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Review

Research progress in methods of acquisition, structure elucidation, and quality control of Chinese herbal polysaccharides

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ABSTRACT

The therapeutic efficacy of traditional Chinese medicine has been widely acknowledged due to its extensive history of clinical effectiveness. However, the precise active components underlying each prescription remain incompletely understood. Polysaccharides, as a major constituent of water decoctions—the most common preparation method for Chinese medicinals—may provide a crucial avenue for deepening our understanding of the efficacy principles of Chinese medicine and establishing a framework for its modern development. The structural complexity and diversity of Chinese herbal polysaccharides present significant challenges in their separation and analysis compared to small molecules. This paper aims to explore the potential of Chinese herbal polysaccharides efficiently by briefly summarizing recent advancements in polysaccharide chemical research, focusing on methods of acquisition, structure elucidation, and quality control.

1. Introduction

Chinese herbal materials have a long history of treating cancer, infectious diseases, immune disorders, and other ailments in China and neighboring countries [1-4]. Despite extensive clinical experience, the complex theories of traditional Chinese medicine present challenges for integration into modern medical systems. A primary point of contention is the identification of active ingredients in traditional Chinese medicines. To gain broader acceptance and more effective application, it is crucial to elucidate these active compounds. Historically, small molecular compounds were considered the main effective molecules, while polysaccharides, the primary component of water decoctions, were often overlooked or removed as impurities. However, as research progresses, natural product-derived polysaccharides are found to have diverse clinical applications. For instance, carragelose, a linear iota-carrageenan polysaccharide from *Chiondrus crispus*, can be used to treat influenza [5, 6]. A polysaccharide vaccine comprising polysaccharides from *S. enterica* subsp. enterica Typhi (*S. Typhi*), Typhim Vi, received FDA approval in 2014 for typhoid fever prevention [5, 7]. Similarly, lentinan, a polysaccharide from *Lentinus edodes*, has been approved for treating diseases such as cancer in Asian countries [8]. Additionally, numerous studies report polysaccharides with biopotentials, including enhancing immunity and treating cardiovascular diseases, cancers, and colitis [9-12]. Consequently, polysaccharides may be major contributors to the efficacy of traditional Chinese medicine [13], which has increased researcher interest in these compounds. Data from the Web of Science Core Collection shows a significant increase in articles about traditional Chinese medicine polysaccharides over the past decade (Fig. 1), suggesting that polysaccharides from medicinal herbs are a rich source for new drug discovery and development. However, compared to their small molecular weight counterparts, research on traditional Chinese medicine polysaccharides lags behind. This is partly due to the more complex and challenging processes of extraction, separation, purification, and structural analysis of macromolecular compounds. Additionally, the quality of herbal materials from different origins and batches is not always consistent.

Despite the challenges mentioned previously, the extensive potential of Chinese herbal polysaccharides in treating various diseases has led to increased research efforts in this field. Currently, most reviews on Chinese herbal polysaccharides focus on either the extraction process and bioactivity development of specific species or the application of particular techniques in extraction or characterization processes. A recent review summarized qualitative and quantitative analysis methods for polysaccharides [14] but omitted information about their preparation, which forms the foundation for subsequent analysis. Additionally, while some researchers have extensively explored the relationship between polysaccharides' biofunctions and their structure [15], this is not the primary focus of this paper. The scarcity of comprehensive reviews systematically summarizing the entire process of chemical research on Chinese herbal polysaccharides has made it challenging to identify key scientific questions and follow research frontiers. Consequently, this paper aims to provide a concise overview of the advances in this field over the past five years.

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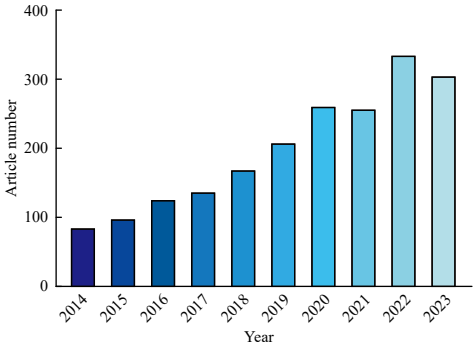

Fig. 1 The published article number of Chinese herbal polysaccharides. Data are collected from the Web of Science Core Collection, using the search terms 'polysaccharides' and 'Chinese medicine' in the topic field

2. Extraction, separation and purification methods

Traditional Chinese medicinal preparations typically undergo water decoction prior to patient administration. Polysaccharides, being predominantly water-soluble compounds, are likely to constitute a significant pharmaceutical component of these decoctions. Consequently, the extraction of polysaccharides from Chinese herbs and the elucidation of their chemical structures hold considerable importance in the screening of lead compounds and the identification of novel target molecules.

2.1. Extraction

Polysaccharides exhibit water solubility while remaining insoluble in organic solvents. This property allows for their extraction using water and subsequent precipitation with water-soluble organic solvents, including methanol, ethanol, isopropanol, and acetone. Hot water extraction (HWE) is commonly employed due to its simplicity, cost-effectiveness, and accessibility. Key parameters for this method include temperature, duration, number of repetitions, and solid-to-solvent ratio. Cold water extraction is also utilized to obtain polysaccharides from herbal materials, mitigating potential degradation and bioactivity reduction associated with high temperatures. For instance, *Grifola frondosa* was extracted in ultrapure water at 4 °C for 12 h, yielding 1.16% of polysaccharides [16]. However, these conventional methods often face criticism for their time-consuming nature, need for repeated extractions, and low yields. Consequently, emerging techniques such as microwave, ultrasound, and enzymatic treatments are being incorporated into extraction systems to address these limitations.

2.1.1. Microwave-assisted extraction

Microwave-assisted extraction (MAE) utilizes microwave radiation as its heating source, which can effectively create perforations on cell membrane and cell wall surfaces. This process allows more external solutions to penetrate cells, facilitating rapid dissolution and release of polysaccharides. It is crucial to carefully consider microwave power, extraction temperature, duration, and liquid-to-solid ratio in specific experiments to prevent adverse effects on polysaccharide structures and bioactivities. When extracting polysaccharides from *Panax ginseng*, MAE significantly enhances production compared to HWE. Specifically, optimal conditions of 30∶1 mL·g^{-1} liquid-to-solid ratio, 550 W, 6 min, and 70 °C yielded a maximum of 41.6% ± 0.09%, whereas optimized HWE produced 28.5% ± 1.62%. Additionally, MAE-extracted polysaccharides demonstrated superior antioxidant activities [17]. For *Hippophae rhamnoides* L., optimal conditions of 600 W, 85 °C, 6 min, and 10∶1 liquid-to-solid ratio resulted in a yield of 0.264% ± 0.005%. These polysaccharides exhibited enhanced DPPH and hydroxyl radical scavenging capabilities compared to those extracted *via* heat reflux [18]. However, MAE's inherent limitations of uneven heating and inconsistent power can potentially damage polysaccharide structures and compromise extraction repeatability [19]. Consequently, this method is more appropriate for materials with high thermal stability.

2.1.2. Ultrasound-assisted extraction

Similar to MAE, ultrasound-assisted extraction (UAE) is characterized by its low energy and time consumption. The intense energy generated by ultrasound can rapidly disrupt cell walls, facilitating the swift release of intracellular polysaccharides into the extracellular environment, thereby enhancing extraction efficiency. When extracting polysaccharides from *Crataegus pinnatifida* Bunge, UAE significantly increased the yield from 5.88% ± 0.19% to 7.47% ± 0.05% compared to hot water extraction, with minimal impact on polysaccharide composition and bioactivity [20]. However, in certain instances, ultrasound application may result in the loss of active functional groups and polysaccharide degradation [21-23]. For example, *Flammulina velutipes* polysaccharides modified by ultrasound exhibited a less stable triple helix structure compared to those obtained solely through hot water extraction [22]. Additionally, ultrasound irradiation substantially reduced the molecular weight and particle size of yellow tea polysaccharides, potentially influencing their antioxidant activities [23].

2.1.3. High pressure-assisted extraction

High pressure serves as an additional method to enhance extraction efficiency, achievable through multiple approaches.

2.1.3.1 Pressurized liquid extraction

Pressurized liquid extraction (PLE) facilitates the extraction of targeted molecules from a solid matrix *via* elevated temperature and pressure conditions. Typically, dried herb material powder is mixed with diatomaceous earth in a specific ratio before being transferred into an extraction cell. The extraction process then proceeds under predetermined pressure and temperature parameters [24, 25]. This method significantly reduces extraction time, typically to between 20 and 40 min. Moreover, PLE yields a higher quantity of polysaccharides compared to conventional extraction techniques [26, 27].

2.1.3.2 Subcritical water extraction

Subcritical water extraction (SWE) utilizes high pressure to maintain water in a liquid state at temperatures significantly above its boiling point [28]. Under these conditions, water exhibits a lower dielectric constant and viscosity, enhancing the solubility of polysaccharides and other macromolecules [29, 30]. Zhang et al. applied SWE to extract polysaccharides from *Sagittaria sagittifolia* L. and determined that optimal yield (25.5%) was achieved at pH 7, 170 °C, 16 min extraction time, and a liquid-to-solid ratio of 30∶1 mL·g^{-1} [31]. In a similar study, the optimal conditions for extracting pumpkin polysaccharides *via* SWE were established at 150 °C, 15∶1 liquid-to-solid ratio, and 10 min extraction time. The resulting polysaccharides demonstrated significant potential in treating type 2 diabetes mellitus [32].

