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REVIEW

Technologies for the Extraction and Post-extraction of *Stevia rebaudiana* Leaves

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Abstract

Due to the perceived link between sugar and diseases such as diabetes, obesity, and cardiovascular diseases, the market for low-calorific sugar alternatives or sweeteners has intensified in recent times. Stevia (*Stevia rebaudiana Bertoni*) is an herbaceous perennial shrub and a member of the *Asteraceae* family. Stevia has found use as an alternative sugar product in beverages and other foods due to its low cost, low calorific content, and health benefits. This study is a review of various technologies that have been employed by various researchers in the processing of stevia leaves into syrup, powder, or crystals. The merits and demerits of each technology for the extraction process were also discussed. It was observed that its leaves contain the highest concentration of sweeteners and that solvent extraction is the most widely adopted extraction technique by various researchers, which has been reported to give a stevioside yield of more than 90%. The use of green-assisted extraction techniques, such as supercritical fluid extraction, ultrasonic-assisted extraction, microwave-assisted extraction, pressurized fluid extraction, and enzyme-assisted extraction, offers a higher yield, better recovery, and higher purity of the steviosides. However, these technologies are yet to be utilized on an industrial scale. Post-extraction technologies such as chromatography have been reported to give an extract purity of about 97%. Other post-extraction technologies include adsorption and membrane technology for purification and/or concentration of the extract. The study also identified potential knowledge gaps that might help drive future research in the field.

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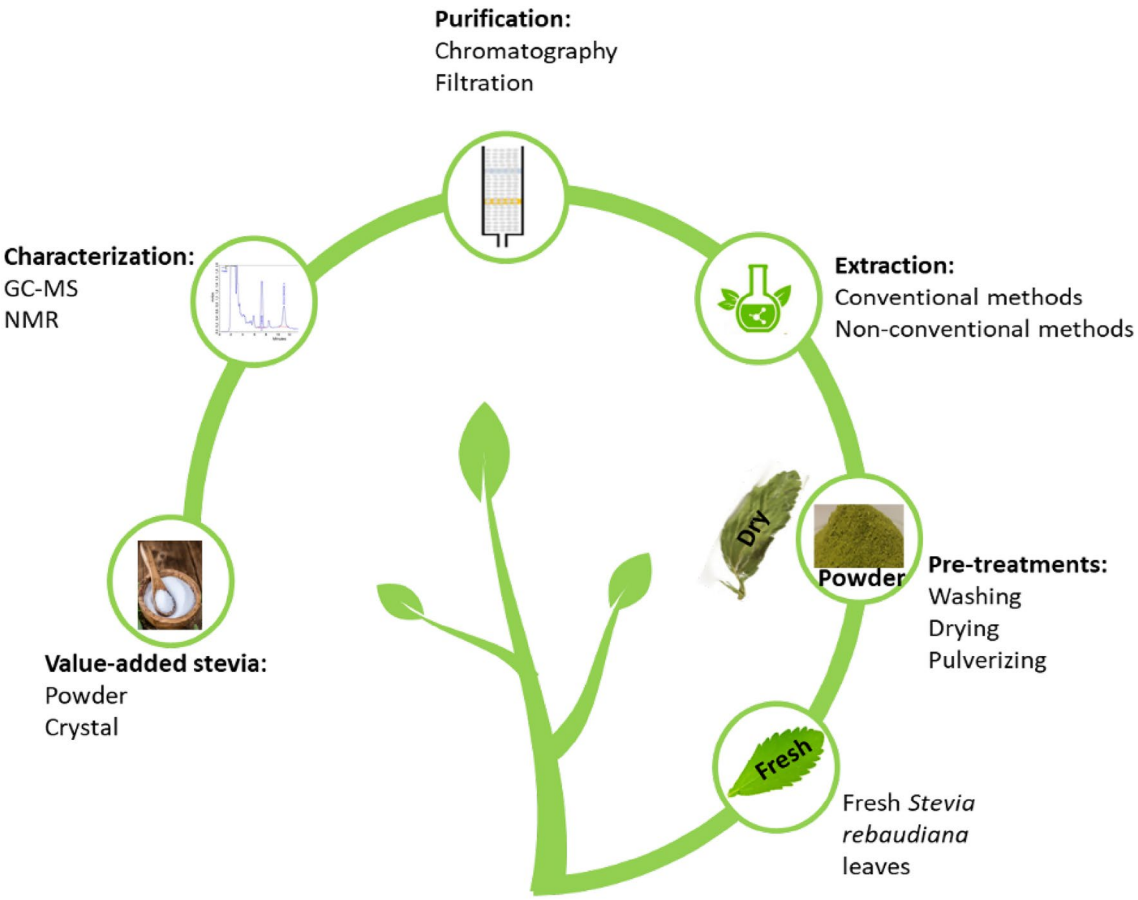
Graphical Abstract



Keywords Green technology · Food industry · Rebaudiosides · Stevia powder · Stevioside

1 Introduction

Since ancient times, sugar has been the preferred sweetening agent in the food sector [1–3]. The rise in the consumption of sugar has, however, been reported to have caused diabetes, obesity, and cardiovascular diseases as well, as several other medical and nutritional issues [4]. In recent years, customers have started using natural and dietetic food products as a result of increased health consciousness. Therefore, low-calorie sweeteners have been researched as sugar substitutes. Sweeteners from synthetic and natural sources are both available in the market. Examples of such synthetic sweeteners include sucralose, acesulfame-K, saccharin, and neotame [4]. Such artificial sweeteners lack the authentic sweetness of sugar due to their frequent metallic flavor [4, 5]. The metallic taste is attributed to the presence of certain chemical groups in their molecular structures. These sweeteners contain functional groups, such as sulfonamides, amides, and chlorine atoms, which are responsible for the metallic aftertaste. When these compounds come into contact with taste receptors on the tongue, they can trigger an unusual sensory response, resulting in the perception of a metallic flavor. A high number of consumers are enthusiastic about sweeteners that have low calorific value and a sugar-like taste profile [6]. Hence, natural source sweeteners made from stevia have attracted a lot of attention recently since they have several of these desired characteristics [6].

Stevia (*Stevia rebaudiana Bertoni*) is an herbaceous perennial shrub, a member of the *Asteraceae* family, and is indigenous to specific parts of South America, including the Parana Estate in Brazil and the Amambay Mountain Range in eastern Paraguay [4, 7]. It consists of roughly 150–200 species of perennial shrubs and herbs, most of which occur in semidry highlands at elevations of 500–3000 m [8]. The mature plant can reach a height of 1.8 mm when grown on more fertile soils. Additionally, the species can flourish in sub-alpine regions, scrubby woods, and grasslands [4]. For the expanding natural food sector, this plant is predicted

to rise in importance and become a significant source of high-potency sweeteners. This plant is safe, poses no risk to human life or health, and offers great promise for the creation of a calorie-free sweetener with positive health effects [4]. In fact, Stevia is Generally Recognized As safe (GRAS), a regulatory review process category used by the U.S. Food and Drug Administration (FDA).

Stevia is most well-known for the buildup of a highly sweet-tasting (calorie-free) combination of diterpene glycosides [9], recognized as steviol glycosides in its leaves, which are centered on the ent-kaurene skeleton referred to as steviol [8, 10]. The steviol glycosides quantities depend significantly on the production environment and genotype, and it has been linked to the high sweetness in stevia leaves, which is 250–300 sweeter than sucrose [4, 9, 11]. The main compositions of the compound glycosides include stevioside, dulcoside A, rebaudiosides (A, B, C, D, E, F), and steviolbioside [7, 9, 11, 12]. According to research, stevioside (4–13%) is the most prevalent steviol glycoside present in the plant leaves, followed by rebaudioside-A (2–4%), rebaudioside-C (1–2%), and dulcoside-A (0.4–0.7%). Other compounds, including steviolbioside and rebaudioside B, D, E, and F, were also found in leaf extracts, but they were relatively minor components [6, 7, 11]. Aside from Stevia's ability to sweeten, its leaves have significant therapeutic advantages, which is why there is growing interest in using this aqueous extract [11]. The chemical found in stevia leaves has antioxidant, antifungal, antibacterial, antitumor, duretic anti-hypertensive, anti-inflammatory, antidiarrehic, antihyperglycemic activities and diabetic patients may take it safely [7, 9, 11]. Hence, consuming dried Stevia leaf infusions directly or incorporating them into a variety of food combinations, which include juices, pastries, jams, and confectionary goods, could improve the functional qualities of these goods [11].

Various researchers have reported several methods, including both conventional and modern extraction techniques, that have been employed to extract, separate, and purify the active compounds within the stevia plant [11, 13–17]. Steviosides have been extracted from stevia plants by numerous researchers, and their findings, based on a multitude of processes, have been well documented, including within various review articles. For instance, Bursać Kovačević et al. [18] and Raspe et al. [19], explored advanced extraction methods for valuable compounds from stevia leaves. However, these studies neglected to utilize simpler technologies, such as solvent extraction, for achieving the same purpose. Similarly, in another study, Wang et al. [20] reviewed the application of various engineering techniques for the extraction and post-extraction of stevia. Regrettably, this study failed to delve into the intricate technical aspects surrounding these diverse techniques and was further limited in its scope of covered techniques. To the best of the authors' knowledge, a comprehensive study that thoroughly discusses both simpler and advanced technologies for steviosides extraction from stevia, while elucidating their strengths and limitations, remains conspicuously absent. Bridging this gap is a fundamental objective of the present study.

In this regard, this study is a review of literature that has been published on extraction and post-extraction technologies for obtaining stevioside from stevia leaves. The objectives of this study is, therefore, to compare and critically analyze the different technologies used for the extraction of stevioside from stevia plants, their merits and limitations, post-extraction technologies for the production of stevia syrup, powder, and crystals. The study also discusses various characterization techniques that have been utilized for the analysis of stevia extract.

2 Production of Value-Added Stevia

In order to increase the value of stevia, the leaves are processed into syrup, powder, or crystals. The process is summarized in Fig. 1. The leaves are first pre-treated, then the glycosides are extracted with the use of conventional methods (solvent extraction) or non-conventional methods (green-assisted extraction methods). Thereafter, the extract is purified, concentrated, and crystallized, depending on the finished product of interest.

The pre-treatment stage is the preliminary phase in any consumable or high value added product extraction operational procedure, including the extraction of stevia glycosides. To start the process, the plant matrix is cleaned with distilled water to get rid of any dirt and other impurities. The plant matrix is then dried using either air drying, shade drying, freeze drying, microwave vacuum drying, convection drying, or oven drying [15, 21–38]. The dried plant matrix is next pulverized with the aid of mechanical or electrical grinder to create powdery mote with a better surface-volume ratio which improves the effectiveness of extraction by increasing the interaction of the material with the solvent [15, 21–23, 25, 31, 35, 36, 39]. However, some scholars may argue that even though the pulverization of the leaves increases its surface area, it could pose some problems such as extended filtration duration, loss of volatile compounds as the mechanical or electrical grinding process might generate heat, and degradation of sensitive compounds sensitive to the mechanical forces during grinding, and increased energy consumption [40].

