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Antioxidant activities and emulsification properties of the new model systems of whey proteins and reduced sugars

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Abstract

The final products formed from the various systems of the Maillard reactions possess different functional properties such as browning intensity, antioxidant activity, and emulsion stability. To study these properties and activities, different systems of whey proteins reaction with glucose and fructose at different concentrations to form a new model system of Maillard reaction products (MRPs) was observed. Results showed that high optical densities (peaks) at 280 and 420 nm indicated the formation of the intermediate stages of MRPs and the formation of advanced MRPs, respectively. Additionally, results showed that these Maillard reaction model systems possessed different antioxidant activities as demonstrated by DPPH and reducing power assays (20 - 93.2% and 40 - 90%, respectively) depending on the type and concentration of sugar, and the incubation time. The whey protein-fructose model system possessed high antioxidant activity (93.2%), and had the highest percentage on the emulsionwas found to possess a stability index (75.4%). The whey protein-fructose model systems comprised the highest number of the studied model systems to form MRPs, and had highly powerful antioxidant activity and emulsifying index.

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Introduction

The Maillard browning reaction is considered as a complex cascade reaction between proteins (amino acids) and reducing sugars, which result in a variety of by-products, intermediates, and brown products (melanoidins) that contribute markedly to the aroma, taste, colour, and antioxidant power of foods (Jing and Kitts, 2002). It is considered an important method for protein modification (Mastrocola and Munari, 2000; Kleekayai *et al.*, 2022). The most beneficial effects of Maillard reaction products (MRPs) are high antioxidant, antihypertensive, antimicrobial, anticarcinogenic, and antimutagenic activities (Rufian-Henares and Morales, 2008). However, the disadvantage of the formed products may be the formation of some toxic compounds, and the loss of the nutritional value of the products (Somoza, 2005; Price *et al.*, 2022).

In general, MRPs exhibit high antioxidative effects in food systems, and are considered a common food additive for enhancing stability and texture (Thongzai *et al.*, 2022). Many studies have reported the benefits associated with advanced MRPs, including effective natural antioxidants in the model systems in some foods (Benjakul *et al.*, 2005), as well as antimicrobial (Rufian-Henares and Morales, 2008), antihypertensive (Rufian-Henares and Morales, 2007), and anticarcinogenic and antimutagenic properties (Lee and Shibamoto, 2002). The Maillard reactions involve stable free radicals as well which may interact with lipid-free radicals to cause an inhibition of lipid peroxidation.

Whey protein has numerous nutritional advantages, and plays a role in different physiological functions. Whey protein beverages are gaining popularity among athletes, bodybuilders, and other individuals (Jeong *et al.*, 2022). The main protein constituent of whey is α-lactoalbumin and β-lactoglobulin, and it is considered a high-protein food ingredient due to its high contents of essential amino acids (*e.g.* lysine) (Morgan *et al.*, 2005). Whey powders contain some carbohydrates especially lactose (73%), and approximately 12% of proteins

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with a high percentage of lysine. Different conditions may affect the formation of the Maillard reaction such as the concentration of the proteins and reducing sugars, temperature, pH, water content, and the presence of other substances (Franzen *et al.*, 1990). The Maillard reaction may occur naturally in foods through processing, storage temperature, packaging during storage, and concentration of atmospheric oxygen.

Physical and chemical properties of proteins can be modified by enzymatic or non-enzymatic methods. Chemical modification may involve an increase in hydrophobicity and emulsifying properties by acylation, succinylation, derivatisation, or glycosylation (Chao *et al.*, 2022). In Maillard reactions, protein modification by glycosylation enhances the functional properties such as the stability and emulsifying properties of the proteins in foods (Haque and Roos, 2004). During thermal processing, glycosylation may take place in foods when the side chain of proteins is modified in the presence of reducing sugars to form covalent bonds (N-glycosides), which then form browning products that are classified under the term Maillard reaction. These conjugates of whey proteins and reducing sugars may enhance the emulsifying properties of proteins (Knudsen *et al.*, 2002; Li *et al.*, 2020).

