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Effects of drying methods on the physicochemical characteristics and bioactivities of polyphenolic-protein-polysaccharide conjugates from *Hovenia dulcis*

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ABSTRACT

Four drying methods, including freeze drying, hot air drying, vacuum drying, microwave drying at 400, 600, and 800 W, were applied to dry the peduncles of *Hovenia dulcis*. Then the effects of different drying methods on physicochemical characteristics and bioactivities of polyphenolic-protein-polysaccharides conjugates extracted from *H. dulcis* (PPPs) were investigated and compared. Results showed that different drying methods affected the physicochemical characteristics and bioactivities of PPPs. Noticeable variations in extraction yields, contents of total proteins, contents of total polyphenolics, contents of total flavonoids, contents of uronic acids, molecular weights, apparent viscosities, molar ratios of constituent monosaccharides, ratios of constituent amino acids, and degrees of esterification were observed in PPPs obtained by different drying methods. Besides, a total of 13 phenolic compounds in PPPs were identified by UPLC-ESI-QTOF-MS. In addition, PPPs, especially PPP-M6 and PPP-V dried by microwave drying at 600 W and vacuum drying, respectively, exhibited remarkable antioxidant activities, antiglycation activities, and inhibitory activities on α-amylase and α-glucosidase. Results suggested that the microwave drying and vacuum drying techniques could be appropriate drying methods before extraction of PPPs with high bioactivities for applications in the functional food and medicine industries.

1. Introduction

Natural conjugates extracted from edible and medicinal plants, such as polyphenolic-polysaccharide conjugate, polyphenolic-protein conjugate, and polyphenolic-protein-polysaccharide conjugate, possess various health benefits [1], which have attracted increasingly interests in recent years. For instance, the polyphenolic-polysaccharide conjugates extracted from medicinal plants exhibit strong antioxidant [1,2] and anticoagulant activities [3,4]. The polyphenolic-protein conjugates also exert strong antioxidant activity [5]. Furthermore, the polyphenolic-protein-polysaccharide conjugates exhibit remarkable antioxidant [6], antibacterial [6], antifungal [6], and antitussive activities [7,8]. Therefore, the discovery and characterization of natural conjugates from edible and medicinal plants as safe compounds for medicine and health foods are necessary, which has become a hot research spot worldwide.

Hovenia dulcis Thunb. (Rhamnaceae), known as "Zhi Ju" or "Guai Zao" in China, is a deciduous tree native to East Asia and mainly distributed in China, Japan and Korea [9]. It is a medicinal and edible fruit, which has been used for the treatment of various diseases such as alcoholism and liver diseases in China. Generally, the leaves, seeds, peduncles, barks, and roots of *H. dulcis* are capable of promoting digestion, quenching thirst, producing saliva, and relieving the symptoms of hangover and antifebrile [10]. Previous studies have revealed that phenolic compounds extracted by methanol/ethanol from *H. dulcis* are identified as natural antioxidants [11,12] and hypoglycemic agents [13]. In addition, polysaccharides are also regarded as one of the most important bioactive components in *H. dulcis* [9,10,14–18], which possess multiple bioactivities such as antioxidant activity [9,14–16], hypoglycemic activity [16], and immunostimulatory activity [17]. However, as far as we know, studies on the polyphenolic-protein-polysaccharide conjugates extracted from *H. dulcis* are limited. Therefore, the investigation of physicochemical characteristics and bioactivities

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of polyphenolic-protein-polysaccharides (PPPs) from *H. dulcis* is necessary, which is beneficial to well understand the chemical characters and bioactivities of *H. dulcis*.

The fresh peduncles of *H. dulcis* contain more than 50% of water, which are susceptible to deterioration. Therefore, fresh peduncles of *H. dulcis* require appropriate drying to prolong shelf-life, maintain quality, and reduce nutrient loss. Considering the drying efficiency, convenience, time consuming, cost, and environmental influence, different drying techniques possess their own advantages and disadvantages. Several drying methods have been used in food processing and preservation, such as freeze drying (FD), hot air drying (HD), vacuum drying (VD), and microwave drying (MD) [19–22]. FD preserves the original nutrition compositions, bioactive ingredients, and color, which is based on the dehydration by sublimation of a frozen product [23]. HD is low cost and easily controlled, which is solid to be dried through a continuous flowing hot stream of air [23]. VD can avoid the structure changes caused by the high temperature and presence of oxygen, which is also a frequently used drying technique [24]. MD has a high drying rate, homogeneous energy delivery on the material, and good process control [20]. Previous studies have revealed that different drying methods have significant effects on the structure and biological activity of bioactive compounds in fruits and vegetables [19–22]. However, as far as we know, effects of different drying methods on the physicochemical characteristics and biological activities of PPPs extracted from *H. dulcis* have never been investigated. Whether the physicochemical characteristics and bioactivities of PPPs extracted from *H. dulcis* are influenced by different drying procedures remains unknown.

Therefore, in the present study, effects of different drying methods, including freeze drying, hot air drying, vacuum drying, and microwave drying at 400 W, 600 W, and 800 W, on the physicochemical characteristics and bioactivities of polyphenolic-protein-polysaccharide conjugates extracted from *H. dulcis* were systemically investigated and compared.

2. Materials and methods

2.1. Material and chemicals

The fresh peduncles of *H. dulcis* were collected from Ankang City, Shaanxi Province, China.

Trifluoroacetic acid (TFA), bovine serum albumin, soluble starch, α-amylase (1000 U/mg), α-glucosidase (100 U/g), acarbose, monosaccharide standards (rhamnose, mannose, glucuronic acid, galacturonic acid, glucose, galactose, xylose and arabinose), amino acid standards (aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine), gallocatechin, rutin, myricetin, quercetin, kaempferol, 1-phenyl-3-methyl-5-pyrazolone (PMP), *m*-hydroxydiphenyl, griess reagent, vitamin C (VC), sodium nitroprusside (SNP), 2,2′-azino-bis(3-ethyl benzthiazoline-6-sulphonic acid) (ABTS), butylated hydroxytoluene (BHT), 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), and 4-nitrophenyl β-D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and chemicals used were of analytical grade.

2.2. Drying experiments

Fresh peduncles of *H. dulcis* were washed and sliced into small pieces, and then used for drying. Four drying methods, including freeze drying (FD), hot air drying (HD), and vacuum drying (VD), as well as microwave drying (MD) at the microwave power of 400, 600, and 800 W, were performed. Briefly, for the FD, peduncles of *H. dulcis* were freeze-dried at −40 °C for 96 h (SCIENTZ-12N, Ningbo Scientz Biotechnology Co., Ltd., China). HD was carried out by using a hot air drying oven (101A-3, Shanghai Experimental Instrument Factory Co., Ltd., China) at 75 °C for 9.5 h according to the previous study [21]. VD was carried out by using a vacuum drying oven (DZF-6050, Shanghai San Fa Scientific Instruments Factory Co., Ltd., China) at 50 °C for 33 h according to the previous study [25]. Microwave drying was carried out by using a microwave drying oven (MKJ-J1-3, Qingdao Makewave Microwave Applied Technology Co., Ltd, Shandong, China) at the microwave power of 400, 600, and 800 W for 12.5, 7.5, and 3.5 min, respectively. Finally, the moisture content of all dried samples reached approximately 5% (wet basis), and the moisture content was detected by a moisture meter (XY-105 W, Qingdao Tuo Ke Instruments Factory Co., Ltd., China). After drying, the peduncles of *H. dulcis* dried by different drying methods were grounded into powder, and passed through a 60-mesh sieve, and sealed in airtight plastic bags before use.

