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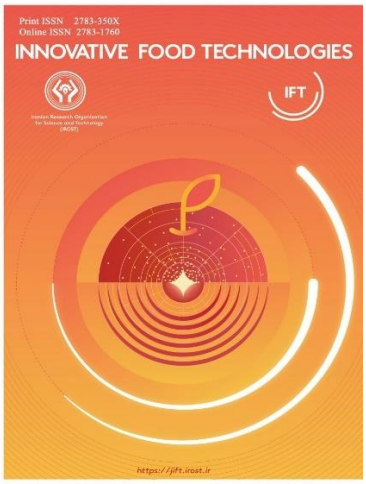

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Improving the oxidative stability of purslane seed oil via emulsions stabilized by whey protein isolate-inulin mixtures and conjugates

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Abstract

The principal purpose of this study was to investigate the antioxidant ability of whey protein isolate-inulin mixtures and conjugates to inhibit the oxidation of purslane seed oil. The conjugation variables were WPI to inulin ratios (1:1, 1:2, and 2:1w/w) and incubation temperatures (60 and 80°C) at 79% relative humidity for 24 h. The reaction was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Fourier transform infrared spectroscopy. Functional properties of the samples, including emulsion stability, rheological behavior, and thermal stability were evaluated. Differential scanning calorimetry results showed that the glass transition temperature (53.3 to 78.1 °C) and denaturation temperature (63.1 to 86.9 °C) of the conjugates increased in comparison with other samples. The results of zeta-potential, particle size and emulsion stability indicated that the WPI-inulin conjugates were the most influential factor at stabilizing O/W emulsions compared with mixture samples. The oxidative stability of Purslane seed oil increased with increasing the incubation temperature of conjugates, so the emulsions stabilized by conjugates produced at 80°C showed lower levels of conjugated dienes and anisidine value. Results indicated that conjugation of WPI with inulin led to improved thermal stability, antioxidant capacity and emulsion stability of the samples.

Keywords Purslane *(Portulaca oleracea)* seed oil; Maillard reaction, Whey protein isolate-inulin conjugate; Antioxidant activity; O/W emulsions; Oxidative stability.

1. Introduction

Purslane *(Portulaca oleracea)* seed oil is rich in polyunsaturated fatty acids (PUFA) consisting mainly of linoleic acid (33.80%) and α-linolenic acid (34.64%). It has been indicated as rich source of omega-3 and omega-6 fatty acids [1]. Lipid oxidation is a major cause of deterioration in foods containing PUFAs and affects the sensory and nutritional values of food products [2, 3]. Microencapsulation represented an impressive approach to protect the PUFAs against environmental stressors including oxygen, heat, moisture and light [4]. Emulsion systems are one of the most acceptable techniques for the delivery of PUFAs [4]. Emulsions are not stable thermodynamically and tend to break down over time due to different mechanisms, such as gravitational separation, coalescence, flocculation, particle coalescence, Ostwald ripening, and phase separation. To enhance their long-term stability, stabilizers like emulsifiers, texture modifiers, ripening inhibitors, and weighting agents are added to emulsion formulations. Emulsifiers are the most crucial stabilizers used in any emulsion formulation [5]. Inulin-type fructans are linear oligo- or polysaccharides with 2–60 fructose units linked by β (2→1) glycosidic bonds, with normally one glucopyranose unit at the reducing end. It is widely known as a dietary fiber, a modified texture and fat/sugar replacement in the food industry [6]. Whey protein isolate (WPI) is a by-product of the dairy industry. The major protein components in whey are β-lactoglobulin (β-LG), glycomacropeptide (GMP), α-lactalbumin (α-LA), immunoglobulins (Igs), bovine serum albumin (BSA), lactoferrin (LF), lactoperoxidase (LP) and protease peptone (PP) [7]. It is widely used as a nutrient and functional ingredient in food products [8]. WPI used as texturizers, stabilizers, emulsifiers, gelling agents, foaming and flavor binding agents. However, It is unstable under several undesirable processing conditions such as temperature (Temperatures > Tm), pH (pH near pI), high ionic strength and high concentrations of salt [9, 10].

Maillard reaction, which is widely renowned as non-enzymatic browning in foods, refers to a complex series of chemical reactions that occurs naturally among the amino groups of proteins and the carbonyl group of reducing sugars in food thermal processing and during storage, spontaneously [11, 12]. Maillard reaction also considered as the primary method to rectify protein [12]. Traditionally, glycation of proteins by polysaccharides can be conducted by either dry heating or wet heating conditions. Also, novel approaches are increasingly employed for the maillard reaction. Dry heat treatment was found to be the most common technique, the research

has shown the wet heating method has slow reaction yields [13]. Temperature, relative humidity and incubation time also the protein/polysaccharide ratio and pH are important factor in the maillard reaction of proteins and polysaccharide. The percentage of conjugation substantially increase from 26 to 40% with an increase of 10 °C (from 50 to 60 °C). Similarly, an increase in the RH from 50 to 80% led to a growth in the glycation of proteins [13]. Through glycation of protein with polysaccharide via Maillard reaction the functionality of native protein is greatly enhanced by forming the covalently conjugated products [14]. Maillard reaction products (MRPs) showed metal-chelating ability, reducing power, and radical scavenging [15-17]. Therefore, they can inhibit unfavorable oxidation of PUFAs in emulsions, whilst acting as emulsifiers. The adsorbed Maillard conjugates at the interface of O/W can restrict oxidation reaction [18].

In this study, antioxidant emulsifiers were prepared using the Maillard reaction by conjugating WPI and inulin to control the lipid oxidation of purslane seed oil. The WPI-inulin conjugates were synthesized by the dry- heating method (60 and 80°C) under relative humidity of 79% for 24 h. The emulsifying activity, creaming index, emulsion viscosity, droplet size and zeta-potential of the conjugates (1:1, 1:2 and 2:1 w/w) and mixtures (1:1, 1:2 and 2:1 w/w) of WPI - inulin was evaluated and compared. Due to the important impacts of parameters such as temperature, relative humidity and incubation time on the Maillard reaction, we selected the different parameters to comparing our results with previous studies in section of WPI glycation.

2. Materials and methods

2.1. Materials

WPI (Protein in dry matter: 92.16%, lactose: 1.56%, fat: 0.92%, ash: 3.19%, water:4.48%; Mw: 14,175 to 70,000 kDa) was obtained from Sachsenmilch (GermanProt, Germany). Inulin (HPX: DP ≥32; Mw: 5.0×10^2 to 1.3×10^4 Da) was purchased from Beneo (Germany) and Purslane seed was obtained from a local market. All other chemicals used in this study, including potassium bromide (KBr), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium dodecyl sulfate (SDS), para-anisidine, β-mercaptoethanol, isooctane, isopropanol and methanol, were purchased from Sigma-Aldrich. Other chemical reagents were of analytical grade.

