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ABSTRACT

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However, the application of supercritical fluid in the extraction of phytosterols has offered a promising green technology in overcoming the limitations of conventional

extraction.

KEYWORDS: Techniques, phytosterols, conventional and non-conventional extraction, health benefit

Additional information

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Techniques for the extraction of phytosterols and their benefits in human health: a review

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ABSTRACT

This review summarizes the information on the health-promoting effects of phytosterols and the techniques for their extraction. The extraction and analysis processes of phytosterols are complex and have not been fully established. Phytosterols have significant roles in the areas of foods, nutrition, pharmaceuticals, and cosmetics. Free phytosterols extracted from plant sources are widely used in fortified foods and dietary supplements. Most phytosterols are extracted from plant matrices using organic solvents which are health and environmental hazards. However, the application of supercritical fluid in the extraction of phytosterols has offered a promising green technology in overcoming the limitations of conventional extraction.

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Introduction

Plant sterols, generally known as phytosterols, are natural and bioactive compounds representing a diverse group of triterpenes. Phytosterols are present in all plant cell membranes and are especially enriched in vegetable oils and fats, cereals and cereal products, vegetables, fruits, and berries.[1] In addition to herbaceous plants and oilseeds, the waste of industrial processing of softwood and hardwood (tall pitch and sulfate soap) are important sources of phytosterols. These phytosterols contain 10 to 15% phytostanols.[2–4] Almost unexplored new natural sources of phytosterols are at present gaining much attention such as microalgae.[5] Phytosterols are essential components of the cell membrane lipid bilayer.[6] They consist of 28 or 29 carbon atoms in the main structure and resemble cholesterol both in structure (four ring steroid nucleus, 3β-hydroxyl group, and often a 5,6-double bond) and function (stabilization of phospholipid bilayers in cell membranes). Most phytosterols have a side chain composed of nine to ten carbon atoms whereas cholesterol has a side chain composed of eight carbon atoms. More than 200 types of phytosterols have been found in various plant species.[7] Among them, β-sitosterol, campesterol, and stigmasterol are more abundant in nature. Other phytosterols, such as brassicasterol, Δ^5-avenasterol, sitostanol, and campestanol, are found in minor quantities and are present in almost all plants. In nature, plant sterols exist as free sterols or conjugates of fatty acid esters, glycosides, and acetylated glycosides.[8,9] As a natural component of plant lipids, phytosterols have gained much attention in reducing the serum cholesterol level in humans, as well as the risk of heart disease.[10,11] In addition, phytosterols have anti-inflammatory, antibacterial, antiulcerative, and antitumor properties.[12–14] Plaza et al.[15] reported that foods enriched with phytosterols promote consumer health and prevent different diseases.

Various chromatographic techniques can quickly analyze bioactive compounds. However, their efficiencies may depend on the extraction methods, including various extraction parameters, the nature of the compound of interest, and the plant matrix. Qualitative and quantitative studies of bioactive compounds from plant materials mainly rely on the selection of proper extraction methods.[16] Until now, extraction techniques have been investigated to recover valuable natural

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compounds from plants for commercialization. Since phytosterols are lipids in nature, various extraction methods can be used to extract these non-polar compounds. Currently, various conventional and non-conventional techniques are used to extract compounds of interest from different plant matrices. Among the conventional extraction methods, Soxhlet extraction is the most favorable method and is still considered a reference to newly developed methods. However, the organic solvents used in conventional extraction techniques are harmful to human health as well as the environment. As a result of the use of large amounts of organic solvents in conventional extraction, the demand for new technologies has increased to reduce the use of organic solvents and provide some advantages over conventional extraction techniques. The extraction techniques have extensively been reviewed elsewhere with the emphasis on the extraction of bioactive compounds other than phytosterols from plant matrices.[17–19] Therefore, the objective of this review is to summarize the beneficial effects of phytosterols and the techniques for their extraction and analysis with some favorable indications for commercialization using environment-friendly technology.

Benefits of phytosterols

Figure 1 shows the benefits of phytosterols for human health. The physiological effects of phytosterols both in humans and animals have been assessed in many studies.[20–22] It has been well established that phytosterols have a remarkably beneficial effect in the reduction of serum cholesterol, as well as the risk of heart disease in humans.[23–27] Phytosterols also have significant roles in the areas of pharmaceuticals (production of therapeutic steroids), nutrition, and cosmetics. As a result of the health benefits, they are usually utilized in the food and cosmetic industries as value-added

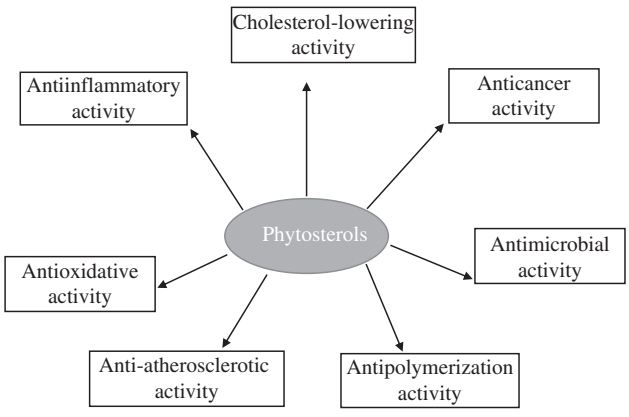

Figure 1. Benefits of phytosterols for human health.

additives.[28] The mode of action of phytosterols is not accurately understood. However, two theories have been considered for lowering the cholesterol level in blood plasma. The first theory states that the marginally soluble cholesterol in the intestines is precipitated by the presence of added phytosterols and can, therefore, not be absorbed by the intestinal cells. The second theory declares that for absorption into the blood stream through intestinal cells, cholesterol has to enter mixed micelles consisting of bile salts and phospholipids in which cholesterol is marginally soluble and is displaced by phytosterols resulting in the prevention of cholesterol absorption.[9,25] A recent report showed that phytosterols also inhibit the synthesis of 27-hydroxycholesterol and, hence, reduce cholesterol absorption.[29] In another study by Francavilla et al.,[5] a neuromodulatory action of *Dunaliella tertiolecta*-derived phytosterols was found in selective brain areas of rats. Recently, Morgese et al.[30] investigated the effects of an acute oral administration of the lipid extract of *Chlorella sorokiniana*, on cognitive, emotional, and social behavior in rats, analyzing possible underlying neurochemical alterations. Their results showed improved short-term memory in *Chlorella sorokiniana*-treated rats compared with controls. In their study, neurochemical investigations revealed region-specific effects, leading to an elevation of noradrenaline and serotonin content in the hippocampus.