2.3. Hot water extraction of polyphenolic-protein-polysaccharide conjugates

Hot water extraction of polyphenolic-protein-polysaccharide conjugates (PPPs) from the peduncles of *H. dulcis* was performed according to a previously reported method with slight modifications [16]. Briefly, the dried *H. dulcis* powders (10.0 g) were firstly refluxed with 100.0 mL of 80% (*v*/*v*) ethanol at 80 °C for 2 h to remove most of the small molecules. PPPs in the extracted residues were then extracted twice with 300.0 mL of deionized water at 95 °C for 3 h. After removing the starch and proteins by heat stable α-amylase (1.0 U/mL) and pancreatin (1.0 U/mL), respectively, the supernatants were precipitated with three volumes of 95% ethanol (*v*/*v*) overnight at 4 °C. Then, the precipitates were redissolved in deionized water, and transferred to an Amicon ultracentrifugal filter device (molar mass cutoff: 3 kDa, Millipore, Billerica, MA, USA). The low molecular weight compounds (below 3 kDa) were removed by centrifugation (3500*g* for 25 min) for three times. Finally, PPPs obtained from the peduncles of *H. dulcis* dried by freeze drying (FD), hot air drying (HD), vacuum drying (VD), and microwave drying at 400 W (M4), 600 W (M6), and 800 W (M8) were coded as PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, PPP-M8, respectively.

2.4. Physicochemical characterization of PPPs

2.4.1. Physicochemical properties analysis of PPPs

The chemical compositions, including the contents of total polysaccharides, uronic acids, and proteins in PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8, were analyzed by the phenol-sulfuric acid method, the m-hydroxydiphenyl method, and the Bradford's method according to previously reported methods [26]. Furthermore, the contents of total polyphenolics and flavonoids were determined by the methods according to the previous study [27].

2.4.2. Determination of molecular weights, apparent viscosities, and constituent monosaccharides of PPPs

The absolute molecular weights (M_w) and polydispersities (M_w/M_n) of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were measured by high-performance size-exclusion chromatography coupled with a multi-angle laser light scattering and refractive index detector (HPSEC-MALLS-RID, Wyatt Technology Co., Santa Barbara, CA, USA) according to our previously reported method [26]. The Shodex OHpak SB-806 M HQ (300 mm × 8.0 mm, i.d.) column was utilized for the separation of PPPs at 30 °C. Furthermore, the apparent viscosities of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6,

and PPP-M8 were also measured by a Discovery Hybrid Rheometer-1 (DHR-1, TA Instruments, New Castle DE, USA) equipped with a parallel steel plate (40 mm diameter, 1.0 mm gap) according to our previously reported method [28]. Moreover, constituent monosaccharides of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were measured by high performance liquid chromatography (HPLC, Agilent Technologies, Santa Clara, CA, USA) according to our previously reported method [28].

2.4.3. Fourier transform infrared (FT-IR) spectroscopy analysis of PPPs

The fourier transform infrared (FT-IR) analysis of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 was also performed by a Nicolet iS 10 FT-IR (ThermoFisher scientific, Waltham, MA, USA) according to our previously reported method [26].

2.4.4. Determination of amino acid compositions of PPPs

Amino acid compositions of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were measured according to a previously reported method with some modifications [29]. Briefly, the sample (20.0 mg) was hydrolyzed with 5.0 mL of 6 M HCl at 110 °C for 24 h in a sealed tube. The hydrolysates were dried by evaporation under reduced pressure, and redissolved in 2.0 mL of 0.02 M HCl. The amino acid compositions were analyzed by using an automated amino acid analyzer (HITACHI L-8900, Amino Acid Analyzer, Japan).

2.4.5. Identification of phenolic compounds of PPPs

In order to release phenolic compounds from PPPs, PPPs were firstly degraded by heat-stable α-amylase, glucoamylase, pectinase, and trypsin. Then, the released polyphenolic fractions from PPPs were analyzed by using an UPLC 1290 series system coupled with a high resolution quadrupole time-of-flight mass spectrometer (G6545 Q-TOF-MS, Agilent Technologies, Palo Alto, CA, USA). Meanwhile, a ZORBAX SB-C18 column (2.1 mm × 50 mm, 1.8 μm, Agilent Technologies, Palo Alto, CA, USA) was utilized for the separation of phenolic compounds. The mobile phase consisted of two solvents: water (A) and acetonitrile (B), and the linear gradient solvent system was as follows: 0–10 min, 5–20% B; 10–15 min, 20–60% B; 15–20 min, 60–90% B; 20–21 min, 90–5% B. Parameters for Q-TOF-MS analysis were set using negative ion mode with spectra acquired over a mass range from 50 to 3000 m/z. The optimum values of the ESI-MS parameters were: capillary voltage of +4.0 kV; drying gas temperature of 350 °C; drying gas flow of 12.0 L/min; nubilizing gas pressure of 40 psi. Moreover, the pseudomolecular ions $[M-H]^-$ were selected as precursor ions. The Data Acquisition software (Agilent Technologies, Palo Alto, CA, USA) and Qualitative Analysis 10.0 software (Agilent Technologies, Palo Alto, CA, USA) were utilized for data acquisition and analysis, respectively. Then, the Personal Compound Database Library Manager B. 08.00 software (PCDL, Agilent Technologies, Palo Alto, CA, USA) and Traditional Chinese Medicine Database (TCM-DATABASE, Agilent Technologies, Palo Alto, CA, USA) were used for the identification of compounds.

2.5. Evaluation of in vitro antioxidant activities of PPPs

The ABTS, DPPH, nitric oxide (NO) radical scavenging activities, and ferric reducing antioxidant powers (FRAP) of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were measured according to our previously reported methods [30,31]. Both BHT and vitamin C were used as positive controls. The ABTS, DPPH, NO radical scavenging activities of PPPs were measured at five different concentrations, and the IC_{50} values (mg/mL) were calculated based on a logarithmic regression curve. Afterwards, the FRAP was expressed as the absorbance at 593 nm.

2.6. Evaluation of in vitro antiglycation activities of PPPs

Antiglycation activities of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were measured by BSA/glucose model according to a previously reported method with slight modifications [32]. In brief, a total 5.0 mL of reaction mixture consisted of 5% BSA (*w/v*), glucose (500 mmol/L), sodium azide (0.1%), phosphate buffer (200 mmol/L, pH 7.4), and PPPs at different concentrations (0.25–2.00 mg/mL). Aminoguanidine (AG) was used as the positive control. All the mixtures were incubated at 37 ± 0.5 °C for 14 days. The fluorescence of the glycated solution was determined using a Sanko 930 A spectrofluorometer at an excitation/emission wavelength of 370/440 nm, which is characteristic of advanced glycation end products (AGEs). The inhibition activity on AGEs was measured at different concentrations, and a logarithmic regression curve was established to calculate IC_{50} values (mg/mL).

2.7. Evaluation of in vitro α-amylase and α-glucosidase inhibitions of PPPs

Both α-glucosidase and α-amylase inhibitory effects of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were measured according to our previously reported method with slight modifications [33]. The α-amylase solution (10 U/mL, dissolved in 0.1 M, pH 6.8 phosphate buffer), α-glucosidase solution (3.0 U/mL, dissolved in 0.1 M pH 6.8 phosphate buffer), and pNPG (8 mM, dissolved in 0.1 M pH 6.8 phosphate buffer) were used in this study. Acarbose standard was used as a positive control. The α-amylase and α-glucosidase inhibitions were measured at five different concentrations, and a logarithmic regression curve was established to calculate IC_{50} values (μg/mL).

2.8. Statistical analysis

All experiments were conducted in triplicate, and data were expressed in means ± standard deviations. Statistical analysis was performed using Origin 9.0 software (OriginLab Corporation, Northampton, Mass., USA). Statistical significances were carried out by one-way analysis of variance (ANOVA), followed by Duncan's test. Values of $p < 0.05$ were considered as statistically significant.

