Current Pharma Research
ISSN-2230-7842
CODEN-CPRUE6
www.jcpronline.in/

Review Article

Advances of supercritical fluid chromatography in lipid profiling.

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Received 14 July 2019; received in revised form 28 August 2019; accepted 02 September 2019

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ABSTRACT

Supercritical fluid chromatography (SFC) has great favour due to its high efficiency, low organic solvent consumption, and specialty for the identification of the isomeric species. It is describes the advances of SFC in targeted and untargeted lipid profiling. The advancement of the SFC instruments and the stationary phases are summarized. Applications of SFC to targeted and untargeted lipid profiling are discussed in detail. The perspectives of SFC in the lipid profiling are also proposed. It is useful and promising tool for investigating lipids in vitro and in vivo, SFC will predictably obtain further development. In the recent of supercritical fluid chromatography (SFC), it was categorized as high-pressure or dense gas chromatography (HPGC or DGC) and low boiling point hydrocarbons were used as supercritical mobile phase. Various liquids and gases were examined, however, by the late 1970s, carbon dioxide (CO_2) became the most preferred fluid because it has low critical temperature (31.1^0C) and relatively low critical pressure (7.38 MPa); in addition, it is non-toxic, non-flammable and inexpensive. A prototype of a modern packed-column SFC instrument appeared in the late 1970s. Most of the applications are related to the pharmaceutical sector, but interest in food and natural products is growing fast. Lipid and carbohydrate extract are example of starting material employed to purify these relevant compounds. SFC fulfils high demands with respect to selectivity, versatility and sensibility. Fractionation or purification by SFC of high-valued compounds from natural sources is an interesting option, the relevance of which will increase in the future.

KEYWORDS

Supercritical fluid chromatography, online SFC technique, untargeted lipid profiling, targeted lipid profiling.

1. INTRODUCTION

Recently, supercritical fluid chromatography (SFC), is alternative technique to liquid chromatography (LC) and extension of gas chromatography (GC), has aroused extensive attention due to its high efficiency and environment protecting [1]. Fig.1 shows the number of articles on SFC published each year from 2008 to 2018. SFC uses supercritical fluid as the mobile phase, these fluids with low viscosity and high diffusivity, which is incorporates the features of both liquid and gas [2]. Carbon dioxide (CO_2) is used as the preferred supercritical fluid (SF), it is easily pressurized and heated beyond its critical points (31^0C, 74 bar) (Fig. 2 shows the phase diagram of CO_2). The viscosity and diffusivity of the supercritical fluid are very close to those of gas, which results in high separation efficiency at high mobile phase velocity. The density and diffusivity for the analytes [3]. Incorporating both the features of liquid and gas, SFC is regarded as hybrid of GC and high-performance liquid chromatography (HPLC) [4] it has advantages as high separation efficiency, low organic solvent consumption and short separation time [5]. A lot of attention has been paid to the detection and quantification of lipids, such as the endogenous lipid analysis in vivo [6], and the nutritious lipid analysis in oils [7] or plants [8].

Separation of the lipid carried out by GC, HPLC and GC analysis of the lipids also derivatization step is needed and thermal degradation of analytes would happen due to the high temperature. HPLC-MS based on the methods without derivatization are widely used in the analysis of lipids. The very important advantage of HPLC over GC is has greater sensitivity as the enhanced chromatographic selectivity achieved with a rich variety of packed HPLC columns. HPLC for the lipids analysis always sales a long time and organic solvent consuming. In recent years, SFC has met with great favour in targeted and untargeted lipid profiling due to its high efficiency, low organic solvent consumption, and the specialty for unambiguous identification of the isomeric species of some lipids [9].

This paper outlines are the recent advances of SFC in targeted and untargeted lipid profiling. First, the development of SFC is summarized. Targeted and untargeted lipid profiling by SFC is described and discussed in detail. Furthermore, the online SFC technologies applied in lipid profiling are summarized. Perspectives of SFC applied in lipid profiling are also proposed.