Other health-promoting effects of phytosterols such as anticancer, antioxidative, anti-inflammatory, and anti-atherosclerotic activities have been reported. In many studies, phytosterols have exhibited toxic effects on breast, colon, and prostate cancer cells.[31–34] Epidemiological studies have demonstrated a reduction in the risk of common cancers, such as cancers of the lung, stomach, colon, breast, and prostate through implementing phytosterol-enriched diets.[26] Phytosterols also protect low-density lipoproteins from peroxidation.[35] Some researchers have reported that phytosterols, especially Δ^5-avenasterol show antioxidant activity and anti-polymerization properties.[36,37] Recently, Burg et al.[38] reported that dietary intake of phytosterol blends mainly containing stigmasterol might be beneficial in preventing Alzheimer's disease. Sundarraj et al.[39] reported that γ-sitosterol exerts potential anticancer activity because of growth inhibition, cell cycle arrest, and cancer cell apoptosis. In another study, Balamurugan et al.[40] showed that γ-sitosterol reduced hyperglycemia in STZ-induced diabetic rats through increased insulin secretion and inhibition of gluconeogenesis. Phytosterols also have an adverse impact on the human body. Excess phytosterols in the diet may reduce the blood levels of carotenoids

and vitamin A that are essential for the visual function of the eye.[41] At present, free phytosterols extracted from various plant sources are widely used in fortified foods and dietary supplements. Some commercially available products such as margarine, yogurt, yogurt drinks, and orange juice contain plant sterols.

Extraction techniques for phytosterols

The extraction techniques are capable to separate the soluble plant metabolites in a selective solvent. The isolation techniques for phytosterols rely on the nature of the matrix and the form of phytosterols (free, esterified, and glycosylated).[7] Diverse conditions for each extraction technique should be applied to obtain the appropriate extract yield from the plant source. The quality of an extract is substantially influenced by various factors such as plant material, solvent, extraction procedure, and other factors.[18] During extraction, all parameters have to be optimized to obtain a high quality extract with high quantities of the desired compound. As mentioned above, there are conventional and non-conventional extraction techniques. The most common objectives of these techniques are: (a) to extract the bioactive compound of interest from plant matrices, (b) increase bioassay sensitivity by increasing the concentration of target compounds, (c) enhance selectivity of analytical methods, (d) convert the bioactive compounds into a more suitable form for detection and separation, and (e) provide a robust and reproducible method that is independent of variations in the sample matrix.[16] Figure 2 shows the method involved in the extraction and analysis of phytosterols. Roiaini et al.[42] studied the effect of various phytosterol extraction methods such as Soxhlet, ultrasonic, supercritical carbon dioxide, and supercritical carbon dioxide with cosolvents on cocoa butter. The authors reported that the highest phytosterol content obtained using supercritical carbon dioxide with a cosolvent. A process for extracting sterols from corn fiber using ethanol was developed by Abbas et al.[43] They claim that the extracted phytosterols are selected from the group consisting of α, β, and γ forms of sitosterol, sitostanol, stigmasterol, stigmastanol, campesterol, campestanol, spinasterol, phytosterol esters, phytostanol esters, and mixtures thereof.

Saponification

Saponification usually provides a concentrated sterol fraction, which simplifies the analysis of total and individual phytosterols. In plant matrices, sterols are also present in ester form. These steryl esters have to be hydrolyzed into free sterols, which are unsaponifiable. Hydrolysis of esters can be performed in two ways: either by heating water with plant matrices at high temperatures (200–260°C) and pressures (1.5–50 MPa) or by heating the sample with sodium or potassium hydroxide at temperatures ranging from 90 to 120 C while stirring. The latter has the advantage of combining hydrolysis and saponification.[44]

Acid hydrolysis and alkaline saponification with alcohol have been applied to determine phytosterols from cereals.[45,46] The combination of acid hydrolysis and alkaline hydrolysis gives better results for the determination of phytosterols compared with alkaline

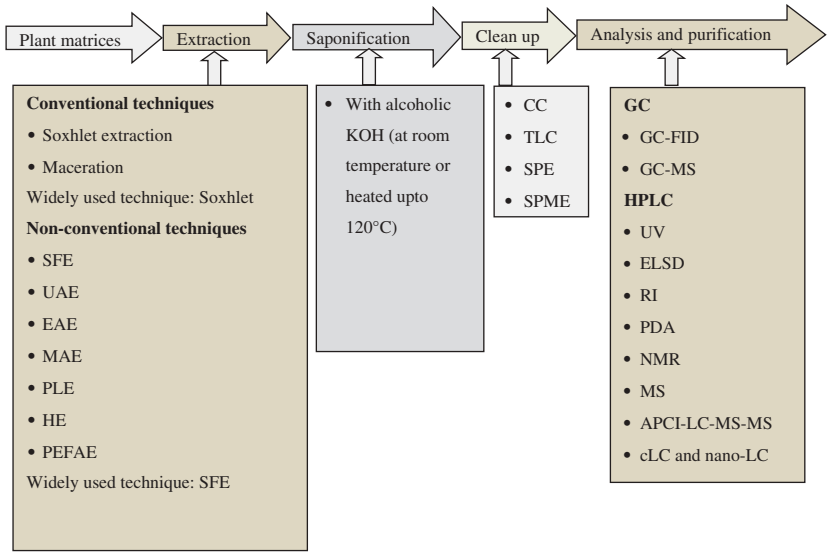

Figure 2. Methodologies for extraction and analysis of phytosterols.

saponification alone. Saponification has also directly been used to determine the phytosterols from different samples, especially foods.[47,48] Saponification alone cannot hydrolyze the acetal bond between the sterol and carbohydrate moieties and thereby fails to quantify steryl glycosides.[49] Moreover, saponification is a time-consuming step. On the contrary, acid hydrolysis cannot be used to determine Δ^7-phytosterols as they are decomposed or isomerized after short periods of hydrolysis.[50]

Saponification with alcoholic potassium hydroxide is possible either at room temperature or through heating. At room temperature, the lipid extract can be saponified overnight with 1 M alcoholic potassium hydroxide while stirring. For hot saponification, the lipid extracts are heated with alcoholic potassium hydroxide. Several internal standards including betulin, cholestane, 5α-cholestan-3β-ol, and 5β-cholestan-3α-ol have been used for saponification.[51–54] Internal standards can diminish the analytical errors that may arise from instrument handling and other experimental variables. After saponification, the unsaponifiable matter is separated and analyzed by chromatography. The unsaponified materials are isolated through multiple extractions of organic solvents such as diethyl ether, petroleum ether, *n*-hexane, or heptanes, and subsequently, the solvents are evaporated at low temperatures.