With the advancement of extraction technologies, the nutraceutical and medicinal value of stevia has drawn more attention [18]. Extraction is an instantaneous and effective method employed to separate and concentrate the extracted substances, whereby one or more substances are

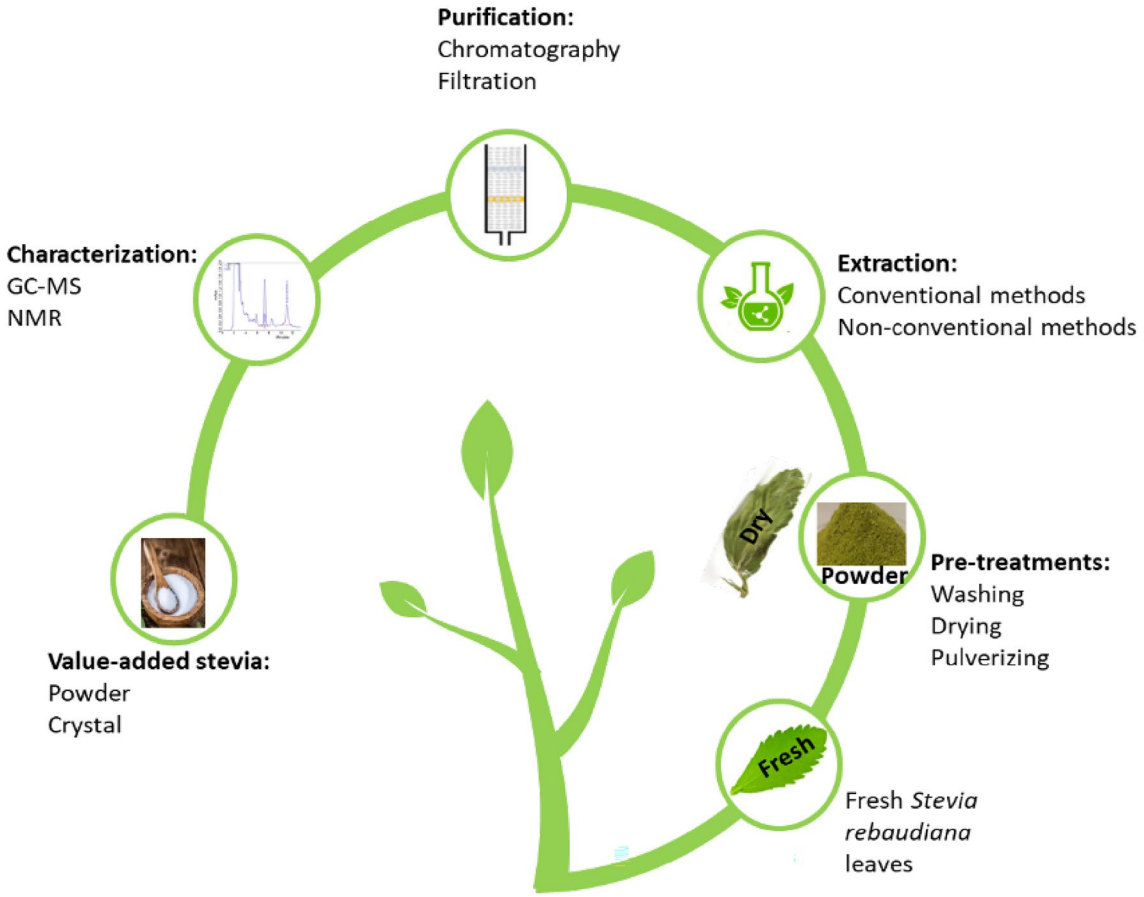


Fig. 1 From matrix to finished product process

transferred from the matrix in which they are present, to the liquid phase of an extraction solvent. It is regarded as one of the chief crucial phases in herbal products production because it has an impact on the active ingredients in the sample both qualitatively and quantitatively [41–44]. Generally, extraction depends on a variety of factors, such as the extraction method (Fig. 2), raw materials, extraction solvent physicochemical profile (polarity, viscosity, volatility, stability, toxicity and purity), solid–liquid ratio, extraction temperature, pH and time. Thus, for productive extraction of stevia, it is crucial to optimize these processing parametric conditions as they invariably affect the yield, physicochemical properties, uses and nutraceutical functional activities of the targeted sweetener compound of interest [19, 20, 43, 45–54]. After proper extraction, the next is further purification, and characterization of the substances from the liquid phase using various techniques, in order to remove impurities and other unwanted ingredients (such as essential oils, tannins, and flavonoids in the crude extracts that are partly responsible for some of the off-tastes of the finished product) [18, 22, 44, 48, 55–58].

Furthermore, it is imperative to say that since the method of extracting bioactive from medicinal plants plays a vital role in providing consumerists a high-quality product, various conventional extraction procedures have already been established for the extraction of both liquid and powdery stevia. However, these conventional procedures have some downsides and in order to eliminate these limitations, novel assisted green extraction techniques have been proposed and established for eco-efficient extraction of both liquid and powdery stevia with better yield in short time and recent advances of these methods are discussed below [19, 44, 59–65].

Rajasekaran et al. [66], through their study using hot water solvent extraction technique, has confirmed that all organs of stevia plant (leaves > shoots > roots > flowers) contain the natural sweetener compounds, even though it was greatly upheld that the leaf part contain the highest concentration (64.80 g steviolbioside kg^{-1} dried plant material), and this implies that they serve as the chief organ for both synthesis and principal accumulation of stevioside compounds, and makes stevia leaves to be the

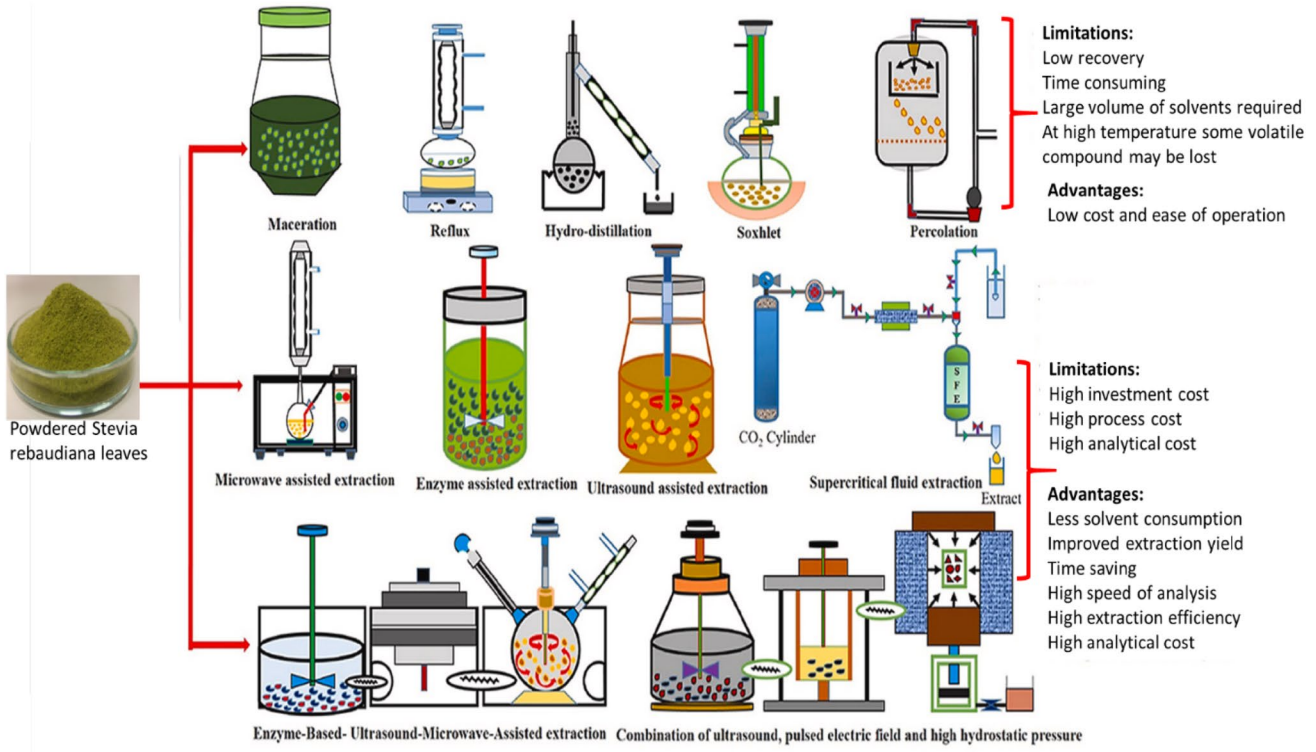


Fig. 2 Commonly used techniques for the extraction of stevia [44]

most widely used part in the production of the high value added steviol glycosides [66]. In a similar manner, Serfaty et al. [8] also investigated the potential impact of agro-technical parameters (planting period, plant density in the field, and harvesting regime) on the percentage yield. According to their research, the plants that were picked in September, just as flowering was beginning, had the highest stevioside yields. Specifically, in early September, the stevioside output peaked at 40 g/m^2, which is determined by dividing the stevioside concentration in leaf matrix by the aggregate dry weight of the leaf matrix. This may be because the process of flowering in Stevia plants triggers certain changes in their physiology. When the plant starts to flower, it undergoes a shift in stevioside allocation from leaf growth to reproductive development. As a result, the stevioside output may decrease. However, by picking the plants just as flowering commences, the stevioside concentration in the leaves remains high. Figure 3 illustrates that although the stevioside concentration in leaf topped out in June at 12.5%, this concentration was paired with the lowest leaf yield of 0.08 kg/m^2, which led to a low stevioside yield. However, the maximum stevioside production was achieved in the very beginning of September owing to the still high stevioside concentration (11.7%) and the high leaf biomass output of 0.35 kg/m^2[8].

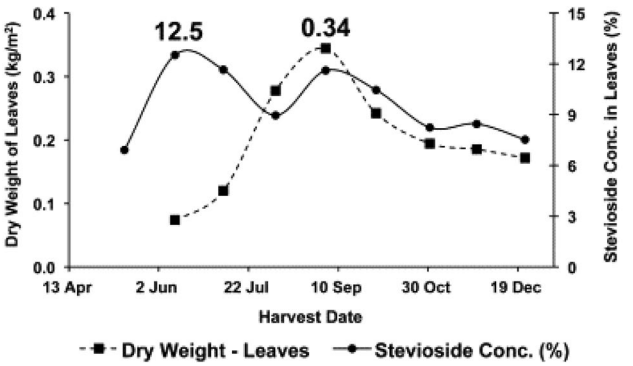


Fig. 3 Stevioside concentration in leaf and leaf dry weight during sequential harvest periods [8]

3 Solvent Extraction

Historically, solvent extraction is a conventional method of extraction that was first used to extract the steviol glycoside natural sweetener from stevia leaves, and this was patented on the account of Persinos in 1973 [67]. Shortly, as described in recent time by Gunasena et al. [63], in the solvent extraction procedure, glycosides are first extracted into hot water and then into an organic solvent that is water immiscible, like chloroform or hexane. To obtain the solid

mass, the organic phase is separated, concentrated, and then dissolved in warm methanol. Steviol glycosides crystallize as a result of cooling and are then cleaned with ice-cold methanol. To obtain high purity steviol glycosides, they are finally recrystallized from methanol or water [63].

Rai et al. [7] used response surface approach to conduct a hot water extraction for the extraction of steviosides from dried stevia leaves matrix, while examining the impact of the ideal operating conditions on the extraction output. Over the course of the investigation, 6.64 to 10.75% of the dried stevia leaf were extracted as stevioside. However, it was discovered that the ideal operating conditions for stevioside extraction are temperature of 78 °C, heating period of about an hour, and solid to liquid ratio of 1:4 (g/mL). This is because at these settings, 10.45 g of stevioside is extracted from 100 g of dried stevia leaves [7]. The extraction protocol and yield was somewhat similar to that obtained by Rajab et al. [68]. In a similar vein, the two significant glycosidic sweeteners contained in *S. rebaudiana bertoni* were extracted, separated, and purified by Kumari et al. [69] using a straight-forward, cheap, reflux apparatus, thin-layer chromatography, and column chromatography procedures. Since these two substances have polar behaviors due to their glycosidic nature, they were extracted using polar solvents such as methanol at 65 °C for roughly an hour by simple extraction method shown in Fig. 4 [69]. Similar to this, ethanol was used with reflux apparatus by Shukla et al. [70] but a percentage yield of 4.5% (w/w) was obtained.