2.2. Mixture and Conjugates preparation

WPI and inulin (6% w/w) were dispersed in 0.1 M phosphate buffer solution (pH=7) with stirring the solution at ambient temperature for 1h. The solutions were stored at 4 ℃ overnight to ensure complete hydration. WPI and inulin solutions were mixed (1:1, 1:2 and 2:1 (on a weight basis)). The samples were freeze-dried (Operon FDB-5503, Korea); ground and sieved. The powders were incubated (Memmert UNE400, Germany) at 60 and 80 °C for 24h with 79% RH provided by saturated KBr in a screw cap container. The mixture and conjugate samples were stored at -18 ℃ for further analysis.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli [19] using 15% acrylamide separating gel and a 5% stacking gel with 10% (w/v) SDS. Samples (0.025 g) were prepared in a 0.5M Tris–glycine buffer (pH 6.8) containing 4% SDS, 0.001% bromophenol blue and 5% β-mercaptoethanol. Electrophoresis was run at 50 V for 60 min and then at 110 V for 120 min. After electrophoresis, the gel sheets were stained with Coomassie brilliant blue R-250.

2.4. Fourier transform infrared (FT-IR) spectroscopy

The freeze-dried samples were blended with KBr, pressed to form appropriate tables, and then analyzed using a Tensor II, Bruker FT-IR spectrometer (USA), with a scan range of 500-4000 cm^{-1} and resolution of 4 cm^{-1}.

2.5. Differential scanning calorimeter (DSC)

Thermal behavior of samples were characterized using differential scanning calorimetry (ZF-DSC-D2H, METTLER TOLEDO. USA). Approximately 10 mg of sample was loaded in an aluminum pan, and then heated in a calorimeter from 30 ^{o}C to 300 ^{o}C at a heating rate of 10 ^{o}C/min.

2.6. 2, 2- Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity of WPI-inulin mixtures and conjugates were evaluated by Noshkam and Madadlou [20] with slight modification. One mL of each sample (6 mg/ml) mixed with 2 mL 0.12 mM DPPH in methanol and shaken well. The solution placed in a cabinet that was away from light for 30 min at ambient temperature. Then centrifuged at 750 g for 10 min and the absorbance of the supernatant was determined at 517 nm. The percentage of DPPH radical scavenging activity of the samples calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1- (A_s - A_c)/ A_b] \times 100 \quad (1)$$

where A_b is the absorbance of methanol added instead of samples, A_s is the absorbance of samples and A_c is the absorbance of methanol added to samples instead of DPPH.

2.7. The purslane seed oil extraction

The purslane seed oil is extracted using the hydraulic cold pressing machine at room temperature (Wuhan Hdc Technology, China). Then, extracted oil was filtered and afterward was centrifuged at 3500 rpm for 20 min. After filtration, oil was stored in dark bottles at refrigerator temperature until used. The composition and amount of fatty acids of purslane seed oil were evaluated by gas chromatography (G-M 815/ GOW-MAC Co.) The sample was injected into HP-88 column BPX-70 (50 m, 0.25 mm, 0.32 μm). The injector and detector (FID) temperatures were 250 °C. The carrier (N2) gas flow was 1.7 ml/min [21].

2.8. Emulsion preparation

The preparation of the oil-in-water (O/W) (10:90 w/w) emulsions was carried out according to a method reported by Chen et al. [22] with some modifications. The mixture and conjugate powders (4% w/w) were dissolved in 0.1 M phosphate buffer solution (pH=7) with stirring at ambient temperature for 2 h and kept overnight at 4 ^{o}C to hydrate fully. O/W emulsions were contained 10% w/w Purslane seed oil as dispersed phase, 4% biopolymer mixture and conjugates as emulsifier and 86% w/w phosphate buffer (continuous phase). The solution was homogenized (SilentCrusher M, Heidolph, Germany) at 13,000 rpm for 5 min, and then emulsified using an ultrasonic homogenizer (Samkoon, IRAN) at 400 kHz for 5 min.

2.9. Zeta-potential and size measurements

The zeta-potential and particle sizes of the samples were carried out using a Malvern ZS Particle Sizer (Malvern Instrument Ltd., UK). Samples were diluted in deionized water at a ratio of 1:100 (v/v).

2.10. Rheological properties

The apparent viscosity of emulsions was determined by Brookfield viscometer (DV-II+ Pro, Brookfield, USA) at the shear rate of 100 s^{-1} at ambient temperature, using an LV spindle (S00).

2.11. Emulsion stability (Creaming test)

10 mL of each emulsion was poured into a glass test tube and was sealed immediately after preparation then kept at ambient temperature (25 ◦C) for 21 days. The total emulsion height (H_1) and top cream phase and, or bottom serum phase (H_2) were measured [23]. The stability of the emulsion was reported based on the following equation:

Emulsion stability (%) = $(H_2/H_1) \times 100$ (2)

2.12. Lipid oxidation measurements

The oxidative stability of Purslane seed oil in O/W emulsions was determined by measuring the conjugated dienes (CD) and anisidine value (AV).

2.12.1. Conjugated Dienes (CD): An aliquot of 20 µL of the emulsion was mixed with 1mL of isooctane/isopropanol (2:1, v/v), followed by vortexing for 1 min and then centrifuged for 6 min at 6000g. The supernatant was filtered using a 0.2 µm syringe filter to remove the probable derangement of the proteins. The absorbance was measured at 232 nm by using a UV/VIS spectrophotometer (Biochrom WPA, UK). The amount of CD in the oxidizing emulsions was calculated on the basis of linoleic acid which has a molar absorptivity (ε) of 27,000 [22]. The CD content was calculated by Equation (3).

CD = A233nm/[oil] ×Ɛ ×L (3)

[oil], the concentration of oil, kg/L; L, optical path of colorimetric ware, 1 cm; Ɛ, molar absorptivity of conjugated dienes, 27,000 $M^{-1}cm^{-1}$.

2.12.2. The para-anisidine value (pAV): The para-anisidine value (pAV) was modified according to Sun et al. [24]. 0.5 mL emulsion was added into a volumetric flask (25 mL) and

made up to the mark with isooctane. The samples were vortexed and centrifuged (Hettich EBA 200, Germany) at 5000 rpm for 10 min. The absorbance (A1) was measured at 350 nm (UV/VIS spectrophotometer, Biochrom WPA, UK) using isooctane as blank. Then, 5 mL of supernatant was mixed with 0.2 mL para-anisidine solution (0.25% w/v solution in acetic acid) and vortexing for 10s. After 10 min, the absorbance was measured at 350 nm (A2) against the isooctane blank containing para-anisidine. The pAV values calculated using the following equation:

$$pAV = 25(1.2A2 - A1)/\text{sample mass} \quad (3)$$

2.13. Statistical analysis

All the samples measured in triplicate. For each case three replicas were prepared. Statistically significant differences were determined at $P < 0.05$ level using the software SPSS version 21 (IBM, USA), by Duncan's multiple range tests.

