OP-236 [Beekeeping Technology and Quality]

Ultrasound-Augmented Conjugation of Whey Protein Isolate and Propolis: Structural Insights and Functional Enhancements

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Propolis has been extensively utilized as a traditional food and medicinal source owing to its potent antioxidant activity. Polyphenols, including total phenolic and total flavonoid, are acknowledged as the primary antioxidant components in propolis. However, propolis exhibits inherent limitations including low water solubility, restricted oral bioavailability, and unfavorable taste and odour profiles during processing and storage, which limit its application. To enhance these functional properties of propolis, this study aimed to develop a conjugation between whey protein isolate (WPI) and propolis through free radical reactions, both with and without ultrasound treatment. Notably, subjecting the reaction to ultrasound at a power of 270 W for 20 minutes accelerated the conjugation process, leading to significantly elevated levels of total phenolic and total flavonoid contents. This enhancement may be attributed to the favorable structural modifications induced by ultrasound treatment. To better understand the conjugation mechanisms under the influence of ultrasonic waves, the structural properties of various WPI-propolis conjugates were investigated with the aid of Fourier transform infrared spectroscopy, circular dichroism and fluorescence spectrometry. Comparative analysis revealed distinct structural differences between conjugates prepared via ultrasound and the traditional free radical method. Specifically, the ultrasound-treated conjugates exhibited reduced α-helix structures but showcased higher contents of β-turn and random coil. Additionally, these conjugates displayed enhanced fluorescence intensity and surface hydrophobicity. Furthermore, the integration of ultrasound exhibited a multifaceted impact on the bioactivity and functionality of the conjugates. It significantly improved antioxidant activity, thermostability, water solubility and emulsifying stability. These results highlight the potential of WPI-propolis conjugates, formed through ultrasound-assisted free radical reactions, providing new insights into broader applications in the food industry.

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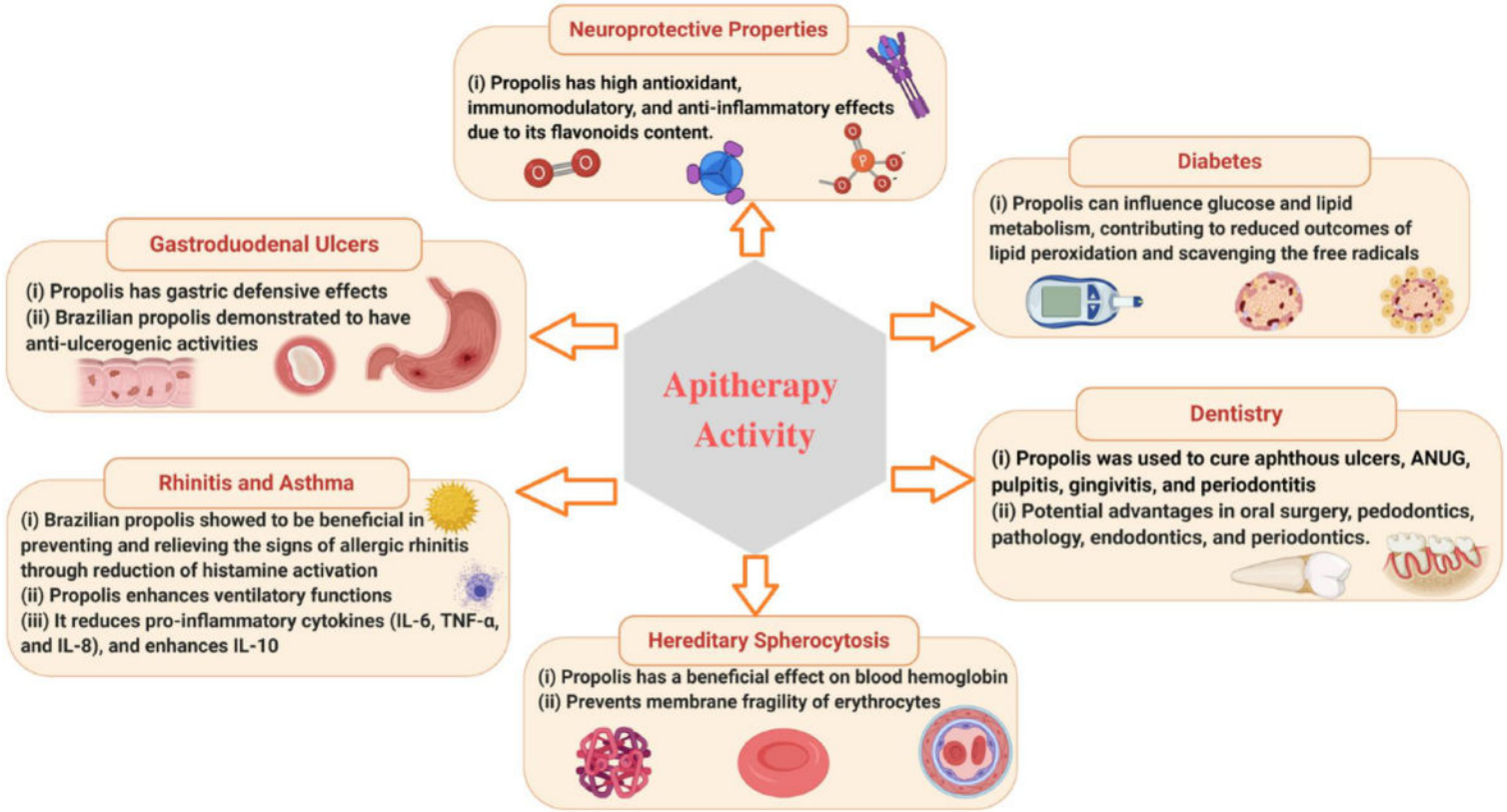
Introduction