Therefore, the present work aimed to produce a partially glycosylated whey protein with enhanced antioxidant model system of MRPs. In the present work, the modification of whey proteins by glycosylation, with glucose and fructose at different concentrations, at 75°C and pH 8.0, to form a new whey protein-sugar model system (MRPs), was investigated. The intermediate compounds, the final products, and the antioxidant activities of the new MRP model system also were investigated.

Materials and methods

Chemicals and materials

D–Glucose and D-Fructose were purchased from BDH Laboratory Supplies (Poole, BH15 1TD, England). Trichloroacetic acid (TCA) and potassium ferricyanide were purchased from Gainland Chemical Company (GCC) (Sandycroft, Deeside, U.K). 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and SDS were purchased from Sigma Aldrich (Germany). Methanol was purchased from Scharlau Barcelona (Spain). Whey was purchased from a local market (Irbid, Jordan). Other reagents used in the present work were of analytical grade.

Protein determination

The total protein concentrations were determined in triplicate by the Biuret method using bovine serum albumin (BSA) as standard (Nowotny, 1979). The amount of soluble protein was calculated from the standard curve as mg of protein per mL of the test sample.

Spectroscopic studies and browning intensity

The UV-vis optical density and browning intensity of MRPs were measured according to the Ajandouz *et al.* (2011). The MRPs were diluted with distilled water and the absorbance was measured at 250 - 700 nm, and specifically at a wavelength of 280 and 420 nm by UV-vis Thermo Electron Model BioMate 3 spectrophotometer (USA).

Preparation of MRPs at different reaction times

The model system in our experiments comprised whey (proteins), glucose, and fructose at different concentrations (0.2 and 0.6 M). Whey and sugars were dissolved in 50 mL of distilled H_2O, in a 10 mL cap-glass tube that was adjusted to pH 8.0 with aqueous KOH solution. They were incubated in a water bath at 75°C and at different intervals: 0, 24, 48, 72, and 96 h. The first sample (control) was taken at zero time (*i.e.* no incubations), and the other samples were taken at the appointed time, and the absorbance was read at 250 - 700 nm, and specifically at 280 and 420 nm.

Determination of DPPH radical-scavenging activity

DPPH radical scavenging was determined by the colorimetric method (Singh and Rajini, 2004; Bal *et al.*, 2021). Briefly, 80 µL of MRP sample was treated with 2 mL of 0.12 mM DPPH (in methanol) and 320 µL of distilled water. The solution was mixed and allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm. For the control sample, gallic acid was used. The radical scavenging activity (percentage) was calculated using Eq. 1:

$$\text{Radical scavenging activity (percentage)} = [1 - (As / Ac)] \times 100 \quad \text{(Eq. 1)}$$

where, As = absorbance of sample, and Ac = absorbance of the control.

Determination of reducing power

The reducing power of the MRPs was determined by the colorimetric method (Gu *et al.*, 2009) with modifications. Briefly, 1.0 mL of MRPs was mixed with 1.0 mL of 0.2 M sodium phosphate buffer (pH 7.0) and 1.0 mL of potassium ferricyanide. The reaction mixture was incubated in a water bath at 50°C for 20 min, followed by the addition of 1.0 mL of 10% trichloroacetic acid. Then, the mixture was centrifuged at 1,500 rpm (Hermle Z 230 A, Germany) for 10 min at room temperature. Next, 1.0 mL of distilled water and 200 µL of 0.1% $FeCl_3$ were then added to 1.0 mL of supernatant. The absorbance was read at 700 nm.

Emulsification property measurement

The emulsification properties of the MRPs were determined using the emulsion stability index (ESI) by the turbidimetric method (Hao *et al.*, 2011). The emulsion was formed by thoroughly mixing 10 mL of corn oil and MRP solution. Next, 100 µL of the emulsion was added to 10 mL of SDS (0.1%) solution, immediately (zero time, T_0), and then homogenised after 10 min (T_{10}). The absorbance was measured at 500 nm (at T_0 and T_{10}). ESI was calculated using Eq. 2:

$$ESI\ (min) = (T_0 \times t) / T_{10} \quad \text{(Eq. 2)}$$

where t = time between 0 and 10 min.

