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The Use of supercritical Fluid Chromatography as an Isomeric Separation Technique in Food Analysis

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Abstract

Supercritical fluid chromatography (SFC) is an advanced technique having wide applications in the research and analytical field. This technique is ideal for the separation of thermally labile substances and isomeric compounds in varied matrixes from nonpolar to polar analytes. The use of carbon dioxide in supercritical fluid chromatography as a mobile phase makes this technique economically cheap and environmentally friendly due to its non-toxic and inert nature. Initially, this technique had limited application in the food matrix because carbon dioxide does not efficiently elute very polar and ionic compounds; however, the use of modifiers like methanol and ethanol was successful in overcoming this challenge. Environmental contaminations from industrial pollutants and various other sources, releasing hazardous chemicals into the food causing bioaccumulation of chemicals and their metabolites. Food as a whole is very complex and analytes of interest are generally present at trace levels. A supercritical fluid chromatography system coupled with the diode-array detector, Flame Ionization Detector, Electron Capture Detector, and mass spectrometer are appropriate for diverse applications in food analysis. The diffusion coefficient of the solute in an SFC was found to be 10 times greater than conventional liquid chromatography techniques. This can be used for the analysis of enantiomeric residues in the food matrix using specific chiral columns. Supercritical fluid chromatography (SFC) has been used for the separation of isomeric compounds such as pesticides, synthetic pyrethroids, and insecticides in various food matrices. SFC has advantages over other techniques due to its higher productivity and better results in terms of chromatographic parameters such as better peak resolution, higher sensitivity, and highest peak separation of isomeric compounds, etc. This cost-effective and less hazardous technique has promising results in the analysis of food.

Introduction

Supercritical fluid chromatography (SFC) is an innovative technique finding application in analytical chemistry. In this technique, supercritical fluids are used as a mobile phase to carry the analytes through the column. A supercritical fluid is generated when the temperature and pressure of a gas or a liquid exceed their critical phase where a distinct gas or liquid phase does not exist. A critical phase is a state of thermodynamics where the critical temperature (T) and critical pressure (P) margin disappears. The figure 1 shows the phase diagram of super critical fluids. The improved efficiency in separating analytes attracts their application in various fields like food, pharmaceuticals, cosmetics, and environmental industries. Supercritical fluid chromatography techniques utilize the combination of gas chromatography (GC) and high-performance liquid chromatography (HPLC) with column chromatography. A supercritical fluid is the phase of a substance at critical temperature and pressure, resulting in a combination of properties of liquid and solid. SFC with a dynamic equilibrium can have characteristics like diffusivity, viscosity, and solvation with intermediate features of gas and liquids. (**Figure 1**)

The analytes in supercritical fluids extraction get separated based on kinetic properties such as varying molecular size, diffusion coefficient, polarity, supercritical fluids, etc., due to its ability to adjust solubilizing power. This technique is ideal for the extraction of thermally labile analytes and can be used for faster extraction having application in various types of samples. SFC can be used for the analysis of amino-acid, carbohydrate, lipid components, such as phospholipids, fatty acids, cholesterol, glycolipids, proteins, some of the vitamins, natural antioxidants, alkaloids, phenolic acids; flavonoids, terpenes Stilbenes. Food products are diverse in nature and analytical chemists find it challenging to develop techniques to separate and quantify them with the common analytical techniques. Currently, supercritical fluid chromatography (SFC) is seen as a promising technology for the analysis of the natural product with advantages like short run time, sensitivity, selectivity and being economically feasible [1]. Supercritical fluid extraction technology has been widely used in the food processing industry.

Various pesticides, which include insecticides, growth regulators, and fungicides, are used in agriculture for the protection of crops. The lethal doses of these pesticides present in agricultural products cause various health ailments to humans. The estimation of these pesticide residues is becoming very crucial due to bioaccumulation and continuous use of them to produce food. Supercritical fluid chromatography (SFC) is used for the determination of pesticides in food commodities like fruits and vegetables. In this technique, the pesticides are extracted using a supercritical fluid extraction process (SFE), followed by concentration and analysis by SFC [2]. The SFC technique has a great impact on the analysis of isomers in food. This chapter will focus on principles, current advancements, and applications of supercritical fluid chromatography in various sectors.