- Propolis, a natural resinous product collected from flowers, tree buds, pollen and exudates, also incorporates enzymes secreted by bees

Introduction

- a protective material for honeycombs against microbial contamination
- a traditional food and medicine source for human beings



Introduction

- Polyphenols are the main bioactive components in propolis
  - --flavonoids (such as flavones, flavonols, and flavonones)
  - --phenolic compounds (such as caffeic acid, chlorogenic acid and cinnamic acids)
  - --Phenolic compounds' esters (such as caffeic acid phenethyl ester)

Propolis Administered	Model/Diabetes Type	Identified Compounds	Effects
China	STZ-induced type 1 diabetic rats	Caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, pinobanksin, quercetin, kaempferol, apigenin, pinocembrin, chrysin, CAPE, galangin.	Attenuates diabetes via directly decreasing the levels of fasting blood glucose and HbA1c; reduces MDA, ROS and RNS.
Mexico	STZ-induced type 1 diabetic mice	Pinocembrin, quercetin, naringin, naringenin, kaempferol, acacetin, luteolin, chrysin.	Decreases blood glucose and loss of body weight; increases plasma insulin levels and activities of the enzymes SOD, CAT, and GPx in serum.
Brazil	Skeletal muscle cells (L6 myotubes)	Artepillin C, coumaric acid, and kaempferide	Promotes GLUT-4 translocation and glucose uptake activity by PI3K and AMPK phosphorylation in a dose-dependent manner.

Propolis Administered	Cell Lines	Identified Compounds	Effects	Ref.
Poland	Me45, HCT-116	Pinostrobin, kaempferol, galangin, chrysin, apigenin, quercetin, gallic acid, ferulic acid, caffeic acid, coumaric acid and benzoic acid.	Presents antitumor activity in a dose-dependent manner; HCT-116 cells are more susceptible and showed typical changes for cell necrosis; Me45 cells death was mainly through apoptotic pathway.	[180]
South Poland	Caco-2, DLD-1, HT-29, H23, A549, MCF-7, MDA-MB-468, LN18, U87.	Aromatic acids, fatty acids, flavonoids, chalcones and some esters.	Strong antitumor effect on Caco-2 cells; decreases cell numbers in a dose-dependent manner in cells H23, A549, MCF-7, MDA-MB-468, LN18 and U87.	[49]
Brazilian (red, green and brown propolis from Northeast, South, Southeast and Midwest)	B16F10, SF-295, OVCAR-8, HCT-116, HL-60, Jurkat, MOLT-4, K562	Coumaric acid, *p*-coumaric acid, gallic acid, caffeic acid, ferulic acid, cinnamic acid, artepillin C, genistein, kaempferol, catechin, epicatechin, daidzein, naringenin, pinobanksin, formononetin, pinostrobin, rutin, quercetin, luteolin, apigenin, terpenes, tocopherol.	Presents antitumor activity.	[34,181–183]

Nelly Rivera-Yañez et al., *Nutrients*, 2020

Scientific question

- Despite the proven health benefits of propolis, its integration into foods remains constrained
- The inherent limitations of propolis during processing and storage