3. Results and discussion

3.1. SDS-PAGE

SDS-PAGE was used to confirm the covalent bonding of WPI to Inulin due to the formation of Maillard conjugates with high molecular weight. As can be seen in Fig1 the lanes from 2 to 4 correspond to WPI- inulin mixture (1:1, 1:2 and 2:1), lanes from 5 to 7 to those obtained at 60 °C correspond to WPI- inulin conjugates (1:1, 1:2 and 2:1) and lanes from 8 to 10 were obtained at 80 °C correspond to WPI-inulin conjugates (1:1, 1:2 and 2:1), lanes 1 and 11 which correspond to the native WPI and marker, respectively. No new bond was detected in mixtures of WPI-inulin (lanes 2-4), the unique bands of WPI appeared, meaning that the covalent bonds did not form between WPI and inulin. A similar phenomenon was reported by Chen et al. [9] for the whey protein isolate-gum acacia mixture. The WPI protein bands tended to vanish when samples incubated at 60 °C. Hence, new compounds with higher molecular weight started to form, which indicates that the glycosylation occurs between WPI and inulin. Compared with the pattern of native WPI, the Conjugate formation resulted in a decreased intensity band, instead, a band appeared that indicated the high molecular weight compounds due to the covalent binding between the protein and carbohydrate [25]. By increasing incubation temperature (80°C), the

degree of glycosylation increased gradually, and the high molecular weight emerged in lanes 8-10. The new bands at the top zone of the gel lanes revealed new substances with higher molecular weight as protein-polysaccharides conjugates. Possibly, the polymeric structure of inulin led to the formation of conjugates with wide molecular weight distribution. A similar phenomenon was reported by Schmidt et al. [26] for whey protein isolate-citrus pectin conjugate formation. There were no significant changes in the band patterns regarding the different ratios of WPI- inulin. The results showed that the temperature is much more effective than the protein-polysaccharide ratio in the characteristic bands of WPI - inulin.

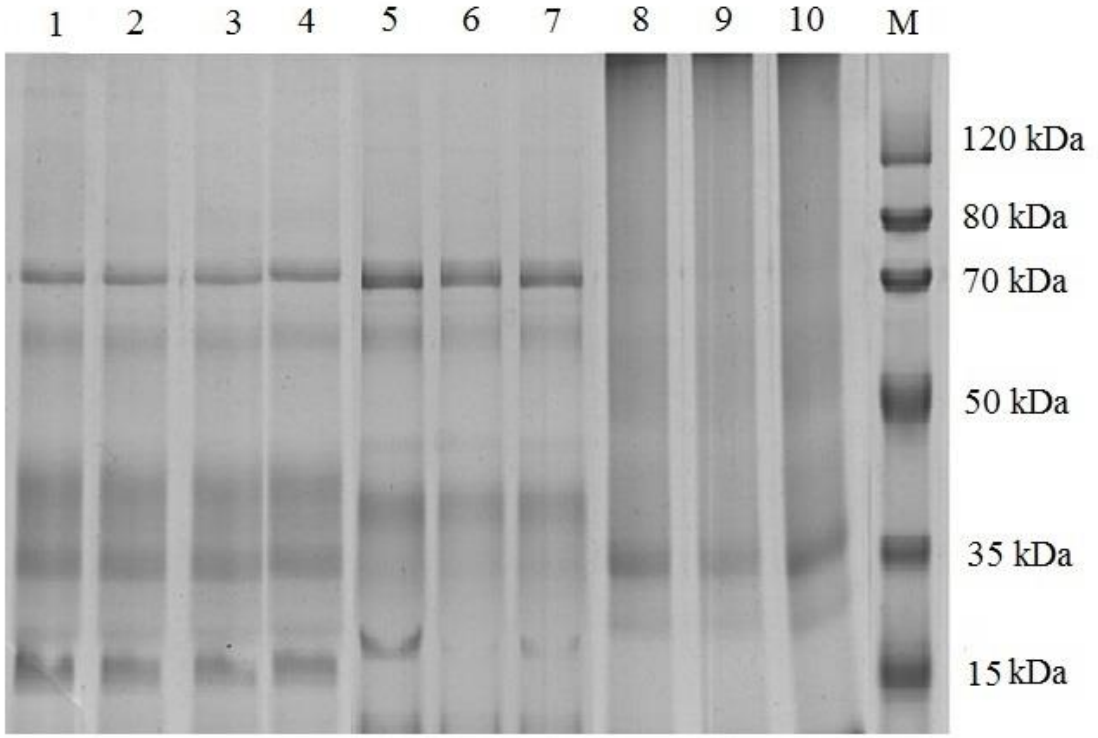

Fig 1. SDS-PAGE of WPI native (1); WPI – Inulin (1:1, mix) (2); WPI – Inulin (1:2, mix) (3); WPI – Inulin (2:1, mix) (4); WPI – Inulin (1:1, 60°C) (5); WPI – Inulin (1:2, 60°C) (6); WPI – Inulin (2:1, 60°C) (7); WPI – Inulin (1:1, 80°C) (8); WPI – Inulin (1:2, 80°C) (9); WPI – Inulin (2:1, 80°C) (10) and marker (M).

3.2. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy can be a valuable technique for studying the molecular structure, and protein-carbohydrate interactions and the characteristics of Maillard reaction conjugates [27, 28]. The FT-IR spectra of inulin, WPI and WPI-inulin mixtures and conjugates are shown in Fig 2.

The peak at around 1034 cm^{-1} is due to the stretching vibration of C-O in the tetrahydrofuran ring of fructose and C-C of the pyranose ring [29, 30], the intensity and wavenumber of this band changed after mixing and glycation with WPI and shifted to lower wavenumber. With increasing incubation temperature, the peak shifted to lower wavenumbers and its intensity also increased. The peak of amide I at 1630 cm^{-1} in the spectrum of WPI is attributed mainly to C=O stretching vibration with minor contributions from the vibrations of N–H bending and C–N stretching [31]. The results show that the mixing and conjugation of WPI and inulin, the wavenumber of this peak shifted to higher wavenumbers. The temperature and ratio of protein to polysaccharide play an essential role in changing in the wavenumber of these bonds. The lowest wavenumber of this peak observed with the ratio of 2:1 whey protein isolate - inulin for mixed and conjugated samples. The temperature affected the position and intensity of the amide I peaks, so that the conjugates produced at 80°C showed sharper peaks in this area. Higher temperatures change the secondary structure of proteins, which causes changes in the peak position and intensity [32].

The intrinsic shape of the amide I band (1630 cm^{-1}) is characteristic of the secondary structure of proteins [33]. The peak located at 1515 cm^{-1} was related to the N – H bending and C – N stretching vibrations in peptide bonds and it is named amide II band [33-35]. The peak position of the amide II band of the WPI-inulin mixtures and conjugates shifts to a higher wavenumber, so that, conjugates produced at 60 °C showed the highest wavenumber (1538 cm^{-1}). This phenomenon indicates that the β-sheet structure of the protein is reduced and the protein structure is stretched. This may be a sign of the secondary structure of WPI was changed, which changed from ordered to disordered. This may also denote the breaking of disulphide bonds,suggesting wpi has undergone a greater degree of denaturation [36]. The peak intensity increased with increasing incubation temperature (80°C), It might be due to the hydrolysis of peptide bonds [33]. When the ratio of inulin increased (1:2), the peak shifted to the higher wavenumber. Generally, the formation of hydrogen bonds increases the wavenumber of N-H bending vibration [31]. Reduction of some functional groups such as NH2 of WPI and formation of new groups such as Amadori compounds (C = O), Schiff base (C = N) and pyrazines (C – N) may appear during the Maillard reaction. The vibrations of these new groups and C – O, N – H and C – H bands of the amide group are located in a range of 1800–800 cm^{-1} [20]. An band at 2926 cm^{-1} was due to anti-symmetric stretching of –CH2 for WPI [37]. In the inulin spectrum,