3. Results and discussions

3.1. Physicochemical characteristics of PPPs

3.1.1. Chemical compositions of PPPs

The moisture contents of the peduncles of *H. dulcis* dried by FD, HD, VD, M4, M6, and M8 were determined to be (4.79 ± 0.23)%, (3.67 ± 0.19)%, (4.13 ± 0.35)%, (2.02 ± 0.33)%, (3.74 ± 0.59)%, and (4.98 ± 0.64)%, respectively. The extraction yields and chemical compositions of PPPs are summarized in Table 1. As shown in Table 1, different drying methods had obvious effects on the extraction yields of PPPs, which ranged from (2.61 ± 0.07)% to (5.20 ± 0.14)%. Indeed, the highest extraction yield was observed in PPP-M4 among all PPPs extracted from *H. dulcis* dried by different methods. The high extraction yields of PPP-M4, PPP-M6, and PPP-M8 obtained by microwave drying might be due to the fact that the intense heat generated from the microwave creates a high vapor pressure and temperature inside plant tissue, resulting in the disruption of plant cell wall polymers [34]. Moreover, there was no significant difference observed in contents of total polysaccharides in PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8. The total polysaccharides in PPPs ranged from (38.15 ± 1.38)% to (41.85 ± 2.59)%, which were similar with the sugar contents in the crude

Table 1
Chemical compositions of PPPs.

Chemical compositions	PPP-F	PPP-H	PPP-V	PPP-M4	PPP-M6	PPP-M8
Extraction yields (%)	2.61 ± 0.07^{d}	3.03 ± 0.09^{c}	3.16 ± 0.10^{c}	5.20 ± 0.14^{a}	4.58 ± 0.16^{b}	5.09 ± 0.21^{a}
Total polysaccharides (%)	38.86 ± 1.33^{a}	38.15 ± 1.38^{a}	40.41 ± 1.82^{a}	41.85 ± 2.59^{a}	41.84 ± 3.16^{a}	39.90 ± 3.82^{a}
Total uronic acids (%)	9.06 ± 2.10^{ab}	10.22 ± 2.50^{a}	7.46 ± 0.50^{ab}	4.44 ± 0.65^{c}	6.51 ± 1.24^{bc}	4.65 ± 0.84^{c}
Protein contents (%)	29.57 ± 0.76^{b}	33.93 ± 0.85^{a}	26.67 ± 0.82^{c}	22.90 ± 1.08^{d}	22.55 ± 1.05^{d}	24.03 ± 1.19^{d}
Degree of esterification (%)	8.53 ± 0.25^{b}	13.98 ± 0.46^{a}	6.22 ± 0.12 ^{cd}	6.43 ± 0.16^{c}	5.89 ± 0.21^{d}	4.78 ± 0.17^{e}
TPC (mg GAE/g)	213.21 ± 3.83^{c}	177.55 ± 0.92^{d}	249.14 ± 2.61^{b}	256.926 ± 2.60^{a}	257.89 ± 2.50^{a}	256.93 ± 4.25^{a}
TFC (mg RE/g)	151.77 ± 2.68^{b}	111.92 ± 1.65^{e}	194.36 ± 6.19^{a}	144.11 ± 3.39^{c}	133.82 ± 3.97^{d}	138.36 ± 1.92 ^{cd}

PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, PPP-M8, polyphenolic-protein-polysaccharide conjugates extracted from the peduncles of *H. dulcis* dried by freeze drying, hot air drying, vacuum drying, microwave drying at 400 W, microwave drying at 600 W, and microwave drying at 800 W, respectively.
TPC, total polyphenolic content; **mg GAE/g**, mg of gallic acid equivalent per gram of PPPs; **TFC**, total flavonoid content; **mg RE/g**, mg of rutin equivalent per gram of PPPs. Values represent mean ± standard deviation, and superscripts (a-f) differ significantly ($p < 0.05$) among PPPs; Statistical significances were carried out by ANOVA, followed by Duncan's test.

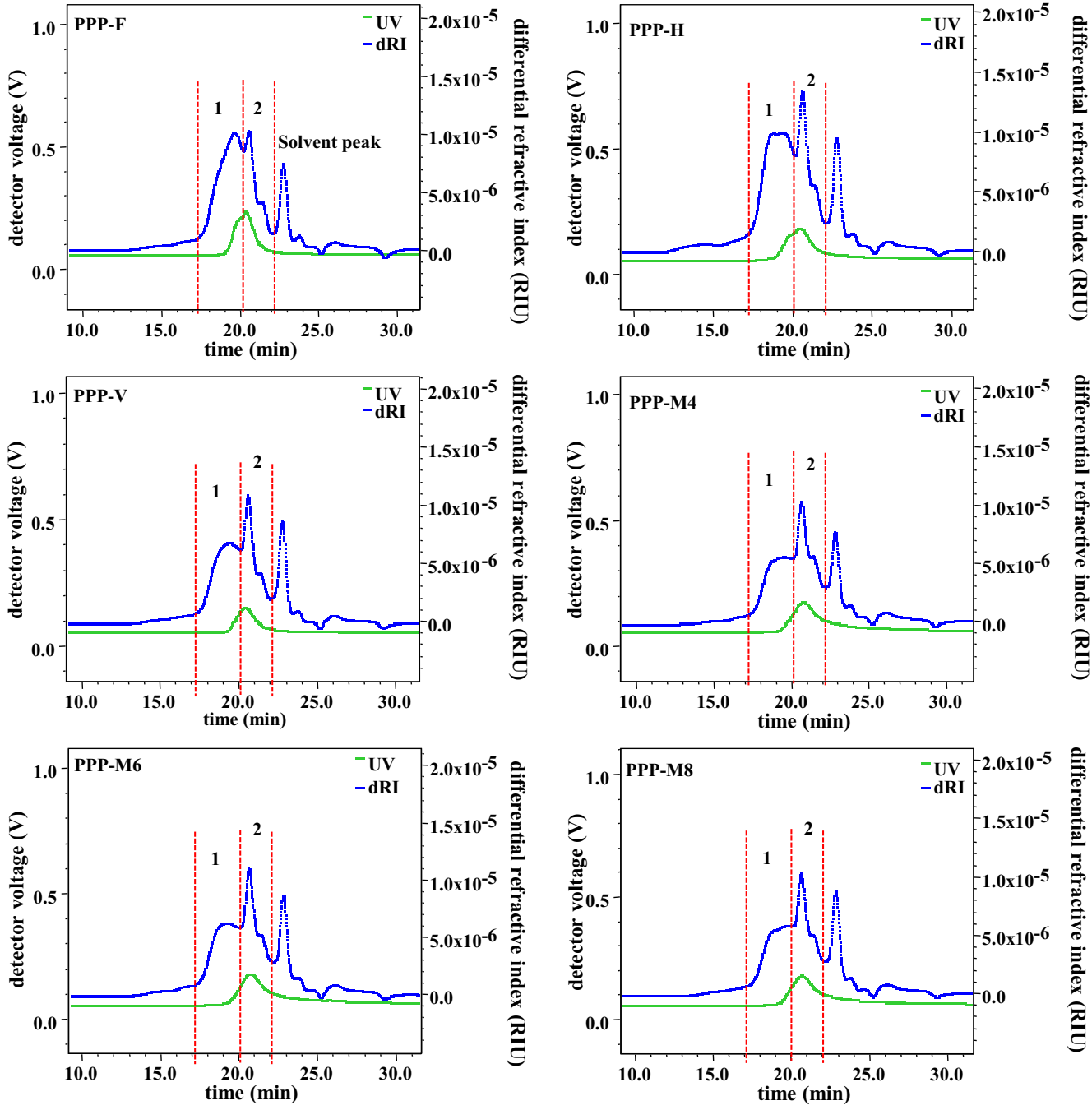

Fig. 1. High performance size exclusion chromatograms of PPPs. PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, PPP-M8, polyphenolic-protein-polysaccharide conjugates extracted from the peduncles of *H. dulcis* dried by freeze drying, hot air drying, vacuum drying, microwave drying at 400 W, microwave drying at 600 W, and microwave drying at 800 W, respectively.