A new method for the derivatization of phytosterols has been developed by Liu and Ruan.[55] In this derivatization method, the solution of phytosterols in tetrahydrofuran is added to the mixture of benzoyl chloride and pyridine, and then the reaction is stirred at room temperature. The benzoyl group is incorporated into the phytosterols. A small column of aluminum oxide in the form of a solid phase extraction (SPE) or a solid phase microextraction (SPME) has been used to avoid solvent extraction. A minimal amount of phytosterols presented in the sample can be extracted by SPE and SPME. In saponified samples, the potassium salts of free fatty acids are tightly bound to aluminum oxide forming insoluble aluminum soaps. Through elution with ether, the unsaponifiable materials are collected from the column.[56]

Conventional techniques

Conventional techniques include Soxhlet extraction, maceration, heating under reflux, percolation and hydrodistillation. Among these conventional techniques, Soxhlet extraction (hot continuous extraction) and maceration (single continuous extraction at room temperature) methods have usually been used for the extraction of phytosterols.[57–59] Table 1 shows the conventional methods for phytosterol extraction from various plant matrices.

Soxhlet extraction

Among the traditional techniques, Soxhlet extraction has been extensively and widely used to extract various compounds of interest from plant matrices. It is still used as a reference technique for evaluating the performance of other conventional and non-conventional methods although it was initially designed for lipid extraction.[60] In Soxhlet extraction, plant materials are placed in a thimble holder that is then placed in a distillation flask. The distillation flask is filled with the solvent of interest. When the solvent reaches the overflow level, the solution in the thimble holder is aspirated and loaded back into the distillation flask by a siphon. The solution carries the extract into the bulk liquid. Extracted solutes are kept in the distillation flask and the fresh solvent goes back to the thimble holder of plant material. This process is continued until the extraction is complete.[17]

A suitable solvent should be chosen for the extraction of targeted compounds using the Soxhlet extraction method. The yield and compositions of the extract vary because of the use of different extracting solvents. The most widely used solvents for the extraction of phytosterols are *n*-hexane,[61] petroleum ether,[62] ethanol,[63] and methylene chloride.[64] Soxhlet extraction is time-consuming and involves large amounts of organic solvents which are health and environmental hazards.

Maceration

Maceration is a simple and inexpensive procedure to extract phytosterols.[65] This method can be used for both initial and bulk extraction. The suitable solvents are added to the pulverized plant materials in a closed container at room temperature. Stirring the solvent can enhance the speed of extraction. The extraction of the compound stops when equilibrium is developed between the concentrations of solute in the extract and plant material. After extraction, the liquid is separated from the residual plant material (marc) by decantation. The marc is pressed to recover the remaining extract. These liquid extracts are filtered to remove impurities. Centrifugation is often needed if the plant material is too small to be filtered. To ensure the complete extraction, the marc is repeatedly extracted with fresh solvents.[66] Maceration is a time consuming method in which several hours to a few weeks are needed for complete extraction. It is not efficient enough to extract poorly soluble compounds at room temperature.

Table 1. Extraction of phytosterols using conventional techniques.

Plant matrices	Extraction technique	Used solvent	Detection method	Internal standard	Main analyzed phytosterols	Contents of phytosterols (µg/g dry wet)	Total lipid (%)	References
Olive drupes or leaves	Maceration	Dichloromethane-Hexane 2:1	GC-MS Cholesterol		β-sitosterol, campesterol Stigmasterol Brassicasterol	137.1–475.1 3.9–28.8 1.3–8.2 0.1–0.3	-	Orozco-Solano et al. [58]
Leaves of piper gaudichaudianum Kunth	Soxhlet	Petroleum ether or ethanol	GC-MS	Perylene	β-sitosterol Stigmasterol	58.9–106.9 183.6–964	2.79–20.34	Péres et al. [62]
Anise, coriander, caraway, white mustard, nutmeg seeds	Soxhlet	Hexane	GC	5α-cholestane	Sterols	-	7.4–25.3	Kozłowska et al. [61]
Ripe pulp of mango	Soxhlet	Dichloromethane	GC-MS	Tetracosane	Fucosterol β-sitosterol Campesterol Stigmasterols	23–146 237–692 52–174 24–82	-	Vilela et al. [57]
Sea buckthorn seed	Cold Pressing		GC-FID and GC-MS	5α-cholestane	Campesterol Clerosterol Sitosterol + Lanosterol Δ^5-avenasterol+ obtusifoliol	139 84 4622 973	-	Li et al. [68]
C. nutans	Soxhlet	Ethanol	HPLC	-	Total phytosterols B-sitosterol	470 230	-	Mustapa et al. [63]
Pumpkin seed	Cold pressing		GC-MS	Cholesterol	β-sitosterol and spinasterol Δ^7-avenasterol,	406–514 16–94		Rabrenović et al. [69]
Corn, sesame, oat and peanut	Hexane extraction	Hexane	UPLC-APCI-MS	6-ketocholestanol	β-sitosterol Campesterol Stigmasterol Ergosterol	754–2753 63–1498 21–1185 0.3		Lu et al. [123]
Pumpkin seed	Soxhlet	Hexane or petroleum ether	GC-MS	Betulin	β-sitosterol Campesterol Stigmasterol Desmosterol	51.9–95.7 23.8–28.6 7.8–32.4 43.7–59.8	43.37–44.65	Hrabovski et al. [70]
Roselle seeds	Soxhlet	Petroleum ether	GC-FID	5α-cholestane	β-sitosterol, campesterol, stigmasterol, cholesterol and Δ^5-avenasterol	5072.4	14.6	Nyam et al. [118]