Statistical analysis

Analysis was carried out using Microsoft Excel 2016. Data were expressed in mean of three replicates for the MRPs model system ± standard deviation (mean ± SD). $p < 0.05$ was considered statistically significant.

Results and discussion

Protein content

The protein content in the whey sample was measured by the Biuret method using BSA as a standard protein. Results showed that the whey sample contained 2.66 ± 0.37 mg/mL protein.

Browning intensities of the MRPs

The initial step of the Maillard reaction cascade is the reaction between the free amino group of a protein and the reducing end of sugars, which leads to the modification of proteins (glycation) (Cardoso *et al.*, 2018). The browning reaction in the heated foods can be evaluated by using different Maillard reactions of different model systems, between amino acids and sugars or proteins, under specific conditions. These systems would exhibit various physical, biological, and chemical characteristics. The early and final stages of the MRPs were studied for their spectrophotometric measurements at 280 nm for the first stages of Amadori rearrangement and heterocyclic compounds, and at 420 nm for browning intensity detection for the by-product compounds such as glucosamine, ketosamine, and diketosamine, and others.

The difference in browning and optical densities between the MRPs model systems may be due to the glycation of whey proteins due to several parameters such as the type of carbohydrate (glucose and fructose), their concentrations, temperatures, and pH levels.

Figure 1 illustrates the UV-vis spectra of MRPs solutions at 250 - 700 nm after dilution with distilled water and incubation at 0, 24, 48, 72, and 96 h.

The peaks of optical densities (browning intensities) at 280 and 420 nm were observed for all the MRPs model systems. The browning colour intensity is considered as an indicator of the stages of the formation of MRPs in foods (Morales and Jiménez-Pérez, 2001). In Figure 1, the optical densities increased for all the model systems, and the highest peak spectrum of browning was observed in the whey-fructose (0.6 M) MRPs model system when the heating time was increased. Kim and Lee (2010) obtained similar results. The optical densities at 280 nm could be used to determine the intermediate compounds of MRPs. The optical densities at 420 nm suggest the formation of melanoidins (Ajandouz *et al.*, 2011).

Furthermore, the absorbance of all the model systems was found to increase with increasing concentrations of reactants. The condensation of the amino group of proteins and a hydroxylic group of sugar fragments form the heterocyclic and polymer compounds, which then form brown pigments (melanoidins) with high absorbency.

The present work demonstrated that the best model systems of MRPs were obtained by using the whey (proteins) with fructose at 0.6 M, followed by fructose at 0.2 M. The MRPs model systems with glucose (0.2 and 0.6 M) had less optical densities.