While high-pressure extraction can significantly enhance yield and reduce extraction time, it often adversely affects the bioactivities of polysaccharides. In obtaining polysaccharides from *Fucus virsoides* and *Cystoseira barbata*, PLE yielded the highest output compared to conventional methods and MAE. However, polysaccharides extracted *via* this method exhibited lower antioxidant capacity [33]. Moreover, prolonged exposure to high temperature and pressure may compromise the polysaccharide structure. For instance, in the extraction of *Grifola frondosa* polysaccharides, *β*-glucans may degrade at temperatures exceeding 100 °C, and the triple-helix structure of *Lentinus edodes* tends to deteriorate when extraction time surpasses 10 min at 150 °C [29, 34].

2.1.4. Pulsed electric field-assisted extraction

Pulsed electric field-assisted extraction (PEFE) employs two electrodes to generate high-voltage pulses, inducing cell rupture and facilitating rapid polysaccharide release from herbal materials. For instance, when extracting polysaccharides from orange peels, PEFE was conducted using the EX-1900 PEF equipment with parameters of 6 $kV \cdot cm^{-1}$ for electrical field intensity, 1 Hz for frequency, 30 for pulse number, and 20 μs for pulse width [35]. Under these conditions, the yield of soluble dietary fiber from orange peel reached 238 $mg \cdot g^{-1}$, with the extracts demonstrating enhanced antioxidant activities compared to those without PEFE treatment. PEFE is considered an environmentally friendly and effective method, as it eliminates the need for toxic solvents and mitigates temperature-induced damage to polysaccharides [36]. However, the relatively high cost of this method limits its widespread adoption in chemical laboratories.

2.1.5. Enzyme-assisted extraction

Herbal materials typically contain not only polysaccharides but also proteins and other components. These additional components can be degraded by enzymes, facilitating the release of targeted polysaccharides. Enzyme-assisted extraction generally requires minimal energy and toxic reagents while preserving the polysaccharide structures. Commonly employed enzymes include cellulase, hemicellulose, pectinase, papain, and trypsin [37]. However, the use of enzymes increases the cost of this method compared to others. Furthermore, due to the selectivity of enzymes, obtaining sufficient polysaccharides in a short period solely through this method can be challenging. Consequently, this technique is often combined with other extraction methods, such as UAE and MAE [38, 39].

2.1.6. Supercritical fluid extraction

Supercritical fluid extraction (SFE) is an emerging method that offers advantages such as high efficiency, enhanced purity, and reduced requirements for organic reagents. Supercritical fluids are substances maintained above their critical pressure and temperature, exhibiting properties between those of liquids and gases, which can significantly increase the solubility of polysaccharides [40, 41]. Carbon dioxide is the most commonly used supercritical fluid due to its low cost, low toxicity, and favorable critical points [40]. In a study extracting polysaccharides from *Grifola frondose* using supercritical CO_2, the optimal extraction conditions were determined to be 34.5 MPa, 36.7 °C, and 116.3 min, resulting in a yield of 4.61% [42].

2.1.7. Dilute acid/alkali-based extraction

For polysaccharide extraction, dilute acid or alkali solutions are commonly employed to disrupt cell walls and hydrolyze linkages between cell wall proteins and glucans, facilitating the release of intracellular polysaccharides. Extracting pectin from cell walls presents challenges due to its complex interactions with cellulose and proteins. However, appropriate hot acid solutions can effectively disrupt these networks and maximize pectin extraction. For instance, pomelo peel powder was dissolved in 50 $mmol \cdot L^{-1}$ HCl, stirred at room temperature for 30 min, and then heated in a microwave at 1100 W for 2 min, yielding 20.5% ± 0.6% [43]. In addition to breaking down glycopeptide linkages in glycoproteins, dilute alkali extraction can enhance the solubility of acidic polysaccharides by forming salts with them. Traditional water extraction of *Lentinus edodes* polysaccharides yielded only 0.12%, whereas an optimized alkaline extraction using 0.1 $mol \cdot L^{-1}$ NaOH at 60 °C for 2 h increased the yield to 7.11% [44]. Similarly, extracting polysaccharides from Fuzhuan brick tea with 0.1 $mol \cdot L^{-1}$ NaOH solution at 60 °C for 4 h resulted in a yield of 19.86% ± 1.48%, surpassing that of HWE at 15.36% ± 0.3%. Notably, the alkali-extracted polysaccharides demonstrated superior immunomodulatory potential both *in vitro* and *in vivo* [45]. It is important to note that the type and concentration of acid or alkali must be carefully controlled during the process to prevent polysaccharide degradation [46, 47].

2.1.8. Aqueous two-phase extraction

Aqueous two-phase extraction (ATPE) is an environmentally friendly method capable of separating, concentrating, and partially purifying polysaccharides from herbal materials. Aqueous two-phase systems (ATPS) form through phase separation of an aqueous mixture containing two incompatible polymers or salts at concentrations above critical levels. A previous review comprehensively describes the underlying dynamics [48]. In polysaccharide extraction applications, factors such as solute type and concentration, pH, and temperature significantly influence extraction efficiency. An ATPS combined with ultrasound was employed to extract and separate solanine and polysaccharides from *Solanum nigrum* unripe fruit. The optimized conditions were 36% ethanol, 0.21 $mg \cdot mL^{-1}$ K_2CO_3, 15 °C with 50 min ultrasonic pretreatment, resulting in average separation efficiencies of 2.07 and 8.15 $mg \cdot g^{-1}$ for solanine and *Solanum nigrum* polysaccharides, respectively [49]. Using single-factor experiments and response surface methodology, Hu et al. determined that a system comprising 17.86% $(NH_4)_2SO_4$ (*W/W*) and 28.86% ethanol (*W/W*) with a 1∶30 solid-to-liquid ratio, 8000 $r \cdot min^{-1}$ tissue-smashing power, and 4 min extraction time effectively extracted *Lycium barbarum* L. polysaccharides, yielding 24.79 $mg \cdot g^{-1}$ [50]. Similarly, an ethanol/ammonium sulfate aqueous two-phase extraction system was utilized for *Lycium barbarum* L. polysaccharides extraction, with two distinct polysaccharides extracted *via* microwave-assisted method. This approach significantly enhanced extraction efficiency and selectively extracted various polysaccharides compared to conventional methods [51].

Among these methods, extraction with boiling water remains the most prevalent, despite its high energy and time requirements and relatively low yield. Microwave-assisted extraction and aqueous two-phase extraction generally require the shortest processing time, with the latter operating at room temperature, which is typically the lowest operating temperature, followed by enzyme-assisted extraction. Furthermore, to obtain polysaccharides efficiently and with simple operations, there is a growing trend toward using combined extraction methods for herbal materials. For instance, ultrasound-enzyme-assisted extraction was employed to extract *Armillaria mellea* polysaccharides. This combined method yielded 6.32%, higher than UAE and EAE individually, and demonstrated significant anti-diabetic effects in a mouse model [38]. Another study utilized a combination of EAE, ATPE, and MAE to extract Purple-heart Radish polysaccharides. Specifically, 7.697 g $(NH_4)_2SO_4$, 19.43 mL deionized water, and 12.07 mL ethanol were mixed to form a stable ATPS. Subsequently, 0.5 g sample and 0.1225 g papain were added before microwave processing at 68 °C for 8.4 min, resulting in a final yield of 9.107% [39]. It is important to note that extraction significantly influences the entire research process of Chinese medicinal polysaccharides, largely determining their yield, physicochemical properties, monosaccharide composition, conformation, and bioactivities [52-54]. However, optimal conditions for the highest yield do not necessarily ensure optimal bioactivity performance. Our previous research indicated that different extraction methods affect the composition, structure, and immunological activities of *Lycium barbarum* polysaccharides. Specifically, HWE produced the highest total sugar and acidic polysaccharides, while MAE was more suitable for obtaining polysaccharide-protein complexes. Furthermore, polysaccharides obtained by PLE, UAE, and HWE exhibited better immunomodulatory activities than those from MAE [53]. Therefore, careful consideration must be given to extraction reagents, methods, and their combinations. Addi-

tionally, innovative extraction technologies such as homogenate extraction, vacuum extraction, and nanoparticle-involved extraction have been applied to polysaccharide extraction, warranting further exploration and application [29].

2.2. The separation and purification

2.2.1. The removal of small molecules and proteins

The crude polysaccharides frequently contain numerous extraneous components derived from raw materials, including small molecules and proteins. These impurities may interfere with subsequent structural analysis and functional property investigations of the polysaccharides. To mitigate this issue, it is essential to initially remove these contaminants from the crude polysaccharides.

Generally, small molecules can be readily eliminated through dialysis or ultrafiltration using membranes with specific *Mw* cut-off values. For pigment removal, several methods are available, including H_2O_2 treatment, activated carbon adsorption, anion exchange microporous resin application, and organic solvent successive rinse. Among these, H_2O_2 is the most widely used due to its accessibility. However, the use of activated carbon particles and organic solvent successive rinse is limited by their inefficiency and toxicity, respectively. In comparison to other methods, anion exchange microporous resin offers a milder and more effective approach [55].

