should be done. For apolar stationary phases, SE-54 (5% phenyl–95% methyl) and OV-17 (50% phenyl–50% methyl) an additional crosslinking is suggested (160°C overnight) [29,30].

The retention order of fat soluble vitamins (A, E, D2, D3, K1 and K2) was modified by the polymer nature [13,27,28]. Vitamin A, which coelutes with vitamin D2 using Carbowax 20M is retained more with a cyano-substituted liquid crystal polysiloxane phase while better separation between vitamin E and K1 is obtained using the Carbowax 20M phase. Moreover, the cyano group seems to have specific interaction with double bonds because of the retention order inversion between vitamins D2 and D3 which only differ by one double bond.

An interesting study has been done on selectivity of different phases used for tocopherol separation [29]. As in capillary SFC, the tocopherol retention order was inverted by going from apolar to polar stationary phases. However, the non-polar stationary phases (ODS and SE-54) (5% phenyl–95% phenyl-silicone) were unable to separate β- and γ-tocopherols. This result seems opposite to that obtained by Snyder [21] in cSFC. The great difference between the column lengths (25 cm in capillary packed SFC, 10 m in capillary SFC), i.e. in the chromatographic efficiency, could explain this result variation.

A total separation of tocopherols was obtained by using a Carbowax phase (polyethylene glycol) with capillary packed column (Fig. 7). Ibanez et al. have studied the effect of the amount of polymer used in coating particles. Increasing the coated Carbowax amount from 3 to 10% mainly increases selectivity between β- and γ-tocopherol, without modifying retention [11,29].

A additional increase, from 10 to 20%, increases both retention and selectivity for this isomer couple, but decreases the resolution through a major loss in efficiency. For the tocopherol separation 10% of Carbowax was selected as the best amount. However, the analysis of SFE olive extract shows the

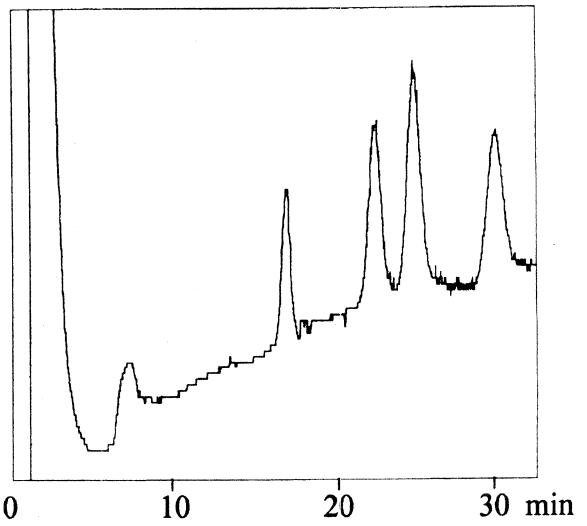

Fig. 7. SFC chromatogram of tocopherol with Carbowax capillary packed column. Column: silica particle coated with 10% CW 20M (25×0.05 cm); mobile phase: neat CO_2; T=60°C; pressure gradient from 200 to 300 atm at 3 atm/min (reprinted with permission from Ref. [29]). Peak identification: (1) α-tocopherol; (2) β-tocopherol; (3) γ-tocopherol; and (4) δ-tocopherol.

coelution of squalene with γ-tocopherol [11]. This coelution was not observed with the same Carbowax stationary phase in capillary SFC [16].

Applied to the analysis of carotenoids, an additional silanol deactivation before coating seems required [30]. Polymethylhydrosiloxane [26] or octamethyl-tetrahydrosiloxane [30] can been used as silylation reagent. High temperature and long reaction time are required for performing the silica deactivation [30,31]. Three stationary coated phases were investigated for the carotenoid separation: SE-54 crosslinked, OV-17 crosslinked and deactivated silica.