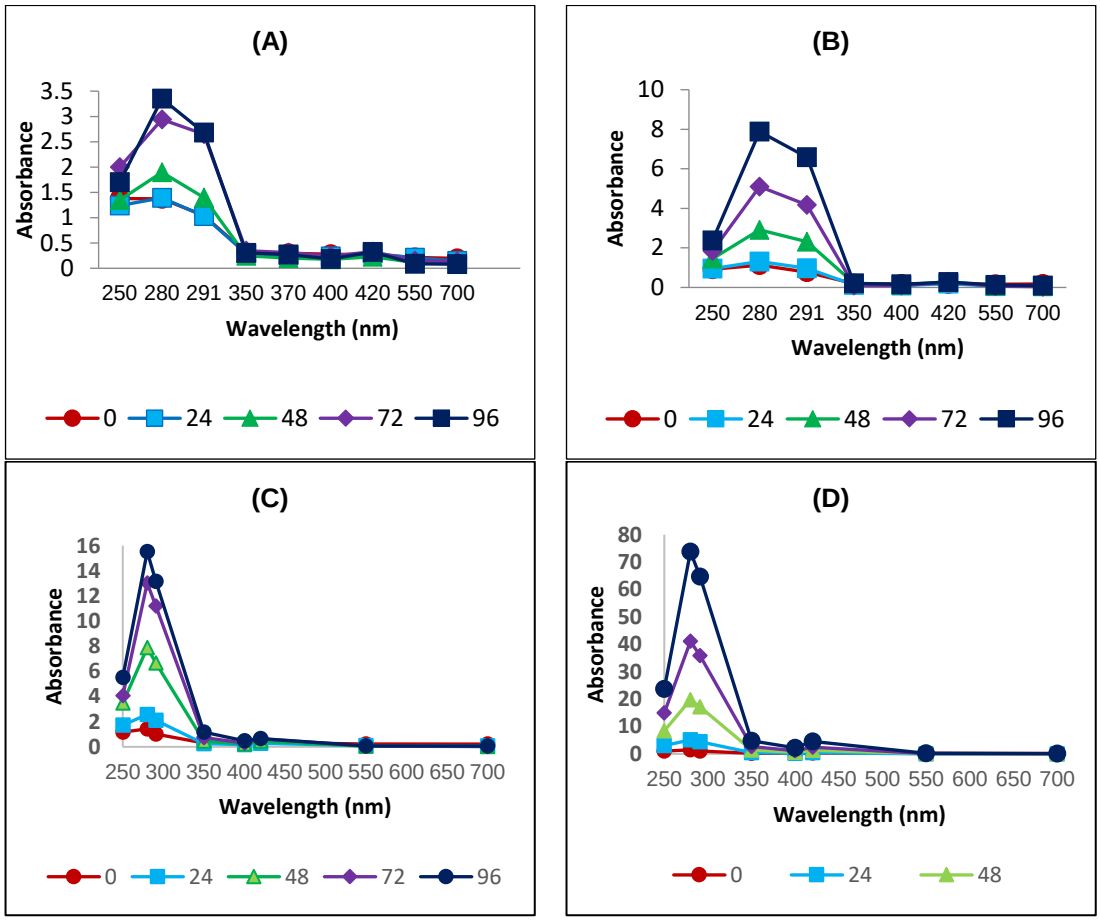

Figure 1. Browning intensity (absorbance) of the MRPs solutions of the whey protein-sugar model systems at 250 - 700 nm, and at different incubation times (0, 24, 48, 72, and 96 h). **(A)**: whey protein-glucose (0.2 M); **(B)**: whey protein-glucose (0.6 M); **(C)**: whey protein-fructose (0.2 M); and **(D)**: whey protein-fructose (0.6 M). Each point is mean ± SD of triplicate, $n = 3$.

Therefore, the sort of reducing sugar involved in the reaction significantly influenced the MRPs formation rate.

Glycation of proteins depends on the N-terminal α-amino group of amino acids, especially lysine and arginine residues of proteins. It also depends on the carbohydrate types, isomers, and concentrations (Ledesma-Osuna *et al.*, 2008), which influence the initial nucleophilic attack due to differences in reduction.

Temperature is also a significant factor on Maillard reaction. A temperature rise can cause glycation enhancement within the Maillard (Cheison *et al.*, 2013), and consequently cause the formation of assorted intermediate compounds and products like melanoidins (Pinto *et al.*, 2012). Another factor that affects the Maillard reaction formation is pH (Czerwenka *et al.*, 2006). At a specific pH, the sugar aldehyde group and the lysine amino group may be protonated or deprotonated. Therefore, it may influence or prevent the formation of protein glycation or protein-sugar conjugate. A slightly alkaline or at pH 8.0 has been widely used for whey protein conjugation and glycation (Deng *et al.*, 2017).

Antioxidant activity of MRPs

The MRPs have exhibited an efficient natural antioxidant in model systems and a few foods (Ajlouni and Pan, 2014) by delaying, retarding, or preventing the oxidation processes. As an example, MRPs of xylan and chitosan exhibited powerful antioxidative preservations for lipid food storage (Cui *et al.*, 2014). Furthermore, MRPs contributed and exerted effects on the aroma, colour, taste, flavour, and antioxidant activity. The obtained results of the antioxidant activities of MRPs in various whey protein-sugar mixtures indicated high antioxidative activity by chelating metals and scavenging DPPH radicals (Dittrich *et al.*, 2003).