Instrumentation

The SFC instrumentation includes a pump, an extraction chamber, an oven, a column, a recovery chamber, a restrictor, and a collection device. The supercritical fluid produced from compressed carbon

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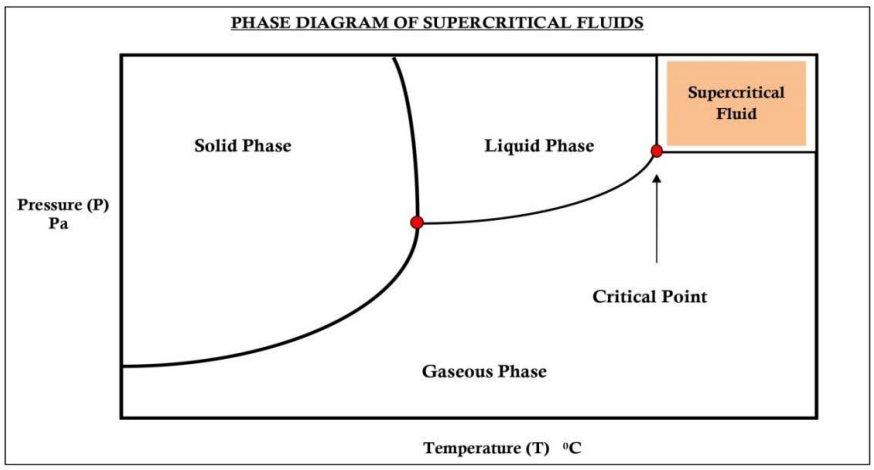

Figure 1: Phase diagram of super critical fluids.

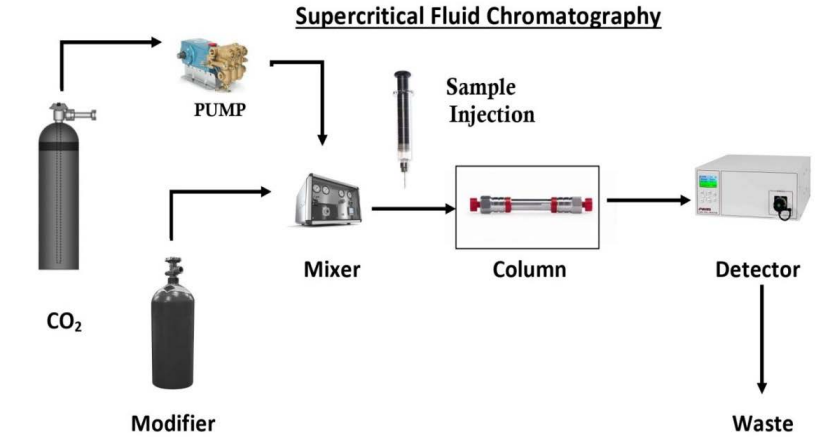

Figure 2: A Basic flow chart of a Supercritical fluid chromatography system.

dioxide is pressurized above its critical point, is pumped into the extraction chamber. The analytes of interest dissolves in the critical fluid and passes through a column placed in the oven. The temperature-controlled oven and restrictor help to attain the required conditions. The sample is introduced through the injection valve into the system. The instrumentation of a basic supercritical fluid chromatography is given in figure 2. (Figure 2)

The backpressure regulator controls the pressure and the column oven controls the temperature to attain the critical point to form the supercritical mobile phase. The modifiers placed in separate containers are pumped and mixed with carbon dioxide as per the composition ratio. The mobile phase takes the sample through the high-pressure cell of an optical detector. The detectors can be mass spectrometers, flame ionization detectors, or evaporative light scattering detectors. A portion of the sample is allowed to pass through the detector using a splitter connected to a pressure control valve.