polysaccharides extracted from the peduncles of *H. dulcis* by hot water extraction [16]. Results indicated that polysaccharide was one of the major biological components in PPPs. In addition, the contents of proteins in PPPs ranged from (22.55 ± 1.05)% to (33.93 ± 0.85)%, which suggested that protein was also one of the most important components in PPPs. Furthermore, the contents of total uronic acids in PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6 and PPP-M8 were significantly affected by different drying methods, which were determined to be (9.06 ± 2.10)%, (10.22 ± 2.50)%, (7.46 ± 0.50)%, (4.44 ± 0.65)%, (6.51 ± 1.24)%, and (4.65 ± 0.84)%, respectively. Results suggested that pectin-like acidic polysaccharides existed in *H. dulcis* [26,35]. Moreover, although the small molecules were removed by ethanol extraction, ethanol precipitation, and ultrafiltration, polyphenolics and flavonoids were also observed in PPPs. The contents of total polyphenolics in PPPs ranged from 177.55 ± 0.92 mg GAE/g to 257.89 ± 2.50 mg GAE/g, which suggested that natural polyphenolic-protein-polysaccharide conjugates might exist in PPPs extracted from *H. dulcis* [6]. The formation of polyphenolic-protein-polysaccharide conjugates might be mediated by H-bonding and hydrophobic interactions, as well as by the existence of hydrophobic cavities and crevasses [6]. The highest content of total polyphenolics was observed in PPP-M6, which might be partially due to the fact that the intense heat generated by microwaves produces a sudden high pressure and temperature inside tissue matrix, leading to more prompt inactivation of oxidative and/or hydrolytic enzymes, and greater cell disruption than other drying treatments [36]. In addition, the contents of total flavonoids in PPPs ranged from 111.92 ± 1.65 mg RE/g to 194.36 ± 6.19 mg RE/g. Results showed that the contents of total polyphenolic and flavonoids were significantly affected by different drying methods.

3.1.2. Molecular weights, apparent viscosities, and constituent monosaccharides of PPPs

The molecular weights (M_w), viscosities, and constituent monosaccharides are important properties of polysaccharides that influence their bioactivities, and different drying processes can affect their molecular weights, and constituent monosaccharides [20,31,37]. Therefore, molecular weights, apparent viscosities, and constituent monosaccharides of PPPs obtained by different drying methods were investigated and compared. Fig. 1 showed HPSEC-RID-UV chromatograms of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8. Results suggested that different drying methods significantly affected molecular weight distributions of PPPs. Two fractions were observed in PPPs and the solvent peak was detected at 22 min. In addition, the UV (280 nm) signal was observed in both fraction 1 and fraction 2, which further indicated that polyphenolics and proteins might bond on polysaccharide fractions in PPPs. As shown in Table 2, molecular weights of fraction 1 and fraction 2 in PPPs ranged from 3.60×10^4 Da to 5.16×10^4 Da, and from 0.31×10^4 Da to 1.78×10^4 Da, respectively, which were lower than that of previous studies (from 5.3×10^3 Da to 5.794×10^6 Da) [14,16,17]. The peak areas of fraction 1 and fraction 2 in PPPs ranged from 46.6% to 64.7%, and from 35.3 to 53.4% (Fig. 1), respectively, which indicated that the high M_w fraction (fraction 1) of PPP-M4, PPP-M6, and PPP-M8 were degraded and converted into low M_w fractions (fraction 2) during microwave drying [30,35]. Furthermore, the polydispersities of fraction 1 and fraction 2 in PPPs ranged from 1.42 to 1.91, and from 1.04 to 1.10, respectively, which were in accordance with the HPSEC-RID chromatograms.

Moreover, the influence of shear rate on the apparent viscosity of each sample with the concentration of 10.0 mg/mL at 25 °C were shown in Fig. 2A. The apparent viscosities of PPPs depended on the shear rate, which showed decreasing viscosity with the increase of shear rate under steady-shear conditions. Moreover, the PPPs solutions showed non-Newtonian shear thinning behaviors at low shear rate range (0.01–50 s^{-1}), but nearly Newtonian flow behavior at high shear rate range (50–100 s^{-1}). Previous studies have revealed that the shear-thinning behavior is significantly related to the aggregation or dispersal states of the molecules in the shear flow [28,38]. Furthermore, PPPs obtained by different drying methods showed the similar apparent viscosities (Fig. 2A), which indicated that different drying methods had no significant effects on the apparent viscosities of PPPs.

Furthermore, it is generally accepted that biological activities of polysaccharides were influenced by their compositional monosaccharides and molar ratios [39]. Therefore, constituent monosaccharides of the polysaccharide fractions in PPPs extracted from the peduncles of *H. dulcis* dried by different methods were investigated and compared. Fig. 2B showed that the HPLC-UV profiles of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were similar. The constituent monosaccharides of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were measured as GalA, Gal, Man, Rha, GlcA, Glc, and Ara. Molar ratios of GalA, Gal, Man, Rha, GlcA, Glc, and Ara in PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 are summarized in Table 2. Results suggested that different drying methods had no effects on the types of constituent monosaccharides in PPPs, but their molar ratios varied by different drying methods. Furthermore, GalA, Gal, and Ara were determined as the dominant monosaccha-

Table 2
Molecular weights (M_w), polydispersity (M_w/M_n), and constituent monosaccharides of PPPs.

	PPP-F	PPP-H	PPP-V	PPP-M4	PPP-M6	PPP-M8
$M_w \times 10^4$ (Da, error)						
Peak 1	3.60 (±0.41%)^f	5.16 (±0.43%)^a	3.79 (±0.41%)^e	4.68 (±0.32%)^d	5.00 (±0.26%)^b	4.86 (±0.29%)^c
Peak 2	0.44 (±4.08%)^e	0.76 (±1.87%)^d	0.31 (±3.73%)^f	1.50 (±1.11%)^c	1.66 (±1.12%)^b	1.78 (±1.09%)^a
M_w/M_n						
Peak 1	1.91	1.69	1.86	1.55	1.47	1.42
Peak 2	1.04	1.06	1.10	1.05	1.08	1.06
Constituent monosaccharide and molar ratios						
Galacturonic acid	1.00	1.00	1.00	1.00	1.00	1.00
Galactose	1.80	1.47	1.96	1.51	1.59	1.64
Arabinose	0.67	0.64	0.73	0.65	0.65	0.74
Mannose	0.10	0.06	0.12	0.10	0.09	0.08
Rhamnose	0.39	0.39	0.41	0.34	0.36	0.37
Glucuronic acid	0.02	0.02	0.02	0.02	0.02	0.02
Glucose	0.64	0.15	1.04	0.85	0.51	0.19

PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8, polyphenolic-protein-polysaccharide conjugates extracted from the peduncles of *H. dulcis* dried by freeze drying, hot air drying, vacuum drying, microwave drying at 400 W, microwave drying at 600 W, and microwave drying at 800 W, respectively.
Values represent mean ± standard deviation, and superscripts (a-f) differ significantly ($p < 0.05$) among PPPs; Statistical significances were carried out by ANOVA, followed by Duncan's test.