Non-conventional techniques

The qualitative and quantitative analyses of target compounds from natural sources have been performed after exhaustive extraction of the sample using conventional extraction techniques. However, conventional extraction techniques have some limitations such as a long extraction period, requirement of an extra pure solvent, the necessity of solvent evaporation, a lower selectivity of extraction, and thermal degradation of heat sensitive compounds.[60] A wide range of efficient and promising techniques have been introduced in the past decades to overcome these limitations. The most promising techniques for the extraction of bioactive compounds are microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE), pulsed electric field-assisted extraction (PEFAE), hydrotropic extraction (HE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE).[19,67] Among these non-conventional techniques, PLE and SFE have been performed under pressurized conditions. SFE, PLE, EAE, and MAE have typically been used to extract sterols from various plant matrices. Table 2 shows the non-conventional techniques for the extraction of phytosterols from different plants.

Microwave-assisted extraction

MAE is a simple and cost-effective technique to extract bioactive compounds from plant materials.[76] Microwaves are electromagnetic fields with a frequency of 300 MHz to 300 GHz or wavelengths of 1 cm to 1 m. They are made of electric and magnetic fields, which are perpendicular to each other. Electromagnetic energy is converted into heat through two mechanisms ionic conduction and dipole rotation.[77] Since the flow of ions is inhibited by the medium, heat is generated by the ionic conduction mechanism. In contrast, dipole rotation is caused by the alignment of the molecules possessing a dipole moment in both the solvent and solid sample in the electric field. This frequent change of direction produces collisions between particles resulting in liberation of heat energy. The particular advantage of microwave heating in extraction is the enhanced breakdown of weak hydrogen bonds by the dipole rotation of the molecules.[78]

Microwave energy is absorbed by the sample components based on their dielectric constants.[79] When microwave radiation is run through the solvent carrying sample, it directly reaches the solid sample without being absorbed by the solvent. This causes quick heating of the moisture in the solid sample resulting in evaporation creating a high vapor pressure. The high vapor pressure ruptures the cell wall of the matrices and releases the components into

Table 2. Extraction of phytosterols using non-conventional techniques.

Plant matrices	Extraction technique	Used solvent	Detection method	Internal standard	Main analyzed phytosterols	References
Grape seeds	SFE	SC-CO_2	GC-FID	Dihydrocholesterol	β-sitosterol, campesterol, and stigmasterol	Beveridge et al. [71]
Roselle seeds	SFE	SC-CO_2	GC-FID	5α-cholestane	β -sitosterol, campesterol, stigmasterol, and Δ^5-avenasterol	Nyam et al. [118]
Corn bran and germ	ASE (PLE)	Hexane, isopropyl alcohol, or ethanol	HPLC-UV-Vis and ELSD	-	Total phytosterols	Moreau and Hicks [72]
Sea buckthorn seeds	SFE	SC-CO_2	HPLC-ELSD	-	β-sitosterol	Sajfrtová et al. [120]
Berry seeds	SFE	SC-CO_2	HPLC-ELSD	-	β-sitosterol	Sovová et al. [73]
Tomato seed	SFE and ASE (PLE)	SC-CO_2 and hexane and ethanol	GC-MS	Cholestane	Brassicasterol, sitosterol, campesterol, and stigmasterol	Eller et al. [74]
Lotus bee pollen	SFE	SC-CO_2	GC-FID	-	β-sitosterol, campesterol, stigmasterol and β-amyrin	Xu et al. [75]
Corn, sesame, oat and peanut	SFE	SC-CO_2	UPLC-APCI-MS	6-ketocholestanol	β-sitosterol, campesterol, stigmasterol and ergosterol	Lu et al. [123]
Pumpkin seed	SFE	SC-CO_2	GC-MS	Betulin	β-sitosterol, campesterol, stigmasterol and desmosterol	Hrabovski et al. [70]
Olive drupes or leaves	UAE	Dichloromethane hexane	GC-MS	Cholesterol	β-sitosterol, campesterol, stigmasterol and brassicasterol	Orozco-Solano et al. [58]
Leaves of piper gaudichaudianum Kunth	UAE and PLE	Petroleum ether and ethanol	GC-MS	Perylene	β-sitosterol and stigmasterol	Péres et al. [62]
Rape seed	PEF	Hexane		-	β-sitosterol, campesterol and sitostanol	Guderjan et al. [101]
Marine algae	MAE	Ethanolic KOH	HPLC-UV and GC-MS	-	Fucosterol and 24-methylenecholesterol	Xiao et al. [82]
Goldenberry pomace	EAE	n-Hexane and H_2O	GC-FID	-	B-sitosterol, campesterol, Δ^5-Avenasterol, stigmasterol	Ramadan et al. [85]

solvent.[18] Therefore, MAE includes three sequential steps: i) separation of solutes from the sample matrix under increased pressure and temperature, ii) solvent diffusion across the sample matrix, and iii) release of solutes from the sample matrix to the solvent. The simple mechanism of MAE method is described in Fig. 3. MAE can be performed in two different modes of operation. The first one is a pressurized microwave-assisted extraction (PMAE), which is carried out in closed vessel under controlled pressure and temperature. Another is a focused microwave-assisted extraction (FMAE), which is operated in an open vessel with surrounding pressure.[80] MAE has several advantages over conventional extraction techniques, such as a quick extraction of the compound of interest, less use of organic solvents, reduced thermal gradients, and small equipment.[81] Xiao et al.[82] extracted phytosterols from algae by developing an efficient method using MAE coupled with high-speed counter-current chromatography.