To eliminate proteins from crude polysaccharides, several methods are commonly employed, including chemical reagents, protease treatment, or repeated freeze-thaw cycles. Chemical reagents frequently utilized include Sevag reagents, trichloroacetic acid, hydrochloric acid, trifluorotrichloroethane, and calcium chloride. While hydrochloric acid demonstrates the most effective deproteinization, it results in significant polysaccharide loss. Other reagents offer milder and more controllable conditions with lower polysaccharide loss rates, although they may require repeated applications to achieve the desired outcome. However, the introduction of toxic reagents may pose challenges in removal and potentially impact subsequent processes [56-58]. The protease method represents an eco-friendly and straightforward deproteinization approach, requiring minimal time and causing minimal structural alterations to polysaccharides. However, protease is relatively costly, and its efficacy may be limited compared to other methods due to enzyme selectivity [56, 59]. Repeated freeze-thaw cycles offer another environmentally friendly method that avoids toxic reagents. This technique, requiring no organic solvents or expensive equipment, can effectively remove proteins from polysaccharide samples, making it potentially scalable for food and medicinal industries [60]. Nevertheless, this method is relatively time-consuming, and optimization of freezing and thawing temperatures and freezing duration is necessary to maximize deproteinization efficiency.

2.2.2. Separation and purification

The polysaccharides extracted from raw materials and subsequently deproteinized often lack homogeneity and require further separation and purification. Several methods can be employed for this purpose, including precipitation-related techniques, ultrafiltration, dialysis, and column chromatography.

2.2.2.1 Precipitation methods

Polysaccharides of varying molecular weights exhibit different solubilities in specific concentrations of alcohols or ketones, such as methanol, ethanol, and acetone. This property enables the use of step-wise precipitation for initial purification. The process involves gradually adding alcohols or ketones to the extract, increasing from low to high concentrations, followed by thorough stirring, overnight settling, and centrifugation to precipitate the polysaccharides. Generally, polysaccharides with higher molecular weights precipitate first. For instance, using 60%, 70%, 80%, and 90% ethanol to precipitate *Sagittaria sagittifolia* L. extract solution yielded four distinct polysaccharides with corresponding molecular weights of 52.0, 294.9, 230, and 229.4 kDa [61]. In a similar approach, the supernatant of pretreated *Flammulina velutipes* was precipitated with ethanol to final concentrations of 40%, 60%, and 80% (*V/V*), resulting in polysaccharides FVY-40, FVY-60, and FVY-80, respectively. These polysaccharides had molecular weights of 2377.04, 18.32, and 3.84 kDa, with FVY-80 demonstrating the strongest antioxidant effect [62]. The critical aspect of step-wise precipitation is avoiding coprecipitation, making this method particularly suitable for separating polysaccharides with significant differences in solubility.

Additionally, certain long-chain quaternary ammonium salts and metal ions can be employed for polysaccharide precipitation [63]. Long-chain quaternary ammonium salts, such as cetyltrimethylammonium bromide, can form complexes with acidic polysaccharides or those with high molecular weight [64]. Conversely, polysaccharides can form complexes with metal ions like Cu^{2+}, Ba^{2+}, Ca^{2+}, and Pb^{2+} to precipitate [65]. Although these methods are straightforward and cost-effective, they may potentially alter the polysaccharide structures and yield relatively low quantities. Consequently, these techniques are not widely utilized in polysaccharide purification [63].

2.2.2.2 Membrane filtration

Membrane filtration techniques, particularly dialysis and ultrafiltration, are effective in separating polysaccharide fractions based on their molecular weight. These methods facilitate the isolation of distinct polysaccharide components.

Dialysis membranes vary in their permeability to substances of different molecular weights and configurations. This characteristic enables dialysis to serve dual purposes: desalting throughout the process and separating polysaccharides based on their molecular weights. Additionally, this method minimally affects the functional groups of polysaccharides. However, it typically requires extended periods for completion [66, 67].

Ultrafiltration is a pressure-driven separation process utilizing membranes with specific pore sizes [68]. This technique enables the isolation of polysaccharides with varying molecular weights and is more time-efficient compared to dialysis. In a study involving *Brasenia schreberi*, three distinct polysaccharides were obtained using ultrafiltration membranes of 100, 50, and 10 kDa sizes, operating at 0.5 MPa pressure and 30 $L{\cdot}h^{-1}$ velocity. This method offers advantages such as operational simplicity, cost-effectiveness, and environmental sustainability. However, subsequent research has indicated that it may alter the secondary structure of polysaccharides [69]. Tang et al. developed a comprehensive membrane separation system incorporating microfiltration, ultrafiltration, and nanofiltration to purify polysaccharides from *Lentinus edodes* water extract. The process involved initial treatment with a polypropylene filter to remove insoluble matter, followed by sequential treatment with 2.5, 5, and 10 kDa ultrafiltration membranes, and concluding with a nanofiltration membrane (350 Da molecular weight cut-off). This procedure yielded three polysaccharide fractions, designated as LE-UF-1/2/3. Scanning electron microscopy revealed diverse morphologies: LE-UF-1 exhibited various forms, including lump-like, rod-like, and sheet-like structures, LE-UF-2 appeared as lump-like particles, and LE-UF-3 displayed sheet-like formations. These structural differences may contribute to their varying immune-enhancing properties [70].

2.2.2.3 Column chromatography

While the aforementioned methods are straightforward and effective, obtaining homogeneous polysaccharides through these techniques alone remains challenging for researchers. Consequently, it becomes necessary to combine these approaches

with column chromatography. As a highly commercialized technique, column chromatography has gained widespread adoption in the extraction and purification of homogeneous polysaccharides due to its operational simplicity and high purification efficiency. Currently, the most prevalent column chromatography methods include anion exchange column chromatography, gel permeation column chromatography, and affinity column chromatography. A comprehensive overview of chromatography applications in polysaccharide separation and purification has been well-documented in a recent review [65].

(1) Anion exchange column chromatography

Acidic polysaccharides can be adsorbed on anion exchange columns, while neutral polysaccharides typically cannot, enabling the separation of these two types. Additionally, in alkaline buffers, neutral polysaccharides can behave as weak acids and adsorb to the column. After adsorption, eluents with varying ionic strengths or pH levels can be used to elute polysaccharides of different acidic degrees, facilitating the separation of various polysaccharides. For instance, Wu et al. subjected crude *Glycyrrhiza* polysaccharides to ion-exchange column chromatography, utilizing NaCl solutions of different concentrations to elute the polysaccharides, resulting in two fractions: GPS-E1 and GPS-E2 [71]. The binding capacity of a polysaccharide to an ion exchange column is related to its structure. Generally, the adsorption ability of polysaccharides increases with the number of acidic groups or the molecular weight of the polysaccharide molecules, while it decreases if the polysaccharides have numerous branches [71]. Diethlaminoethyl (DEAE) is commonly used in anion exchange chromatography for polysaccharide separation, including DEAE-cellulose, DEAE-sepharose, and DEAE-dextran. As an example, a DEAE sepharose™ fast flow column was employed to separate polysaccharides from *Lycium ruthenicum*, with the LRP3 fraction eluted using 0.2 mol·L^{-1} NaCl solution [72].

(2) Gel permeation column chromatography

Gel permeation column chromatography, also referred to as size exclusion chromatography, is a technique capable of separating polysaccharides based on their molecular weight and size. Generally, polysaccharides with higher molecular weights elute more rapidly. In practice, this method is frequently combined with anion exchange chromatography to obtain homogeneous polysaccharides [73-77]. For example, homogeneous polysaccharides from Glehniae Radix were purified using a Sephadex G-75 gel filtration column (1.6 cm × 60 cm) following fractionation by a DEAE-cellulose 52 column (5.0 cm × 30 cm) [74].

(3) Affinity column chromatography

Molecules capable of binding with targeted polysaccharides can serve as ligands for column loading. Subsequently, pre-separated samples are passed through the column, allowing the targeted polysaccharides to adsorb while other polysaccharides flow through. The targeted polysaccharides are then obtained by altering the mobile phase's ionic strength and pH, which dissociates the ligands and polysaccharides. However, due to the scarcity of universal affinity ligands, this method is less prevalent than the two previously mentioned techniques [63, 65].

3. The structure elucidation methods

The diverse structures of polysaccharides form the foundation for their wide-ranging biological functions. To gain a comprehensive understanding of the efficacy of Chinese herbal medicine and facilitate the secondary development of active compounds, it is crucial to elucidate the chemical structure of polysaccharides derived from Chinese herbs.

3.1. Analysis of monosaccharide composition

3.1.1. Acquisition monosaccharides-complete hydrolysis

Prior to analyzing monosaccharide composition, polysaccharide samples must undergo complete hydrolysis into monosaccharides. This process presents a dual challenge: ensuring sufficient hydrolysis for complete depolymerization of polysaccharides while carefully controlling hydrolysis conditions to maintain the stability of released monosaccharides [78]. Research has demonstrated that basic sugars resist hydrolysis but remain stable post-hydrolysis; acidic sugars hydrolyze more readily but are unstable and prone to degradation after hydrolysis, while neutral sugars exhibit intermediate characteristics [79]. Consequently, different polysaccharides necessitate tailored hydrolysis conditions.