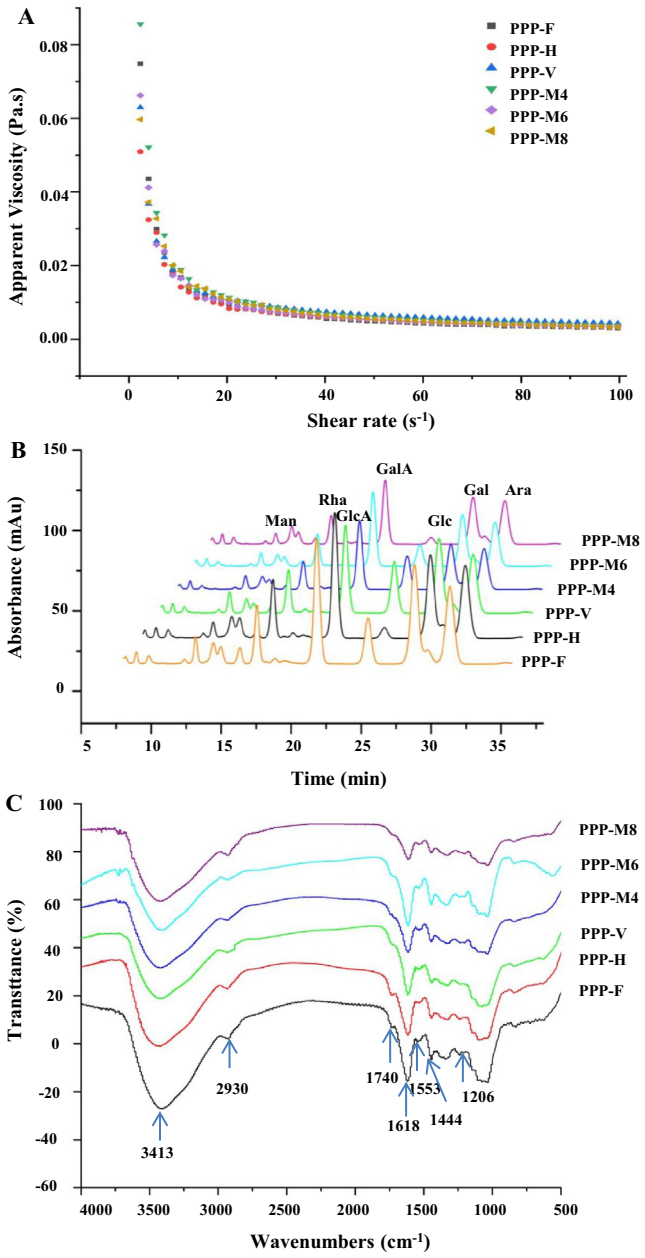

Fig. 2. Dependence of apparent viscosity on the shear rate (A), high performance liquid chromatography profiles (B), and FT-IR spectra (C) of PPPs. PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, PPP-M8, polyphenolic-protein-polysaccharide conjugates extracted from the peduncles of *H. dulcis* dried by freeze drying, hot air drying, vacuum drying, microwave drying at 400 W, microwave drying at 600 W, and microwave drying at 800 W, respectively; **Man**, mannose; **Rha**, rhamnose; **GlcA**, glucuronic acid; **GalA**, galacturonic acid; **Glc**, glucose; **Gal**, galactose; **Xyl**, xylose; **Ara**, arabinose.

rides in PPPs extracted from the peduncles of *H. dulcis*, which were similar with the previous study [9]. Results further confirmed that polysaccharides in PPPs were pectic-polysaccharides [26,33]. According to the constituent monosaccharides in PPPs, results suggested that rhamnogalacturonan I (RG I), homogalacturonan (HG), and arabinogalactan (AG II) might exist in PPPs extracted from the peduncles of *H. dulcis* [9,17,31].

3.1.3. Amino acid compositions and phenolic compositions of PPPs

A total of 16 amino acids were detected in the protein fractions of PPPs (Table 3). As presented in Table 3, contents of amino acids varied by different drying methods. The ratios of essential amino acids (EEA) in PPPs ranged from 21.17% to 29.78%. The predominant amino acids of PPPs were determined as arginine, aspartic acid, serine, glutamic acid, alanine, threonine, and glycine. The ratios of arginine, aspartic acid, serine, glutamic acid, alanine, threonine, and glycine in PPPs ranged from 9.44% to 22.03%, from 10.59% to 12.02%, from 7.94% to 9.03%, from 6.86% to 10.23%, from 5.92% to 8.66%, 6.31% to 7.19%, and from 5.99% to 7.08%, respectively. The similar trend was measured in polyphenolic-protein-polysaccharide conjugates extracted from *Cystoseira barbata*, in which threonine, aspartic acid, glutamic acid, serine, glycine, alanine, and arginine were the major amino acids [6].

Furthermore, multiple polar functional groups of polyphenols interact selectively and unselectively with bioactive macromolecules such as proteins and polysaccharides, which may affect the stabilization and consequently, change the bioactivities of these macromolecules [16]. Therefore, phenolic compositions of PPPs extracted from the peduncles of *H. dulcis* dried by different methods were investigated and compared. As shown in Fig. 3, a total of 13 compounds were detected in PPPs (PPP-M6 was selected as the representative sample), and their retention times, molecular ions, formulas, and scores obtained by PLCD were summarized in Table 4. Finally, 13 of phenolic compounds were identified by UPLC-ESI-QTOF-MS according to the *m*/*z* of compounds, TCM database, scores obtained by PLCD, several reference standards (gallocatechin, rutin, myricetin, quercetin, and kaempferol), and previous studies [40,41]. These compounds were identified as protocatechuic acid, gallocatechin, p-hydroxybenzoic acid, ampelopsin, quercetin-7,4′-diglucoside, dihydroquercetin, rutin, myricitrin, myricetin, quercetin, kaempferol, 5-methylmyricetin, and naringenin (Table 4). Indeed, some phenolic compounds, including protocatechuic acid, gallocatechin, p-hydroxybenzoic acid, ampelopsin, rutin, myricetin, quercetin, kaempferol, and naringenin, have been found in ethanol/methanol extracts from *H. dulcis* in previous studies [40,41]. These phenolic compounds might be associated with proteins and/or polysaccharides spontaneously by ionic interactions, hydrophobic interactions and hydrogen bonding, which happened in heating and other treating processes [42]. Furthermore, in this study, the contents of total polyphenolics in PPPs were measured before and after centrifugation by Amicon ultracentrifugal filter device, which had no significant difference. Results suggested that phenolic compounds were bonded on the conjugates.

3.1.4. FT-IR spectra of PPPs

The FT-IR spectra were used for determination of the structural features of PPPs. As shown in Fig. 2C, the FT-IR spectra of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 were similar, which indicated that PPPs extracted from the peduncles of *H. dulcis* dried by different methods had similar chemical structures. Briefly, a strong absorption at 3413 cm^{-1} represents the stretching vibration of hydroxyl group, while the weak peak at 2930 cm^{-1} is assigned to the stretching vibration of C-H [9,43]. The absorption band at 1740 cm^{-1} is assigned to be the C═O stretching vibration of esterified groups [26]. Furthermore, the intense peak that appeared at 1618 cm^{-1} is assigned to be the C═O asymmetric stretching of COO^{-}, suggesting the existence of uronic acids in PPPs [16,31]. The bands assigned to the amide I and amide II regions at 1618 cm^{-1} and 1553 cm^{-1} were visible in the spectra of PPPs, which indicated that polysaccharides were complex with proteins [6]. In addition, the band at 1444 cm^{-1} is assigned to be the C—H stretching vibrations or O—H deformation vibrations [33]. The peak at about 1206 cm^{-1} confirmed the presence of phenyl-OH structures [1,44]. Furthermore, the degrees of esterification (DEs) of PPPs were also investigated by FT-IR spectroscopy analysis, which varied by different drying methods (Table 1). As shown in Table 1, the DEs of PPPs ranged from 4.78% to 13.98%.