Pump

The pumps used in SFC are generally positive displacement pumps that are highly accurate and reproducible. These pumps can reach up to a pressure of 10,000 psi with varied flow rates up to 24 ml/min. various types of pumps are available like the fixed flow pump, low pressure, and manually pressure controllable pumps. Two major pump systems commonly used in the SFC system are syringe pumps and piston pumps, and these pumps liquefied carbon dioxide [3].

Mobile Phase

This chromatographic technique with enhanced separation capabilities resulted by exchanging the common mobile phase (organic solvents) with compressed gases. Gases like carbon dioxide, nitrous oxide, and ammonia with co-solvents like ethanol, methanol, isopropanol, and acetonitrile are commonly used in supercritical fluid chromatography (SFC). The viscosity and density of supercritical fluids are closer to gas and the diffusion coefficient is intermediate between liquid and gas. Temperature and pressure are the major factors influencing the state of supercritical fluids.he critical parameters of various compounds used in SFC systems are listed in table 1. (Table 1)

Carbon dioxide gas is widely used in supercritical chromatography as it's easily available with inert, stable, and non-toxic properties. Supercritical Carbon dioxide is a lipophilic solvent having properties of both liquid and solid forms with a critical temperature at 31.0°C and critical pressure at 73.8 bar [4]. The separation of analytes occurs at the subcritical point and the modifiers help to increase the critical point. A binary pump is the most used pump in an SFC system, due to its flexibility in selecting mobile phase ratios. Isocratic and gradient modes are used widely in SFC. Carbon dioxide along with methanol as a modifier is used since it is more soluble than other solvents like acetonitrile and water. The solubility of water is the lowest among

Table 1: Critical properties of various solvents.

Solvent	Molecular mass g/mol	Criticaltemp K	Critical pressure atm	Criticaldensity g/cm³	Critical volume cm³/mol	triplepointtemp °C
Carbon dioxide (CO_2)	44.01	304.1	72.8	0.469	94.12	-56.6
Water (H_2O)	18.015	647.09	217.7	0.322	56	0.01
Methane (CH_4)	16.04	190.4	45.4	0.162	98.63	−182.47
Propane (C_3H_8)	44.09	369.8	41.9	0.217	200	-187.63
Ethylene (C_2H_4)	28.05	282.4	49.7	0.215	130.4	−169.2
Propylene (C_3H_6)	42.08	364.9	45.4	0.232	181.0	-185.19
Methanol (CH_3OH)	32.04	512.6	79.8	0.272	114	−97.7
Ethanol (C_2H_5OH)	46.07	513.9	60.6	0.276	167.1	−123
Nitrous oxide (N_2O)	44.013	306.57	72.5	0.452	97.4	−90.81

these solvents and for using water as a modifier the mobile phase saturated with water is used. The modifiers are used in supercritical fluid chromatography because they help in increasing the density of the mobile phase and improving the solvent strength.

The modifiers with a low polarity that are added along with primary modifiers are known as secondary modifiers; they are also known as additives. The additives can be grouped as acidic additives like acetic acid, trichloroacetic acid, etc. And basic additives like isopropylamine [5]. The carbon dioxide is pumped into the liquid form by adding helium; this increases the cylinder pressure and decreases the Solvation power, and thereby dissolves the carbon dioxide. [6]. The syringe pumps used in SFC have a fixed volume, whereas the piston pumps work basis on the liquid volume of the gas liquid-supplied cylinder. Modifiers are either introduced into the system as a premix with definite concentrations or by a binary pump system wherein one pump takes the mobile phase and the other pump carries the liquid modifier. The premixed modifier has various disadvantages like poor mixing which results in deviations in modifier concentrations. [7]. In the binary fluid pump system, the liquid carbon dioxide and modifiers are mixed in a volume-to-volume ratio to form the mobile phase, providing flexibility in analysis. The major concern in the formation of supercritical fluids is the difference in compressibility of different fluids used as mobile phase and modifiers [8].