Based on the β-carotene Hildebrand solubility parameter (8.71 $cal^{1/2}/cm^{3/2}$) [32], temperatures and pressures were calculated by using the Giddings equation [33] to reach a CO_2 density (0.98 or 1.04 g/ml) having the same solubility parameter. Temperature was maintained at 10°C in order to operate at pressures lower than 30 MPa, i.e. near the chromatograph pressure limit. The chromatograms of β-carotene and lycopene seem to be noisy (FID detection), with peak tailing. The authors noticed that

the peak shape for β-carotene is improved with deactivated silicas while β-carotene does not possess groups able to interact with residual silanol. However, it is well known that silica is unable to separate cis/trans isomers, that could explain the increase in the peak symmetry.

It is interesting to note that the different treatments applied to the silica produce important chromatographic modifications: (i) the carotene retention is greater on the deactivated silica than with the apolar SE-54 phase and OV-17; (ii) the retention order follows that observed with apolar phase in RPLC on the SE-54 and OV-17; and (iii) this retention order is inverted on the deactivated silica.

3.3. Packed columns

The use of packed columns involves numerous differences in comparison with capillary or packed capillary columns. To favor the compound elution and to improve the peak shape, modifiers are added to CO_2. This addition avoids the use of a pressure gradient during analysis. Moreover, the nature and percentage of the modifier influences selectivity which often improves separations. The high eluent strength reached with a modifier allows work at ambient or subambient temperatures, which reduces the degradation of thermolabile compounds. At low temperature, the compressibility of the fluid is also reduced which limits efficiency modifications in the column. This addition requires the use of a UV detector instead of FID.

However, working with pure CO_2, UV and FID detectors have been used for the analysis of cholesterol from human serum [34]. By using an inert ODS silica gel, no modifier was required to improve the peak shape of cholesterol, even if this peak showed a slight tailing due to interactions with trace amounts of the silanol groups.

The FID detector was connected by splitting the mobile phase between the separation column (ODS; 250×4.6 mm) and the UV detector. The sensitivity of the UV detector was improved by a decrease in the wavelength down to 190 nm. The comparison of quantification between the two detectors shows better reproducibility (variation coefficient) with the UV detector.

Specific detection can be carried out with a UV detector, since the different compounds do not absorb at the same wavelengths. With an ODS silica column with ethanol as modifier, the crude palm oil has been fractionated into several families: free fatty acids (215 nm), tocopherol and tocotrienols (290 nm), diglycerides (215 nm), triacylglycerols (215 nm) and carotenoids which overlapped with the triacylglycerols (400 nm) [16]. However, a pure carotenoid fraction was obtained with a silica column instead of an ODS column, because of the retention inversion between the two previous overlapped families.

From the ODS separation, the tocopherol fraction was isolated and analysed by using the silica column. Four main compounds were completely separated: α-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol.

The effect on the tocopherol retention of the amount of modifier in the CO_2 has also been studied with an ODS silica column [35]. With pure CO_2, the retention order of the four tocopherols was identical to that observed in HPLC with ODS stationary phase, but the positional isomer β- and γ-tocopherols were surprisingly separated. The addition of methanol (from 0.5 to 15%) both improves the tocopherol peak shapes and decreases their retention. However, following the addition of methanol, a rapid inversion in the retention order of β- and γ-tocopherols was reported, which strongly reduces the isomer separation. SFC quantification of tocopherols from different oils has shown that δ and α are often the main tocopherols in vegetable oils (Fig. 8).

Numerous studies have been performed on carotenoid separation. They are classified into two families, xanthophylls which are mono (β-cryptoxanthin) or di-hydroxylated (lutein, zeaxanthin), and carotenes which are non-oxygenated (lycopene, α- and β-carotene). Both lutein and β-carotene were found in olive oil [8], while at least ten carotenes are in crude or fiber palm oil [16,36].