Global sales of healthy food products are estimated to have reached $1 trillion by 2017 according to Euromonitor [10] . Some of the compounds that will be reviewed in this paper have an expected compound annual growth rate (CAGR) >>10%, for example 14.9% for the omega-3 market and 25.8% for nuts and seeds, during the period 2016-2022 [11] and 19.1% during the period 2015-2020 for the phospholipids market [12]. International markets are progressively looking towards natural and organic products versus synthetic [13]. Natural products are not only used in foods, with the ageing population resulting in an increase in the demand of cosmetics and antioxidant products for improving aesthetic appeal. Natural products have provided value to the pharmaceutical industry over the past half century. The therapeutic areas of infectious diseases and oncology have benefited from numerous drug classes which are derived from natural product sources [14].

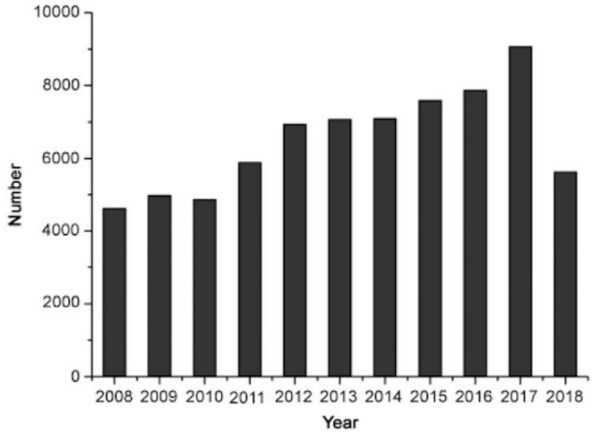

Fig. 1. The number of publications on the applications of supercritical fluid chromatography.

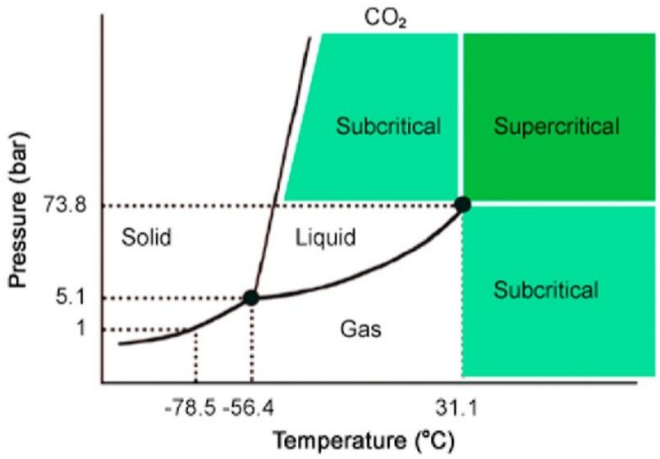

Fig. 2. Pressure-temperature phase diagram of co_2 Reproduced from reference, with the permission of MDPI.

2. Development of SFC

2.1. Advancement of SFC instruments [16-21]

Traditional SFC technique acquired attention from the 1960s to the 1970s [16] and the heat faded because of the rapid growth of HPLC. In the 1980s, development of SFC met with its second act. The development of instruments and column led to the commercialization of SFC [1]. Open tubular capillary SFC was first introduced by Novotny [17], which was developed from GC and was usually coupled with flame ionization detectors (FID) [18]. Fig. 3A shows a schematic diagram of a typical GC-like open tubular column SFC system. Even though cSFC was popular in the 1980s as the extension of GC, it disappeared in the 1990s because of its limit use for hydrophobic compounds only [19].

In 1982, Gere et al [20] transformed a Hewlett-Packard (HP) HPLC system into an SFC system by adding a back pressure regulator (BPR, in order to keep pressure constant whatever the mobile phase flow rate) and other devices, which was the first commercial SFC instrument. Fig. 3B shows a schematic diagram of a typical LC-like packed column SFC system with an automated backpressure regulator [21].