Abou-Arab et al. [71] extracted stevioside from Stevia dry leaves by three approaches. In their first method, hot universal solvent (water at 65 °C) was used for solvent extraction at various leaf-to-water ratios (1:15–1:75). It was discovered that the ideal ratio was 1:35, which had the highest stevioside content (7.53%), and highest recovery (80.21%). As opposed to the hot water solvent extraction, the second approach used methanol for extraction at a 4:1 ratio, yielding greater stevioside recovery (94.90%). In the third procedure, an extraction was carried out using a 4:1 binary solvent mixture of methanol and water. The recovery of stevioside (92.34%) using the binary solvent mixture was higher than that of regular hot water extraction. They came to the conclusion that methanol is best for isolating stevioside from Stevia leaves since it demonstrated the maximum extraction efficiency. However, stevioside's purity for methanolic extraction, though, lagged behind that of water extraction. According to the Total Soluble Solids "TSS" and depigmentation results, water has a higher capacity to extract soluble solids than methanol and methanol/water extraction, which may explain the difference in purity. Additionally, same impact was seen when more pigments were extracted using water as opposed to methanol or a combination of methanol and water. They concluded that while water extraction required more steps for purification, methanol and methanol/water extraction only required

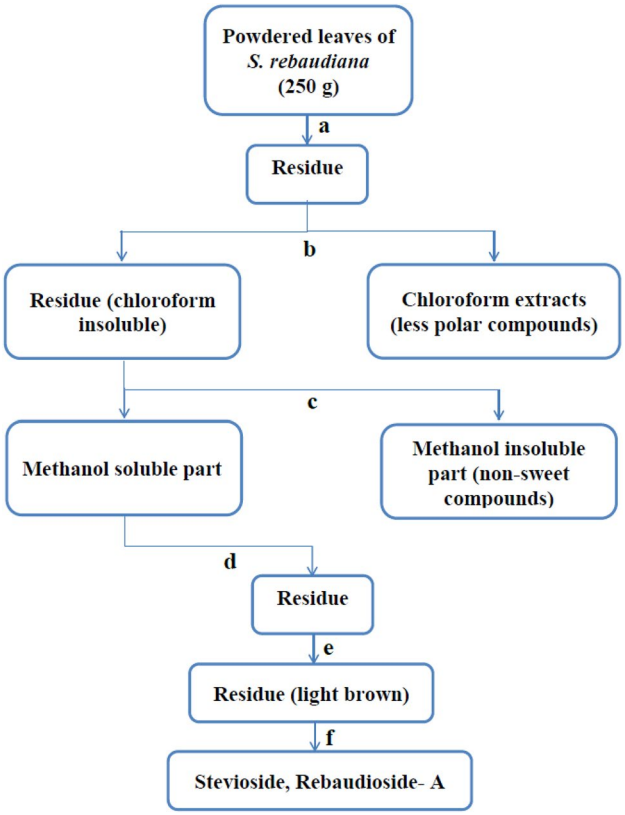


Fig. 4 Extraction of major stevia compounds. **a** Repetitive extraction with methanol via refluxion (21 h). Filtered each time, combined the filtrates, and distilled off the solvent; **b** chloroform refluxion; **c** dissolved in a small amount of methanol and refrigerated overnight; **d** distilled off methanol; **e** added water (0.2 L), extracted with n-butyl alcohol (3.20 L), combined all n-butyl alcohol layers, and distilled off solvent rotavapor; **f** column chromatography [69]

two processes, showing that both methods were far more straightforward and less complicated than water extraction. This is because water is a universal solvent and can extract a wide variety of compounds from the plant material, including sugars, organic acids, and other hydrophilic substances. This diversity of extracted compounds can make the extract more complex and require additional purification steps to isolate the desired target compound, such as stevioside in the case of *Stevia rebaudiana*. On the other hand, solvents like methanol or a combination of methanol and water tend to extract more specific and targeted compounds, such as steviosides, with less interference from other unwanted compounds. This selectivity simplifies the purification process as there are fewer impurities to remove, resulting in a more straightforward and efficient extraction method. Although the usage of methanol is nevertheless of major concern from an eco-economic perspective [71].

This claim about methanol's performance was similarly affirmed by Afandi et al. [72]. Due to the issue of hydrolyzable components and the challenges associated

with solvent removal in the customary solvent extraction, they performed the extraction, utilizing the soxhlet apparatus in their investigation. Among the variety of polar to non-polar solvents (Methanol, Ethanol, Acetone, water, Petroleum Ether, and n-Hexane) employed, methanol was shown to be the best solvent based on its efficiency of extraction and glycosides content. The better extraction yield of rebaudioside-A obtained using methanol and other polar organic solvent was due to the hydrophilic hydroxyl group's ability to enhance stevia's dissolution. However, they came to the conclusion that the non-polar solvents might be the most effective at removing the unwanted components because they produced an infinitesimal dark green powdery yield, which is a sign of substantial removal of chlorophylls and its undesirable allies, and because the yield from polar solvent extraction is time- and temperature-dependent (Figs. 5A and B), beyond which rebaudioside can oxidize and purity and extraction efficiency can also decline [72].

4 Green-Assisted Extraction Methods

Recent studies have shown that the yield, recovery and purity of steviosides by novel green-assisted methods is higher than that achieved by usual conventional solvent extraction using different solvents and processing conditions. Although their large industrial scaling up outbreak is still embryonically work in progress, but many feat has been accorded to laboratory and small industrial scale of these eco-benign methods.

4.1 Supercritical Fluid Extraction (SCFE)

As shown in Fig. 6, supercritical fluid extraction is a novel, eco-sustainable process that uses homogeneous solvent(s) created at temperatures and pressures above their critical points, where liquid and gas phases become indistinguishable, to extract the constituents of interest from the plant material [49, 73–81]. These types of supercritical homogeneous solvents which are non-condensable at temperatures and pressures above their critical point, can diffuse through solids like a gas and dissolve materials like a liquid, among others (hexane, nitrous oxides, sulfur hexafluoride, trifluoromethane toluene, pentane etc.). The most commonly employed supercritical solvent is CO_2 due to its numerous physiochemical qualities, heat transfer characteristics, thermodynamical profile, harmless reputation, nobleness, non-corrosiveness, colourlessness, inodorousness, inexpensiveness, inflammability and eco-benign demeanor [19, 73, 76, 77, 82–92]. CO_2 with its critical temperature (32 °C) and pressure (74 bar) can also be explored with co-solvents such as methanol, and water (subcritical fluid) in order to accelerate effusion rate, shorten extraction time and enable modulation of CO_2 non-polarity profile for effective extraction of polar compounds like the non-polar compounds [19, 49, 92–94].

For a long time, supercritical fluid extraction has been regarded as an effective method for various extraction studies due to its better selectivity and product purity, infinitesimal thermal denaturation, and ease of construction and design. It was employed for the first time in 1969 by Zosel to extract high-value-added compounds on an industrial scale following the first scholastic report on the solvation properties of supercritical fluids by Hannay and Hogarth in 1879

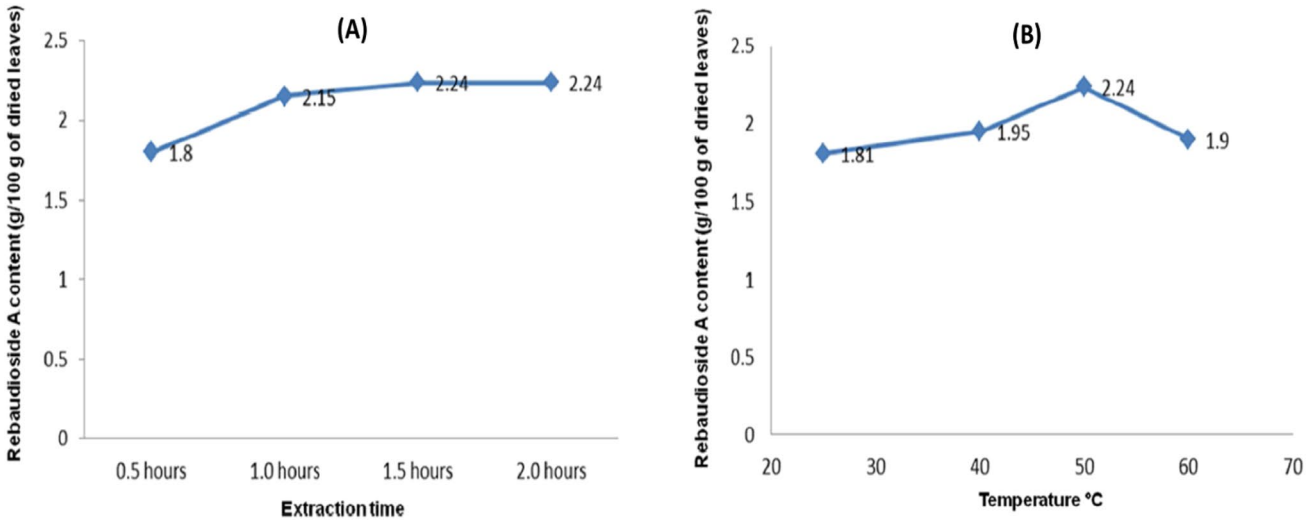


Fig. 5 **A** Influence of extraction time on rebaudioside A content. (T = 50 °C, 1:15 mass of dry leaf matrix: solvent ratio and three times extract); **B** Influence of extraction temperature on rebaudioside A content by absolute ethanol [72]

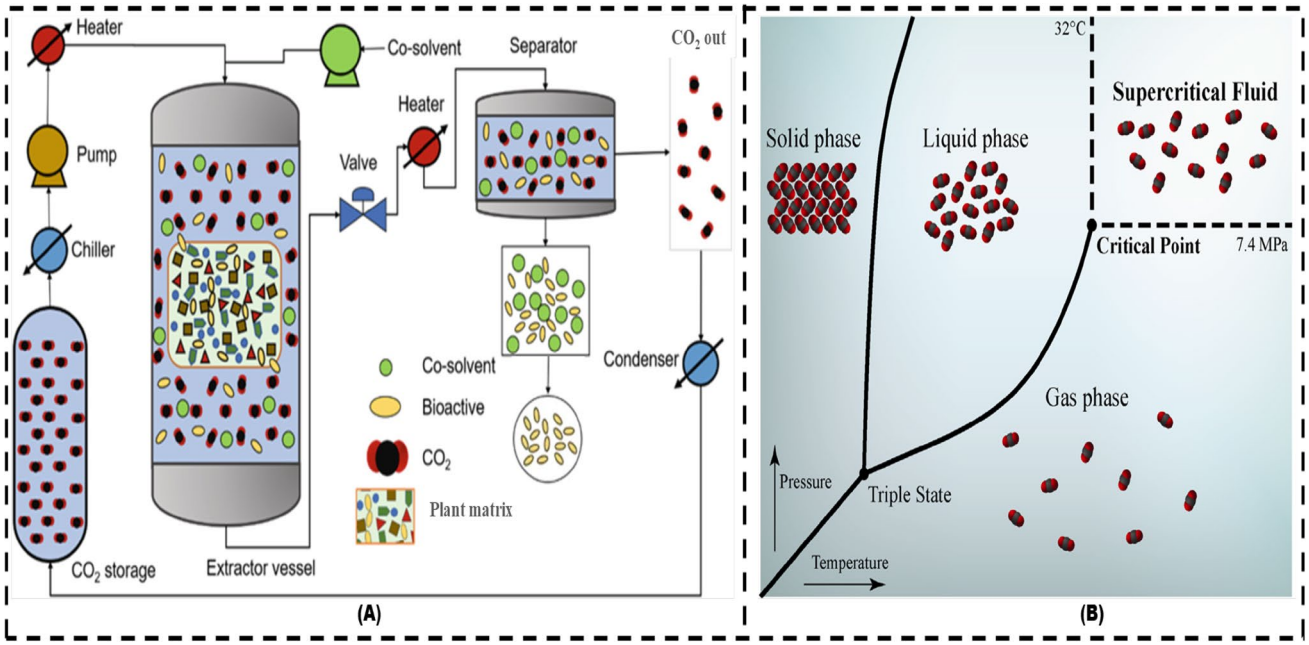


Fig. 6 **A** Operational procedure for the SCF extraction process [78]; **B** Phase diagram for a pure compound in a close-system. The triple point indicates the critical pressure & temperature of CO_2 [77]

[23, 85, 95–97]. However, patently speaking as regarding its utilization for stevia product, SCF for stevia extraction was first record in Japan by Tan et al. [98]. Thereafter, it has been used extensively as a far superior extraction system for extracting *S. rebaudiana* products that do not consist of unwanted or flavor-marring components [4, 16]. For instance, Water [99] in early 2000, had followed the patent by Tan et al. [98] to work on extracting steviol glycosides with better quality. In their investigation, it was shown that the overall average output for pre-treated stevia leaves with SCFE at 200 bar and 30 °C was 3.0 percent (m/m), and that almost 63 percent of this total output was achieved using the conventional method. In contrast, yields for SCFE using co-solvent were less than 0.50%, with an exception at 120 bar, 16 °C, and 9.5% H_2O. For this condition, the overall extraction output was 3.4%, and the traditional extraction was successful in obtaining up to 70% of the whole glycosidic fraction. Thus, it can be seen that using untreated or pretreated leaves did not significantly affect the yield for the conventional method. The value of the glycosidic component was better for the SCFE in terms of its potential as a sweetener in regards to the corresponding proportion of stevioside and rebaudioside A, which ultimately proved to be the success of their research [99]. A very similar SCFE procedure was also used by Yoda et al. [100] and the process extracted approximately 50% of the original stevioside and 72% of rebaudioside A [100].