Table 3
Amino acid compositions of PPPs.

Amino acids	PPP-F (%)	PPP-H (%)	PPP-V (%)	PPP-M4 (%)	PPP-M6 (%)	PPP-M8 (%)
Aspartic acid	11.78	10.59	12.02	11.05	11.47	11.94
Threonine	7.09	6.89	7.07	6.31	6.54	7.19
Serine	8.46	9.03	8.19	7.94	8.64	8.86
Glutamic acid	6.86	7.63	7.10	10.23	8.81	9.18
Proline	6.44	5.38	6.65	6.08	6.18	6.34
Glycine	6.14	6.83	6.25	5.99	7.08	7.04
Alanine	5.92	7.24	6.46	7.20	8.66	8.59
Cystine	4.80	2.50	3.24	2.98	0.88	3.79
Valine	3.52	4.04	4.25	3.88	3.46	4.52
Isoleucine	3.33	3.65	3.21	3.77	2.36	4.53
Leucine	4.78	5.19	5.81	5.07	3.44	6.14
Tyrosine	1.59	1.73	2.19	5.38	4.21	3.92
Phenylalanine	2.53	3.57	3.57	3.36	2.51	3.99
Lysine	5.75	4.48	5.87	3.00	2.86	3.35
Histidine	0.73	1.10	1.35	0.60	0.87	1.17
Arginine	20.29	20.15	16.76	17.15	22.03	9.44
Essential amino acids	27.00	27.82	29.78	25.39	21.17	29.72
Non-essential amino acids	73.00	72.18	70.22	74.61	78.83	70.28

PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, PPP-M8, polyphenolic-protein-polysaccharide conjugates extracted from the peduncles of *H. dulcis* dried by freeze drying, hot air drying, vacuum drying, microwave drying at 400 W, microwave drying at 600 W, and microwave drying at 800 W, respectively.

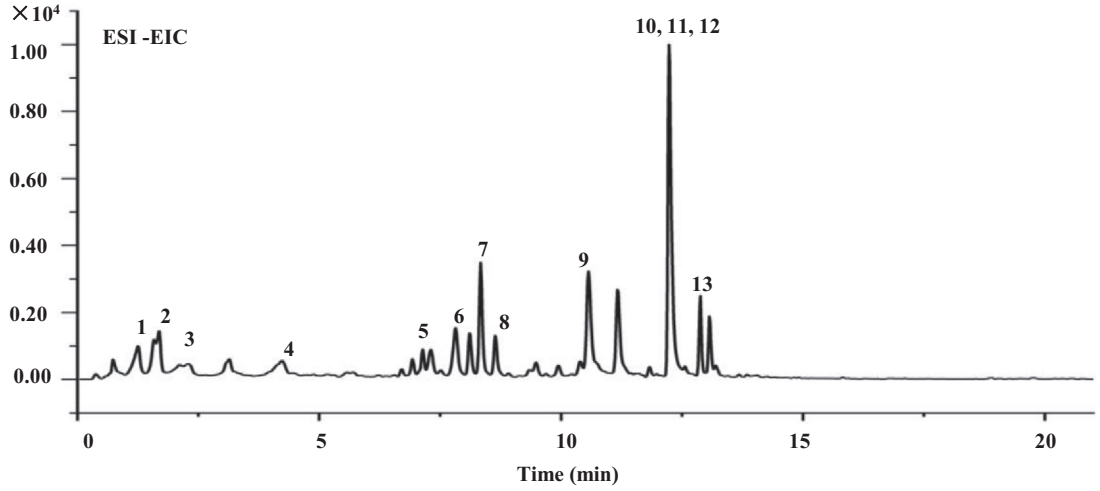

Fig. 3. Extracted ion chromatogram of the representative polyphenolic-protein-polysaccharide conjugate (PPP-M6) obtained by microwave drying at 600 W.

Table 4
Phenolic compositions of the representative polyphenolic-protein-polysaccharide conjugate (PPP-M6) obtained by microwave drying at 600 W.

No.	Time (min)	Formula	Molecular ion $[M-H]^-$	Error (ppm)	Score (DB)	Score (MFG)	Identifications
1	1.248	$C_7H_6O_4$	153.0192	−0.98	99.78	99.79	Protocatechuic acid[a,b]
2	1.680	$C_{15}H_{14}O_7$	305.0668	0.50	99.49	99.49	Gallocatechin[a,b,c]
3	2.296	$C_7H_6O_3$	137.0245	3.17	86.46	86.48	p-Hydroxybenzoic acid[a,b]
4	4.243	$C_{15}H_{12}O_8$	319.0462	0.54	99.47	99.44	Ampelopsin[a,b]
5	7.139	$C_{27}H_{30}O_{17}$	625.1414	0.59	99.24	99.25	Quercetin-7,4′-diglucoside[b]
6	7.821	$C_{15}H_{12}O_7$	303.0513	0.80	99.25	99.31	Dihydroquercetin[b]
7	8.337	$C_{27}H_{30}O_{16}$	609.1467	1.00	98.33	99.53	Rutin[a,b,c]
8	8.636	$C_{21}H_{20}O_{12}$	463.0885	0.57	99.6	99.69	Myricitrin[b]
9	10.567	$C_{15}H_{10}O_8$	317.0307	1.29	97.91	99.35	Myricetin[a,b,c]
10	12.231	$C_{15}H_{10}O_7$	301.0372	5.23	99.21	87.90	Quercetin[a,b,c]
11	12.248	$C_{15}H_{10}O_6$	285.0409	1.57	98.54	98.52	Kaempferol[a,b,c]
12	12.298	$C_{16}H_{12}O_8$	331.0463	1.14	99.27	99.24	5-Methylmyricetin[b]
13	12.880	$C_{15}H_{12}O_5$	271.0615	1.62	96.92	96.90	Naringenin[a,b]

[a]Compared with literatures; [b]Compared with TCM-database; [c]Compared with an authentic standard.
Compounds in this table were corresponding to compounds marked in Fig. 3.

3.2. Effects of different drying methods on the bioactivities of PPPs

3.2.1. Antioxidant activities of PPPs

Pharmacological studies have shown that polysaccharides extracted from *H. dulcis* exert obvious antioxidant activities [9,14,16,26]. However, antioxidant activities of polyphenolic-protein-polysaccharide conjugates and effects different drying methods on their antioxidant activities have never been investigated. Therefore, antioxidant activities of PPPs were investigated and compared. Fig. 4 showed the ABTS, DPPH, and NO radical scaveng-