the peak around of 2930 cm^{-1} was attributed to C-H stretching [38]. After mixing and conjugating WPI and inulin, the position and intensity of these bands greatly decreased, which may be due to intermolecular interactions (Hydrogen bonding and hydrophobic interactions) that reduce vibrations of the methylene group. These findings are in agreement with the reports of Dong et al. [37] for WPI- Flaxseed Gum conjugates and mixtures. The peak of WPI located at wavenumbers 3271 cm^{-1} was attributed to the free and bonded O–H group with N–H and O–H [31, 36]. The peaks of inulin located at wavenumbers 1653 cm^{-1} were attributed to asymmetric carboxylic acid and –OH groups [38, 39]. It disappeared after mixing and glycation WPI- inulin. This was attributed to the interactions between the protein and the polysaccharide and indicated the low capacity of carbohydrate molecules to form intramolecular hydrogen bonding in the presence of the protein [40]. The peak at 1390 $cm^{-1}$is assigned to the COO^- symmetric stretching or CH_3 of WPI [41]; the intensity of this band decreased after glycation and mixing WPI with inulin.

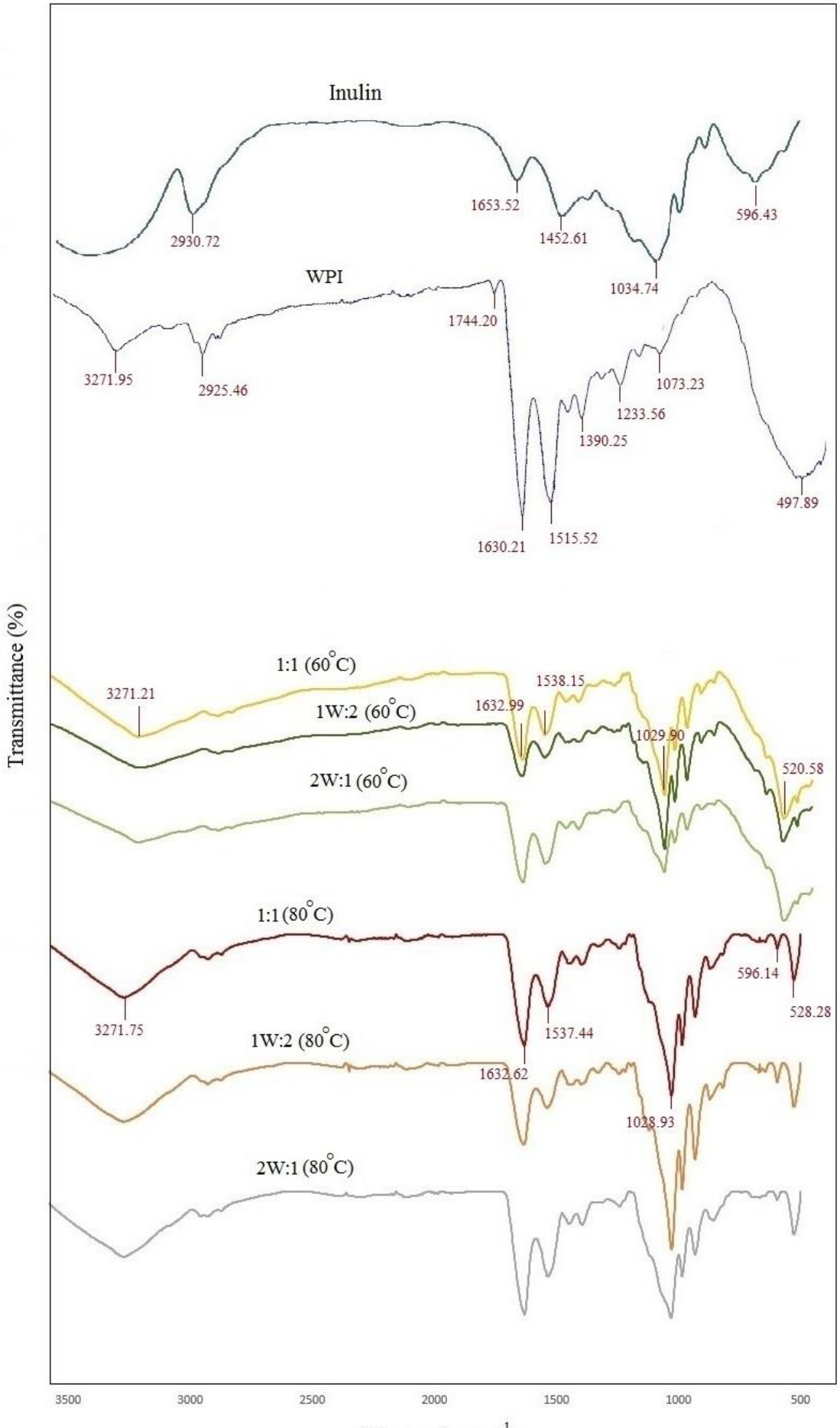
Inulin
2930.72
1653.52
1452.61
1034.74
596.43
WPI
3271.95
2925.46
1744.20
1630.21
1515.52
1390.25
1233.56
1073.23
497.89
Transmittance (%)
1:1 (60°C)
3271.21
1538.15
1632.99
1029.90
520.58
1W:2 (60°C)
2W:1 (60°C)
1:1 (80°C)
3271.75
1537.44
596.14
528.28
1W:2 (80°C)
1632.62
1028.93
2W:1 (80°C)
3500
3000
2500
2000
1500
1000
500

Fig 2. FTIR spectra of Inulin, WPI and WPI-Inulin mixtures and conjugates (60 and 80°C)**.**