In the same light, Erkucuk et al. [16] reported their investigation on supercritical fluid CO_2 extraction of glycosides from *S. rebaudiana* leaves. They examined the effect of varying pressure (150–350 bar), temperature (40–80 °C), and ethanol–water combination concentration (70:30) as co-solvents (0–20%) using CO_2 flow rates of 15 g/min for 60 min. The results showed that pressure above 300 bar had no discernible effect on the yield of the compounds, although temperature and co-solvent were more effective. It was further observed that when temperature rises, both the upsurge in solid volatility and the reduction in solvent denseness begin to vie for attention. In that, if the density effect was predominant, the solubility of the glycosides in the supercritical phase will decrease at higher temperatures and in a case where the vapor pressure is overwhelming, the solubility of the glycosides will increase with the increase in the vapor pressure. Meanwhile, as CO_2 is a non-polar solvent, the separation practice did not exhibit any unique selectivity irrespective of the temperature, whereas the dissolution rate of the glycosides in the supercritical phase decreased at elevated temperature when the density influence is dominant, whereas when the vapor pressure was dominant, the dissolution rate of the glycosides rose as the vapor pressure rose. In contrast, the addition of ethanol–water mixture as a polar co-solvent increased the solubilization of glycosides because polar co-solvents prompt alterations in the structure of the cellular matrix via intracrystalline and osmotic swelling and tear analyte matrix ties by contending with polar interactions between matrix and the compounds. Specifically, according to the Box-Behnken Design (BBD) results obtained as shown in Fig. 7**,** optimum

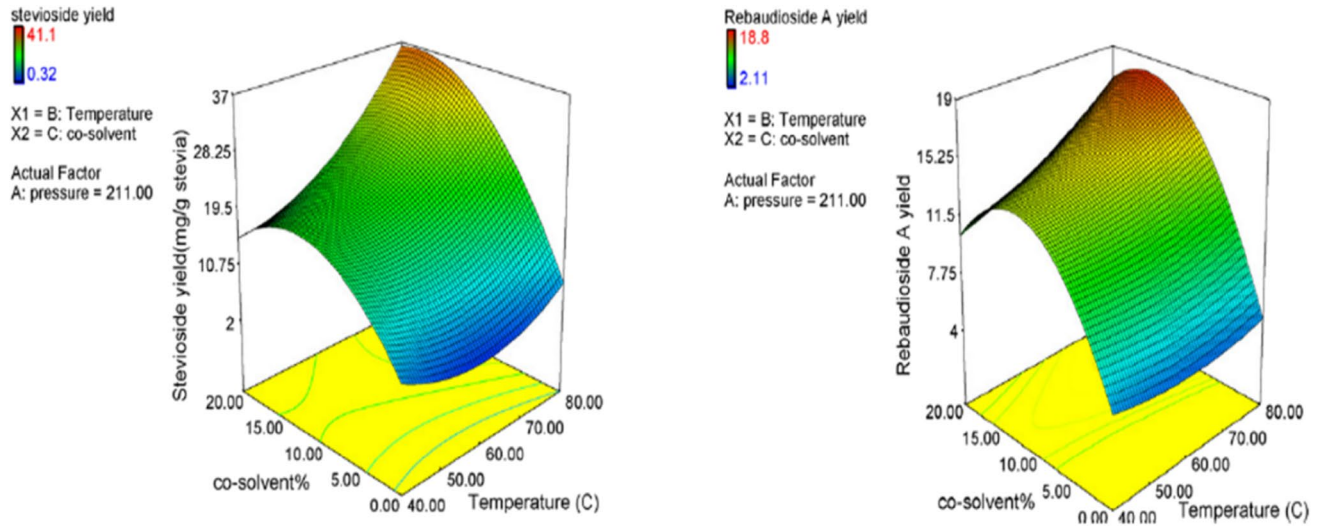


Fig. 7 Effect of co-solvent and temperature at constant pressure (211 bar) [16]

extraction conditions were prompted as 211 bar, 80 °C, and 17.4% co-solvent ratio, which gave rise to 36.66 mg/g and 17.79 mg/g for stevioside and rebaudioside A, respectively. The overall glycosides content was somewhat greater than ethanol extraction (48.60 mg/g) but comparable to those produced by means of classical water extraction (64.49 mg/g), highlighting the difficulties in applying SFE on an industrial scale [16].

Contrary to the supercritical fluid extraction methods that have been reported for the extraction of stevia glyocosides, Yildiz-Ozturk et al. [101] developed a faster subcritical water extraction system for the extraction of the two chief sweetener compounds. In comparison to the total steviol glycoside composition of water extracts reported in the literature, this method gave a higher yield (38.67 mg/g stevioside and 35.68 mg/g rebaudioside A, at optimum extraction conditions of 125 °C, 45 min, and flow-rate of 4 ml/min) and good extraction efficiency which was firmly ascribed to a comb of both improved solubility, faster mass transfer and dynamic supply of fresh water to wet and penetrate the plant matrix deeper at high temperatures. Owing to the fact that at a higher temperatures, physicochemical profile (viscosity, surface tension, and fast diffusion) of water were greatly influenced and thus reduces solvent strength of water close to non-polar compounds.

4.2 Ultrasonic-Assisted Extraction (UAE) and Microwave-Assisted Extraction (MAE)

Ultrasonic and microwave assisted extraction is among the most widely used promising green alternative technology for the extraction of bioactive compounds owing to their avalanche meritorious benefits such as low-cost, enhanced bio-accessibility of bioactive compounds, ease of unification with other techniques, less usage of organic solvents, industrial scale plausibility, low energy requirement, improved extraction yield at short duration and ease of operation [23, 49, 51, 81, 102–118]. From the mechanistic point of view as shown in Fig. 8A, during the ultrasonic assisted extraction, introduction of high frequency ultrasound waves of more than 20 kHz causes an upset in the matrix-solvent inter-mixture, resulting in cell wall breakdown and solvent permeation. More specifically as depicted in Fig. 8B, the mobility of ultrasonic waves that produce high intensity cavitation froth carrying solvent vapor arbitrates the extraction process. These cavitation eventually dissipates, transforming ultrasonic waves into mechanical energy. The cell wall is then torn by the mechanical energy, which also causes the particle size to decrease due to intra-molecular forces destroying the particle–particle connection. Thereafter, the bioactive compounds are released as the cell wall breaks [23, 106, 108, 109, 119–131].

For the microwave-aided extraction method, a dipole moment forms between the matrix and the solvent in the microwave when electric and magnetic fields are introduced, which causes heat transfer and mass transfer [85, 104, 132–135]. To put it in another perspective as shown in Fig. 9, the MAE approach uses microwaves to create high temperature and the evaporation of intracellular extracts, which causes the cell wall to breakdown and ultimately cause the release of intracellular active biomolecules into the solvent [23, 131, 136].

Carbonell-Capella et al. [138] reported an electro-technologically assisted method for the hydro-ethanolic extraction of steviol glycosides compounds from *Stevia rebaudiana* leaves. Their findings showed that when water (100%)

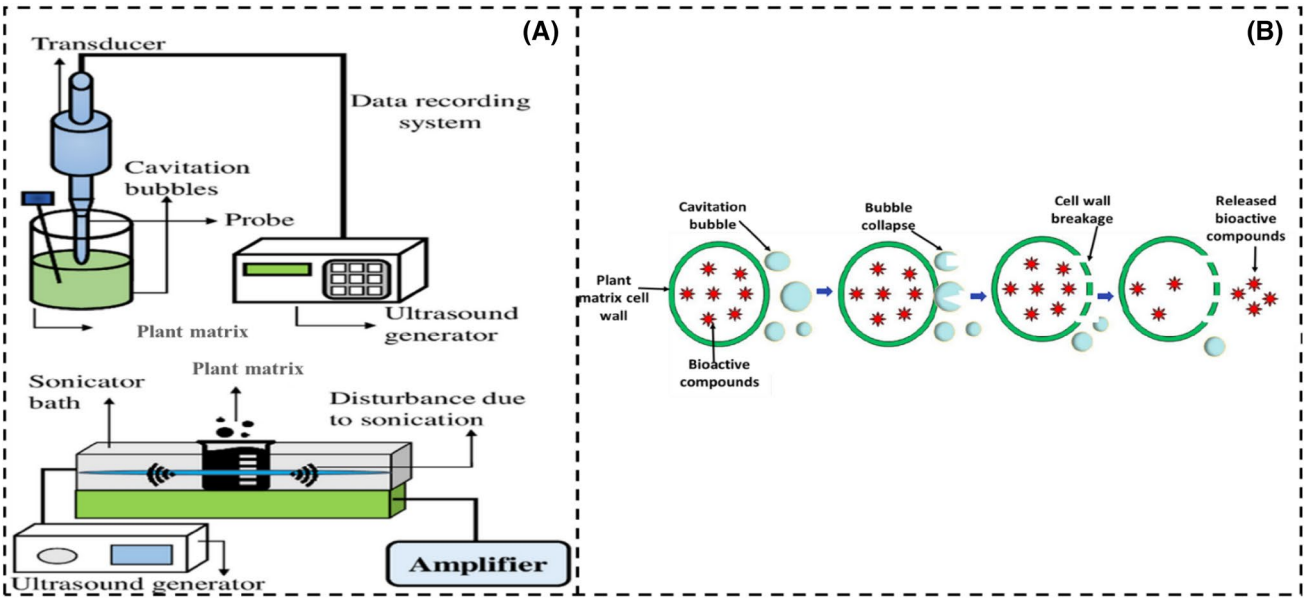


Fig. 8 Typical ultrasonic assisted extraction operational setup[85] (**A**) and its mechanism (**B**) [126, 128, 129]