Acid hydrolysis is the most widely employed method for depolymerizing polysaccharides. Trifluoroacetic acid (TFA), sulfuric acid, and hydrochloric acid are commonly utilized in this process. Among these, TFA exhibits mild hydrolysis capability and is easily controlled and volatilized, allowing for simple removal through evaporation with minimal impact on subsequent operations, thus finding more extensive application [73, 77, 80, 81]. However, TFA faces challenges in hydrolyzing certain stable polysaccharides such as cellulose, *β*-glucan, and chitin. Sulfuric acid, being a stronger acid, demonstrates effective hydrolysis for cellulose, pectin, and other stable polysaccharides [82-84]. Nevertheless, samples treated with sulfuric acid require prompt analysis to prevent unexpected monosaccharide hydrolysis. Additionally, the difficulty in removing sulfuric acid necessitates consideration of how to mitigate its residual effects on subsequent experiments. Hydrochloric acid, another strong acid, can be utilized for complete hydrolysis [85]. Post-hydrolysis, sodium hydroxide can neutralize hydrochloric acid to prevent excessive degradation of released monosaccharides or interference with subsequent chromatographic separation. A comprehensive discussion of the acid hydrolysis reaction mechanism and influencing factors has been well documented in a previous review [86].

When a single acid hydrolysis proves ineffective, a two-step acid hydrolysis process can be considered. For polysaccharides rich in uronic acid, this two-step approach can achieve more comprehensive monosaccharide release. In a specific example, samples from carrots, okra, and purslane underwent initial hydrolysis with 0.09 mol·L^{-1} TFA at 79 °C for 1.5 h, followed by a second hydrolysis at 100 °C with 2 mol·L^{-1} sulfuric acid for 2 h. High-performance anion exchange chromatography (HPAEC) detection revealed that the total monosaccharides from acidic polysaccharide samples treated by this two-step hydrolysis were more numerous than those obtained through a single-step acid hydrolysis [87]. Furthermore, to enhance the efficiency and completeness of monosaccharide release, researchers have incorporated microwave technology and enzymes into acid hydrolysis processes [88, 89]. One study reported that fucoidan, chondroitin sulfate A, heparin, and four other polysaccharides could be completely hydrolyzed by microwave-assisted HCl solution in just 10 min, significantly reducing the hydrolysis time for polysaccharides [88].

3.1.2. Analysis methods of monosaccharide composition

3.1.2.1 Thin layer chromatography analysis

Thin layer chromatography (TLC) is an efficient analytical technique characterized by its simplicity of operation and equipment. To analyze the monosaccharide composition of hydrolyzed polysaccharides using TLC, it is essential to investigate an appropriate development system, visualization method, and specific retention factor value. However, this method has limitations in terms of resolution and is challenging to apply for quantitative analysis [90].

3.1.2.2 Capillary electrophoresis-involved analysis

Capillary electrophoresis (CE) possesses characteristics of rapid analysis, excellent separation, and minimal requirement for toxic organic reagents, which have garnered significant attention

in polysaccharide analysis. Among various CE modes, capillary-zone electrophoresis and micellar electrokinetic chromatography are the two most frequently employed. To analyze monosaccharide composition, CE can be further coupled with diverse detection methods, including UV, fluorescent detectors, electrochemical detectors, and mass spectrum detectors [91]. However, due to their inherent electroneutrality, sugar samples after complete hydrolysis cannot be directly analyzed by CE without undergoing time-consuming pre-column derivatization. An alternative approach involves using strong alkali as background electrolytes, enabling sugar dissociation and charging. Additionally, borate buffer can address this issue, although the sensitivity of this method is limited [92].

3.1.2.3 Gas chromatography-involved analysis

Gas chromatography (GC) offers several advantages for determining monosaccharide composition, including rapid sample transfer in the gas phase, a diverse range of stationary phases, and sensitive detectors. These features contribute to its good selectivity, high resolution, strong sensitivity, and fast analysis speed. However, the primary challenge in applying this method is the low volatility of most polysaccharides, necessitating their conversion into volatile and heat-stable derivatives prior to analysis. Common derivatives used in GC analysis of polysaccharides include acetates, trimethylsilyl derivatives, alditol acetates, aldononitrile acetates, and oxime-derived compounds [93]. Flame ionization detector (FID) and mass spectrometry (MS) are typically coupled with GC as detectors [94]. While MS detectors offer greater selectivity, they are more frequently employed for peak identification rather than quantification of monosaccharide derivatives. GC-FID is generally preferred for quantification due to its ease of operation and high sensitivity [92]. Nevertheless, these methods face criticism for the potential loss of polysaccharide details and sample waste resulting from unsuccessful derivatization, indicating areas for further improvement.

3.1.2.4 High-performance liquid chromatography (HPLC)-involved analysis

HPLC is an effective technique for separating monosaccharides. However, most polysaccharides lack charges, chromophores, and fluorescent groups, making them difficult to detect at ultraviolet and visible light wavelengths. Consequently, commonly used HPLC detectors such as ultraviolet, photodiode array, and fluorescence detectors cannot be directly employed for monosaccharide analysis. To address this limitation, a standard approach involves derivatizing sugar samples to meet UV detection requirements and enhance sensitivity and selectivity [95]. Frequently used derivatization reagents include 1-phenyl-3-methyl-5-pyrazolone (PMP), 2-aminobenzamide (2-AB), and *O*-aminobenzoic acid [96]. Alternatively, certain detectors are compatible with HPLC for polysaccharide determination, such as evaporative light scattering detector (ELSD), charged aerosol detector (CAD), and refractive index detector (RID) [97-99]. These methods offer advantages over derivatization techniques, as they eliminate the need for complex sample preparation while enabling simultaneous impurity separation and more sensitive monosaccharide analysis.

HPAEC coupled with pulsed amperometric detection (PAD) is a powerful method for analyzing monosaccharide composition. This technique can identify nearly all monosaccharides and most oligosaccharides without derivatization, saving time and avoiding the use of toxic derivative reagents [88, 100]. HPAEC offers excellent separation capabilities, while PAD provides high sensitivity, detecting sugars at concentrations as low as pmol levels. Furthermore, both techniques have a wide range of commercially available products. Consequently, HPAEC-PAD has become the most widely used method for monosaccharide composition analysis. However, it is important to note that PAD requires a strongly alkaline environment, which can lead to gradual electrode degradation and signal loss [101]. Additional information regarding constituent monosaccharides can be found in Table S2.

3.1.2.5 Supercritical fluid chromatography-involved analysis

Supercritical fluid chromatography (SFC), an emerging chromatographic technique, typically employs supercritical carbon dioxide as the mobile phase, in contrast to the liquid or gas used in traditional chromatography. Supercritical carbon dioxide, existing in a state between liquid and gas, possesses characteristics of high solubility, low viscosity, and high diffusion rate. These properties confer advantages such as high efficiency, rapid analysis, and reduced solvent consumption [102]. A monosaccharide composition analysis method utilizing SFC was developed using an ultra-high-performance SFC system equipped with a photodiode array detector. This technique was applied to analyze polysaccharides from *Schisandra chinensis*, revealing the presence of 7 neutral monosaccharides and two acidic monosaccharides [103].

3.2. Analysis of sugar residue linkage mode

3.2.1. FT-IR

FT-IR is a valuable analytical tool characterized by its rapid scan rate, high resolution, and sensitivity. It provides essential information about polysaccharide structures, including monosaccharide types, glycosidic linkages, configurations, and functional groups. For instance, in the characterization of polysaccharides from *Craterellus tubaeformis*, FT-IR spectroscopy enables the observation of absorption peaks corresponding to characteristic functional groups and *α/β*-linkages [104]. However, this method has limitations in identifying specific sugar residue connections and determining the precise proportions of monosaccharide types within the polysaccharide repeat unit. Consequently, FT-IR is primarily employed for qualitative analysis of polysaccharides rather than quantitative assessments.

3.2.2. Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is a crucial and powerful tool for analyzing the configuration of anomeric carbon, residue linkage, and sequence of polysaccharides, making it widely applied in confirming polysaccharide structures. ^{1}H NMR is typically employed to determine the configuration of glycosidic bonds and analyze other functional groups, such as sulfate, in polysaccharides [105]. However, due to the frequent overlapping of proton signals in ^{1}H NMR spectra of polysaccharides, these results often require further analysis in conjunction with NMR carbon spectroscopy data. ^{13}C NMR, with its higher resolution, can not only ascertain the position of different carbons but also differentiate molecular conformations [106, 107]. Additionally, two-dimensional NMR spectra play a vital role in the complete attribution of polysaccharide ^{13}C NMR [108, 109]. In ^{1}H-^{1}H correlation spectroscopy (COSY), each cross peak indicates the coupling relationship between adjacent hydrogen nuclei, with the intensity of the cross peak directly related to its corresponding ^{3}J value. Heteronuclear singular quantum correlation (HSQC) reflects the coupling relationship between directly connected ^{1}H and ^{13}C nuclei. When combined with partial acid hydrolysis, the heteronuclear multiple bond correlation spectrum (HMBC) can provide structural information of the glycan skeleton by associating the ^{1}H nucleus with the long-range coupled ^{13}C nucleus.