Enzyme-assisted extraction (EAE)

EAE of biologically active compounds from plants is considered a potential alternative to conventional solvent extraction methods. EAE is based on the capability of enzymes to catalyze reactions with high specificity and can function under mild operating conditions.[83] Some phytochemicals in the plant cell are present as bound to cell wall components, which are not easily extracted by the conventional solvent extraction methods. However, pretreatment of the sample with an enzyme is an effective way to release some bounded compounds into the solvent and thereby increases the total yield of the compound of interest. Enzymes such as cellulase, hemicellulase, α-amylase, and pectinase enhance the extraction of compounds by rupturing the cell wall and cleaving the structural polysaccharides and lipid bodies.[84] The compounds present in the cell are easily comes out into the solvent due to the breakage of the plant cell walls by enzymatic treatment. Figure 4 shows the mechanism of the EAE method. EAEs have been presented as enzyme-assisted aqueous extraction (EAAE) and enzyme-assisted cold pressing (EACP).[84] The EAAE method is used to extract oil from different parts of plants especially from seeds.-[85,86] In the EACP method, enzymes assist the hydrolysis of the cell wall. It is considered an alternative for the extraction of bioactive compounds from oil seeds because of its non-toxic and non-flammable properties.[84] Different factors such as enzyme types and concentrations, water to substrate ratio, pH of the sample suspension, the particle size of the sample, and treatment time mainly influence the yield of extract by EAE.

Ramadan et al.[85] performed the extraction of phytosterols from goldenberry pomace oil by EAE using *n*-hexane as a solvent. It was found that phytosterols and total oil yields were increased by EAE when compared with those obtained by conventional solvent extraction. Phytosterol recovery from oil deodorizer distillates using enzymes has been reported.[87] Panpipat et al.[87] carried out the enzymatic recovery of phytosterols from rapeseed and soybean oil deodorizer distillates mixture via ethanolysis using Lipozyme. More than 95% of phytosterols were recovered from the oil deodorizer distillates.

Ultrasonic-assisted extraction

The UAE technique is facilitated by ultrasound, which is a high-frequency sound wave. The sound wave with more than 20 kHz (up to 100 MHz) passes to the solvent. There are several possible hypotheses for ultrasound enhancement of extraction such as disruption of the cell wall, increase of penetration and swelling, and hydration processes among others. Under ultrasonic

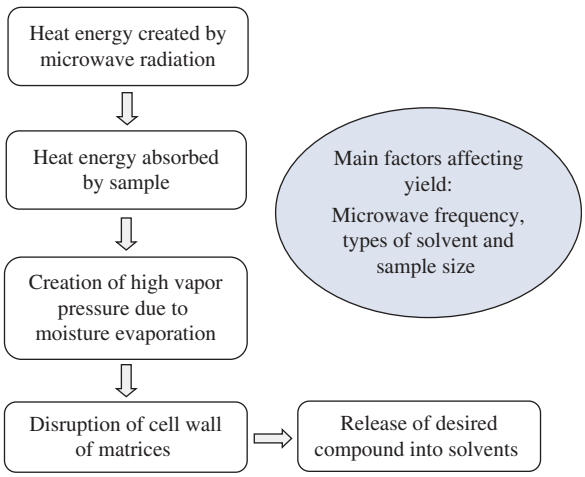

Figure 3. Mechanism of the microwave-assisted extraction (MAE) method.

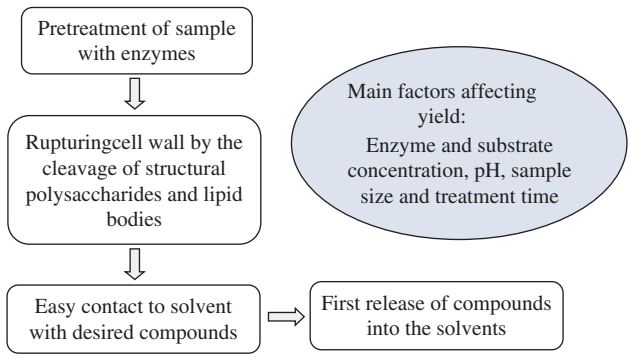

Figure 4. Mechanism of the enzyme-assisted extraction (EAE) method.

wave, solid and liquid particles are vibrated and accelerated and then solute from the sample quickly comes out from solid phase to the solvent.[88] The intensity of ultrasound reduces intermolecular forces resulting in a breakdown of the molecular structure. This process is called cavitation and includes production, growth, and the collapse of bubbles.[89] The collapse of bubbles causes the disruption of cell membranes to facilitate the release of extractable compounds and also increases the penetration of solvent into cellular materials and enhances the mass transfer.[88,90] The mechanism of the UAE method is shown in Fig. 5. The most important factors to achieve an efficient and effective extraction are moisture content and particle size of the sample, solvent, milling degree, frequency, and time of sonication. Moreover, temperature and pressure are involved as governing factors for the ultrasound action.

UAE does not require complex or expensive instrumentation. It can be applied both in an analytical and commercial scale.[91] The main advantages of UAE are the reduced extraction time as well as the lower energy and solvent use. Furthermore, ultrasound wave energy creates reduced thermal gradients and extraction temperatures, faster energy transfer, selective extraction, reduced equipment size, quick start-up, increased production, and eliminates process steps.[92] UAE of phytosterols from olive drupes and leaves has been carried out by Orozco-Solano et al.[58] UAE provides three times higher efficiency for the extraction of phytosterols than the conventional maceration method using the same solvent, dichloromethane-hexane. Libo et al.[93] also reported the UAE of phytosterols from pumpkin seeds.

Pulsed electric field-assisted extraction

Pulse electric field-assisted extraction (PEFAE) is considered to be an emerging technology that has attracted attention for enhancing mass transfer, especially in the food industry.[94,95] During the last decade, it has been used to improve the pressing, drying, extraction, and diffusion processes.[96,97] This method is based on the use of external electric fields, which provoke the electroporation of biological cell membranes enhancing the diffusion of solutes. In PEFAE, a suspension of living cells is placed in a treatment chamber, which consists of two electrodes with a simple circuit producing exponential decay pulses. An electric potential passes through the biological membrane in the suspension and thereby separates cell membrane molecules depending on their dipole nature. When the transmembrane potential exceeds the critical value of approximately one volt, repulsion is induced between the charged molecules. This leads to an electrical breakdown and changes in the cell membrane structure resulting in a drastic increase of permeability.[98] Figure 6 shows the mechanism of the PEFAE method. PEFAE can be operated as a continuous or batch mode depending on its design. The efficiency of PEFAE depends on several parameters, such as electric field strength, specific energy input, the number of pulses, the time between the pulses, treatment temperature, treatment time, and sample properties.[99,100] The advantage of PEFAE is that it minimizes the degradation of thermolabile compounds because of its ability to function at moderate electric field strengths.