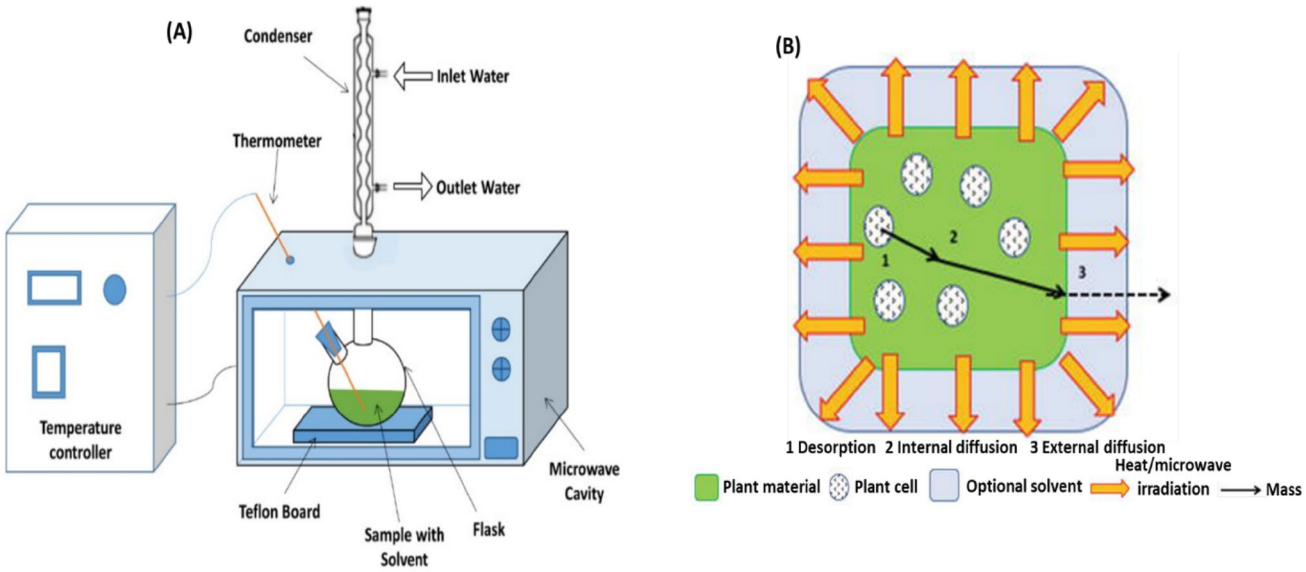


Fig. 9 **A** Typical microwave assisted extraction operational setup [137] and **B** its mechanism [133]

was used as the solvent, ultrasound-assisted extraction produced the highest yield of rebaudioside A (22.760.1 mg/g), while microwave allowed for the highest stevioside recovery (50.86 mg/g). Figure 10 shows that pulsed electric field technology was the most effective at recovering stevioside (44.260 mg/g) and rebaudioside A (22.460 mg/g) when employing equal ratio of water–ethanol solvent. This analytical result demonstrates that both procedures and ethanol percentage had a substantial impact on steviol glycosides recovery, and as shown in Fig. 10, the use of ethanol caused a reduction in extraction output in comparison to extraction employing water as solvent, indicating that water was the optimum solvent for steviol glycosides extraction. It was also speculated that the exceptional behavior of Pulsed Electric Field (PEF) could be strongly linked to the application of electric fields, which appear to trigger permanent pore growth in plant membranes, enhancing the extractability of steviol glycosides by releasing the solutes into the solvent. Even though some pores will not seal, a 50% ethanol concentration during the

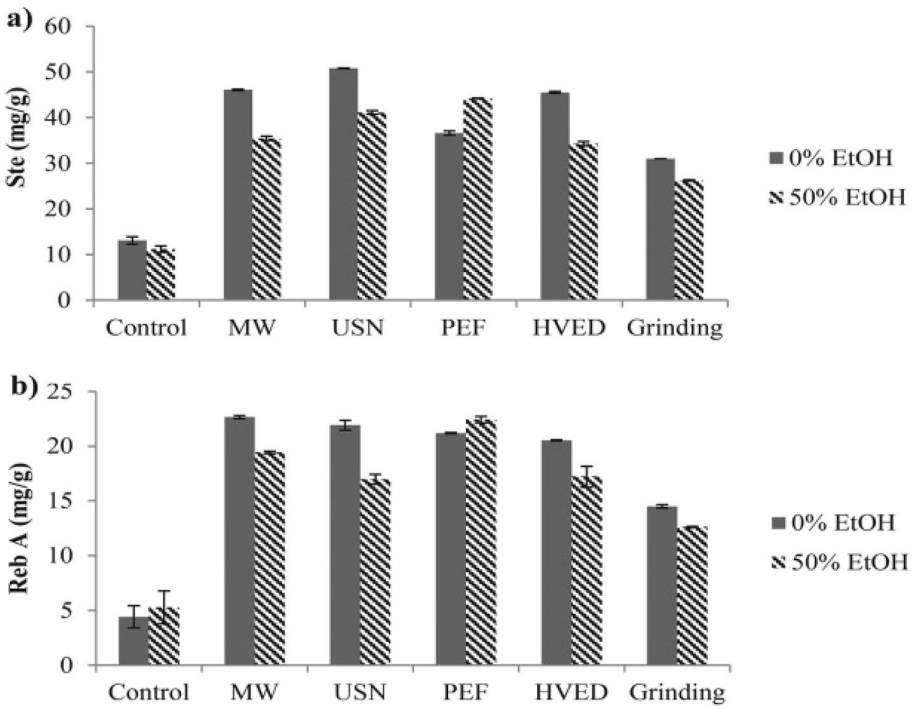


Fig. 10 Steviol glycosides recovery; **a** stevioside, **b** rebaudioside A, in Stevia extracts after diffusion (Control), microwave (MW), ultrasounds (USN), pulsed electric fields (PEF), high voltage electrical discharges (HVED), and grinding assisted extraction [138]

diffusion process may enhance the cellular contents leak. In terms of microwave treatment, the improved stevioside recovery was affirmed to be due to effective microwave heating and destabilization of weak hydrogen bonds fostered by molecule dipole retention, which enhances mass transfer through diffusion inside the solid and also reinforces liquid mixing. Meanwhile, High Voltage Electrical Discharges (HVED) assisted extraction can induce turbulation of suspension and pill fragmentation caused by electrical discharges, necessitating further research into HVED prior to actual upgrading of the methodology to a commercial scale like other methods [138]. Analytically speaking, a similar methodological comparative trend of result was obtained by Periche et al. [11] and Jaitak et al. [15] (Fig. 11), which further corroborate the industrial potential of both microwave and ultrasonic assisted green extraction method using water or hydro-alcoholic solvents as compared to the well-known time-consuming conventional method, which is better for extraction of antioxidant bioactive molecules in stevia rather than steviol glycosides [11].

Liu et al. [14] and his team used a sonic power of 60 W at 68 °C for the extraction of stevia, and achieved a stevia yield that is almost twice as high as that of the usual traditional extraction method within a substantially shortened extraction time of 32 min. In addition, they were also able to mechanistically establish that the extraction yield increases with increasing sonic power which is due to the cavitation produced in the solvent by the passage of an ultrasonic wave and diffusion through the cell walls of the stevia leaves. They conclusively predicted that the lower extraction temperature contributes to the improved purity of the sweet compounds of interest [14].

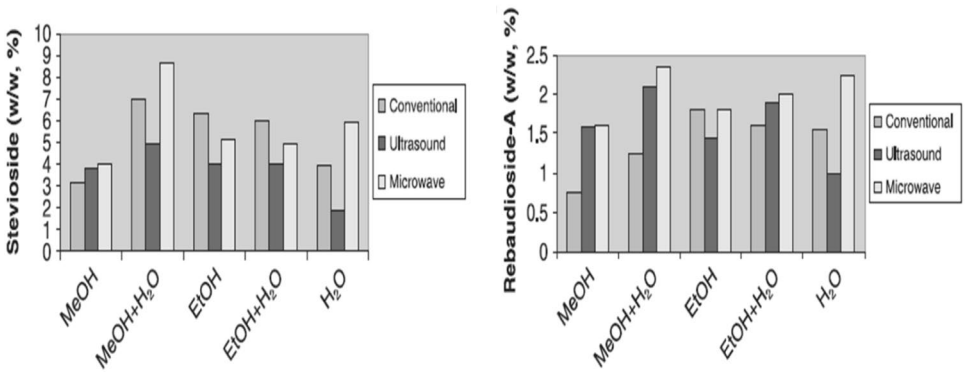


Fig. 11 Comparison of effect of solvents (MeOH, MeOH + H_2O (80:20), EtOH, EtOH + H_2O (80:20), H_2O) in conventional, ultrasound and microwave-assisted extraction of stevioside and rebaudioside-A [15]

4.3 Pressurized Liquid Extraction (PLE) or Pressurized Fluid Extraction (PFE)

Antecedently, Richter et al. [139] was the first to establish pressurized fluid extraction as a novel approach for the extraction of diverse biological substances by the immix of high pressure and high temperature with solvents which usually ranges from 50 to 200 °C and 3.5 to 20 MPa, correspondingly in order to keep the solvent in liquid state [66, 75]. Within the above PFE kinetic parameter, the extraction yield is enhanced owing to amplified diffusion rate and solubility of metabolites in the solvent, ensuing from disruption of analyte-matrix interactions initiated by van der Waals forces, hydrogen bonding, dipole attraction, reduced viscosity and surface tension of the solvent [19, 51, 140]. The myriad merits of pressurized liquid extraction include: simplicity, quickness, inexpensiveness, automaticity, needs little solvent, saves time, boosts efficiency, enhances repeatability, and dramatically reduces exposure to harmful solvents. All of these benefits fully gratify the goal of accomplishing a sustainable environ and thus birth its experimental trial in extracting stevia products [23, 141–143].

For instance, Rao et al. [144] developed a methodology to establish a simple, in-expensive and eco-friendly process in obtaining pure steviosides. In their study, steviosides were extracted from leaves using a pressurized hot water extractor (operating conditions: 100 kPa pressure, 120 rpm, and temperatures of 100–110 °C for 10 min). The steviosides and rebaudioside A contents after the final purification was 9.05 g and 0.2 g of stevia leaf per 100 g, respectively. When compared to other commercially available steviosides, this technique also enhanced the sweetness and palatability profiles [144]. The potential of pressurized hot water extraction was similarly upheld by Pól et al. [5]. However, it is interesting that through their comparative strong cation-exchange (SCX) single and double column liquid chromatography separation, they were able to provide better insight to the separation of the glycosides that are accountable for the taste in stevia products owing to the fact that it was not viable for them to pinpoint which of two chromatogram peaks with *m/z* 965.4 belonged to rebaudioside A and rebaudioside E as discussed in the next section [5]. This same research team, Pól et al. [145] later investigated the effect of methanolic pressurized extraction, and it was discovered that methanol has significantly higher extraction capability for isolating stevioside from *S. rebaudiana* leaves than water within the range of 110–160 °C and 50 bar pressure because the increased solvation power and diffusivity of subcritical methanol were probably sufficient for releasing stevioside from the stevia plant matrix across almost all the temperatures, while the hot water extraction system yield increased continuously up to 110 °C and then a linear uptick was observed after that. Although steviosides significantly degraded in the presence of both solvents or had a lower extraction yield in water at higher temperatures, the authors still hold little reservation for methanol usage compared to the universal readily available green solvent (water) [145]**.** Teo et al. [146] also shared the same view as above when they investigated the pressurized water extraction of *S. rebaudiana* leaves at a temperature of 100 °C for 50 min using a flow rate of 1.5 ml/min and a pressure of 11–13 bar, where 1436 mg/100 g steviosides and 1433 mg/100 g rebaudioside A were successfully extracted.

4.4 Enzyme-Assisted Extraction (EAE)

In the face of the potential methods of obtaining stevia discussed in the foregoing paragraphs, due to the few limitations of the aforementioned non-enzymatic extraction technologies, the use of enzymes in the biotech industry to extract bioactive components from plants has also emerged as a promising field [4]. Enzymes are excellent catalysts that aid the extraction, or conversion of intricate and simple bioactive compounds from natural sources. Enzymes have been successfully utilized in the extraction of vanillin from vanilla green pods, polysaccharide from *sterculia*, oil from grape seed, carotenoids from marigold flower, flavonoids from citrus peel waste, catechins from tea beverage, capsaicin from chilli, Oligosaccharide from Rice bran, lycopene pigment in tomatoes, proteins from lentils and white beans, pectin–polysaccharide from mangosteen, lignin from flax, polyphenols from grape pomace, sugar from grapefruit peel waste, and other biomolecules closely related to stevioside. EAE is a viable and cost effective technique that can be utilized to develop comparable processes, which can pave the way for improved stevioside production while minimizing pollution, high energy consumption, and totally eradicating the use of chemicals in any form [147–162].

Mechanistically, as shown in Fig. 12, enzyme-aided extraction accelerates cell wall disruption. Enzymes like cellulase and pectinase degrade the herb cell wall, consequently making intracellular constituents more easily reachable for extraction (increasing cell wall permeability) i.e. the application of hydrolytic enzymes such as cellulases, hemicellulases, pectinases, proteases and their comb prior to the real extraction process catalyze a variety of hydrolytic reactions and thus facilitate the breaking of plant cell wall which in turn enhances the release of bioactive molecules from matrix [4, 12, 131, 163–174].