In the process of determining polysaccharide structure, ^{1}H and ^{13}C NMR spectroscopy are typically employed initially to assign chemical shifts to the anomeric hydrogen or carbon of each residue, respectively. Subsequently, ^{1}H-^{1}H COSY is utilized to confirm the chemical shifts of H2−H6. Following this, in conjunction with HSQC, the chemical shifts of C2−C6 can be assigned. Lastly, HMBC serves as a tool to elucidate the potential connection modes of each residue. Comprehensive discussions on the applic-

ation of NMR in the structural analysis of polysaccharides have been thoroughly presented in previous reviews [110, 111].

3.2.3. Mass spectrum-involved analysis

Methylation is a crucial technique for determining the linkage patterns of monosaccharide residues in polysaccharides, with complete methylation being essential for accurate analysis. The fundamental principle involves initially methylating all free hydroxyl groups in the polysaccharide's monosaccharide residues, followed by hydrolysis of the glycosidic bonds to yield partially methylated monosaccharides. Notably, the locations of unmethylated hydroxyl groups indicate the original connection points of the monosaccharide residues [112]. Moreover, the relative proportions of different methylated monosaccharides can be used to deduce the percentage of specific linkages. However, this method's application can be constrained by its substantial polysaccharide requirements and time-intensive nature. Furthermore, acidic polysaccharides present additional challenges due to their limited solubility in DMSO and susceptibility to β-elimination, making complete methylation difficult to achieve [113]. Consequently, the analysis of such polysaccharides often necessitates combining methylation results with other analytical techniques to elucidate their chemical structures. Various methylation methods exist for polysaccharides, including the classic Hakomori method and the Ciucanu & Kerek methylation method. The completion of methylation can be verified by observing the disappearance of the 3700–3200 cm^{-1} -OH stretching band in the FT-IR spectrum.

After methylation, the hydroxyl groups of samples can be acetylated to obtain volatile alcohol acetates, which can be further analyzed by GC-MS. Through the analysis of the peak sequence of gas chromatography and the main ion fragments of the mass spectrum, the linkage type of polysaccharides can be determined with high accuracy [114-116]. While GC-MS is the most widely used technique, MALDI-TOF MS and LC-MS/MS are also effective tools for identifying linkage patterns, as comprehensively reviewed in previous literature [117]. UPLC/QqQ-MS utilizing MRM mode enables rapid separation of isomers and identification of linkage patterns in polysaccharide backbones and branches. However, this method requires the establishment of an extensive linkage standard library and sample pretreatment involving permethylation, acid hydrolysis, and PMP derivatization, making it time-intensive and potentially resulting in sample loss [118]. A recent innovation, logically derived sequence tandem mass spectrometry, based on the dissociation mechanism of hexoses and *N*-acetylhexosamines, has been employed to identify oligosaccharide structures without the need for a mass spectrum library of oligosaccharide standards [119]. Additionally, after acid or enzyme hydrolysis, polysaccharides such as dextran, amylose, and arabinioxylan can be subjected to MALDI TOF/TOF analysis for structural information, using harmine hydrochloride/3-aminoquinoline/α-cyano-4-hydroxycinnamic acid as the matrix, without separation or pretreatment [120, 121].

3.3. Sequencing

Unlike proteins and nucleic acids, polysaccharides are not derived from templates or consist of specific structures. Instead, polysaccharides are a series of macromolecules that share similar monosaccharide composition, degree of polymerization, and branching, making their sequencing challenging. Inspired by proteomics research, the bottom-up approach has been adopted to analyze polysaccharide sequencing [117]. Specifically, polysaccharides are initially mildly degraded into oligosaccharides through acid hydrolysis, enzymatic or oxidative treatments. The degraded oligosaccharides are then separated by chromatography and analyzed using NMR and MS. After structural elucidation, these oligosaccharides are used to reconstruct the overall structure of the original polysaccharides. For instance, fucan sulfate extracted from sea cucumber is of great importance in food and medicine production, but its widespread overlap spectrum in NMR hinders structural analysis. Consequently, researchers cleaved the polysaccharides in three steps through partial acid hydrolysis, providing crucial materials for fucan sulfate structure determination [122]. Amicucci et al. developed a universal depolymerization method named Fenton's initiation towards defined oligosaccharide groups (FITDOG). In this method, Fe^{3+} and hydrogen peroxide are combined to produce reactive radical species that induce oxidative cleavage of the polysaccharide backbone. Subsequently, HPLC-QTOF-MS and UPLC-QqQ-MS are employed for oligosaccharide sequencing and linkage composition analysis, respectively [123, 124]. When characterizing polysaccharides from maize mucilage, a workflow integrating multiple LC-MS techniques was established. TFA was first used to partially hydrolyze polysaccharides into oligosaccharides, which were then characterized by nano-HPLC-chip-QTOF MS and developed into a library [125]. However, even with clearly elucidated oligosaccharide structures, significant challenges remain in reconstructing the parent polysaccharides, such as determining the arrangement of oligosaccharides and the linkages of glucuronic acid residues. Other principles from proteomics research, like the top-down method, may offer an alternative approach for polysaccharide sequencing. However, due to the large size of polysaccharides, obtaining sequencing information through this method remains unfeasible.

The structure of polysaccharides plays a crucial role in their functional properties. Elucidating their structure is the initial step in developing them as potential lead compounds. The widespread adoption of advanced techniques, such as HPLC, MS, and NMR, has significantly enhanced the structural elucidation of polysaccharides. Currently, determining the monosaccharide composition of polysaccharides through complete hydrolysis and chromatography is a relatively straightforward task. Moreover, partial hydrolysis can degrade polysaccharides into measurable secondary polysaccharide and oligosaccharide fragments, which can be further separated by HPLC or GC and precisely analyzed using NMR or high-resolution MS, providing a comprehensive characterization of polysaccharides. Although the complete structural analysis of some complex, high-molecular-weight polysaccharides remains challenging, it is anticipated that with ongoing technological advancements, the structures of an increasing number of polysaccharides will be resolved.

3.4. Conformational study

The biological activities of polysaccharides are intricately linked to their advanced structures in the *in vivo* environment. For instance, lentinan, which possesses a triple-helix structure, demonstrates potent anti-tumor activity. However, this activity significantly diminishes when the triple-helix structure is disrupted [126]. A comprehensive understanding of polysaccharide chain conformations is essential for deeper investigation into their biological activities and the development of more effective clinical treatment strategies.

The conformational analysis of polysaccharides primarily encompasses the examination of chain size and morphology, the interactions between main and side chains, the arrangement of side chains, and the spatial characteristics of polysaccharides. Various research methods are employed for conformational studies, including Congo red tests, circular dichroism analysis, viscosity measurements, light scattering techniques, atomic force microscopy, scanning electron microscopy, X-ray diffraction, and differential scanning calorimetry.

3.4.1. Congo red assay

Congo red assay, widely employed as the most straightforward method for conformation analysis, is utilized to determine whether polysaccharide molecules possess triple-helix struc-

tures [127]. Congo red forms complexes with triple-helix polysaccharides, resulting in a purplish-red coloration. However, the triple-helix conformation can be disrupted when polysaccharides are exposed to an alkaline environment. Following this principle, polysaccharide samples of a specific concentration are typically mixed with Congo red solution initially, followed by the addition of sodium hydroxide to create solutions of varying pH. The absorption spectrum, recorded from 400 to 600 nm, reflects the extent of triple-helix conformation in the polysaccharide [128, 129]. It is important to note that the color change of the solution is not exclusively indicative of triple-helix conformation. In certain instances, polysaccharides with single/double-helix or even random coil structures can also produce similar phenomena [127].

3.4.2. Circular dichroism

Circular dichroism (CD) is a valuable technique for detecting the chiral information of polysaccharides, which can be utilized to analyze their conformational structure. For instance, CD was employed to elucidate the chain configuration of two polysaccharides extracted from steamed ginseng. The CD spectrum of one of these polysaccharides exhibited distinct positive and negative Cotton effects, indicative of its helix/sheet-like structure [130]. Furthermore, variations in ellipticity at different wavelengths can provide insights into the presence of carboxylate n-π^*/π-π^* transitions and the flexibility of the polysaccharides [131].

3.4.3. Viscosity method

The Mark-Houwink equation provides a means to determine the exponent α by estimating the intrinsic viscosity ([η]) and molecular weight. The value of α is intimately linked to the sugar chain conformation. When α exceeds 0.8, the molecules tend to adopt a more extended structure. Conversely, when α falls below 0.5, the chain typically exhibits a branched and compact configuration. For values between 0.5 and 0.8, the chains present as semi-stiff structures [132]. Consequently, determining [η] is a crucial step in this analysis.

3.4.4. Light scattering

Light scattering (LS), comprising dynamic light scattering (DLS) and static light scattering (SLS), is a technique capable of detecting size distribution and hydrodynamic radius (R_h), *Mw* and radius of gyration (R_g), respectively. Furthermore, these data can provide insights into intermolecular interactions, such as hydrogen bonds and Van der Waals forces, and suggest conformations through calculations using the Mark-Houwink equation. While HPSEC-MALLS can also determine molecular parameters detected by SLS, the latter remains irreplaceable for studying polymers with large *Mw* [133, 134].