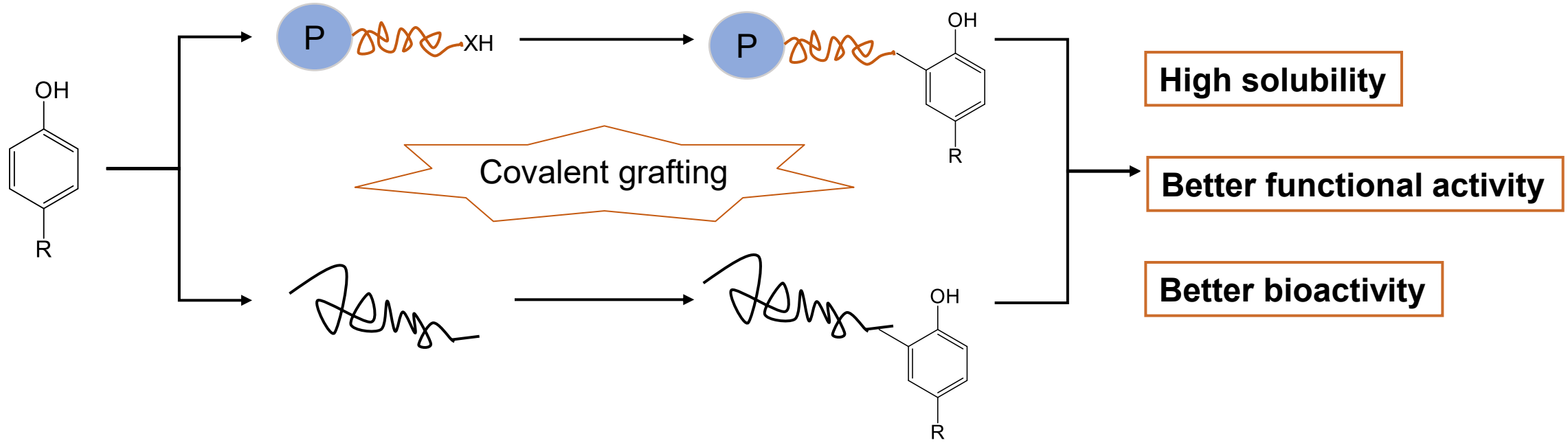
  --low water solubility

  --restricted oral bioavailability

  --undesirable taste and odour profiles

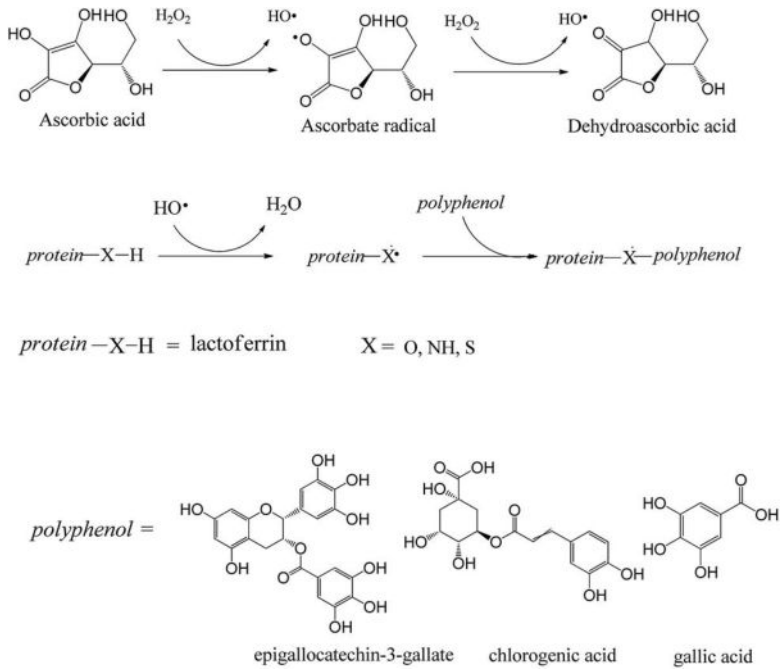
Introduction

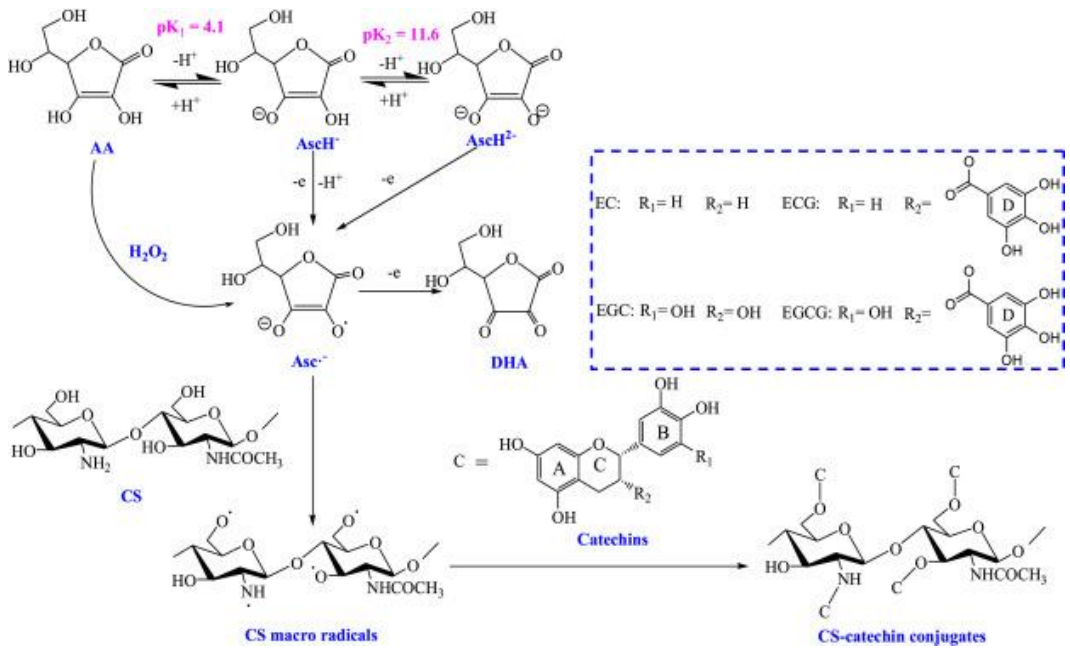
- The conjugation of polyphenols with dietary proteins as a more promising avenue for enhancing the water solubility and stability of the propolis



Introduction

- The free radical method is a green approach due to its avoidance of toxic chemicals, low cost and mild reaction conditions
  - --extended reaction times
  - --a relatively low degree of conjugation





Introduction

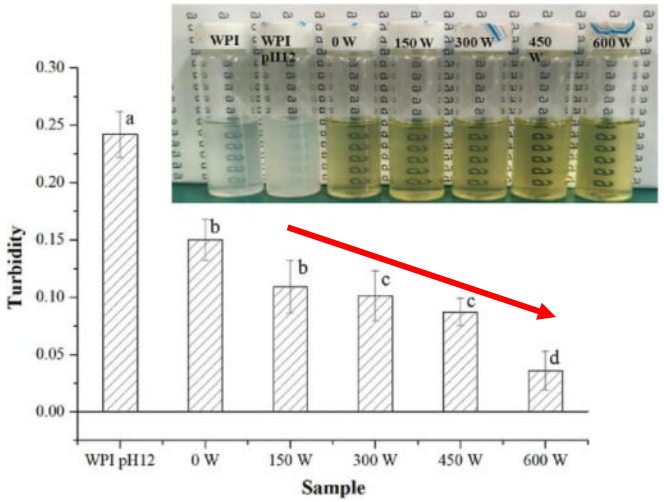