3.3. Differential scanning calorimeter (DSC)

Table 1 represents the DSC characteristics of WPI, WPI-inulin mixtures and conjugates in which the denaturation temperature (T_d), glass transition temperature (T_g) and enthalpy changes of denaturation (ΔH) compared. The glass transition temperature of amorphous or non-crystalline solids is one of the most important physicochemical properties [42]. The Tg of native WPI was around 62.9°C, when WPI was mixed and conjugated with inulin, the glass transition temperature of WPI downshifted to 56.4-61.2°C and upshifted to 64.7-78.1°C, respectively. The results showed that WPI- inulin (2:1) had the highest T_g and T_d among all samples. Increasing the weight ratio of WPI led to a significant increase in the molecular weight of the system, the high molecular weight systems showed a higher glass transition temperature [43]. T_d measures the thermal stability of proteins whereas ΔH evaluates the energy required to disrupt noncovalent interactions during protein denaturation [44]. The T_d and ΔH of native WPI were estimated to be 70.6°C and 7.98J/g at pH= 7, respectively. After mixing with inulin, T_d and ΔH decreased for all mixtures because of the flexible main groups, dissymmetry and nonpolar groups of inulin. Also, the various thermal behaviors of WPI in the absence and presence of polysaccharide would be a sign of WPI- polysaccharide interaction, since the interaction modulated the thermal response of WPI [45]. Mao et al. [45] reported that when WPI mixed with pectin the denaturation temperature of WPI decreased. As shown in Table 1, after glycation, T_d increased for all conjugates (72.8 – 86.9°C), whereas ΔH decreased. Similar results were obtained by He and Vardhanabhuti [46]. The results indicated that conjugation led to the improved thermal stability of the samples and a decrease in ΔH. Our results are in agreement with the research work reported by Pirestani et al. [47] and Spotti et al. [48]. There are various factors that can affect the heat stability of protein after glycation with polysaccharides, including increased steric hindrance, changes of conformation, and surface charge [46]. The grafted polymer can provide steric hindrance, this phenomenon introduced in some studies as the dominant mechanism for improving heat stability of proteins. Also, the effects of steric hindrance might be related to the positive correlation between the length of the grafted polysaccharides and the heat stability of the conjugates, indirectly. Wang and Ismail [49] attributed the improved conjugate heat stability to conformational changes.

Table 1- DSC profiles of Inulin, WPI and WPI-Inulin mixtures and conjugates

Samples	T_g (°C)	T_d (°C)	ΔH(J/g)
1:1 (mix)	56.4±1.04^{j}	67.3±0.12^{h}	6.81±0.09^{c}
1w:2 (mix)	53.3±0.63^{k}	63.1±0.22^{i}	7.69±0.11^{b}
2w:1 (mix)	61.2±0.44^{h}	69.4±0.34^{g}	6.55±0.08^{d}
1:1 (CO60)	65.9±0.23^{e}	72.8±0.32^{e}	5.95±0.09^{f}
1w:2 (CO60)	64.7±0.12^{f}	74.5±0.26^{d}	6.01±0.16^{e}
2w:1 (CO60)	69.5±0.52^{d}	74.8±0.18^{d}	5.42±0.13^{g}
1:1 (CO80)	75±0.39^{b}	84±0.27^{b}	5.43±0.10^{g}
1w:2 (CO80)	73.4±0.29^{c}	81.5±0.19^{c}	5.95±0.11^{h}
2w:1 (CO80)	78.1±0.51^{a}	86.9±1.02^{a}	4.24±0.13^{i}
WPI	62.9±1.28^{g}	70.6±0.16^{f}	7.98±0.07^{a}

3.4. 2, 2- Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity

Maillard reaction products possess superb antioxidant ability. It might be due to the wide variety of mechanisms, including free radical scavenging (e.g., hydroxyl, superoxide, and peroxyl radicals), chelation of metal ions, and breakdown of radical chains and hydrogen peroxide [50]. The results of the DPPH radical scavenging activities of the samples showed in Fig 3. The native WPI (18.46%) and WPI-inulin conjugate (2:1) produced at 80°C (55.3%) showed the lowest and highest free radical scavenging activities, respectively. The higher antioxidant activity of WPI-inulin conjugated compared to its unconjugate counterpart is maybe due to the exposure of electron donating amino acid residues upon WPI denaturation under maillard conditions, higher electron donating capability of the acquired conjugates, perhaps via their hydroxyl and pyrrole groups and hydrogen donating tendency of intermediate or final products of the maillard reaction [50].

The glycation degree and following that the antioxidant capacity of conjugates can be influenced by the incubation temperature of protein- polysaccharide, the type of reactants and their molar ratios [50]. Increasing incubation temperature caused an increase in antioxidant activity of samples ($P<0.05$). There appeared to be the effect of weight ratio, WPI-inulin at 2:1 ratio weight showed the highest DPPH free radical scavenging activity. The antioxidant activity of whey protein isolate is mainly attributed to cyclic amino acids such as tyrosine and tryptophan. These

findings are in agreement with those obtained by Nooshkam and Madadlou [50]. It was reported that dry-heating improved the DPPH, ABTS, and AAPH radical scavenging activity at all ratios with the exception of the samples with WPI- inulin ratios of 3:1 and 2:1 for DPPH radical scavenging activity because of the antioxidant groups in whey proteins might be masked by heating at lower inulin levels and the denaturation and aggregation of whey protein would result in decreased DPPH scavenging capacity.

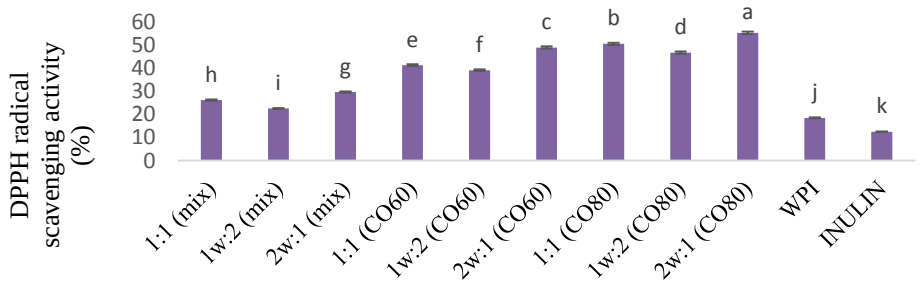

Fig 3. DPPH radical scavenging activities of Inulin, WPI native and WPI-Inulin mixtures and conjugates.

3.5. Purslane seed oil

Purslane seed oil is a rich source of essential fatty acids, particularly omega-3 fatty acids. Encapsulation is an effective method for protecting the oil from oxidation [21]. The extraction yield was obtained 20.14% (w/w). The contents of saturated and unsaturated fatty acids in oil sample were 20.68% and 79.32%, respectively. Linoleic acid followed by linolenic acid were the most unsaturated fatty acids present in purslane seed oil. The extraction yield and the amounts of fatty acids is strongly influenced by the various factors such as plant genotype, growth conditions, extraction method and so on. These findings were consistent with the results of Delfan Hosseini et al. [21] and Petropoulos et al. [1].

3.6. Zeta-potential and size measurements

Zeta-potential has a dramatic effect on the electrostatic repulsion forces between emulsion droplets and the formation of bio-macromolecules in an aqueous phase. It is an important parameter that directly affects the stability of emulsions [51]. According to Table 2, the zeta-potential of emulsions stabilized by WPI was -33.1 mV on the first day, as would be expected