Guderjan et al.[101] reported that β-sitosterol and campesterol are the predominant phytosterols obtained

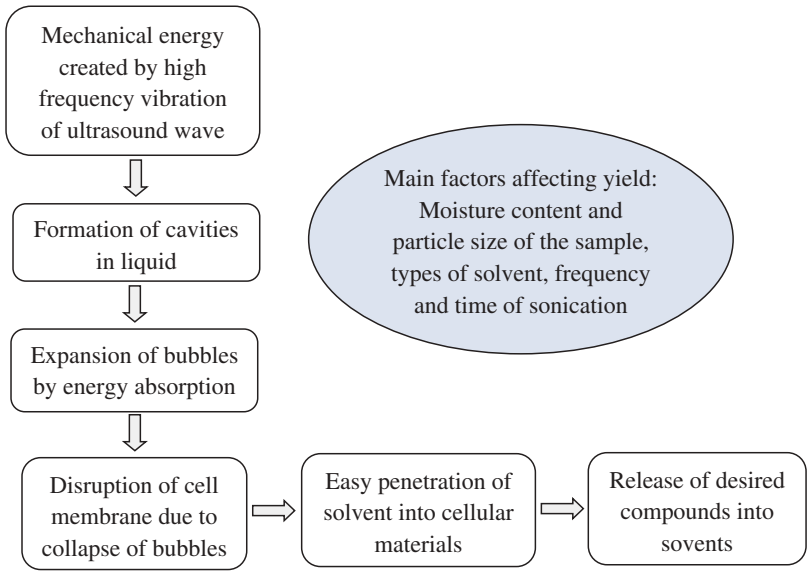

Figure 5. Mechanism of the ultrasound-assisted extraction (UAE) method.

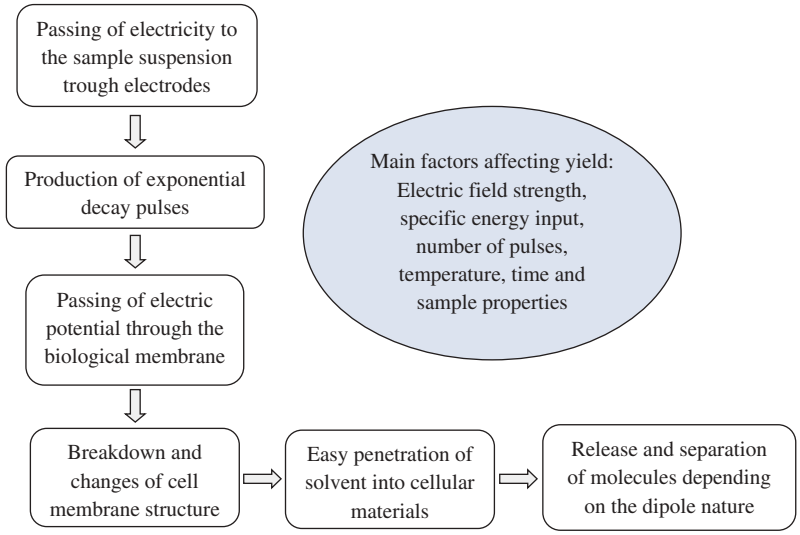

Figure 6. Mechanism of the pulsed electric field-assisted extraction (PEFAE) method.

from rapeseed oil through PEFAE. A pulse electric field is also used as a pretreatment process before conventional extraction for reducing extraction efforts. Guderjan et al.[102] found that the phytosterol yield of maize germs increased by 32.4% with an additional mild application of PEF (0.6 kV/cm).

Pressurized liquid extraction

Nowadays, PLE has been referred to as by various names including pressurized fluid extraction (PFE), subcritical solvent extraction (SSE), accelerated solvent extraction (ASE), high-pressure solvent extraction (HPSE), and enhanced solvent extraction (ESE).[62,103,104] In PLE, elevated pressures and temperatures are used, which drastically increase the extraction process. The main concept is that the increased temperature accelerates the extraction kinetics and the applied pressure, usually 4 to 20 MPa, ensures the solvent remains in a liquid state above their normal boiling point.[103] High pressure also facilitates extraction by driving the solvent into the pores of the sample matrix. In contrast, high temperatures reduce the solvent viscosity and increase diffusivity of the solvent resulting in better penetration of the solvent into the matrix, which enhances the extraction efficiency.[78] The PLE technique can reduce the extraction time and the solvent requirements, because the combination of high pressures and temperatures improve the extraction rate. Several parameters such as pressure, temperature, extraction time, cycle, nature of the solvent, and the sample matrix are the influential factors in PLE.

Péres et al.[62] carried out the extraction of phytosterols and other phytochemicals from the leaves of *Piper gaudichaudianum* Kunth by Soxhlet extraction, UAE, and PLE using petroleum ether and ethanol. The authors reported that the main phytosterols, β-sitosterol, and stigmasterol, were present in higher amounts in the extract obtained by PLE compared with the other extracts. Phytosterol extraction from tea leaves via PLE has been reported by Jacques et al.[105]

Supercritical fluid extraction

Among the non-conventional extraction techniques, SFE is widely used to extract bioactive compounds, including sterols from various plant matrices. Nowadays, the populations are aware of the deleterious effect of organic solvents utilized in the food and pharmaceutical industries. Large quantities of organic solvents are used during liquid solvent extraction and for the isolation of bioactive compounds from natural sources. Moreover, the application of high temperatures during the extraction and evaporation of solvents may degrade thermally sensitive functional compounds. SFE is an effective alternative for organic solvent extraction of functional compounds from natural sources.[106–108] Therefore, it is being used successfully in environmental, pharmaceutical, and polymer applications as well as for food analysis.[109]