3.4.5. Atomic force microscope

Atomic force microscope (AFM) is a powerful tool for visualizing morphology and quantifying structural changes of polysaccharides [135]. Through AFM, the particle size of polysaccharides can be readily determined. For instance, an irregular spherical shape of *Polygonatum cyrtonema* polysaccharides was observed using AFM, simultaneously detecting their molecular height and diameter [136]. Compared to other methods, AFM possesses a high resolution in its vertical direction, providing valuable information about polysaccharide conformation. Based on this capability, a novel method was developed linking chain heights with helix conformation of varying extents. Specifically, the heights of lentinan chains with triple, double, and single helices were measured at approximately 1.746, 1.564, and 1.243 nm, respectively [137].

3.4.6. Electron microscope

Electron microscopy, including transmission electron microscopy (TEM) and scanning electron microscopy (SEM), can also be employed to examine the morphology of polysaccharides. In comparison to AFM, electron microscopy offers higher resolution and provides direct images of polysaccharide surfaces [138], facilitating a deeper understanding of polysaccharide cross-linking [139].

Nevertheless, the concentration of polysaccharides significantly influences their sub-molecular structures, making it nearly impossible to obtain a single polysaccharide suitable for estimation. Consequently, neither electron microscopy nor AFM provides information about the actual state of polysaccharides in solution. Considerable research remains necessary to address this challenge comprehensively.

In conclusion, numerous methods are currently available for investigating the conformation of polysaccharides. Congo red provides a direct indication of triple-helix structures. CD, LS, and viscosity measurements offer insights into size, rigidity, and intermolecular interactions through calculations. The increasing availability of advanced microscopes allows researchers to observe polysaccharides directly, although further consideration is needed to determine appropriate indices for reflecting conformational characteristics. It is worth noting that sample preparation methods may influence the observed structures, potentially differing from their physiological states. Additional techniques for conformational studies include differential scanning calorimetry and X-ray diffraction, which primarily focus on interactions between polysaccharide chains and metal ions or solvent molecules [140-142]. A comprehensive overview of polysaccharide conformational analysis methods has been recently summarized in a review [143].

4. Quality control

The quality control of Chinese herbal polysaccharides involves assessing the composition, purity, and content of polysaccharides in traditional Chinese medicine to differentiate herbal materials from various origins and ensure their efficacy. The 2020 edition of *Chinese Pharmacopoeia* stipulates polysaccharide content requirements for numerous Chinese herbal medicines. For instance, the polysaccharide content (expressed as glucose) in dried *Polygonatum odoratum* products should not be below 6%, while for dried *Ganoderma lucidum* products, it should not be less than 0.9%. In dried seaweed, the polysaccharide content (expressed as fucose) should exceed 1.7%. However, merely evaluating polysaccharide content is often insufficient to guarantee safety and bioactive effects, which are closely linked to polysaccharide structures, including monosaccharide composition, glycosidic linkages, and chain conformation. Consequently, both qualitative and quantitative analyses of polysaccharides are crucial for quality control. Historically, this field has faced challenges such as the absence of specific identification reactions, exclusive content determination methods, techniques reflecting the overall properties of Chinese herbal polysaccharides, quality markers, and reference substances. In recent years, advancements in analytical chemistry have led to continuous enrichment and improvement of polysaccharide quality control methods [144].

4.1. Qualitative analysis methods

Qualitative analysis of Chinese herbal polysaccharides primarily focuses on their structural characterization. While comprehensive structural characterization provides highly accurate identification of polysaccharides from specific sources, as detailed in the previous chapter, its scalability as a general quality control method is limited due to its time- and labor-intensive nature [145]. Alternatively, fingerprinting, a recognized method for the quality control of natural products, serves as a powerful tool for evaluating the authenticity, stability, and consistency of

Chinese herbal polysaccharides. Fingerprint analysis based on molecular weights, constituent monosaccharides, and glycosidic linkages effectively discriminates between different polysaccharides. For instance, HPLC fingerprint analysis of 20 sample batches revealed that glucuronic acid may serve as a specific marker to differentiate white *Flammulina velutipes* from yellow *Flammulina velutipes*, while mannose, rhamnose, xylose, and galactose may be strongly associated with its bioactivity [146]. However, polysaccharide fingerprint analysis typically employs acid hydrolysis as a pretreatment, where results are highly dependent on hydrolysis duration, acid selection, and concentration, compromising the method's accuracy, repeatability, and specificity. Consequently, saccharide mapping, a highly specific and mild digestion method combining endoglycosidase digestion with multiple chromatographic analyses, has been introduced to identify and/or discriminate herbal polysaccharides. The basic procedure is as follows (Fig. 2A): first, characteristic maps of target polysaccharides are established before enzymatic hydrolysis; then, a series of endoglycosidases are used to hydrolyze samples at specific glycosidic bonds; subsequently, chromatographic methods are employed to compare polysaccharide characteristics; furthermore, the enzymatic hydrolysis products of polysaccharides can be separated and analyzed by chromatography and mass spectrometry. In some instances, stable and specifically hydrolyzed polysaccharide fragments can serve as indices for qualitative and quantitative analysis. Compared to other quality control methods, saccharide mapping offers advantages such as good selectivity, high specificity, controllable reaction conditions, and stable products, making it an excellent strategy for polysaccharide quality control.

The identification and analysis of polysaccharides from various sources can be achieved through their distinct responses to targeted endo-enzyme hydrolysis and the chromatographic characteristics of their hydrolysates. For instance, HPSEC-MALLS-RID results (Fig. 2B) demonstrated that after cellulase hydrolysis, peak a of *Cordyceps* polysaccharides decreased while peak b emerged, indicating the presence of *β*-1,4-Glcp glycosidic linkages [147]. Similarly, Fig. 2C illustrates that the HPLC-DAD profiles of polysaccharides from *G. sinense* differed with and without pec-