because at pH above pI (pH = 4.5), the net electrical charge of WPI is negative. The addition of inulin to whey protein isolate slightly reduced the negative charge of the droplets, which represented partial absorption of inulin. The inulin is a non-ionic polysaccharide [6] The presence of hydroxyl groups within its molecular structure may lead to the production of partial negative charges [52]. Xu et al. [53] reported that the zeta potential of the droplets in the emulsions stabilized with WPI decreased when mixed with dextran, which can be explained by the fact that the dextran has no charge. The zeta-potential of the emulsions stabilized by WPI-inulin mixture, WPI- inulin conjugates 60 °C and WPI- inulin conjugates 80 °C changed from -29.2 to - 32.7 mV, -37.9 to -38.9 mV and -38.9 to -45.9 mV, respectively on the first day of production. Our results showed that glycation led to increased zeta-potential and the emulsions formed by conjugates became more negatively charged as the incubation time of conjugates increased. Our results are in agreement with the work reported by Dong et al. [35] which reported that the glycation led to increased zeta-potential, and the conjugate of WPI and flaxseed gum had dramatically enhanced the stability and emulsifying activity in comparison with the mixture. The Maillard reactions lead to the formation of the hydrophilic negatively charged groups such as carboxyl and hydroxyl [54]. Also, free amino group blocking, denaturation of proteins and changing the spatial arrangement of molecules can increase the negative surface charge [55]. During glycation, inulin stick to the lysyl residues of the WPI. Since lysine with pK of 10.6 is a of positive charge, the glycated WPI could increase in net negative charge [45]. The zeta-potential of the emulsions stabilized by WPI- inulin mixture, WPI- inulin conjugates 60 °C and WPI- inulin conjugates 80 °C changed from -30.6 to - 34.9 mV, -39.0 to -41.2 mV and -44.8 to -52.3 mV, respectively at the final day of storage. This might be due to the situation that the changes in the spatial arrangement of molecules led to increasing in the surface charge.

The polydispersity index (PDI) is also used to define the particle size distribution in systems, which also the degree of non-uniformity and heterogeneity index. PDI values greater than 0.7 indicate that the sample has a heterogeneous and wide particle size distribution pattern [56]. Table 2 shows the particle diameter (Z-average) and PDI of emulsions stabilized by WPI, WPI and inulin mixtures and conjugates. On day 1 and day 21, the PDI ranged from 0.257 to 0.546 and 0.263 to 0.604, respectively, which confirms the homogeneous distribution of emulsions. The emulsion stabilized by WPI and conjugate of WPI- inulin conjugate (2:1, temperature 80°C), showed the highest and lowest PDI value, respectively. The arrangement of Z-Average from the

lowest to highest value was as follows: emulsion stabilized with WPI-inulin conjugates 80 °C < emulsion stabilized with WPI-inulin conjugates 60 °C < emulsion stabilized with WPI-inulin mixtures < emulsion stabilized with WPI (the highest Z-Average). Z-Average and PDI values of the emulsions stabilized by the WPI-inulin conjugate were lower than those of the emulsions stabilized by the WPI and WPI-inulin mixture. Since Maillard reaction led to protein denaturation and exposed more hydrophobic groups of WPI, increasing the flexibility of protein structure, and hence effectively reducing the interfacial tension during the homogenization process to form a stable emulsion with a smaller size and more homogenous distribution Nooshkam et al. [57] and Zhou et al. [58]. Similar results reported by others who found that the droplet size of emulsion stabilized by protein-polysaccharide conjugates was smaller than those of emulsions stabilized by mixtures [59-60]. The particle size of emulsions was greatly affected by protein/polysaccharide ratio, so that the particle size of emulsions stabilized with WPI: inulin (2:1) < WPI: inulin (1:1) < WPI: inulin (1:2). This trend observed on the first and 21th day of storage period. The particle sizes decreased with increasing concentration of WPI. The zeta potential results showed that the negative charges of emulsions increased with the increasing WPI concentration, which led to enhanced repulsion between particles. The hydrophilic zone became bulkier when the hydrophilic polysaccharide was absorbed on the protein's surface. So, the filling parameters and curvature showed a decrease and an increase, respectively, which finally led to a decrease in the droplet size of emulsions [61, 62].

Table 2- The average particle size, PDI and zeta potential of emulsions stabilized by WPI native and WPI-Inulin mixtures and conjugates on day 1 and day 21

Samples	Zeta potential (mV) day1	Zeta potential (mV) day21	PDI (day1)	PDI (day21)	Z-Average (nm) day1	Z-Average (nm) day 21
1:1 (mix)	-32.7±0.56^{c}	-33.4±0.17^{de}	0.482±0.002^{c}	0.542±0.002^{bc}	483.0±1.4^{c}	540.8 ±4.5^{bc}
1w:2 (mix)	-29.2±0.47^{e}	-30.6±0.82^{gf}	0.514±0.001^{b}	0.535±0.007^{cd}	505.4±1.3^{b}	552.1±2.4^{b}
2w:1 (mix)	-30.3±0.29^{cd}	-34.9±0.43^{d}	0.450±0.005^{d}	0.510±0.004^{e}	471.1±4.1^{cd}	529.2±1.9^{cd}
1:1 (CO60)	-38.9±0.30^{b}	-40.5±0.71^{c}	0.515±0.004^{b}	0.517±0.002^{e}	422.2±2.5^{e}	477.9±3.8^{e}
1w:2 (CO60)	-37.9±0.51^{b}	-41.2±0.60^{c}	0.507±0.004^{b}	0.531±0.005^{d}	457.1±4.2^{d}	515.1±2.1^{d}
2w:1 (CO60)	-38.4±0.55^{b}	-39.0±0.35^{c}	0.300±0.007^{f}	0.357±0.004^{g}	419.4±1.8^{e}	466.4±3.6^{ef}
1:1 (CO80)	-44.2±0.63^{a}	-44.8±0.39^{b}	0.359±0.005^{e}	0.481±0.001^{f}	346.2±1.5^{g}	432.5±2.6^{g}

1w:2 (CO80)	-38.9±0.19[b]	-52.3±0.28[a]	0.355±0.004[e]	0.551±0.005[b]	401.2±4.0[f]	453.3±5.2[f]
2w:1 (CO80)	-45.9±0.38[a]	-46.1±0.50[b]	0.257±0.009[g]	0.263±0.004[h]	247.6±3.1[h]	411.5±1.8[h]
WPI	-33.1±0.43[c]	-32.0±0.67[ef]	0.546±0.001[a]	0.604±0.005[a]	585.4±2.2[a]	634.7±4.1[a]

3.7. Rheological properties

The rheological characterization of emulsions is influenced by the nature of the interactions between the droplets and can be modified by changing droplet properties for instance charge, hydrophobicity and thickness [63]. The apparent viscosity of the prepared emulsions varied from 0.0014 -0.0027 and 0.0017 – 0.0056 Pa-s at a constant shear rate of 100 s^{-1} (Fig. 4). The emulsion stabilized with WPI exhibited the lowest viscosity during storage for 21 days ($p<0.05$), which indicates the dominant effect of polysaccharides on the viscosity of emulsions. Xu et al. [53] reported that the viscosity of emulsions stabilized by WPI-beet pectin mixture and conjugates was higher compared with the one stabilized by WPI alone. The viscosity of the emulsions increased significantly ($P<0.05$) with increasing incubation temperature. The emulsions stabilized using conjugates 80°C showed the highest viscosity during storage, likely due to the formation of the high molecular weight compounds during glycation at high temperature, which was able to resist the flow [64]. Comparing the different weight ratios on the apparent viscosity of the emulsions revealed that the apparent viscosity of emulsion formed by WPI-inulin (1:2) is higher than other samples. This was probably due to the important role of inulin in increasing the viscosity of emulsions. The binding of WPI to the molecular structure of inulin leads to the formation of hybrid polymer networks that increase the viscosity of the emulsions. The presence of inulin in the emulsion continuous phase caused the droplets to interact strongly through depletion resulting from an increase in the emulsion viscosity [65]. The results showed that the viscosity of emulsions increased after 21 days ($p<0.05$)(Fig.4), indicating that the aqueous phase was trapped in flocculation droplets, which in turn increased the viscosity during the storage period. In general, the rheological properties of emulsion stabilized by conjugates influenced by the WPI- inulin ratio and the temperature incubation. The Maillard reaction can modify the interfacial characteristics of the conjugates. The incubation temperature

of the Maillard reaction favored by the conjugate formation, which produced stable emulsions with higher viscosity.