DPPH radical scavenging

The DPPH radical scavenging activity of various MRPs showed that these model systems actively scavenge DPPH radical by hydrogen donation to form stable DPPH (Yilmaz and Toledo, 2005). The various models of MRPs systems were evaluated for their antioxidants by allowing these systems to react with the stable DPPH molecules. The ability of MRPs to decolourise DPPH will reflect their radical scavenging activity. Figure 2 shows the percentage of the radical scavenging activity of various types of MRPs and gallic acid as a control.

The highest efficiencies of DPPH radical scavenging were obtained in whey-fructose MRPs model systems 0.6 M (93.25%) as compared to whey-glucose 0.6 M (78.5%) (Figure 2).

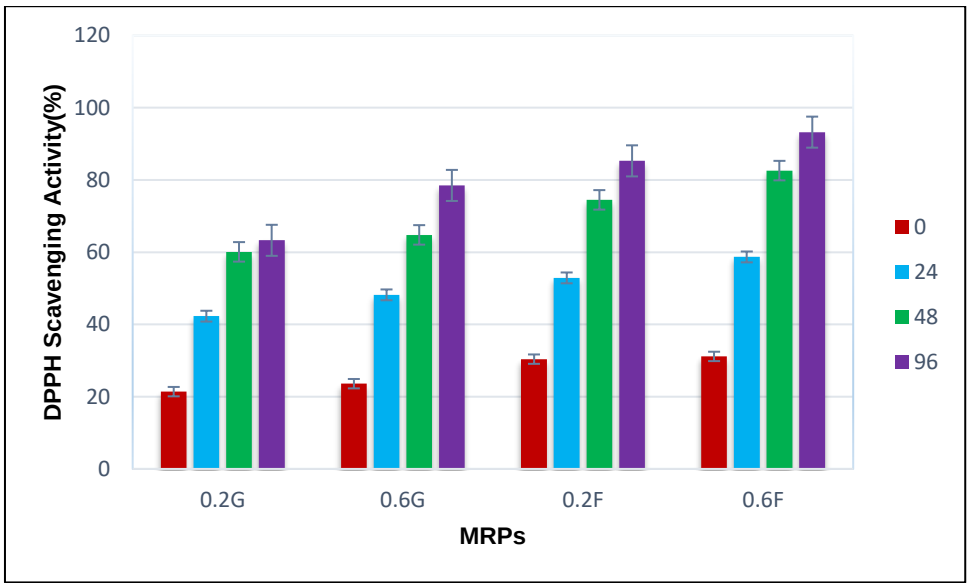

Figure 2. DPPH radical scavenging activity (%) of different MRPs solutions at different incubation times (0, 24, 48, 72, and 96 h). 0.2 G (whey protein-glucose at 0.2 M); 0.6 G (whey protein-glucose at 0.6 M); 0.2F (whey protein-fructose at 0.2 M); and 0.6 F (whey protein-fructose at 0.6 M). Each point is mean ± SD of triplicate, $n = 3$.

Reducing power

The antioxidant activity of many systems and biological samples is often tested by the reducing power method. The presence of certain groups of MRPs model systems such as the hydroxyl and pyrrole groups may act as reducing agents (Yanagimoto *et al.*, 2002). During different stages of Maillard reactions, some of the compounds that are formed exhibited the hydroxyl and pyrroles side chains which then resulted in the formation of brown final products.

Figure 3 shows that all the MRPs had high reducing power; from 85 to 97% when compared with gallic acid as a control (100%).

All MRPs systems had antioxidant activity with a high reducing power activity (approximately 45 - 90%) (Figure 3). The MRPs system of whey protein-glucose at 0.6 M exhibited an occasional power activity (45 - 80%) as compared to the MRPs system of whey protein-fructose at 0.6 M (45 - 90%), which were dependent on carbohydrate concentrations.

Yoshimura *et al.* (1997) reported that heating (100°C) the MRPs for 1 - 6 h would form various compounds that scavenge different radical molecules, and this agreed with the results observed in the present work.