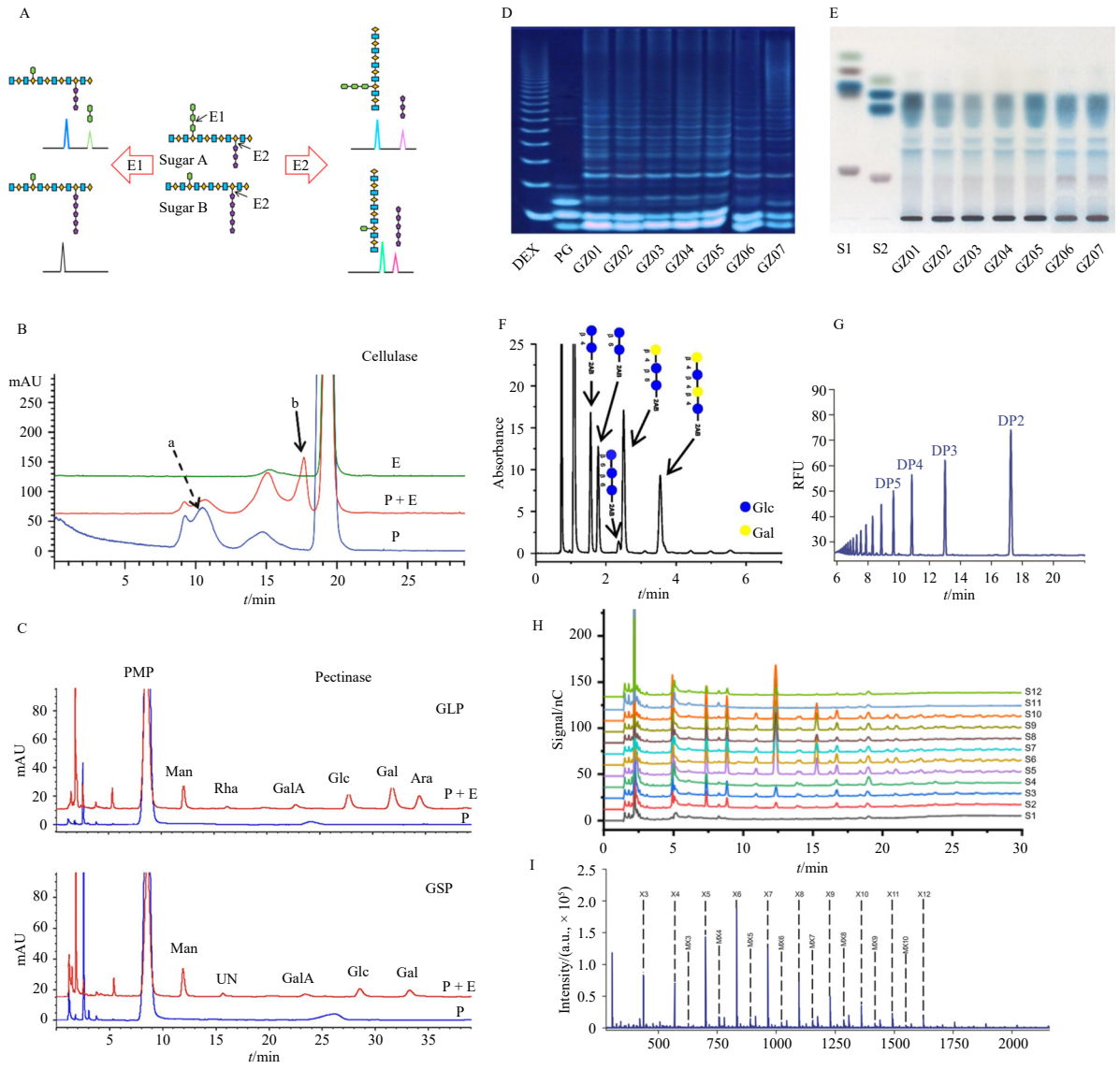

Fig. 2 Application of saccharide mapping in qualitative analysis of Chinese herbal polysaccharides. (A) The schematic diagram of saccharide mapping (E1: enzyme 1; E2: enzyme 2). (B) HPSEC-ELSD of polysaccharides from *Cordyceps* treated with (P + E) or without (P) cellulase (E). Peaks a&b are peaks that changed during cellulose hydrolysis. (C) HPLC-DAD of PMP derivatized hydrolysate of polysaccharides from *G. sinense* (GSP) and *G. lucidum* (GLP) treated with (P + E) or without (P) pectinase (E). (D) PACE of polysaccharides from *Pseudostellaria heterophylla* digested by 1,4-*β*-galactanase (DEX, partial acid hydrolysates of dextran; PG, pectin galactan). (E) HPTLC of polysaccharides from *Pseudostellaria heterophylla* digested by 1,4-*β*-galactanase (from top to bottom, S1: Rha, Xyl, Man, Ara; S2: Fuc, Glc, Gal, and GalA). (F) UPLC-FLR fingerprints of 1,3-*β*-glucanase digested polysaccharides from *L. edodes*. (G) MEKC-LIF of the APTS derivatized dextran ladder. (H) The HPAEC-PAD fingerprints of *α*-amylase hydrolysis of *Grifola frondosa* polysaccharides. (I) MALDI-TOF of commercial xylooligosaccharides.

tinase treatment. Moreover, pectinase-treated saccharide mapping could differentiate between polysaccharides from *G. sinense* (GSP) and *G. lucidum* (GLP), despite their similar compositional sugars [148]. However, more detailed information cannot be discerned through simple comparison. PACE, characterized by simplicity, good repeatability, and the ability to detect multiple samples simultaneously, has been employed in saccharide mapping to distinguish and identify polysaccharides from different origins. As sugars lack ultraviolet or fluorescent groups, hydrolysates require derivatization with acid disodium salt (ANTS) or APTS prior to PACE analysis. To assess the quality of *Salvia miltiorrhiza*, we compared *Salvia miltiorrhiza* polysaccharides (SMP) from different regions in China. PACE results coupled with orthogonal partial least squares discriminant analysis (Fig. 2D) showed that SMPs could be differentiated according to their original regions after digestion by endo-1,5-α-arabinanase [149]. PACE analysis of β-1,4-galactanase hydrolysates from *Polygonatum cyrtonema* polysaccharides after varying steaming durations revealed that β-1,4-Galp were abundant in polysaccharides after steaming but absent in those without steaming, suggesting that steaming significantly influences certain chemical features of this herb [136]. Additionally, this method has been utilized to compare *Dendrobium devonianum* with different appearances [150], characterize polysaccharides from different batches of *Lycium barbarum* [151], investigate natural and cultured *Cordyceps* [152], and compare *Pseudostellaria heterophylla* from different origins [153]. Furthermore, by comparing the biological activity of polysaccharides before and after hydrolysis by different enzymes, the main types of glycosidic bonds affecting the biological activity of polysaccharides can be identified, and the structural characteristics related to the activity of polysaccharides can be analyzed, which aids in improving the quality control of active polysaccharides in traditional Chinese medicine. For example, after being treated with TFA, pectinase, and endo-arabinanase, NO production in RAW 264.7 cells was significantly decreased compared with the original polysaccharides, while the result of polysaccharides treated by 1,3-β-glucanase was similar to that of the original polysaccharides, indicating that α-1,5-arabinosidic linkages and α-1,4-D-galactosiduronic linkages play a crucial role in the immunomodulation effects of *Lycium Barbarum* berries [154].

Although PACE demonstrates excellent separation effects and fluorescence detection sensitivity, the manual gel preparation required before each experiment hinders the method's repeatability. Conversely, HPTLC offers convenience, effectiveness, and high sensitivity without gel preparation. HPTLC-based saccharide mapping can detect polysaccharide responses to glycosidase and analyze their hydrolysates. Using aniline diphenylamine colorimetry, HPTLC detection is more sensitive than commonly used HPLC detectors such as RID and ELSD. Furthermore, HPTLC allows simultaneous analysis of multiple samples, facilitating easy result comparison. However, unlike PACE, which can separate polysaccharides with a degree of polymerization up to 40, HPTLC is limited to oligosaccharides with a degree of polymerization no greater than 15. Consequently, to achieve superior identification and thorough characterization of polysaccharide hydrolysates, researchers often combine HPTLC and PACE. A study comparing *Pseudostellaria heterophylla* from Guizhou, Anhui, and Fujian Provinces using PACE and HPTLC (Figs. 2D and 2E) revealed similar fingerprints for endo-arabinanase, 1,4-β-galactanase, and pectinase hydrolysates, indicating that polysaccharides could serve as standard substances for the quality control of this herb [153].

HPLC has become increasingly significant in saccharide mapping, particularly in the separation of natural products. It is frequently employed to validate the response of polysaccharides to endoglycosidases, thereby indicating the presence of specific glycosidic bonds. The coupling of HPLC with MS has emerged as a valuable tool for analyzing polysaccharide hydrolysates in saccharide mapping. In a study of polysaccharides from *L. edodes*, a UPLC-FLR-MS system was utilized to identify oligosaccharides released by 1,3-β-glucanase as disaccharide, trisaccharide, and tetrasaccharide [155] (Fig. 2F).

Furthermore, CE-LIF is employed for saccharide mapping to achieve enhanced resolution and sensitivity. This method offers significant advantages, including high efficiency, rapid analysis, and minimal consumption of organic solvents and samples. In the identification of *Hericium erinaceus* polysaccharides (Fig. 2G), CE-LIF effectively separates saccharide isomers from enzymatic hydrolysates, with released oligosaccharides from polysaccharides of different origins exhibiting varying quantities [156]. Beyond the aforementioned methods, efforts are being made to develop identification approaches that do not require complex derivatization. HPAEC-PAD enables direct analysis of oligosaccharides following enzymatic hydrolysis. Utilizing hierarchical cluster analysis, *Grifola frondose* polysaccharides can be categorized into distinct groups based on their resources, using complete HPAEC-PAD chromatograms [157, 158] (Fig. 2H). Additionally, a saccharide mapping method utilizing MALDI-TOF-MS has been developed to authenticate commercial xylo-oligosaccharides. This method, requiring no derivatization, can be completed in less than 35 minutes, from sample preparation to obtaining MS profiles [158] (Fig. 2I).

4.2. Quantitative analysis methods

To date, the primary quantitative analysis methods for Chinese herbal polysaccharides encompass colorimetry and chromatography-based techniques. In colorimetry, the phenol-sulfuric acid and anthrone sulfuric acid methods are widely employed for total carbohydrate determination due to their operational simplicity and high sensitivity [159]. These methods share a common principle: under concentrated sulfuric acid, polysaccharides undergo hydrolysis into monosaccharides and dehydration to form furfural. Subsequently, furfural condenses with sulfuric acid or anthrone reagents, producing orange-red or blue-green compounds, respectively. These compounds are detectable at specific maximum absorption wavelengths and quantifiable using glucose standard-based calibration curves. However, the reactivity varies among monosaccharide species, each exhibiting a unique absorbance at the maximum absorption wavelength. Consequently, when glucose is the sole reference compound, the method's accuracy may be significantly affected by the monosaccharide composition. For uronic acid content determination in polysaccharides, m-phenylphenol or carbazole-sulfuric acid methods are utilized, though their accuracy may be compromised due to neutral polysaccharide interference. Zhou et al. developed a non-destructive system for monosaccharide quantification using an attenuated total reflectance-Fourier transform infrared spectroscope (ATR-FTIR). This method successfully determined the monosaccharide composition of various *Atractylodes* polysaccharides without acid hydrolysis. However, its application to other polysaccharide sources remains limited, and the associated chemometrics are complex [160]. Regarding chromatography-based methods, HPLC and GC are frequently employed for quantifying monosaccharides released by complete acid hydrolysis. These methods offer higher sensitivity, stability, and repeatability compared to colorimetry. Nevertheless, minor variations in the hydrolysis process can significantly impact the final results [145].