2.2. Stationary phase of SFC [22,23,24,25,26]

The stationary phases of SFC are very wide ranges are currently available for SFC. The choice of the stationary phases could be explained by the fact that modern SFC covers the application of different chromatographic modes, which has been applied to a wide range polarity of analytes. The column category of SFC includes alkyl bonded phases and polar phases [22]. By using silica or hybrid silica as stationary phases, SFC also is used for the analysis of biologically active substances which display high hydrophilicity [23]. For the lipophilic compounds, SFC has been considered as a powerful tool for lipids analysis using both alkyl bonded phases and polar phases [24]. SFC has been successfully applied to both chiral purity and separation to provide enantiomerically pure compounds. The CSP chemistry for the SFC-based analysis has been developed in these years [25]. SFC has been proven to provide at least two times faster separation than that of normal-phase HPLC on the chromatographic resolution of chiral compounds [5]. Wang et al, [26] achieved a separation of two demethylated nobiletin metabolites, 3'-demethyl-NOB and 4'-demethyl-NOB, via SFC/UV with a chiralpak AD column, which provided a 10 min retention time differences between the nobiletin regioisomer.

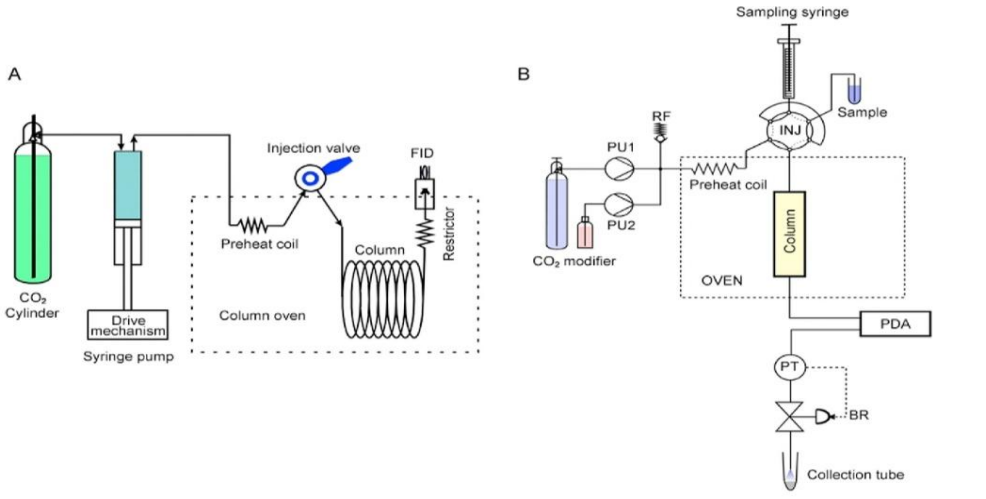

Fig. 3. (A) Schematic diagram of typical GC-like open tubular column SFC system.
(B) Schematic diagram of typical LC-like packed column SFC system with automated backpressure regulator.

2.3. Open tubular capillary column

In 1981, Novotny and Lee's group introduced open tubular capillary column SFC. A typical open tubular capillary column was has a 50 µm inner diameter fused silica capillary tube and the

internal wall was coated with a polymer such as dimethyl polysiloxane that have functioned as the stationary phase. Technically the wide range advantages of SFC over other modes of chromatography such as GC & LC are summarized as in Table 2. In SFC, all three parameters (i.e. pressure, temperature, and modifier content) can independently or cooperatively control the retention, or a gradient method can be applied to all the parameters. These advantages were too heavily emphasized at that time. Therefore, it often misled chromatographers to think that SFC was a type of super chromatography. However, in the case of open tubular capillary SFC, pressure which is the most widely important operating parameter, which is only be varied by changing the flow velocity due to the limitation of the constant restrictor [21] .

2.4. Packed column

All SFC research works were performed using packed columns. Open tubular capillary column SFC it was a GC-like instrument, packed-column SFC was more like LC. In 1982, Gere et al. modified a Hewlett-packed (HP) HPLC system is to operate as an SFC system by adding a backpressure regulator and other devices. They showed that SFC gave higher efficiency with 3, 5 and 10 µm packing materials especially in high flow velocity region. Packed-column SFC was developed almost independently of open tubular capillary column of SFC. Packed-column SFC is become less popular, especially in the US due to the marketing strategy of open tubular column SFC in the middle of 1980s. Fig. 4. shows a schematic diagram of a typical LC-like packed-column SFC system with automated backpressure regulator. It is similar to an HPLC system. However, a backpressure regulator keeps the fluid pressure above the critical pressure and an oven that keeps the fluid temperature above the critical temperature is vital devices specific to SFC [21].