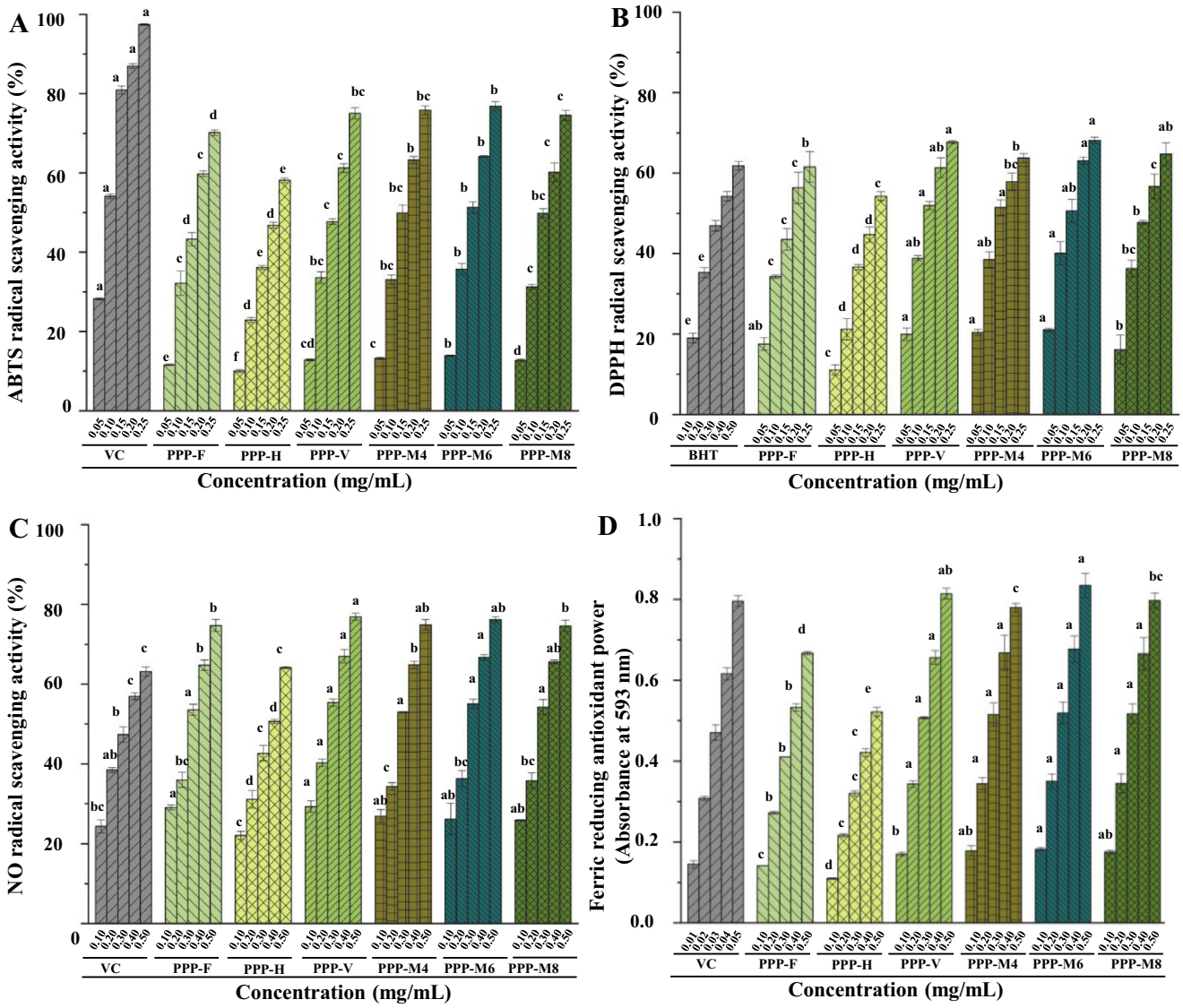

Fig. 4. ABTS (A), DPPH (B), and nitric oxide (C) radical scavenging activities, as well as ferric reducing antioxidant powers (D) of PPPs. PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, PPP-M8, polyphenolic-protein-polysaccharide conjugates extracted from the peduncles of *H. dulcis* dried by freeze drying, hot air drying, vacuum drying, microwave drying at 400 W, microwave drying at 600 W, and microwave drying at 800 W, respectively; **BHT**, butylated hydroxytoluene; ***Vc***, vitamin C; The error bars are standard deviations; Significant ($p < 0.05$) differences are shown by data bearing different letters (a-g); Statistical significances were carried out by ANOVA and Ducan's test.

ing activities, as well as ferric reducing antioxidant powers (FRAP) of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8. The ABTS, DPPH, and NO radical scavenging activities, and FRAPs of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 exhibited a dose-dependent manner, respectively. Results indicated that PPPs exhibited very strong antioxidant activities when compared with the positive controls, and their antioxidant activities varied by different drying methods. The IC_{50} values of ABTS, DPPH, and NO radical scavenging activities of PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, and PPP-M8 ranged from 0.14 mg/mL to 0.21 mg/mL, from 0.14 mg/mL to 0.23 mg/mL, and from 0.23 mg/mL to 0.36 mg/mL, respectively. Furthermore, results showed that PPPs also exerted strong FRAP when compared with the positive control (Fig. 4D). The highest antioxidant activities (ABTS, DPPH, and NO radical scavenging activities, and FRAPs) were observed in PPP-M6 and PPP-V among all tested PPPs, while the lowest antioxidant activities were determined in PPP-H. This condition might be attributed to their contents of total polyphenolics, total contents of uronic acids, and DEs of PPPs [45]. Besides, PPP-M6 also showed higher antioxidant activities than that of other glyco-conjugates, such as feruloyl oligosaccharides extracted from wheat bran insoluble dietary fiber [46], protein-polysaccharide conjugates purified from *Ilex latifolia* Thunb [47], and glycoproteins purified from *Ganoderma atrum* [48]. Considering the drying efficiency, the microwave drying at 600 W could be a potential drying technique before extraction of PPPs with high antioxidant activities for industrial applications.

In general, antioxidant activities of polysaccharides are related to their chemical structures, M_w, and compositional monosaccharides (especially uronic acids) [19,26,49]. For the polyphenolic-protein-polysaccharide conjugates, phenolics and proteins also contribute to their antioxidant activities [6]. In fact, some amino acids, such as tyrosine, histidine, and lysine, have been proved to be capable of donating protons to electron-deficient radicals [50]. Phenolic compounds, especially phenolic acids, also play important roles in the radical scavenging abilities [50,51]. Therefore, the relatively high antioxidant activities of PPPs might be partially attributed to their low M_w, high content of proteins, high content of polyphenolics, and contents of uronic acids.

3.2.2. Antiglycation activities of PPPs

Generally, glycation is a spontaneous non-enzymatic amino-carbonyl reaction between reducing sugars and proteins, which lead to the formation of advanced glycation end products (AGEs) [52]. AGEs can result in many chronic diseases such as aging, arteriosclerosis, and diabetic complications [53]. The mechanism of antiglycation may be related to the antioxidant activity. Therefore, antiglycation activities of PPPs were also investigated in the present study. As shown in Fig. 5A, both PPPs and AG exhibited strong inhibitory effects on the formation of AGEs. The IC_{50} values of inhibition activities on AGEs of PPP-V, PPP-M6, PPP-M8, PPP-M4, PPP-F, and PPP-H were determined to be 0.461 mg/mL, 0.713 mg/mL, 0.746 mg/mL, 0.768 mg/mL, 0.794 mg/mL, and 1.197 mg/mL, respectively. Compared with the positive control (AG, IC_{50} = 0.30 8 mg/mL), PPPs also exhibited strong inhibition activities on AGEs. Obviously, the inhibitory activities on AGEs formation of PPPs were in the order of PPP-V > PPP-M6 > PPP-M8/PPP-M4/PPP-F > PPP-H.

Previous studies have showed that the oxidation is the main cause of the formation of AGEs [32]. In this study, PPPs showed strong antioxidant activities, and could scavenge ABTS, DPPH, and NO radicals. In addition, flavonoids and polyphenolics in PPPs might also contribute to their antiglycation activities [54]. The higher antiglycation activity of PPP-V might be attributed to its stronger antioxidant activities and higher content of total flavonoids.