Injector

The sample is introduced through a valve using a sample loop. The selection of injectors in an SFC system mainly depends upon the type of column. Open tubular columns use a large volume of sample and the packed column uses a lesser volume of sample. The volume of the sample that is injected also depends upon the diameter of the column. The definite volume of the sample is first introduced into the sample chamber using a high-pressure pump and the supercritical fluid then flushes the sample through the column. The method is helpful in diluting the sample to the required concentration and for samples that do not require any dilutions; an in-column injector can be useful. In an in-column injector, the sample is introduced into the column followed by the mobile phase [9].

Stationary phases of SFC

The application of SFC for the analysis of a wide range of analytes with different polarities is achieved by the use of different types of stationary phases. The common types of columns used in an SFC system are the polar phase or alkyl bonded phase columns using silica or hybrid silica [10]. In SFC, polar phase and alkyl bonded phase columns are used for the analysis of lipophilic compounds. [11]. The technique is applied for the separation of pure compounds that are enantiomeric in nature and those which do not separate in normal chromatographic techniques. The separation of two metabolites of demethylated nobiletin namely 3-dimethyl-NOB and 4-dimethyl-NOB was achieved in only 3 minutes using an SFC coupled with a mass spectrometer whereas the separation in a chiral Pack AD column took 10 minutes [12].

Two types of columns generally used in SFC are the packed column and open tubular column. The tubular column is packed with fused silica and has a cross-linkage siloxane coating. The separation of enantiomers has always been challenging in analytical chemistry due to their similarity in chemical and physical properties. The regulatory requirement to detect and quantify the presence of specific enantiomers. In SFC, chiral pairing agents separate isomers by forming diastereomeric adsorbates. The isomers undergo multiple interactions with the enantiomers. The hydrophobic compounds get solubilized due to the non-polar nature of carbon dioxide [13].

Qualitative and quantitative analysis in food

In recent times the technique has been widely used for the extraction, separation, and purification of active constituents in herbal medicines, detoxification, and fractionation of edible oil and fat [14]. Supercritical carbon dioxide is commercially used for the selective caffeination of coffee beans, tea leaves, guarana seeds, cocoa beans, etc. using micro-extraction with low flow rates. The low solubility of caffeine in supercritical carbon dioxides enhanced by the use of co-solvents such as water or ethanol. The pumping of pressurized supercritical CO_2 into coffee beans or tea leaves dissolves the caffeine and extracts them efficiently. The major advantage of this technique is that the whole process is highly selective and only caffeine gets extracted, leaving behind other constituents such as flavors, peptides, fat, etc [15]. Supercritical extraction is being used for the reduction of lipids and cholesterol levels in food products such as meat products, dairy products, egg products, etc. At 45°C temperature and 381 atm pressures, about 89% of lipids and 90% cholesterol were removed from chicken meat powder. While reducing cholesterol and lipid levels, the protein content gets concentrated in the meat product [16].

Various methods have been developed to estimate pesticides using supercritical fluid chromatography coupled to triple quadrupole mass spectrometry. A major limitation in developing a quantitative and confirmatory method for the determination of multi-residue pesticides was the presence of matrix interferences. Matrix interferences from co-extracts were the major hurdle in the accurate measurement of pesticide residues using an LCMSMS or a GCMSMS system. These interferences resulted in 50% of peak suppression in matrices like tomato, whereas the same was less than 20% using an SFC technique. Other advantages of SFC include better retention of polar analytes resulting in good peak shape and better sensitivity. The analysis using an SFC coupled with

a mass spectrometer has higher extraction efficiency and shorter run times. The techniques have higher ionization rates and their capability to remove inferences makes them quantify analytes at low levels [17].

Conventional techniques like normal phase and reversed-phase chromatography are not capable of separating stereoisomers, chiral compounds, and enantiomers. SFC using carbon dioxide as mobile phase with modifiers and additives like methanol, ethanol and acetonitrile, acetic acid, trichloroacetic acid are useful in the separation of isomers by enhancing chiral resolution [18]. The purification of carbohydrates has always been challenging due to the presence of structural isomers like regioisomers. These isomers can be analyzed using a carbon dioxide supercritical fluid chromatography (SFC) method for the separation of glycosides in human milk [19].