2.5. Phospholipids/sphingolipid

Phospholipids (PLs) and sphingolipid (SPs) belong to polar lipids according to their different types of attached hydrophilic groups. PLs, such as phosphatidycholine (PC) and phosphatidyl ethanolamine (PE) are the main lipid type in cell membranes [27]. Lyso-phospholipids are the hydrolysis products of PLs by phospholipase that contribute to signal transduction in various pathophysiological processes [28]. SPs, is structural component of all eukaryotic cell membranes, it plays an important biological roles in membrane fluidity, signal transduction and cell-cell interactions [29]. SFC -MS-based analytical method has been applied to the separation of polar lipids in recent years. A simultaneous and fast determination of 19 polar lipids including PLs, LPLs, and SPs by SFC-MS was performed in 6 min and applied to the analysis of mouse liver [6]. Phospholipids (PPL) are the most important class of polar lipids as they are structural components of living cell membranes and play important role enzyme activation, making them important in nutrition. PPLs consist of a glycerol backbone on which 2 fatty acids occupy the first and second position, and a phosphorous containing moiety is attached to the third position. Analytical techniques traditionally used for phospholipids analysis which is thin layer chromatography (TLC), HPLC, GC and NMR, and are summarized in a number of reviews [30]. In the reverse-phase liquid chromatography (RPLC) , PLs are separated based on their fatty acyl groups, so the isomer with the similar fatty acyl groups may be co-eluted although they

belonging to different lipid classes [31]. in recent days, Yamada et al. [32] developed as SFC which is coupled with an Orbitrap Fourier transform MS (Orbitrap FT-MS) method using a single octadecylsilyl (ODS) column used to analyze various polar lipids in mouse plasma, and isomeric molecules of PLs were successfully separated and identified based on not only their fatty acyl moieties but also their polar head groups.

2.6. Fatty acids

Fatty acid is most important building blocks of complex lipid. Which is usually derived from triglycerides (TGs) or phospholipids in organism? FFAs have a variety of essential functions. For example, FFAs serve as a major provider of physiological energy it is needs during fasting, and constitute cell membranes, and in the some cases act as the key regulators [37]. SFC is commonly used for the effective separation of FFAs and fatty acid methyl esters in oils. Recently Bicchi et al. [38] used SFC with UV detector for the analysis of different fatty acids with the analysis time 50% shorter than that of the corresponding HPLC method. Qu et al [34] UHPSFC-MS method for the determination of 8 FFAs in edible oils with satisfactory correlation coefficients ($R^2 > 0.994$) and good reproducibility (RSD < 15.0%) within only 3 min and no pre-treatment was needed. Additionally, a multidimensional approach was used for the analysis of FAS in fish oil [39].

2.7. Triacylglycerols

Triacylglycerols are the esters which are derived from glycerol and three fatty acids. TGs are present in the blood to enable the bidirectional transference of adipose fat and blood glucose from the liver, and this are the major component of human skin oils [40] FAs are supplied to cardiomyocytes via circulating triacylglycerol (TAG)-rich lipoprotein that are catabolised by lipoprotein lipase on the luminal surface of the coronary vascular endothelium [41] as well as albumin -bound FAs secreted from adipose tissue [42], The lipid droplets are in very close proximity to mitochondria and can be mobilized by lipid hydrolyses in time of energetic need [43]. The Synthesis and hydrolysis of myocardial TGA stores is a highly dynamic process that is tightly regulated and appears to contribute to proper cardiac metabolism and function. Finally the result showed that the retention order of TGs depended on their unsaturation levels and carbon number, and the retention increased following the unsaturation number when the opposite effect was obtained in reverse phase HPLC.