For example, steviosides were extracted enzymatically from stevia leaves by employing hemicellulase, pectinase, and cellulase with varying factors such as enzyme concentration, incubation duration, and temperature [13]. Even though pectinase was the most effective at reducing the incubation time, as shown in Fig. 13, hemicellulase was found to produce the highest amount of stevioside in just

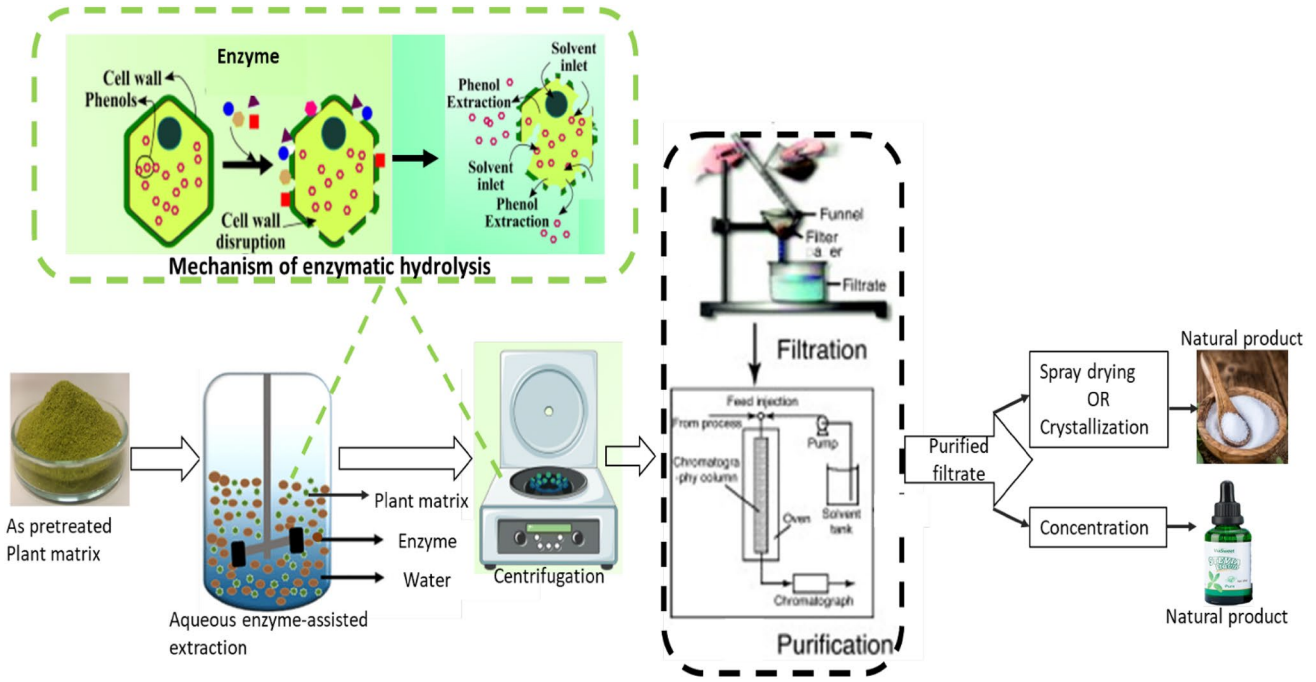


Fig. 12 Typical enzyme-assisted extraction scheme of stevia and its mechanism

one hour when compared to cellulase and pectinase at the same temperature and enzyme concentration. When compared to the conventional solvent-based extraction method, the yield from leaves under optimized conditions increased noticeably (35 times). Based on RSM analysis, the best results were obtained using a mixture of 2% pectinase, cellulase, and hemicellulose, at a temperature of 51–54 °C for 36–45 min. This shows the viability of using enzyme-aided extraction to produce higher yields than the usual solvent extraction method. They further suggested that the extracted stevioside output may be boosted with a comb of enzymatic pre-treatments as seen clearly in Fig. 13. The greatest output of steviosides (9750.4 g) produced utilizing various enzyme combs was thrice greater than the maximum yield (3690.11 g and 3330.5 g) produced using hemicellulose and pectinase as a single enzyme. In addition, it was said that higher temperatures had a favourable impact on the extraction yields, but they can not be maintained ad infinitum due to the compounds' variability (partially hydrolyzing polysaccharides), the potential denaturation of enzymes, and the possibility of denaturing stevia matrix membranes. The above-reported high efficiency was made possible because, mechanistically speaking, pectinase (being pectolytic) has the capacity to destroy pectic compounds, which are available in the middle lamella of primary walls, while cellulase and hemicellulase act on cellulose and cleave beta-1,4 linkages, which are available in the primary wall below the first layer of the middle lamella of the plant cell wall [13].

Contrary to the employment of the above commonly-used enzymes for the extraction of stevia glycosides, Wang et al. [175] carried out an enzymatic synthesis of rebaudioside A from steviosides to enhance the fraction of rebaudioside A to steviosides in steviol glycosides produced, and in order to provide a plausible tactic to enhance the organoleptic profile of the steviol glycoside produced. Their study demonstrates the efficient conversion of steviosides to rebaudioside A by integrating the activities of recombinant UDP-glucosyltransferase UGT76G1 from *S. rebaudiana* and sucrose synthase AtSUS1 from *Arabidopsis thaliana*. The conversion was accomplished by AtSUS1 regenerating UDP-glucose. For UDP-glucose recycling, UDP might be used as the starting material instead of UDP-glucose. In the reaction mixture, UDP concentration can be significantly lowered. With 0.006 mM UDP, 7.2 mM sucrose, and 2.4 mM stevioside, the rebaudioside A production was 78% after 30 h [175].

Among other unpopular methods of stevia production is extraction done with reverse osmosis water that was experimented and concluded by Zhang et al. [6] to be a very good technique for obtaining the natural sweetener stevia glycoside with less impurities and reduced number of unit operations alongside multi-stage membrane process.

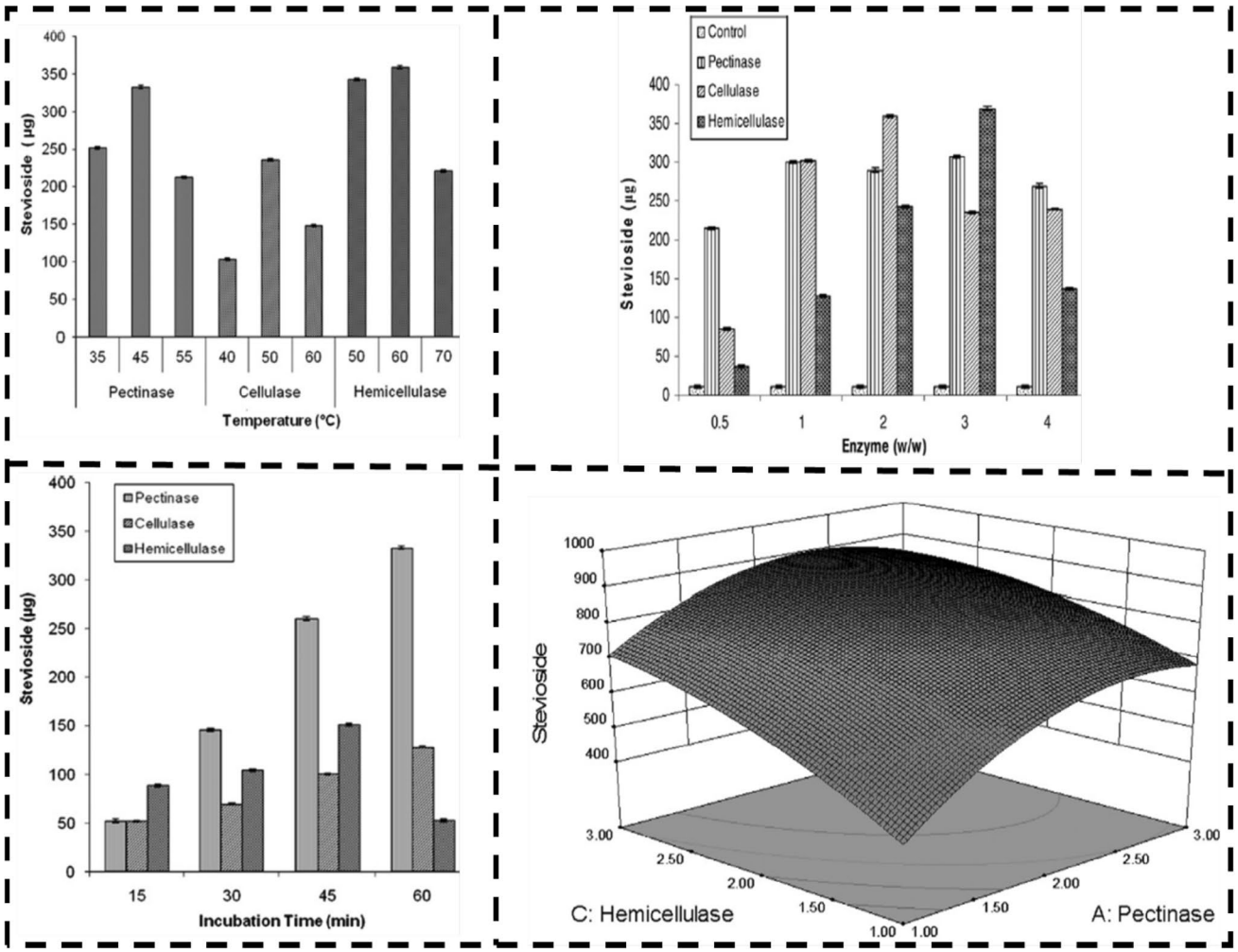


Fig. 13 Influence of temperature, incubation period, and enzyme concentration on enzyme-assisted extraction and 3D response surface contour plots, displaying the influence of pectinase and hemicellulase (%, w/w) on stevioside extraction [13]

5 Post-Extraction Technologies

After the extraction of the various phytochemicals from the stevia leaves, the extract is then processed using a range of technologies, ranging from purification, evaporation or concentration, to filtration, to crystallization, to centrifugation, and then to drying, to convert it into stevia syrup, powder, or crystals. The crude stevia extracts have been associated with a variety of undesirable impurities and chemicals, including sulphates, oil, chlorophyll and their ally pigments and this makes additional purification steps essentially necessary as they afford the final stevia product with aftermath nasty bitter taste. Some of the technologies that have been utilized for the purification of the extract are chromatography, adsorption, and membrane technology.

The use of chromatography as a separation technique has been utilized to obtain a stevia product with less impurities. Rao et al. [144] in their study was able to purify and concentrate sweet glycosides through ultra and nano filtration membrane to remove colour pigments, high molecular impurities, and unpleasant residues which can inevitably give a nasty aftertaste to the final product. A total purity of 97.66% was confirmed to have been achieved through the organic/aqueous washings using HPLC. This percentage purity obtained by [144] was very similar with the one reported by Huang et al. [176] (98.30% for stevioside, 98.50% for rebaudioside-A and 97.60% for rebaudioside-C) using high-speed counter-current chromatography. Kumari et al. [69] also achieved a remarkable purity of 95% for stevioside and 98% for rebaudioside-A through repeated column chromatography on silica gel (60–120 mesh) loaded glass columns using chloroform: methanol (95:5 to 85:15) as the solvent system for elution. In the study, chloroform specifically acted as a decolourizer and remover of other non-polar compounds and greasy materials, while the methanol allowed for better crystallization. This purification output is

consistent with that of Purkayastha et al. [177] and Payzant et al. [178], that used calcium hydroxide and iron chloride (with amberlite FPC23H, amberlite FPA51 and amberlite FPA98Cl) and amberlite XAD-7 resin column, respectively.