These compounds can differ by the position of one double bond (lutein/zeaxanthin; α-/β-carotene) or by the cyclisation of one chain extremity (γ-/α-carotene), and also by the double bond configuration (cis/trans isomers). Their retention behavior has been described both with pure [37] or modified phases [38–41]. With pure CO_2 on ODS silica, the retention

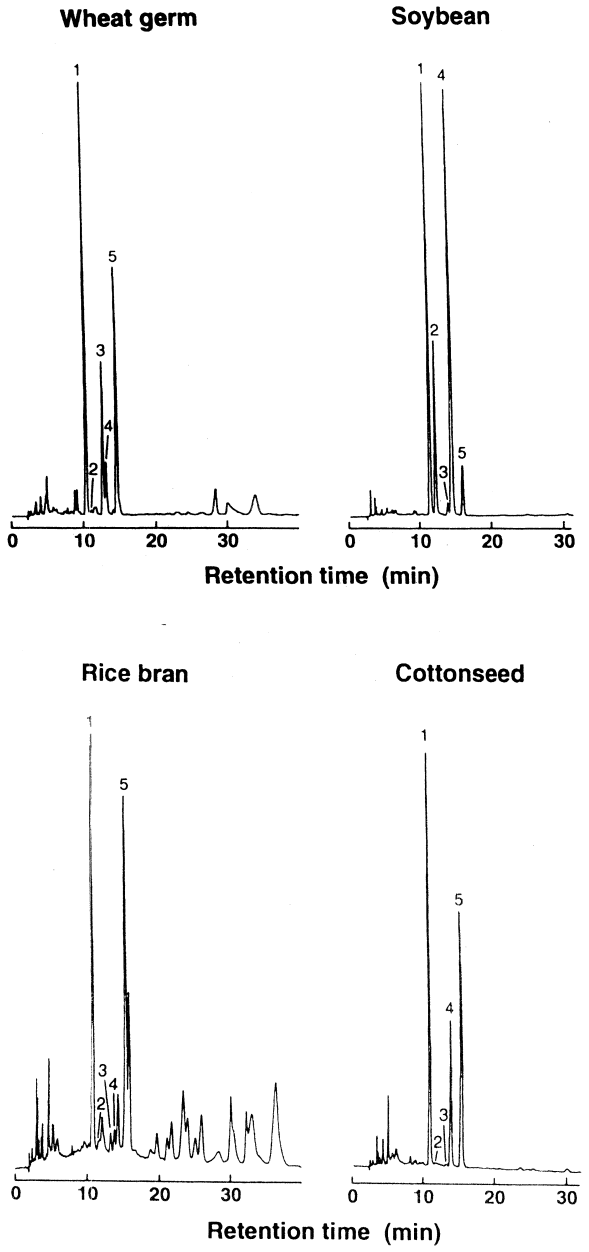

Fig. 8. SFC of tocopherols in vegetable oils. Peak identification: (1) tocol (I.S.); (2) δ-tocopherol; (3) β-tocopherol; (4) γ-tocopherol; and (5) α-tocopherol (reprinted with permission from Ref. [11]).

of β-carotene was clearly related to the pigment solubility into the mobile phase [37]. With the modifiers, retention variations are related to the molar fraction of the modifier (X) following the equation:

$$\log k' = AX^2 - BX + C.$$

However, by using different modifiers, three retention behaviors were observed (Fig. 9) [38]. A satisfactory correlation has been found between these three retention behaviors and the modifier dielectric constant. The modifiers included in the first group (THF, methylene chloride, heptane) induce a rapid decrease in the carotenoid retention. The retention is also reduced by adding modifiers of the second group (ethanol, propanol, acetone) but more slowly than the previous ones. With the modifiers of the third group, having a high dielectric constant (methanol, acetonitrile and nitromethane), the retention first decreases to a minimum, then increases. This particular behavior has also been reported with other compounds [42,43]. With these modifiers, the polarity of the CO_2/modifier mobile phase increases with the modifier percentage.

Since the minimum retention is reached when the solubility of the solute into the mobile phase is maximum, the minimum retention depends on the chemical structure of the carotenes. It occurs with low modifier percentages for the less polar carotenes:

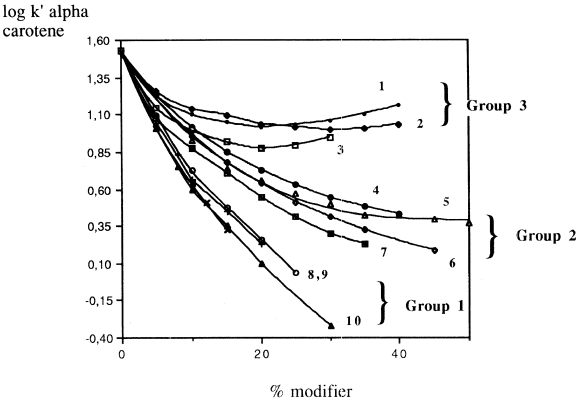

Fig. 9. Variation of the log k' for α-carotene versus the percentage of modifier in carbon dioxide. T=25°C; P=15 MPa; flow-rate, 3 ml/min; λ=450 nm; column Ultrabase UB 225 (Kromasil C_{18}) (25×0.46 cm; 5 μm) (reprinted with permission from Ref. [39]). (1) Acetonitrile; (2) methanol; (3) nitromethane; (4) ethanol; (5) proprionitrile; (6) acetone; (7) 1-propanol; (8) hexane; (9) heptane; and (10) tetrahydrofuran and methylene chloride.

lycopene, γ-, α- and β-carotene (no hydroxyl group), and then the modifier percentage increases depending on the hydroxyl group number (one hydroxyl group for β-cryptoxanthin and two hydroxyl groups for the most polar lutein and zeaxanthin).

The effect of modifiers on the selectivity of the different compounds changes following the chemical differences between the compounds, and does not necessarily depend on the retention behavior. A systematic diminution of resolution between α- and β-carotenes occurs with the modifier addition, and a complete loss of resolution is observed with the modifiers of the first group.

Selectivity between cis/trans isomers of β-carotene is not affected by the increase of methanol, ethanol or nitromethane, is slightly reduced with acetonitrile and acetone, and is completely lost with methylene chloride or heptane. A correlation between the stationary phase organisation, related to mobile phase adsorption onto the stationary phase [44], and this cis/trans selectivity could explain these results.

Since the classical retention order on ODS silica gel is lycopene/γ-carotene/α-carotene, the effect of modifier on the selectivity between γ- and α-carotene and between lycopene and α-carotene is of great interest in reaching a complete carotene separation.

Modifiers that possess the highest dipole moment (nitromethane, acetonitrile, methylene chloride) favor these two selectivities and should be added to the CO_2, when methanol is unable to improve these selectivities. γ- and α-carotene are not resolved with alcoholic modifiers [40]. By reducing the retention of polar carotenoids, the addition of methanol reduces the lutein/zeaxanthin selectivity but increases that between β-cryptoxanthin and lycopene. Finally, the amount in acetonitrile should be high (around 30%) to improve selectivity between lycopene/γ- and α-carotene.

Due to the particular retention behavior, the retention factor obtained with this high amount is no less than that obtained with 5 or 10% of acetonitrile. This isoeluotropic behavior of rich and poor content modifier allows an increase in some selectivities by the addition of modifier in the CO_2 without reducing the resolution by decreasing the retention factor.

To maintain good selectivity between cis/trans isomers and between β-cryptoxanthin and lycopene, a small amount of methanol is also added. The separation of seven carotenoids and of the cis/trans forms is presented in Fig. 10 [40].

Moreover, the type of ODS silica gel (mono or polyfunctional) can change the carotenoid separation [41,45–47]. The greater separations of the β-carotene main mono-cis isomers (9, 13 and 15) are reached on polyfunctional gels, when the separation of the 13 and 15 mono-cis increases following the increase in the bonded density for monofunctional stationary phases [46,47]. However, this increase in the bonded density can induce coelution between 9 cis and all trans β-carotene. The analytical temperature also strongly modifies these cis/trans isomer separations. The retention of the polar carotenes (lutein, zeaxanthin) depends on both the amount of residual silanols and the functionality of the station-