2.8. Sterol lipids

Sterol lipids are the ringed lipids that play an important role in the membrane integrity of eukaryotes cell. The most familiar type of animal sterol lipids is cholesterol that is vital to cell membrane structure, and acts as a precursor to fat-soluble vitamins and steroids hormones. STs is included in plants and which is commonly occurs as mixtures, such as sitosterol, stigmasterol and campesterol [44]. Kim et al employed cSFC-FID for the individual determination of cholesterol and cholesteryl esters in human serum, which achieved an acceptable average relative standard deviation of 2.6% and a detection limit of 4-6 pg. In another work, cSFC with ELSD detector was used for the separation of steroids, which is allowed to the SFC separation developed for the polar, UV transparent compounds that have been ignored [45].

2.9. Prenol lipids

Prenol lipids (PRs) mainly consist of fat-soluble vitamins (i.e. vitamins A, E, and K), carotenoids and ubiquinones. In previous research, cSFC-UV has demonstrated significant potential for the improvement of the separation of carotenoids and their isomer from carrots and tomatoes relative to HPLC-UV. In the recent years technology of pSFC-MS has been used for the analysis of PRs. For example, Bamba et al. established a pSFC-UV-based method for the determination of natural polyprenols, which allowed the advantage of baseline separation of polyprenols and increased the resolution of separation two times higher than conventional RPLC-UV [46].

3. Online SFC technology in lipid profiling

Online coupling of sample preparation techniques with SFC

Along with the comparison of a new generation of instruments, SFC gains to improve their performance, reliability and robustness, and the online coupling SFC system have been booming recently. So, supercritical fluid extraction (SFE) and solid phase extraction (SPE) are the two major sample preparation techniques which have been online coupled with SFC. Fig. 4 shows the schematic diagram of the SFE-SFC-/MS/MS system. The SFE-SFC online system consists of an SFE module and an SFC module. The supercritical extraction is injected directly to the chromatographic column without any pre-concentration. SFE is a physical separation and purification method, which changes the solubility of supercritical CO_2 by balancing the pressure and temperature. The extraction process is composed of extraction and separation. Under the supercritical state, CO_2 is contacted with the substance to be separated, and the components are extracted successively according to their polarities, so as to achieve the purpose of separation and purification. The system contains two six-port valves, one valve which is in charge of SPE procedure while the other is in charge of SFC, and the SPE column and the chromatographic column are connected when the elution begins by the switching of these valves. However, the stability of SFC system might be disturbed by the water matrix after SPE.

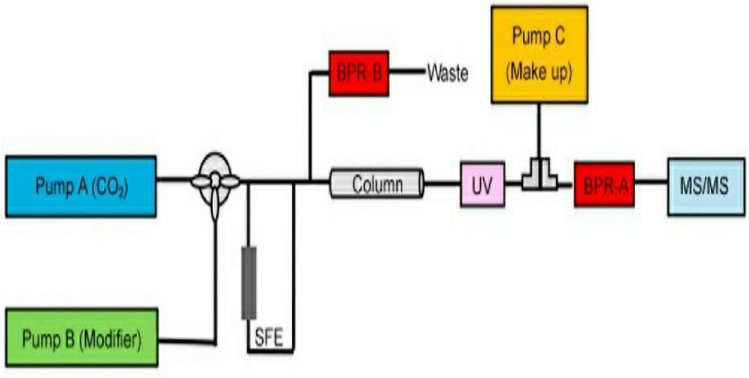

Fig. 4. The schematic diagram of the SEF-SFC-MS/MS system. BPR-backpressure regulator.

3.1. Online SFE-SFC in lipid profiling

In recent years, there have been only a few studies about the online SFE-SFC system which were applied to the lipid profiling. Suzuki et al. used online SFE-SFC to analyze the disease

biomarkers in dried serum spots, which were expected to be applied to disease diagnosis. In this study, four hydrophilic metabolites and 17 hydrophobic metabolites were simultaneously detected within 15 min, and they exhibited comparable diagnostic performance to the serum analysis using LC-MS-MS.

3.2. Sample preparation

A rule-of-thumb is that the molecule will be dissolve in methanol or a less polar solvent is compatible with SFC, including polar solutes. CO_2 has polarity similar to n-heptane at its critical point, but the solvent strength can be increased by increasing density or using a polar co -solvent. In practice, when the fraction of co-solvent is high, the mobile phase is not truly supercritical, but this terminology is used regardless.