Rajab et al. [68] utilized the principle of adsorption for the purification of stevia extract. The purification process was done in two steps. Firstly with activated charcoal to remove phenols, chlorophyll and carotenoids, and followed by treatment with celite in the second step to remove the light yellow colour and obtain white final product. As seen in Fig. 14, a significant decrease in phenol and chlorophyll composition was observed, inferring a profound level of purification. It was empirically established that the fading of the colour signifies the removal of pigments and phenols by charcoal treatment, and the light yellow colour that remained in the extract was further discoloured and refined by treating the extract with celite. This result is in parallel with the two steps purification study reported by Abou-Arab et al. [71]. In their own study, the treatment of stevia extract with $Ca(OH)_2$ removed about 90.18, and 64.30% of carotenoids and chlorophylls and this increased to 97.86 and 97.26% after resin treatment, leading to a final average purity of 97.56%. However, this purification percentage is somewhat higher than that (80.90%) obtained by Zhang et al. [179] using chitosan as the decolourizer, followed by reverse-phase chromatography.

The use of membrane technology, as a purification technique holds great promises. The technology, not only aids in purification but also helps to concentrate the material. Zhang et al. [6], explored this technology. In their study, a ceramic tubular-based microfiltration membrane was first utilized to eliminate the impurities. The permeate from this membrane was then sent through an ultra-filtration membrane, with a trans-membrane pressure of 440 kPa. Thereafter, it was further concentrated in an ultra-filtration membrane with a trans-membrane pressure of 510 kPa at 80 °C. It was also observed that the addition of chemical flocculants in a concentration less than 1% w/w to the microfiltration permeate improved the ultrafiltration flux [6]. In another study, Rao et al. [144] utilized both an ultrafiltration and then a nanofiltration membrane for the separation and concentration of the stevia extract. The effect of feed concentration and pressure was further buttressed in the study.

After the purification and concentration technologies, the concentrated feed can either be packaged as stevia syrup or be converted either into powder or crystals. For the conversion to powdered form, the use of a suitable drying technology, such as spray-drying, can be used. The spray-drying technology has been elaborately discussed by Iwuozor et al. [180]. This technology was utilized for producing stevia powder with a moisture content of 3.9% by Rajab et al. [68]. Process parameters utilized include; inlet temperature of 180 °C, outlet temperature of 95 °C, pump pressure of 500 psi, and air-assisted twin jet nozzle diameter of 0.75 mm. The lower the moisture content of the powder, the higher its shelf-life as it would minimize microbial spoilage. The concentrated feed can also be converted into crystals with the aid of crystallization. Seed inclusion is an important step in this process as it can affect the yield as well as the size of the crystals obtained. Crystallization of stevia extract was performed by Kumari et al. [69]. In their study, the extract residue was dissolved in methanol with minimal heating (This is necessary so as to decrease solubility and increase crystallization). Thereafter, some crystals of steviosides (seeds) were added to initiate the crystallization process. The solution was then refrigerated and the crystallized stevioside was observed after 5–6 days [69].

6 Characterization of Stevia Extracts

The structural characterization of Stevia covers the evaluation of their saccharide constituents, determination of molecular mass, formation of glycosidic linkages and architectural backbones. The major modern instruments employ for this phase as shown in Fig. 15, and they includes: HPLC, NMR,

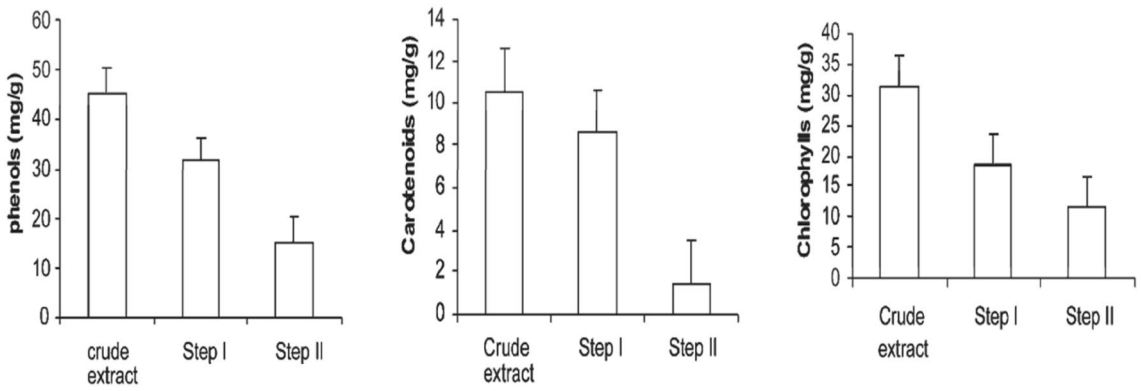


Fig. 14 Reduction in phenols, carotenoids and chlorophyll content after each purification step [68]

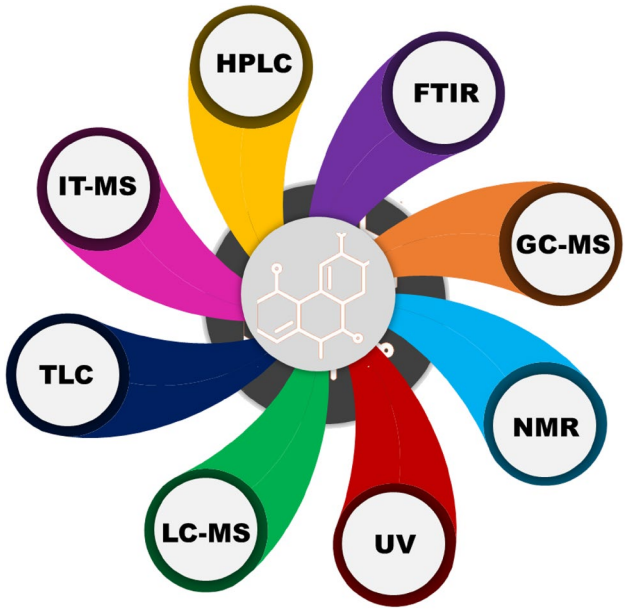


Fig. 15 Major modern analytical instruments employ at characterization stage

FTIR, GC–MS, LC–MS, TLC, Ion trap mass spectrometry (IT-MS), electrospray ionisation time-of-flight mass spectrometry (ESI-TOF-MS) and UV. [4, 23, 181–186]. HPLC is the most widely used of these, owing to its simplicity. Although, according to Pieri et al. [187], since NMR analysis does not depend on the use of reference compounds and is appreciably quicker than HPLC analysis, it is a viable substitute to HPLC-based techniques for quality control of *S. rebaudiana* extracts. Nevertheless, all of the aforementioned methods offer a meticulous comprehension of the shape, crystallization, functional groups, and mechanical stability of the stevia product for fruitful future nutraceutic, biological and therapeutic purposes [4, 23].

Owing to the closely related architectural structures, size, and charge of stevia glycosides, elucidating their structural characteristics is germane as it play a key role in exploring their nutraceutical and biological activity. For instance, it was not possible for Pól et al. [5] to ascertain which of the two chromatograms with m/z 965.4 truly belongs to rebaudioside A and rebaudioside E in their work, because both have the same molecular formula. However, many researchers have been able to successfully isolate and characterize the two major concerned compounds found in Stevia product. For example, Kumari et al. [69] was able to isolate stevioside and rebaudioside-A at retention times of about 11.24 and 17.54 min, respectively as shown in Fig. 16 using amino column and acetonitrile: water ratio of 80:20. These type of distinct resolution peaks was also observed by Jaitak et al. [15] at a retention time of 8.651 and 11.473 min for both stevioside and rebaudioside-A, respectively. In contrast, as opposed the long retention time trend recorded by both Kumari et al. [69] and Jaitak et al. [15], other researchers have recorded a clear peak for the same stevioside and rebaudioside-A at a shorter retention time (3.4 and 4.1 min, respectively [72], and 5.20 and 6.97 min, respectively [14]), and this is consistent with that observed for stevioside, rebaudioside-A and rebaudioside-C by Rao et al. [144], and standard stevioside and rebaudioside-A [68, 72]. Kumari et al. [69] also tried to use water C_{18} column with methanol:

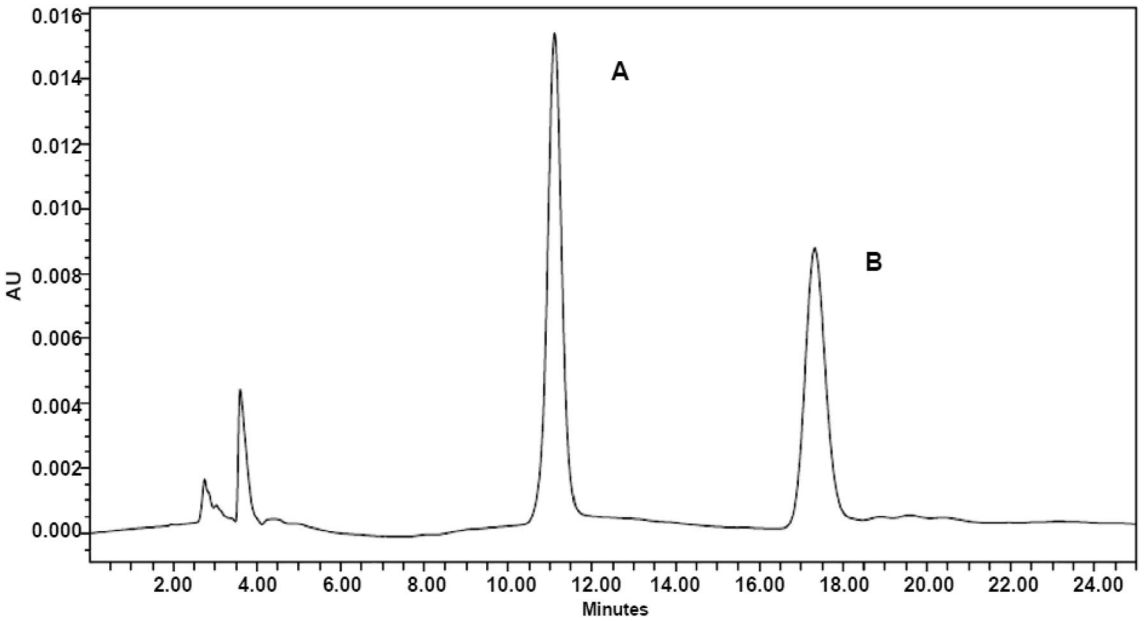


Fig. 16 HPLC chromatogram of **A** Stevioside; **B** rebaudioside-A [69]

water and acetonitrile: water, but they observed a zero peak for all the Stevia glycosides. However, Pól et al. [5] and Starratt et al. [188] differed on this claim as they observed several identifiable peaks with clear resolution using octadecyl siloxane and water, and C_{18} column, respectively. Although like other earlier given reports above, amino column still gave a better retention time (< 17 min) in the same study under similar condition while strong cation-exchange (SCX) gave some of its even not too prominent peak very late as shown in Fig. 17. The various mass to charge ratio of respective HPLC peaks recorded for different Stevia compounds in Fig. 17 by Pól et al. [5] are relatively very much in agreement with HILIC-MS/MS analysis by Well et al. [189], and several NMR analysis as shown in Table 1 [181, 182, 185, 190–194]. Afandi et al. [72] discovered that the intensity of rebaudioside-A peak is a function of Stevia plant material to extracting solvent ratio. In their study, ratio 1:10 gave the best peak, followed by 1:5, 1:20 and then 1:15.