Xanthophyll, lycopene, and beta-carotene were extracted from tomatoes and primary isomers of carotene like α-carotene and β-carotene extracted from carrots were separated using capillary SB-cyanopropyl-polymethylsiloxane and SB-cyanopropyl-50-polymethylsiloxane columns in the SFC technique. The separation was carried out in isocratic mode using carbon dioxide with 1% ethanol as a modifier [20]. The separations of analytes are influenced by factors such as temperature, the concentration of modifiers, and the type of stationary phase used. The optimal separation of the isomeric carotenes was achieved at a temperature of 22-25°C and an increase in temperature led to decreased capacity factor, poor resolution, and peak shape [21].

Techniques were developed using supercritical fluid chromatography coupled with mass spectrometry for the separation of carotenoids such as β-carotene, lycopene, lutein, zeaxanthin, antheraxan-thin, neoxanthin, and violaxanthin on the octadecyl-bonded silica- packed column. Carotenoids with structural/ geometric isomers are found in Trans and Cis forms. An electrospray ionization mode was used to analyze the hydrophobic carotenoids. The monolithic column with methanol and 0.1% ammonium formate as modifier separated the isomers within 15 minutes and the use of mass spectrometer produced better sensitivity, selectivity, and isomeric separation [22]. SFC with mass spectrometer detector and flame ionization detector has been used for the separation and analysis of tocopherols and their analytes. SB-Phenyl-5 and an SB-Octyl-50 capillary column were used in the separation of four tocopherols. The peaks were eluted in the order of delta-tocopherol, beta- tocopherol, gamma-tocopherol, and alpha-tocopherol at 100°C column temperature and 200-240 atm pressure. [23]. The Chemical structures of α-, β-, γ-, and δ-tocopherols figure 3 [24]. (Figure 3)

A rapid method was developed for the determination of polar compounds in tea using Supercritical Fluid Chromatography Coupled with Ion Mobility Quadrupole Time-of-Flight Mass Spectrometry. The multipolar analytes were extracted by microextraction and separated on RX-SIL-packed stationary phases using carbon dioxide as mobile and methanol, trifluoroacetic acid as modifiers. The mobile phase modifiers were added to improve chromatographic separation. Trifluoroacetic acid neutralizes the mobile phase solution by inhibiting the dissociation of analytes with the silicon hydroxyl group of the stationary phase. The method was effective in the separation of seven active polar components from Dendrobium Officinale. The run time of the analysis was completed in eight minutes [25].

A supercritical fluid chromatography coupled with a photodiode array was used for the analysis of eight Sudan dyes in chilly oil. The analysis was carried out using an HSS C18 SB column with acetonitrile, methanol, and formic acid, as the modifiers. The CSH Fluoro-Phenyl stationary phase was successful in better retention of analytes due to the π-π interactions between the aromatic functional groups with the stationary phase. The compounds Sudan IV and Sudan Red B are isomers with a difference in the methyl group bonded to the benzene ring. The analytes were better retained in the HSS-C18 SB stationary phase, which resolved the peaks completely with better separation of the isomers [26].

Optimization of Chromatographic separation

Selections of experimental parameters are crucial in the optimization of chromatographic separation, which includes a mobile phase/modifier Selection, stationary phase of the column, and type of detector used. The other chromatographic parameters include the temperature, pressure, and strength of the modifiers used. The elution power can be adjusted in a normal phase HPLC system by increasing the concentration of methanol. The advantages of using an SFC range of detectors that can be coupled in an SFC system are flame ionization