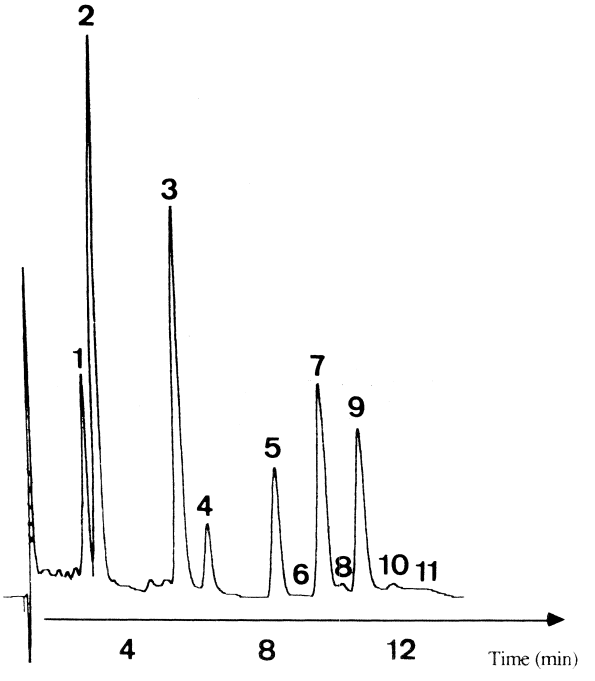

Fig. 10. SFC chromatogram of carotenoids. Column: Ultrabase UB 225 (25×0.46 cm; 5 μm); mobile phase: acetonitrile/methanol/CO_2 (33.25:1.75:65, v/v/v); T=25°C; P=15 MPa; flow-rate, 3 ml/min; λ=450 nm (reprinted with permission from Ref. [40]). Peak identification: (1) lutein; (2) zeaxanthin; (3) β-cryptoxanthin; (4) lycopene; (5) all trans γ-carotene; (6) cis-γ-carotene; (7) all trans α-carotene; (8) cis-α-carotene; (9) all trans β-carotene; (10) 13 cis-β-carotene; and (11) 15 cis-β-carotene.

ary phase. The hydrogen bonds between the hydroxyl groups of polar carotenes and the polar sites of the polyfunctional phases (siloxane groups) favor the retention of these polar carotenes. A retention inversion between zeaxanthin and β-carotene is generally observed by using polyfunctional ODS phases. But unfortunately, because the β-carotene cis isomers are eluted after the all trans β-carotene, coelution between zeaxanthin and the β-carotene cis isomers (13 and 15 mono-cis) can occur.

In this case, coupling between two types of monofunctional columns chosen for their specific selectivities improves the isomer separation. The Kromasil column mainly separates 13, 15 mono cis and all trans β-carotene, while the Hypersil column favors the 9 cis and all trans β-carotene separation. With four columns, 1 Hypersil ODS (25×0.46 cm), 2 Kromasil ODS (25×0.46 cm) and 1 Kromasil ODS (15×0.46 cm), 11 β-carotene cis/trans isomers were separated at 45°C, in 50 min [47].

By reducing the column number twice, the separation of a vegetable oil supplemented by β-carotene was achieved in 30 min (Fig. 11). The triacylglycerols, eluted before carotenoids, are not detected at 440 nm. No peak tailing is noticeable for this direct vegetable oil analysis, which emphasizes the high loadability of packed columns.