In another study, Kitada et al. [195] was able to identify four peaks belonging to dulcoside A, stevioside,

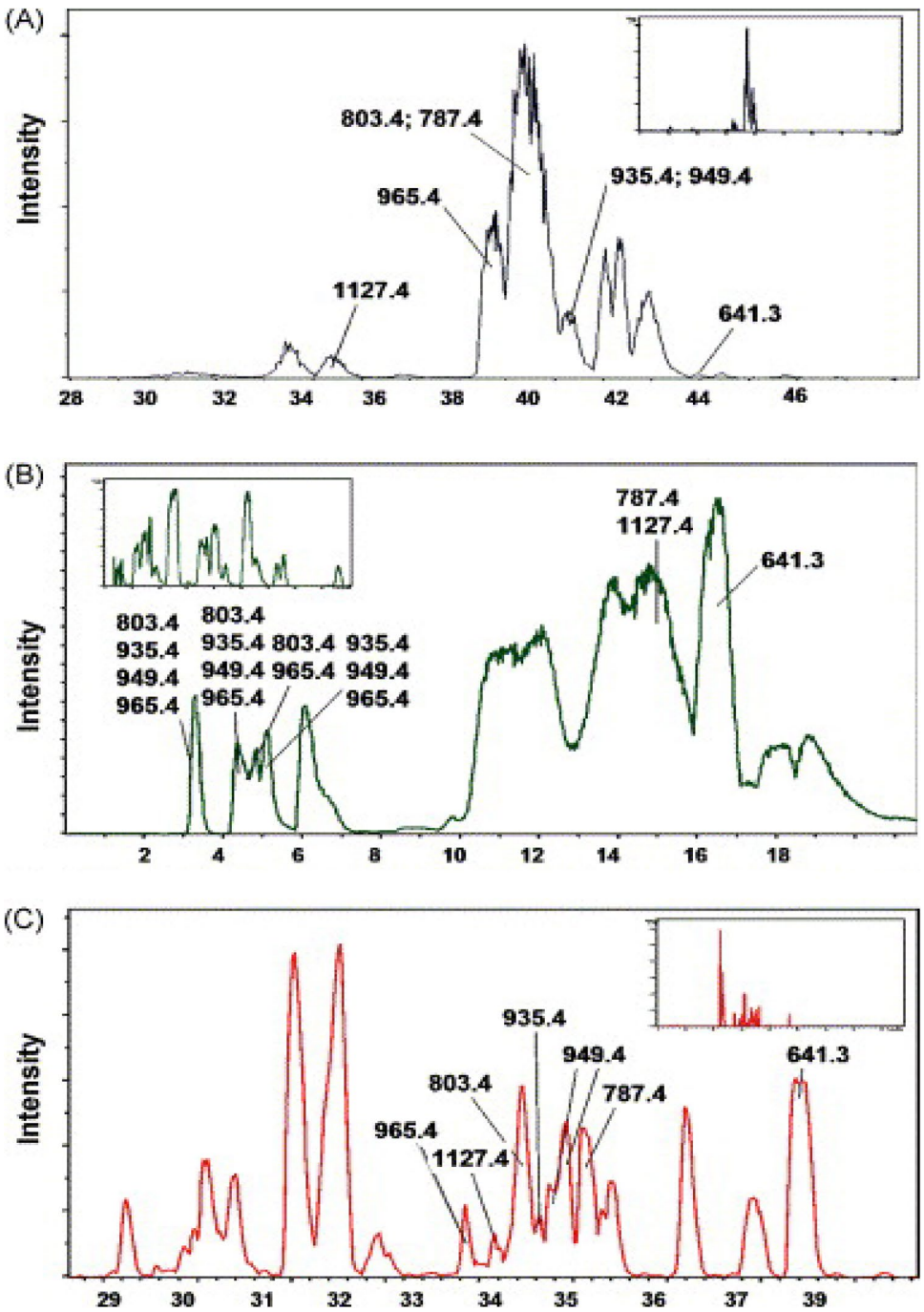


Fig. 17 Separation of aqueous *Stevia* extract by **A** SCX column, **B** Amino column and **C** C_{18} column. TOF–MS trace, Base peak chromatogram at *m/z* 400–1200. *m/z* 641.3-steviolbioside, *m/z* 787.4-dulcoside A, *m/z* 803.4–stevioside and rebaudioside B, *m/z* 935.4-rebaudioside F, *m/z* 949.4- rebaudioside C, *m/z* 965.4–rebaudioside A and rebaudioside E, *m/z* 1127.4-rebaudioside D. Insets in the picture represents overview of the whole chromatogram [5]

Table 1 m/z of different Stevia compounds from various NMR and HRMS analysis. Adapted from Puri et al. [4]

Stevia compound	m/z (g/mol)	References
Rebaudioside A	967.01	[190]
Stevioside	804.87	
Rebaudioside B	804.87	
Rebaudioside C	951.01	[191]
Rebaudioside D	1129.15	
Rebaudioside E	967.01	
Rebaudioside F	936.99	
	959.4	[188]
Rebaudioside M	1289.53	[181]
Steviolbioside	642.73	[192]
Dulcoside A	788.87	[193]

rebaudioside-C, and rebaudioside-A using HPLC and this is in agreement with the report by Pieri et al. [187], Rajasekaran et al. [66] and Dacome et al. [196], although, they observed rebaudioside-B and steviolbioside as opposed to dulcoside-A. Ibrahim et al. [194] also identified three additional known but uncommon compounds (rebaudioside-M, rebaudioside-N, and rebaudioside-O) alongside the already existing-identified rebaudioside-E, and stevioside. The two peaks observed by Woelwer-Rieck et al. [197] using amino-column and HILIC column were very similar in nature as well. Two intense peaks have also been observed in their characterization of Stevia extract using HPLC and it has been affirmed that these two peaks belongs to the two major compounds (stevioside and rebaudioside-A) in Stevia products [16, 175, 198]. This affirmation is also in agreement with the capillary electrophoresis analysis (Fig. 18) where rebaudioside-A peak was seen to be intensely competing with its counterpart (stevioside) [196].

7 Findings and Future Prospects

This study is a review of various technologies that have been utilized in literature for the extraction of glycosides from stevia leaves. The merits and demerits of each technology for the extraction process was also discussed. The study also discussed the post-extraction technologies for the conversion of the stevia extract into syrup, powder, or crystals. The conventional methods used in the stevia industry have been refined over time to meet the demands of large-scale production. Common approach involve the extraction of dried and powdered leaves with a suitable solvent such as hot water, after which a primary clarification is reached by filtration and centrifugation. This method have proven to be effective (it has been reported to give a stevioside yield of more than 90%) but may incur high costs in terms of energy consumption, and waste management. Several emerging extraction techniques have been proposed in the literature to improve the efficiency and sustainability of stevia extraction. These include green extraction methods such supercritical fluid extraction, ultrasonic-assisted extraction, microwave-assisted extraction, pressurized fluid extraction, and enzyme-assisted extraction. These innovations have not yet been applied commercially, though. Adsorption, membrane technology, and chromatography are post-extraction methods used to purify and/or concentrate the extract and have been reported to give a purity above 97%. While these methods offer potential advantages in terms of reduced solvent use, shorter extraction times, and enhanced purity, their economic viability for large-scale production remains an important consideration. During the course of the study, several knowledge gaps were observed, which could form the basis for further works:

- It was observed that most studies engaged more in the extraction of the glycosides present in the stevia leaves, which translates to less work done on the post-extraction technologies.
- For the few authors that researched the post-extraction technologies, very little was done on the characterization of the product obtained. This is necessary as it helps to compare the properties of the stevia product obtained and then link them up with the technologies utilized.
- Future studies should also include a cost analysis of the process. Stevia leaves are yet to be processed in many countries where they are currently grown. This may be due to the presence of a very small plantation base for the raw material, the high capital cost of conventional extraction facilities, and the uncompetitive production cost of small units (as it is susceptible to economies of scale). Researchers must develop low-cost alternatives to conventional and non-conventional technologies that can be used on a small scale.
- Purification and immobilization of UGT76G1 and AtSUS1 should be investigated further, as this will most likely increase the efficiency of rebaudioside-A production from stevioside.
- The use of deep eutectic solvents should be explored in place of commonly used water, alcoholic, and hydro-alcoholic solvents. This could possibly give stevia products better yield and purity with less bitter aftertaste.
- Since ultrasonic and microwave-assisted extraction technologies can enhance the yield and initiate depigmentation of the extract, it is therefore recommended that sun- and oven-drying be avoided at the pre-treatment stage, as this can cause photo-bleaching and the prior destruction of the sweetener product of interest.

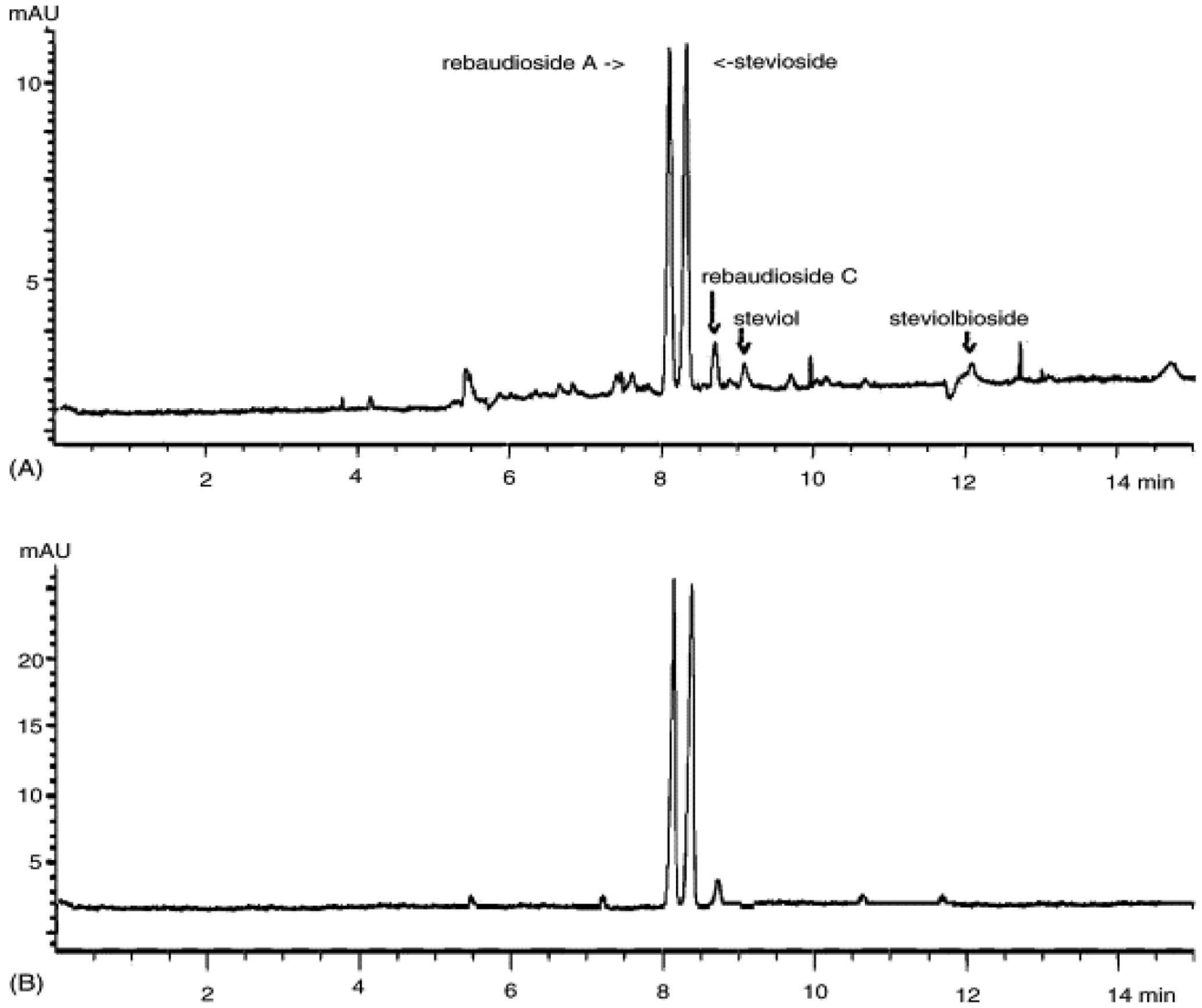


Fig. 18 Capillary electrophoretograms for the stevia compounds from crude (**A**) and purified (**B**) water extracts of *S. rebaudiana* [196]

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Declarations

Conflict of Interest The authors declare that there are no conflicts of interest.

Ethical Approval and Consent to Participate Not applicable.

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