- Ultrasound integrated into the conventional free radical reactions
  - --accelerate the conjugation between proteins and polyphenols.
  - --accelerate the reaction by enhancing the mass transfer of the processing system
  - --bring about modifications in the structural and functional properties of the reactants

Table 2. Degree of polyphenol binding and grafting of the complex

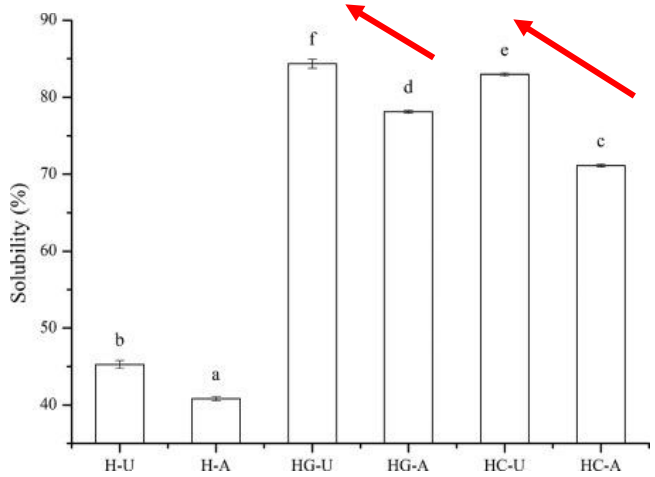
Group	0 W	150 W	300 W	450 W	600 W
Degree of polyphenol binding（%）	79.89±0.40^{d}	81.05±0.08^{c}	83.02±0.23^{a}	81.96±0.08^{b}	83.58±0.17^{a}
Degree of grafting (%)	28.72±2.32^{d}	43.43±2.08^{b}	39.96±1.26^{c}	51.56±1.51^{a}	30.91±2.38^{d}