The bioactivity of polysaccharides is closely related to their molecular weight and distribution, necessitating a method for rapid and accurate quantification of polysaccharides and their biologically functional fractions. Oligosaccharides, containing more structural characteristics reflective of polysaccharides' bio-

logical potential than monosaccharides, may offer a solution to this challenge. The released oligosaccharides potentially exhibit a linear relationship with the content of parent polysaccharides. Research indicates that *β*-(1-3)-glucan-related polysaccharides may be the primary bioactive components in *Hericium erinaceus*. Consequently, *β*-(1-3)-D-glucanase was employed to hydrolyze polysaccharides from *Hericium erinaceus*. Utilizing laminaritriose as an internal standard, enzymatic hydrolysates from nine batches of *Hericium erinaceus* polysaccharides were quantified by CE-LIF with high feasibility [156] (Fig. 3B). Similarly, coupling UPLC with fluorescence and MS enables qualitative and quantitative analysis of polysaccharides based on specific released oligosaccharides. Specifically, *Lentinus edodes* polysaccharides were first hydrolyzed by 1,3-*β*-glucanase, and the released oligosaccharides were derivatized with 2-aminobenzamide and analyzed on HILIC-FLR, using laminaritriose as the standard [155]. Agar, a common adulterant in edible bird's nest (EBN) that is difficult to detect, was addressed by Cheng et al., who established a quantitative method based on an oligosaccharide marker released from the products by hydrochloric acid. Their findings revealed that 60% of commercial Agar was starch, an illegal additive in EBN, highlighting the significance of this method for food quality control [161]. However, these methods require specific oligosaccharides as references, which can be challenging to obtain, limiting their broader application. Our group developed an accurate and rapid saccharide mapping method, HPSEC-MALLS-RID, to quantify polysaccharide fractions without polysaccharide standards. This method employs HPSEC for polysaccharide separation, MALLS for molecular mass determination of polysaccharide fractions, and quantification of polysaccharide fractions using a refractive index detector, based on the universal refractive index increment (*dn/dc*) [150, 162] (Fig. 3A). Application of this method to analyze polysaccharides from *Panax ginseng*, *Panax notoginseng*, and *Panax quinquefolius* revealed differences in molecular masses and contents of target polysaccharides and their fractions, potentially improving the quality control of these three plants [162]. This saccharide mapping-based method is particularly suitable for analyzing polysaccharides with specific structural characteristics and polysaccharide fractions with high bioactivities, as it can accurately and quantitatively determine characteristic fragments in enzymatic hydrolysis products. Coupling this method with biological screening assays to characterize polysaccharides from *Grifola frondose* revealed that fractions of Peak 3 were the major contributors to immune-enhancing activity, emphasizing the importance of its content in quality control [157]. Furthermore, this method can also be utilized for retrospective quantification of active polysaccharide fractions in a traditional Chinese medicine formula containing seven herbs. Specifically, fraction CD2 with a molecular weight of 100–1000 kDa from the complex mixture exhibited the strongest immunomodulatory activity, suggesting its

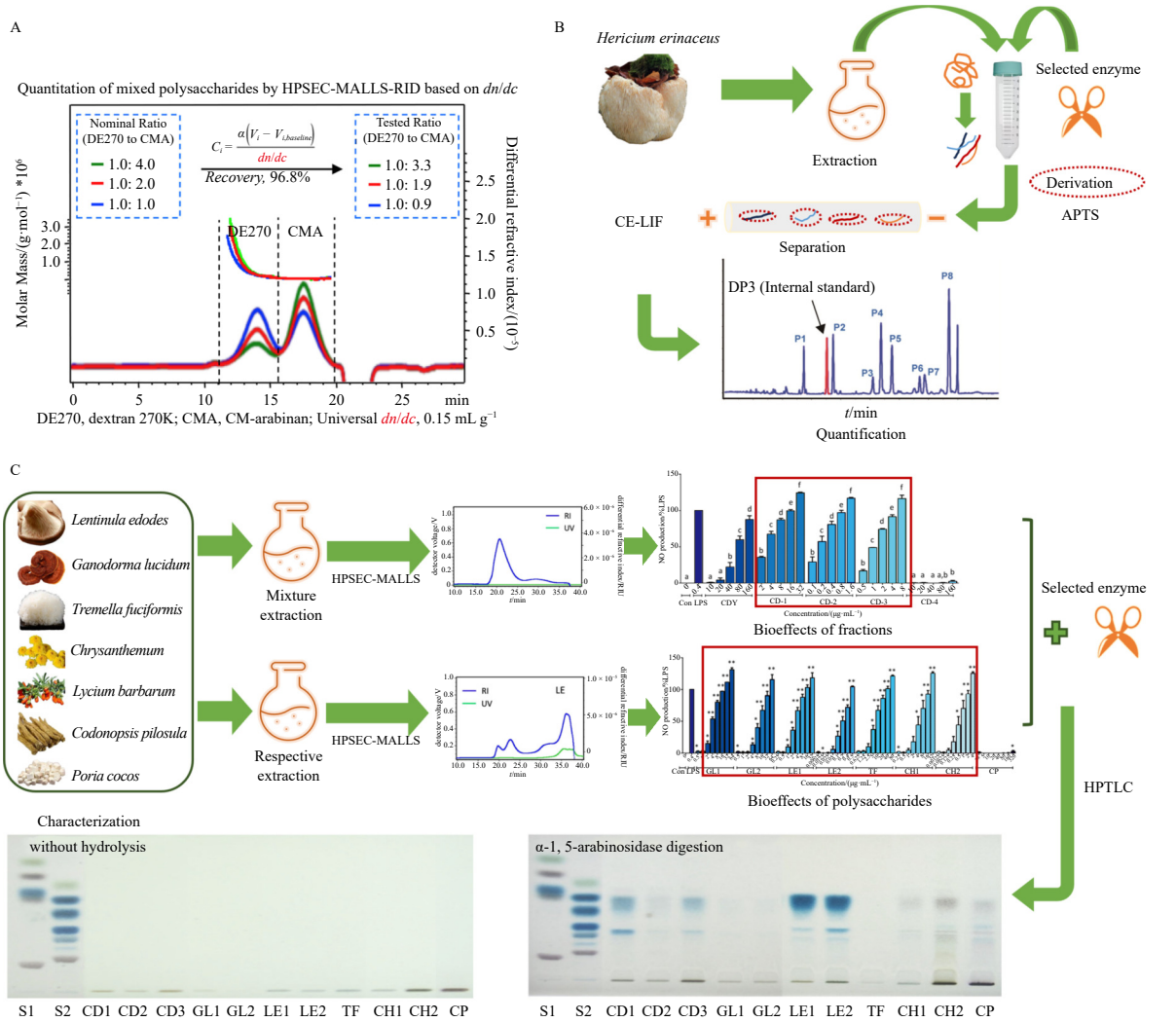

Fig. 3 Application of saccharide mapping in quantitative analysis of Chinese herbal polysaccharides. (A) Quantitative analysis of mixed polysaccharides and their ratios using HPSEC-MALLS-RID with the universal *dn/dc*. (B) Quantitative analysis of polysaccharides from *Hericium erinaceus* by CE-LIF based on an internal standard. (C) Quantitative analysis of polysaccharide fractions with bioactive effects from seven herbs.

potential for improving the quality control of the formula [163] (Fig. 3C).

In comparison to alternative methodologies, saccharide mapping utilizes specific endoglycosidases for polysaccharide depolymerization, offering a relatively gentle and controllable process. Notably, the resulting oligosaccharides can serve as markers for specific polysaccharide types. Concurrently, assessing the biological activity of these oligosaccharides aids in exploring the active structural fragments of polysaccharides. Furthermore, by integrating multiple chromatographic analyses, saccharide mapping enables both qualitative and quantitative determination of polysaccharides, facilitating the distinction and evaluation of Chinese medicinal materials from diverse sources. Moreover, this mapping technique may be applied in elucidating complex polysaccharide structures through high-resolution tandem mass spectrometry and sugar-related database software analysis tools. Consequently, it may play a crucial role in investigating the structure-bioactivity relationship of polysaccharides.

5. Conclusion

Polysaccharides are potentially one of the primary functional components in traditional Chinese medicinal materials, offering a rich resource for novel drug development. Extracting polysaccharides from medicinal herbs is the initial step in utilizing these resources. Currently, the majority of research employs water extraction and alcohol precipitation methods to obtain targeted polysaccharides. Although emerging technologies such as subcritical water, pulsed electric fields, and supercritical fluids have been introduced, the process remains laborious and time-intensive. Moreover, the high pressure or high temperature involved in some methods may compromise the structure of polysaccharides, resulting in high production but diminished bio-functionality, which warrants further investigation. Consequently, developing new methods or introducing technologies from other fields is of significant importance. Regarding the elucidation of polysaccharide structure, a common analysis process has been established, primarily involving the measurement of molecular weight, monosaccharide composition, and linkage modes. However, variations in operational procedures may lead to inconsistent results. Additionally, while electron microscopy, atomic force microscopy, and other methods can provide information about conformation, accurately reflecting the structure of polysaccharides in physiological environments remains challenging and requires further exploration. Saccharide mapping is an emerging method for convenient and accurate quality control of polysaccharides. It is based on enzymatic hydrolysis followed by various chromatographic methods such as HPTLC, PACE, CE, and mass spectrometry. Furthermore, multidimensional chromatographic characteristic analysis of polysaccharide enzymatic hydrolysis can be established to realize glycospectroscopy based on multivariate parameters of polysaccharides, indicating its broad application potential in the quality control of traditional Chinese medicine.

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Declaration of competing interest

These authors have no conflict of interest to declare.

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