Supercritical fluid (SF) defines the state of a substance above its critical temperature and pressure. It contains both the properties of liquid and gas and may, thereby, exist as a fluid instead of either a liquid or gas. Stahl et al.[110] and Taylor[111] have discussed the properties of SF in detail. The gas-like properties of viscosity, diffusion, surface tension, and the liquid-like density and solvating power make SF a suitable solvent

for extracting bioactive compounds in shorter periods of time with higher yields.[112] SF has advantages over liquid solvents such as (a) the solvating power of SF depends on its density, which can easily be adjusted by changing the temperature and pressure and (b) mass transfer is greatly favored because of a higher diffusion coefficient and lower viscosity of SF than the liquid solvents.[17,113]

Carbon dioxide (CO_2) is a non-polar molecule, which makes it an ideal solvent for lipid and lipid-soluble substances, but it is unsuitable for use with most pharmaceuticals and drug compounds. The application of polar solvents such as methanol and ethanol as a modifier has overcome the limitation of the low polarity of CO_2.[114] Generally, an SFE unit consists of several basic parts including a tank for CO_2, a high pressure pump, a chiller for cooling the CO_2, an oven or water bath for controlling the temperature, a cosolvent pump, a back pressure regulator for controlling the CO_2 flow rate, an extractor vessel, and a separator. An SFE system is also equipped with a temperature indicator and gas flow meter.

The application of CO_2 as an SFE has attracted considerable attention because supercritical CO_2 (SC-CO_2) extraction offers further advantages compared with conventional extraction processes.[114] The critical temperature (31.2°C) and pressure (7.38 MPa) of CO_2 are low, which allow operation of SFE using CO_2 at moderate conditions. Moreover, CO_2 is non-toxic, non-flammable, colorless, odorless, inert to most materials, safe, inexpensive, and recyclable.[113,115] Using only a depressurizing process is another advantage offered by using CO_2 as an SFE. SC-CO_2 has been used as a solvent for more than 90% of all compounds extracted from natural sources using SFE.[113]

For an effective SFE, all extraction parameters, such as pressure, temperature, the flow rate of CO_2, extraction time, the solvent to feed ratio, particle size, and moisture content of the sample, have to be optimized.-[116] Other factors, such as sample pretreatment and storage conditions, also affect the yield of the extract as well as the target compounds. Nyam et al.[117] optimized the extraction conditions for phytosterol-enriched oil from Kalahari melon seeds using SC-CO_2. The optimum conditions for the extraction of phytosterols were a pressure of 30 MPa, a temperature of 40 C, and a CO_2 flow rate of 12 mL min^{-1}. Among the extraction parameters temperature significantly affected the phytosterol concentration in Kalahari melon seed oil. In another study, phytosterols were extracted from Roselle seeds using SC-CO_2.[118] The optimal extraction conditions (temperature of 40°C, pressure of 40 MPa, and CO_2 flow rate of 20 mL min^{-1}) were slightly different from the extraction of phytosterols of Kalahari melon seed.

Phytosterol yield may also be influenced by the varieties of plant matrices. Shen et al.[119] performed the SC-CO_2 extraction of phytosterols from rice bran testing various extraction conditions (pressure of 17–31 MPa, temperature of 0–60°C, CO_2 flow rate of 2.5 kg h^{-1}, and extraction time of 6 h). They found that the yield of phytosterols was higher using high pressure and moderate temperature. In less than critical conditions, the yield of phytosterols was lower compared with critical conditions. β-sitosterol has been extracted from sea buckthorn seeds at pressures ranging from 15 to 60MPa and temperatures ranging from 40 to 80°C.[120] The optimal solvent consumption and extraction rate was found using 60 MPa and 40°C.

Analysis of phytosterols

As previously mentioned, phytosterols are present in nature as free sterols, esters of sterols, steryl glycosides, and acylated steryl glycosides. For the determination of total phytosterols, the sample preparation technique should include all possible conjugates of sterols. Since phytosterols are found together with other non-saponifiable components in plant lipids, a reliable analytical technique is needed to evaluate them. Since the discovery of phytosterols, several methods have been developed for quantitative measurement. The first generation approach frequently applied for phytosterol determination was the digitonin precipitation method.-[121] Following that, a more sensitive colorimetric method involving enzymatic oxidation was introduced for the determination of phytosterols. However, this method became obsolete due to the tiresome operation and expensive reagents.[122] The extracts obtained by various techniques have been analyzed by different chromatographic techniques to quantify and characterize the sterol compounds, including gas chromatography (GC), column chromatography (CC), high-performance liquid chromatography (HPLC), and capillary electrochromatography (CEC). Thin layer chromatography (TLC) has been applied for the preliminary assessment of phytosterols qualitatively and quantitatively.

Nowadays, GC and HPLC have been extensively employed in phytosterol determination. HPLC can work at pressures lower than 40 MPa. On the contrary, ultra-performance liquid chromatography (UPLC), a new technology that can be operated at pressures up to 100 MPa, has been used to determine the phytosterols from grains.[123] The detection of

phytosterols can be performed by flame ionization detection (FID), nuclear magnetic resonance (NMR), infrared, ultraviolet (UV) detection, evaporative light scattering detection (ELSD), and mass spectrometry (MS).[124]

GC-FID and GC-MS are widely used to analyze plant sterols. However, better identification and quantification of phytosterols has been achieved by GC coupled with electron impact or chemical ionization MS.[125] The analytical techniques of phytosterols in dietary products have been extensively reviewed by Abidi[124] and Lagarda et al.[7] The combination of methods such as flash liquid chromatography and the flash version of silver ion liquid chromatography has been developed by Francavilla et al.[5] to separate the total sterol fraction and purify the most abundant phytosterols. They claim a purity of 97.87% and a recovery of 98% using these two methods combined. Tables 1 and 2 show the analysis of phytosterols using GC and HPLC. Individual phytosterols have been analyzed by several successive steps such as lipid extraction, saponification and/or acid hydrolysis, and finally purification and quantification.[7] Various chromatographic techniques require the saponification of extracts before phytosterol analysis.