The effect of supercritical CO_2 on the shape of the

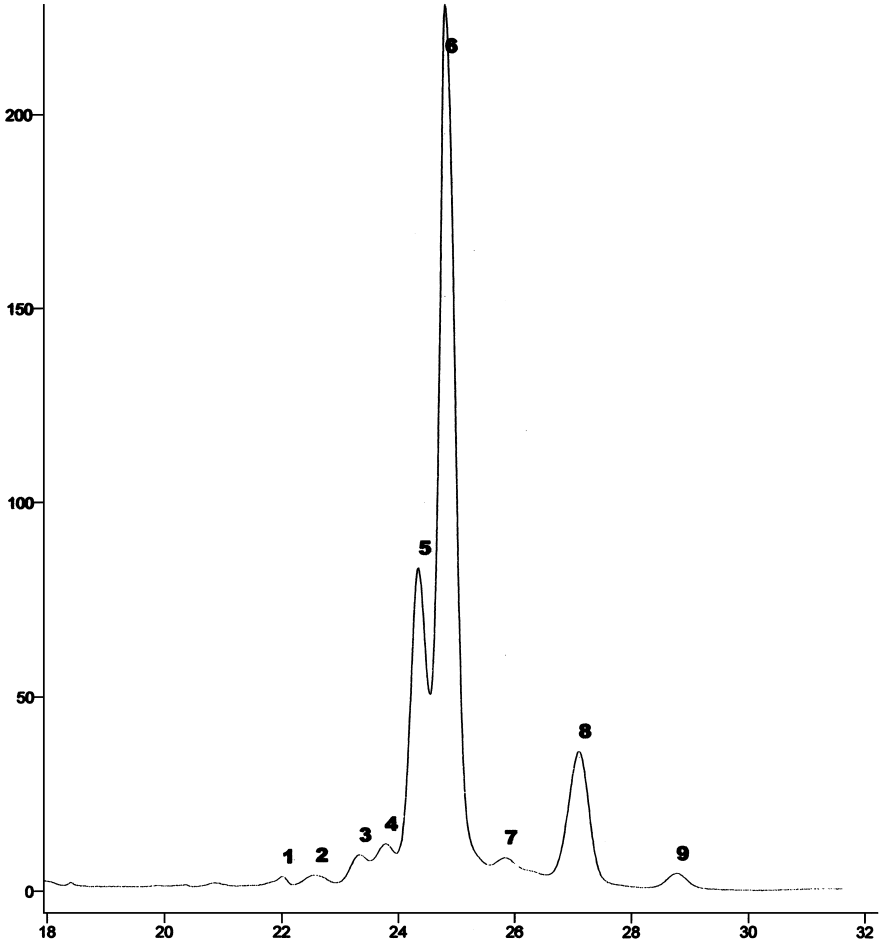

Fig. 11. SFC chromatogram of cis/trans isomers of β-carotene in a supplemented oil. Column: Kromasil C_{18} (50×0.46 cm; 5 μm); mobile phase: acetonitrile/CO_2 (10:90, v/v); T=50°C; P=10 MPa; flow-rate, 3 ml/min; λ=440 nm. Peak identification: (1, 2, 4) unknown cis isomers; (3) 9-9′ di-cis; (5) 9 mono-cis; (6) all trans β-carotene; (7) 9,15 di-cis; (8) 13 mono-cis; (9) 15 mono-cis.

carotenoid absorption spectra has been studied by Hui et al. [48]. They observed that the spectra were similar to those recorded in organic solvents such as hexane, with a shift to shorter wavelength (λ_{max} of all trans β-carotene was 450 nm in hexane and 437 nm in CO_2). Moreover, a modification of the CO_2 density (from 0.7 to 0.95 g/ml), due to a change of pressure or temperature, induces the shift of the λ_{max} of all trans β-carotene. This spectroscopic behavior underlines the modification of the mobile phase/solute interactions.

When polar modifiers are added to CO_2, this spectroscopic behavior is also observed [49]. Unfortunately, the regular increase in the maximum wavelength when adding polar modifiers cannot be correlated to the retention variation of carotenoids.

Whatever these spectroscopic changes, they have to be studied since spectral modifications induced by analytical condition variations hinder the carotenoid identification carried out by comparison of diode array spectra. The temperature regulation of the mobile phase after the column allows increase of the correlation coefficients between spectra and improves the suitability of carotenoid identification [49].

4. Conclusion

Supercritical fluids and methods offer many ways for the analysis of unsaponifiable compounds. In both extraction/concentration and fractionation steps, these fluids provide higher efficiency than other fluids and are more easily eliminated from the extract or the collected fraction than liquids.

Whatever the column type, the high chromatographic efficiencies reached in SFC and the solubility power of the carbon dioxide based mobile phases seems well suited to the analysis of unsaponifiable compounds. Capillary/packed capillary columns with pure CO_2, and packed columns with modifier/CO_2 mobile phases are complementary. The choice between these two methods is mainly governed by either the types of chemical differences between compounds, or by the type of chromatographic detector requested following the chemical structure of compounds.

Sample concentration is another important criterion for this choice, since FID is more sensitive than UV. However, coupling between SFE or supercritical fractionation and SFC by switching systems should allow this limit to be overcome. Moreover, since numerous studies have been carried out in SFC on saponifiable lipids such as triglycerides, ceramides or glyco-lipids, complete lipid analysis from a complex sample by using supercritical fluid will be reached in future.

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