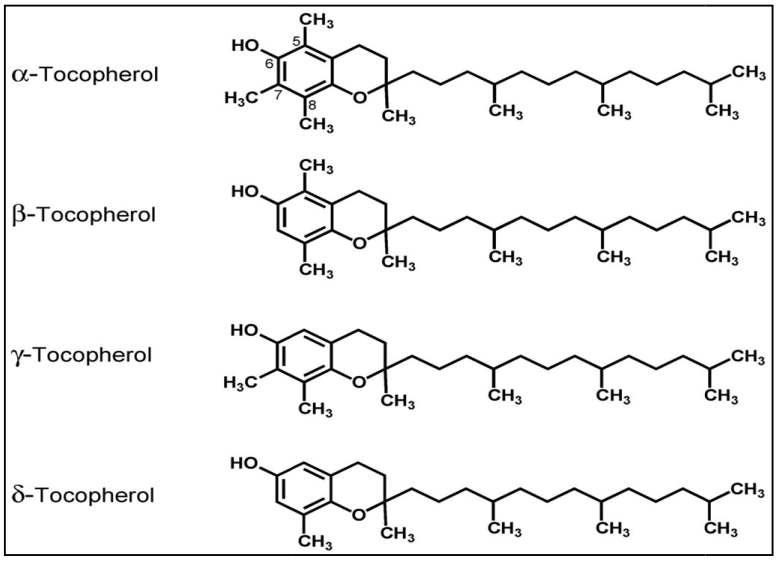

Figure 3: Chemical structures of α-, β-, γ -, and δ –tocopherols.

detector (FID), mass spectrometer, UV-visible spectrometer, IR spectrometer, thermionic detectors, flame photometric detector (FPD), photoionization detector (PID), etc.

The salvation power of the modifiers at specific temperature-specific temperatures is a result of interactions between solvent and solute. The addition of modifiers increases the polarity, aromaticity, and chirality to form a complex of metal ions and the solubility of polar molecules. Modifiers like methanol or ethanol are added to increase the polarity. Toluene is added to induce aromaticity and extracts tri-n-butyl phosphate, dialkylalkyl phosphonates, dibutylbutyl phosphonate, and diamylamyl phosphonate to form a complex of metal ions [27]. The mobile phase additives and hydrogen bond donor analytes, which bind with the stationary phase, are temperature and pressure dependent. The selectivity and efficiency in isomers were dependent on the type of additives used with the mobile phase.

Future Developments

The SFC chemicals are economical, less toxic compared to normal phase and reversed-phase chromatography, making them a greener technique. A single mobile phase in SFC can be used to separate a wide range of analytes just by varying the temperature and pressure. The ability of the SFC to separate isomers and structurally similar compounds without mass spectrometers would promote the use of the SFC system where a normal HPLC system fails. A Gas Chromatography or Liquid Chromatography system would require derivatization to analyze non-volatile or heat-liable compounds, whereas an SFC system can be used without any derivatization just by lowering the temperature. Selecting the stationary phase component of the column, mobile phase and optimization of the method are critical for the analysis of chiral and achiral compounds. The latest improvements in the instrumentation of SFC by using multiple columns have increased its demand in food and pharmaceutical applications. Other than analytical applications, modern SFC instruments have a huge impact on achiral purification in preparative chromatography. The use of these for the analysis of basic compounds, polar compounds, large molecules, and other molecules would make SFC the most preferred technique. It is anticipated that the SFC technique will be widely used in the future due to its extraction capabilities, enhanced recoveries, and use of less toxic chemicals. The use of newly developed hybrid coatings, an advanced composite material in the stationary phase, and the application of surfactants make this technique selective with reduced matrix interference and broader applications.

Summary

Supercritical fluid chromatography is emerging as a mainstream chromatographic technique due to its global demand. The chromatographic technique is extensively used in the separation of isomeric analytes in food and other applications. This technique would surpass other chromatographic methods for the analysis of isomers in food due to its advantage in terms of better extraction, separation, analysis time, and versatility. The cost of instrumentation would be a major disadvantage of using SFC, which can be overcome only by the development of innovative technologies at a cheaper cost. The requirement for a specialized stationary phase for large-scale applications in the food and production industry needs to be addressed. Further, validation of established methods would be required for the assessment of SFC techniques. The SFC has a promising application in research and non-targeted analysis in the coming years.

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