3.2.3. In vitro α-amylase and α-glucosidase inhibitory effects of PPPs

Inhibition of α-glucosidase and α-amylase is one of the main strategies to counteract metabolic alterations related to type 2 diabetes [31]. It is believed that flavonoids and polysaccharides extracted from *H. dulcis* exhibit remarkable *in vitro* anti-diabetic effects [13,16]. Therefore, in order to explore PPPs extracted from *H. dulcis* as functional food ingredients for the prevention of type 2 diabetes, the *in vitro* inhibitory effects of PPPs against α-amylase and α-glucosidase were investigated. As shown in Fig. 5B, PPPs exerted remarkable inhibitory effects on α-amylase and α-glucosidase. The IC_{50} values of α-amylase inhibitions of PPP-V, PPP-F, PPP-M6, PPP-M8, PPP-M4, and PPP-H ranged from 18.39 ± 0.17 μg/mL to 60.72 ± 2.59 μg/mL. Results suggested that different drying methods significantly affected their α-amylase inhibitory effects. The strongest α-amylase inhibition effect was determined in PPP-V among all tested PPPs, while the lowest α-amylase inhibitory effect was measured in PPP-H. Furthermore, compared with the positive control (Acarbose, IC_{50} = 3.56 ± 0.09 μg/mL), PPPs exhibited moderate inhibitory effects on α-amylase, and the order of inhibitory effects on α-amylase of PPPs was PPP-V > PPP-F > PPP-M4/PPP-M8 > PPP-M6 > PPP-H. The α-amylase inhibition effects of PPPs extracted from *H. dulcis* were higher than that of polysaccharides extracted from *Momordica charantia* [19], green tea [55], and etc., and thereby PPPs could be explored as a promising natural α-amylase inhibitor. Furthermore, As shown in Fig. 5C, the IC_{50} values of α-glucosidase inhibition effects of PPP-V, PPP-F, PPP-M6, PPP-M8, PPP-M4, and PPP-H ranged from 23.81 ± 0.12 μg/mL to 70.52 ± 1.67 μg/mL, which were significantly stronger than that of the positive control (Acarbose, IC_{50} = 8494.64 ± 26.40 μg/mL). Results suggested drying methods could also affect the α-glucosidase inhibition effects of PPPs, and the strongest α-glucosidase inhibition effect was also determined in PPP-V among all tested PPPs, while the lowest α-glucosidase inhibitory effect was also measured in PPP-H. The order of inhibitory effects on α-glucosidase of PPPs was PPP-V/PPP-F > PPP-M6/PPP-M8/PPP-M4 > PPP-H. Furthermore, the α-glucosidase inhibi-

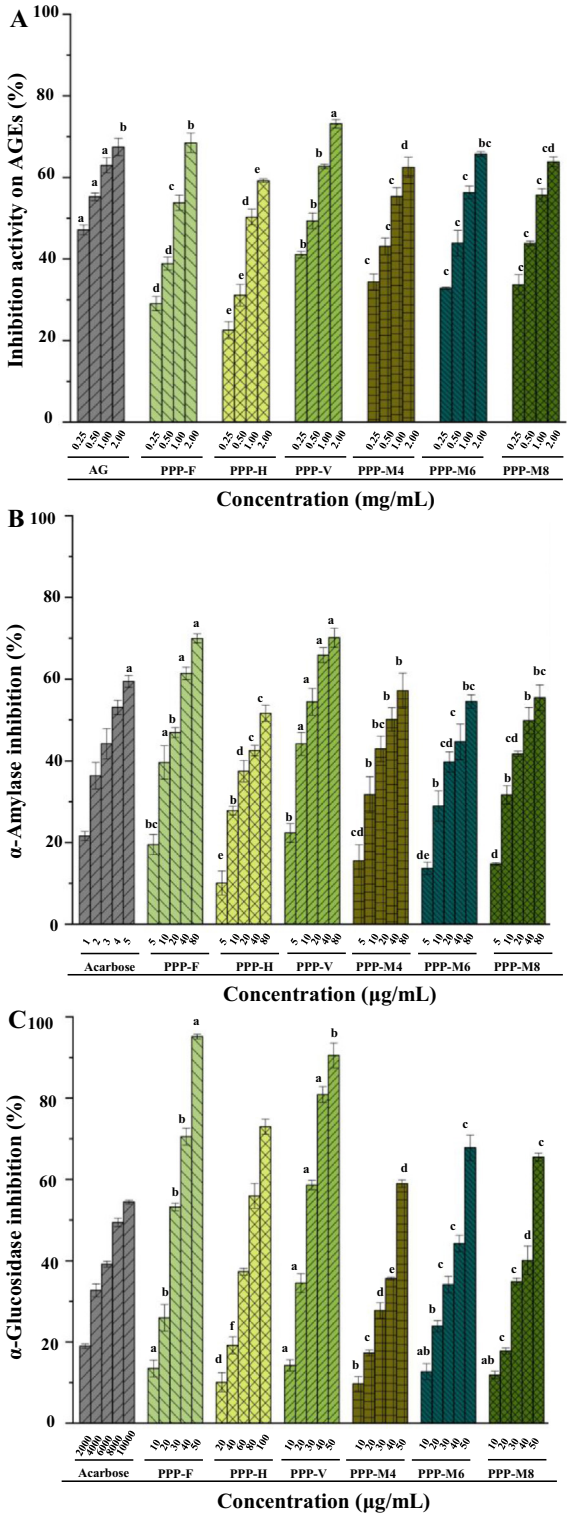

Fig. 5. Inhibitory effects on the formation of AGEs (A), and inhibitory activities on α-amylase (B) and α-glucosidase (C) of PPPs. PPP-F, PPP-H, PPP-V, PPP-M4, PPP-M6, PPP-M8, polyphenolic-protein-polysaccharide conjugates extracted from the peduncles of *H. dulcis* dried by freeze drying, hot air drying, vacuum drying, microwave drying at 400 W, microwave drying at 600 W, and microwave drying at 800 W, respectively; **AG**, aminoguanidine; **AGEs**, advanced glycation end products; The error bars are standard deviations; Significant ($p < 0.05$) differences are shown by data bearing different letters (a-g); Statistical significances were carried out by ANOVA and Ducan's test.

tion effects of PPPs extracted from *H. dulcis* were also higher than that of polysaccharides extracted from *H. dulcis* [16] and green tea [55]. However, the α-glucosidase inhibition effects of PPPs were lower than that of flavonoids extracted from *H. dulcis* [13]. Therefore, the strong α-glucosidase inhibition effects of PPPs might be related to their high contents of flavonoids. Furthermore, the remarkable inhibitory effects of PPPs on α-amylase and α-glucosidase might be attributed to their contents of total flavonoids, and chemical structures and DEs of polysaccharide fractions in PPPs [13,33,56]. Finally, results suggested that PPPs, especially PPP-V and PPP-F, had potential to be explored further as functional food ingredients for prevention and treatment of type 2 diabetes.

4. Conclusion

In this study, PPPs were firstly extracted from the peduncles of *H. dulcis*, which exerted strong antioxidant activities, antiglycation activities, and *in vitro* inhibitory activities on α-amylase and α-glucosidase. Besides, different drying methods significantly affected the extraction yields, molecular weights, apparent viscosities, molar ratios of constituent monosaccharides, contents of uronic acids, degrees of esterification, contents of total polyphenolic, and contents of total flavonoids of PPPs. Results are helpful for the better understanding of the chemical structures of PPPs extracted from *H. dulcis* dried by different methods. Furthermore, bioactivities of PPPs also varied by different drying methods. PPP-M6 and PPP-V exhibited higher antioxidant activities and antiglycation activities than other PPPs, and PPP-V and PPP-F exhibited higher *in vitro* inhibitory activities on α-amylase and α-glucosidase than other PPPs. Results suggested that M6 and VD methods could be potential drying techniques for pre-drying treatment before extraction of PPPs from *H. dulcis* with desired bioactivities, and PPPs could be further explored as functional ingredients for industrial applications.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.10.211.

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