Emulsifying stability of MRPs

Emulsions are thermodynamically unstable systems. The newly formed model systems (MRPs) were used to characterise their behaviour against different destabilisation processes.

Based on Figure 4, the MRPs model systems improved the emulsifying stability (ESI), and the containing fructose exhibited the best ESI. However, the other MRPs models had low emulsion stabilities. Kato (2000) reported that some MRPs that used lysozyme and soy protein as the model proteins exhibited a rise in ESI. The results of emulsification stability indicated that the newly formed systems (MRPs) stabilised the emulsions by reducing tension at the interface of the oil droplets, thus continuing to aqueous phase.

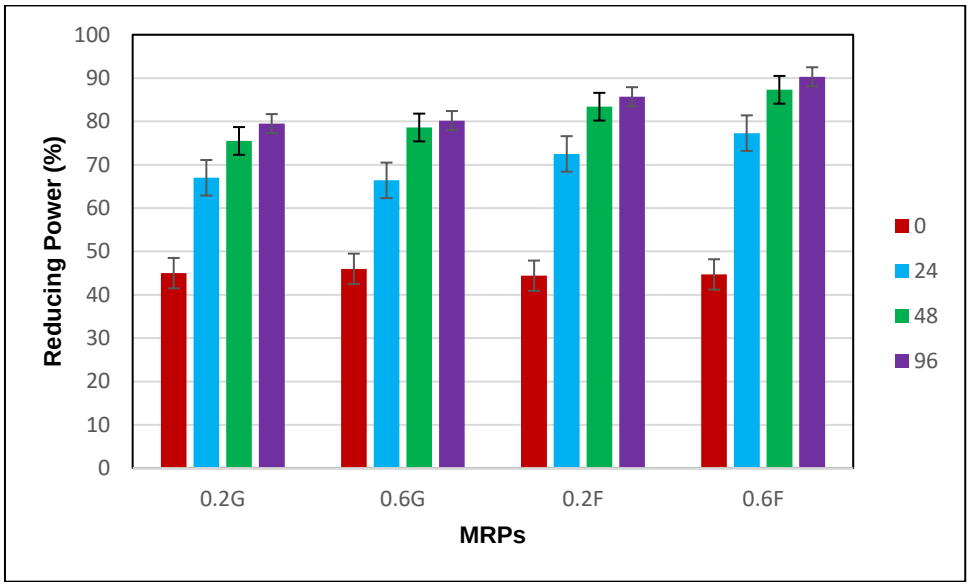

Figure 3. Reducing radical scavenging activity (%) of different MRPs solutions at different concentrations of fructose and glucose at different incubation times (0, 24, 48, 72, and 96 h). 0.2 G (whey protein-glucose at 0.2 M); 0.6 G (whey protein-glucose at 0.6 M); 0.2F (whey protein-fructose at 0.2 M); and 0.6 F (whey protein-fructose at 0.6 M). Each point is mean ± SD of triplicate, n = 3.