3.3. Drawbacks

There have been a few technical issues that have limited adoption of SFC technology, first of which is the high pressure operating conditions.

1. The pressure used is High pressure vessels are expensive and bulky.
2. Difficulty in maintaining pressure (backpressure regulation).But can be overcome by employing currently used automated backpressure regulators.
3. Difficulty in gas/liquid separation during collection of products.

3.4. Advantages

1. SFC is emerging as a separation technique that is superior to both gas chromatography and liquid chromatography for analysis of thermal liable or non volatile compounds.
2. Low viscosity.
3. Lower operating temperature.
4. High diffusion coefficient.
5. High resolution at low temperature.
6. Further advantage is SFC is very clean; mobile phase contaminants are usually trace quantities of other gases.
7. The mobile phase is very free of dissolved oxygen and is not particularly reactive and the mobile phase is easily and rapidly removed.

3.5. Disadvantage

1. A disadvantage of using carbon dioxide as the mobile phase is it does not elute very polar or ionic compounds; this is overcome by using an organic modifier.
2. There are some disadvantage of SFC these include that if molecules are highly polar they are not soluble in the mobile phase.
3. SFC is pressure operating conditions. High -pressure vessels are expensive and bulky.
4. Maintaining pressure in SFC is difficult.
5. Supercritical fluids are highly compressible and their physical properties change with pressure.
6. Cleaning will be time consuming.

3.6. Applications

(1) SFC is used in industry primarily for separation of chiral molecules.

(2) SFC now commonly used for chiral separation and purification in the pharmaceutical industry.

(3) SFC technique has been applied to wide verity of materials, including natural products, drugs, food and polymers etc.

(4) SFC technology has been mainly applied to purification of lipids, but there have been efforts to enrich other compounds.

(5) SFC is alternative separation technique widely used due to its ability to separate cis/trans isomers, especially useful to separate carotenoids.

(6) SFC technology is useful for vitamin fractionation as an alternative to organic solvent based methods for four important reasons: absence of oxygen, light, p^H variations and high temperatures.

3.7. Future Prospects

SFC is well established analytical technique, especially for pharmaceutical applications, but not yet a well established preparative fractionation technique. Not applications are carried out at preparative or larger scale. Pressure drops across the column and cost of specialized stationary phases seem to be practical and economic issues limiting industrial uptake of SFC. Only omega-3 fractionation has reached maturity and is carried out at production level.

Recent development towards enabling larger scale use of SFC is exciting and includes a number of companies offering dedicated SFC columns. Further research and development designing new stationary phases to widen the scope of target compounds that can be fractionated by SFC is still required.

4. CONCLUSION

SFC is considered as a powerful tool for the analysis of the lipid profiling in biological samples. Owing to the advantages of a hybrid of GC & LC, SFC incorporates many features of these two techniques and shows outstanding separation efficiency. And the modifier can flexibly adjust the polarity of the mobile phase in SFC, providing the convenience for simultaneous analysis of various lipids with a wide range of polarities. In recent years, UHPSFC using columns with sub-2μm particles shows a great potential as the comprehensive and high-throughput method for the analysis of the lipid profiling. The development of new stationary phase is conductive to the separation of the specific analytes, especially for the natural isomers in biological samples. The online SFC technologies could greatly shorten the analysis time and simplify the pre-treatment, so they show broad prospects in the lipid profiling. It is believed that the SFC-based method as a promising strategy could provide us many useful insights into lipid metabolism in physiological or pathological studies**.**

5. ACKNOWLEDGEMENTS

I would like to express my special thanks of gratitude to Mr. Vikram Veer, Assistant Professor, as well as Principal Dr. Ashok Bhosale, Shankarrao Ursal College of Pharmaceutical Sciences and Research Centre, Kharadi, Pune-14, Maharashtra, India; who gave me the golden opportunity to do this wonderful review article on the topic "Advances of supercritical fluid chromatography in lipid profiling".

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