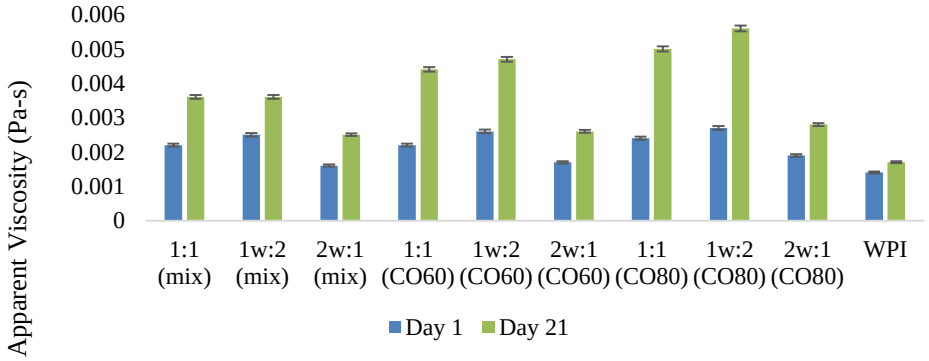

Fig 4. Apparent viscosity (Pa-s) of emulsions stabilized by WPI native and WPI-Inulin mixtures and conjugates on day 1 ■ and day 21 ■ at constant shear rate of 100 s^{-1}**.**

3.8. Emulsion stability (creaming test)

Gravitational or centrifugal forces usually cause creaming and sedimentation. When such forces exceed the Brownian motion of the droplets, a concentration gradient is generated in the system and larger droplets move faster to the top or bottom [66]. The influence of storage time on the stability of emulsion is shown Fig. 5. The emulsion stabilized with native WPI showed the highest creaming index (45.76%) during 21 days of storage ($P<0.05$). Instability of emulsions leads to floating of droplets to the surface, cohesion between droplets, and finally to creaming, aggregation, and phase separation. This phenomenon was observed in the emulsion stabilized with native WPI after third day (0.75%) and increased during the storage time. Polysaccharides play an important role in the stability of emulsions. Polysaccharides increase the viscosity of the continuous phase, and limit the mobility of the emulsion droplets, thus increasing the creaming stability of the emulsion [67]. The emulsion stabilized by the WPI-inulin mixture (2:1) was unstable, showing the cream layer at the top and a turbid serum layer at the bottom formed within 8 days, and it increased by 28.57% during 21days of storage. Creaming and flocculation occur when the electrostatic repulsion force diminished. The droplets coated with WPI and inulin come in contact with each other and the creaming rate increased with particles flocculation. The height of the serum phase in emulsion formed with WPI-inulin mixture (1:2) increased significantly within 6 days, and the phase separation increased to 31.25% on the final day of storage. Depletion flocculation and thermodynamic incompatibility are the two major

mechanisms that can lead to phase separation in the protein-polysaccharide mixture [68]. The stability of O/W emulsions are mostly under the control of the repulsive force and Ostwald ripening. By increasing the repulsion between the droplets, the stability of emulsions increases due to less chances of coalescence [69].

The effects of zeta-potential on stability of emulsions rationalized by Derjaguin–Landau–Verwey–Overbeek theory, which expresses that stability of emulsions depends on the balance between the various forces acting along the interface. Generally, systems with zeta-potential > ±30 mV are stable [70]. According to the results, the arrangement of Zeta- potential of emulsion stabilized with WPI-inulin mixture from the lowest to highest value was as follows: WPI-inulin mixture (1:2) < WPI-inulin mixture (2:1) < WPI-inulin mixture (1:1), which can be related to emulsions stability. Emulsions, like any thermodynamic system, going toward their most thermodynamically stable state and incline to decrease their free energy by reduction the contact area between the oil and water phases, during storage time. therefore, emulsifiers and stabilizers such as maillard conjugates should be used, which enable significant rheological properties in addition to increasing stability over time [71]. The emulsions stabilized by maillard conjugates showed an acceptable stability during 21 days of storage. Maillard reaction leads to denaturation of the protein and increases its surface hydrophobicity and flexibility. The migration rate of the conjugate to the surface of the oil droplets increased and eventually smaller droplets with narrow size distributions were formed by reducing the interfacial tension during the homogenization process [72]. Besides, the protein-polysaccharide conjugates prevent droplet flocculation and coalescence through steric and electrostatic repulsions during the storage period. The maillard conjugate forms a interfacial membrane, which is more effect on preventing partial coalescence during storage time [9]. Other researchers have reported similar results that emulsions stabilized by conjugates could significantly improve emulsifying properties, especially in reducing droplet size and creaming [73, 74].

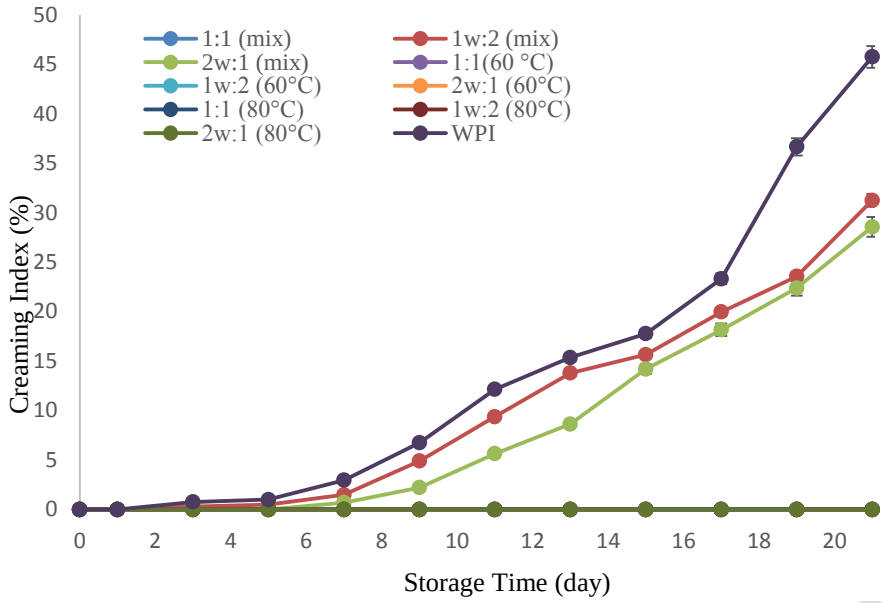

Fig 5. Creaming profile of emulsions stabilized by WPI native and WPI-Inulin mixtures and conjugates during 21 days.