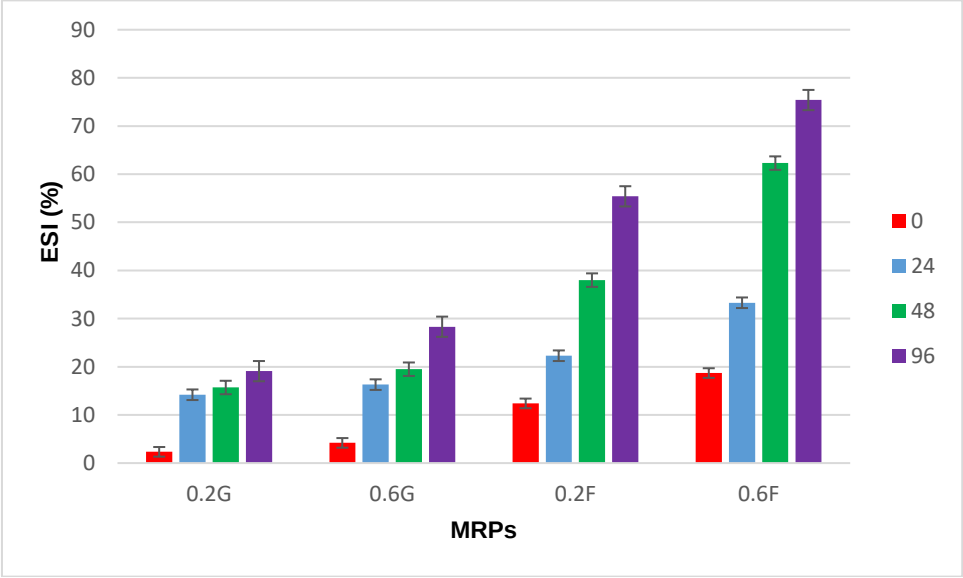

Figure 4. Emulsifying stability (ESI) (%) of different MRPs solutions at different concentrations of glucose and fructose at different incubation times (0, 24, 48, 72, and 96 h). 0.2 G (whey protein-glucose at 0.2 M); 0.6 G (whey protein-glucose at 0.6 M); 0.2F (whey protein-fructose at 0.2 M); and 0.6 F (whey protein-fructose at 0.6 M). Each point is mean ± SD of triplicate, n = 3.

Generally, the fructose-containing model systems showed a higher ESI as compared to the glucose model systems (Figure 4), which may be due to the ketose property of fructose as it forms an open-chain form with a high ability to reduce the tension at the oil droplet interface.

The antioxidant activity of the newly whey model systems (MRPs) of the heated whey protein-carbohydrate mixture was found to correlate well with the browning intensity. Since the MRPs of whey protein-fructose at 0.6 M system had the highest browning intensities, they also had the highest DPPH radical scavenging and reducing power. The present work also showed a correlation between emulsifying stability and browning intensity (Figure 5).

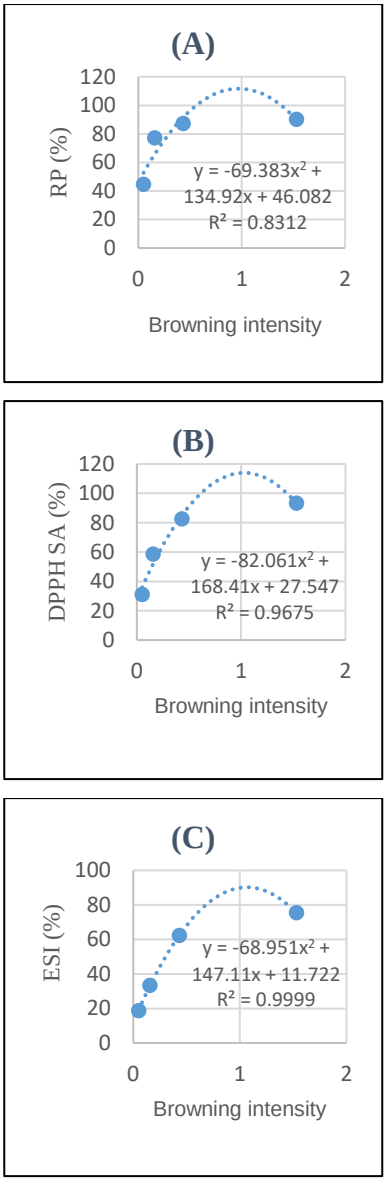

Figure 5. Correlation between the results of browning intensity of the whey protein-fructose (0.6 M) model system of MRPs and the results of reducing power (RP %) **(A)**, DPPH scavenging activity (DPPH SA%) **(B)**, and ESI (%) **(C)**.

Conclusion

The newly formed MRPs model systems of whey showed variable browning intensities, which indicated the formation of different heterocyclic and polymer compounds (melanoidin), depending on the type of the amino acid of whey proteins, the concentrations of glucose and fructose, and the incubation time. The antioxidant activity of the newly formed MRPs model systems of the heated whey protein-carbohydrate mixture, especially whey protein-fructose at 0.6 M system, was found to correlate well with the browning intensity.

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