Gas chromatography (GC)

GC is the most frequently and widely used technique for the determination of sterols. A capillary column provides potential advantages by reducing the time for analysis, improving peak resolution of the components, and offering high thermal stability compared with a packed column.[124] Most of the phytosterols have been determined by GC with a non-polar stationary phase. The stationary phase mainly contains cross-linked polysiloxanes. A slightly polar stationary phase with 5% diphenyl and 95% dimethylpolysiloxane showed high thermal stability and resulted in better resolution of peaks for individual phytosterols. For the GC analysis of sterols, the extracted unsaponified materials are derivatized as trimethylsilyl (TMS). It is hard to improve the peak shape and resolution for individual phytostanols and phytosterols without derivatization.[126] N-methyl-N-(trimethylsilyl)trifluoroacetamide in anhydrous pyridine and bis (trimethylsilyl)-trifluoroacetamide with 1% of trimethylchlorosilane are usually used as derivatized agents. Hexamethyldisilazane with dry pyridine and trimethylchlorosilane are also used for derivatization. Phytosterols are routinely analyzed by GC-FID based on their retention time. On the contrary, GC-MS is utilized to confirm the peak identities of individual phytosterol and their quantities.[7]

High-performance liquid chromatography (HPLC)

Liquid chromatography, especially HPLC, provides some advantages over GC. HPLC can be operated under low column temperatures, and it is possible to analyze and collect the phytosterols as purified forms because of the non-destructive detection conditions. HPLC has been used to analyze phytosterols both on an analytical and preparative scale. HPLC is highly suitable for analyzing thermally sensitive phytosterols. Phytosterols can directly be analyzed by HPLC without loss of sample if the extracted lipids are present in a simple form. Normal phase HPLC has been used to separate and quantify the five major lipid classes of phytosterols (steryl esters, free sterols, steryl glycosides, acylated steryl glucosides and steryl ferulates). In reversed-phase HPLC, adding low volatile polar organic solvents to water makes a rapid equilibration between the bonded silica stationary phase and mobile phase solvents compared with normal-phase HPLC.[124] Octadecyl silica (ODS C_{18}) columns are widely used for the analysis of phytosterols. Various organic solvents such as acetonitrile, methanol, isopropanol, tetrahydrofuran, and acetic acid are used to separate phytosterols in HPLC. Sterols have been detected using UV spectroscopy in the range of 200 to 210 nm, ELSD, refractive detection, and MS.

Phytosterols in *Cissus quadrangularis* L. have been analyzed by HPLC equipped with a C_8 column, PDA, and UV detector.[127] A better peak shape and resolution of β-sitosterol and stigmasterol was found using an isocratic elution with acetonitrile and water (95:5 v/v), which minimizes the variation in the baseline and unexpected peaks. Liu and Ruan[55] reported a new method for quantification of phytosterols based on HPLC by using a benzoyl chromophore as a derivatizing agent. The benzoyl group has been introduced into phytosterols by simple derivatization, which highly improved the UV response at 254 nm.

HPLC-NMR analysis

The HPLC-NMR method has been used to analyze phytosterols in natural extracts obtained by SC-CO_2.[128] A structure-sensitive detection technique such as ^{1}H-NMR can compensate for the lack of a sample standard. Similar signal fingerprints in ^{1}H-NMR have been found for most of the free phytosterols.[129] The conjugated phytosterols show the same signal fingerprint with additional signals for substituents at the C-3 position.[130] Deuterated chloroform and acetonitrile as a mobile phase have been used

in HPLC-NMR for phytosterol analysis. Recently, Sosińska et al.[131] assessed the chemical structure of the dimers formed during thermo-oxidative degradation of β-sitosterol by NMR and IR spectroscopies.

HPLC-MS analysis

HPLC-MS is also used for phytosterol analysis. Notably, atmospheric pressure chemical ionization (APCI) with liquid chromatography-mass spectrometry (LC-MS) has been applied for the analysis of phytosterols from natural sources.[132,133] It is hard to ionize sterols through a conventional electrospray method because of the high lipophilicity of polar functional groups present in phytosterols. Electron ionization (EI) and atmospheric pressure photoionization (APPI) techniques are also fruitful alternatives for lipophilic compounds.[134] Mo et al.[135] introduced a method in which phytosterol analysis has been performed by HPLC based on positive ion APCI tandem mass spectrometry (LC-MS/MS). The most abundant dietary phytosterols and structurally related triterpene alcohols include brassicasterol, campesterol, cycloartenol, β-sitosterol, stigmasterol, and lupeol in edible oils have been measured using APCI LC-MS/MS. This new method provides an advantageous combination of speed, selectivity, and sensitivity for phytosterol analysis.

Capillary liquid chromatography (CLC) analysis

The applications of capillary liquid chromatography (CLC) and nano-liquid chromatography (nano-LC) have increased in the analytical field. The phytosterols in extra-virgin olive oil have been evaluated by nano-LC coupled with UV spectrophotometry and MS.[54] The analysis of phytosterols has also been carried out via supercritical fluid chromatography (SFC) where SC-CO_2 is used as a mobile phase.[136] SFE coupled with SFC provides the advantage of a single operation including extraction, pre-concentration, fractionation, and the quantification of phytosterols.[137,138]

Conclusion

It is well established that phytosterols have beneficial roles in the food and pharmaceutical sectors. The extraction and isolation techniques for phytosterols are still complicated and time-consuming. The increasing demand of phytosterols and other bioactive compounds encourage the development of convenient and efficient methods to extract and isolate them from various plant sources. In conventional extraction, large quantities of organic solvents are needed that require proper disposal from an environmental protection perspective. In non-conventional extraction, several parameters can be controlled simultaneously. Selecting the most appropriate method through optimizing the extraction conditions can reduce solvent consumption. Moreover, some non-conventional methods, such as SFE, using CO_2 could be used without using any hazardous organic solvents. Furthermore, most of the non-conventional methods yielded high amounts of phytosterols with green products. However, the measurement of extraction efficiency is influenced by proper standard methods. Until recently, GC and HPLC were the most commonly used methods for the analysis of phytosterols. Although GC is the best choice for identifying phytosterols in samples, HPLC can also be considered to determine and isolate individual phytosterols. To avoid or reduce organic solvents, SFC can also be effectively used; however, more experiments are required to establish proper working conditions and instrumentation. The increasing demand for these bioactive compounds as a supplement in foods may lead to further innovations in extraction, isolation, and analytical methods that are more efficient, rapid, and environment-friendly.

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Declaration of interest

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