3.9. Conjugated Dienes (CD):

Emulsifiers such as proteins, polysaccharides, and phospholipids used extensively to stabilize emulsions and to protect polyunsaturated fatty acids from oxidation [75]. Analysis of primary lipid oxidation products performed by measuring conjugated dienes (CDs). The results showed that the conjugated dienes value progressively increased throughout storage (72 hours at 65 ° C) (Fig.6). Oil-in-water emulsions can efficiently inhibit lipid oxidation due to the protective effect of the interfacial layer formed by some emulsifiers; this is more evident when comparing the amount of conjugated in emulsions with the bulk oil. The stabilized emulsion by the produced conjugates at 80 °C and the purslane seed oil showed the lowest and highest CDs values throughout storage, respectively ($P<0.05$). The results suggest that the emulsions stabilized by conjugate exhibited stronger oxidation inhibition. The antioxidant activity and inhibition of lipid oxidation of the conjugates increased with increasing the incubation time of WPI-inulin. Maillard reaction products adsorbed at the interface can limit the free radical chain reactions and inhibit oxidation in the next stage, and un-adsorbed Maillard reaction products protect the oil from oxidation at the later stage [76]. The findings agree with Dong et al. [77] who reported the hydrolyzed β-lactoglobulin-glucose conjugates inhibited lipid oxidation in menhaden O/W emulsions. The emulsions formed by mixed and conjugated WPI- inulin at a 2:1 weight ratio

showed the highest antioxidant activity and inhibition of purslane seed oil oxidation, which could be related to the higher molecular weight of the conjugates. The Maillard conjugates with high molecular weight exhibit the better metal chelating ability and prevent oxidative reactions of oils due to their hydroxyl and pyrrole groups [10].

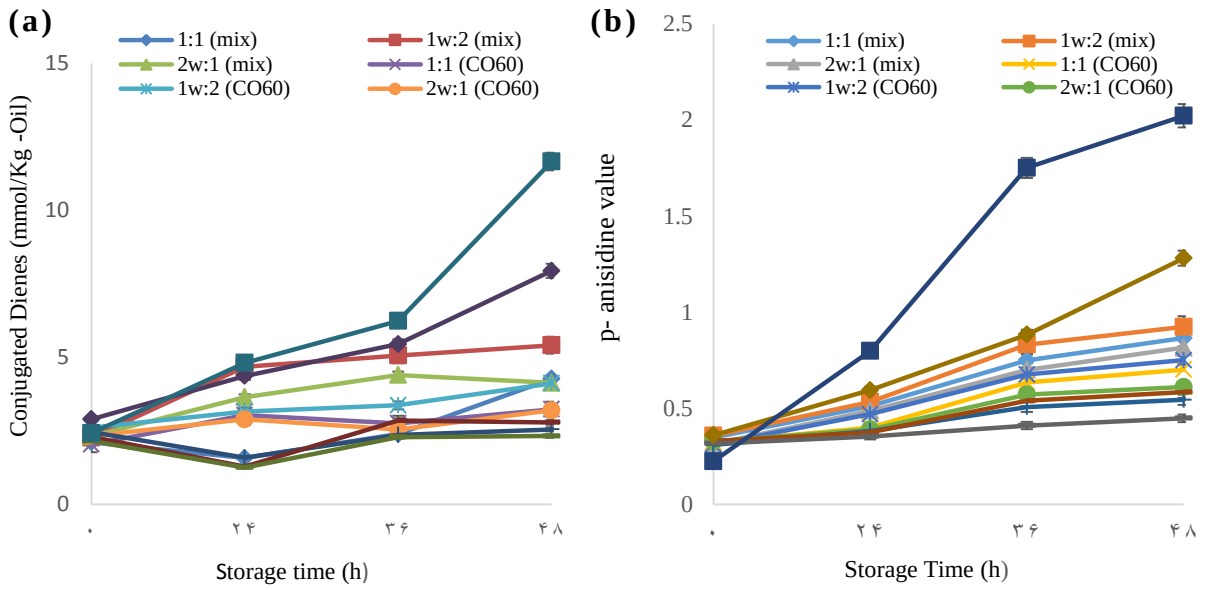

Fig 6. Conjugated Dienes (a) and Para-anisidine values (b) of the samples under accelerated storage conditions (65 °C for 72 h).

3.10. The para-Anisidine value (pAV):

The para-anisidine value (pAV) measures the secondary oxidation compounds (mainly 2-alkenals and 2, 4 alkali dienals) generated during the decomposition process of primary oxidation products (hydroperoxides) [78]. The anisidine index increased throughout storage (Fig. 6). As expected, the Purslane seed oil had the highest pAvs during storage ($P< 0.05$), as it contained a high level of polyunsaturated fatty acids. Compared to the emulsions formed from WPI alone or WPI -inulin mixtures, the rate of conjugated dienes and para-anisidine value formation in the emulsions prepared from WPI -inulin conjugates was lower, indicating that they had better oxidative stability. The results indicated that emulsions stabilized by conjugates especially produced conjugates at 80 °C showed the lower levels of para-anisidine value ($P < 0.05$). By increasing of the protein ratio, the the amount of aldehydes decreased. Hence, 2:1 weight ratio of wpi to inulin showed the lowest amount of para-anisidine value. The free groups of sulfhydryl and the aromatic amino acids in whey protein can also inhibit free radicals [79, 80]. In addition,

whey protein can be absorbed on the oil/water interface, it acts as a physical barrier between lipid droplets and water, which can inhibit lipid oxidation and also prevents the decomposition of hydroperoxides to secondary oxidation compounds [1].

4. Conclusions

The results of the present study showed that the WPI-inulin conjugates had stronger antioxidant activity, thermal stability and emulsion stability than the native WPI and WPI-inulin mixtures. The results of DSC indicated that conjugation led to the improved thermal stability of the samples and a decrease in ΔH value. WPI- inulin (2:1) had the highest T_g and T_d among all samples. The native WPI (18.46%) and WPI-inulin conjugate (2:1) produced at 80°C (55.3%) showed the lowest and highest free radical scavenging activities, respectively. Results showed that glycation led to increased zeta-potential and the emulsions formed by conjugates became more negatively charged as the incubation time of conjugates increased. This emulsion showed an acceptable stability and high viscosity during 21 days of storage. The emulsion containing WPI-inulin conjugate (2:1) exhibited the highest antioxidant activity and inhibition of purslane seed oil oxidation. Results of this study suggest that the oil in water emulsions stabilized with WPI-inulin conjugates can improve the oxidative stability of polyunsaturated fatty acids.

Conflicts of Interest

The authors confirm that they have no conflicts of interest.

Availability of Data and Materials

All data generated or analyzed during this study are included in this manuscript.

Ethic Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author's Contribution

Adeleh Mohammadi: conceptualization, methodology, investigation, formal analysis, writing-original draft, writing-review & editing. Mohammad Ghorbani: supervision, conceptualization, methodology, writing-review & editing. Alireza Sadeghi Mahoonak: conceptualization, methodology. Seid Mahdi Jafari: methodology.
All authors read and approved the final manuscript.

Corresponding author
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Conflict of Interest

The authors have no competing interests.

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