Note: Data in the table are mean ± standard deviation; In the same column, the same letter from a to d means that there is no significant difference ($p>0.05$), but there is a significant difference between different letters ($p<0.05$).

Accelerate WPI-lutin conjugation



Reduce turbidity



Promote solubility

Objective

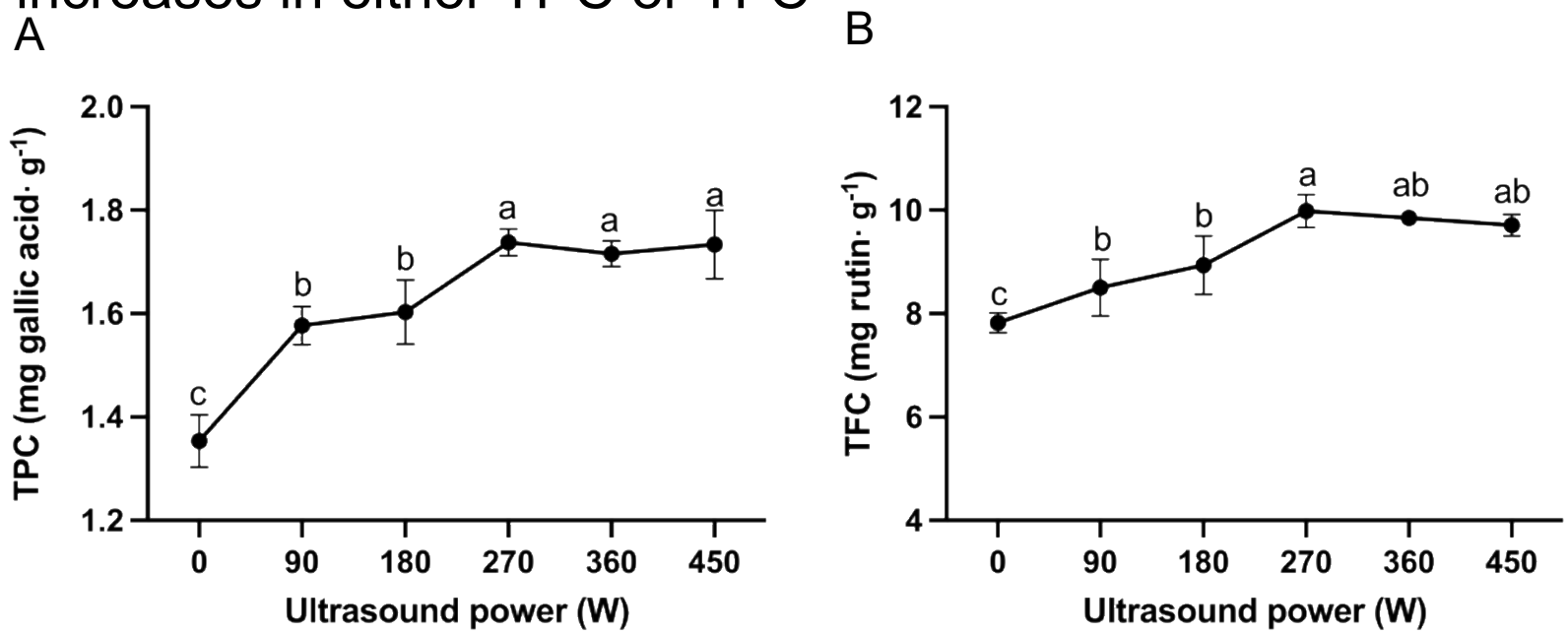
- To establish a protein-polyphenol conjugate under an ultrasonic field

  --the effect of ultrasound on the conjugation efficiency and grafting extent

  --alterations in the structural properties of the conjugates at primary, secondary and tertiary levels, alongside changes in antioxidant activities, solubility, thermal stability and emulsifying properties of the conjugates.

Effects of ultrasound conditions on the TPC and TFC

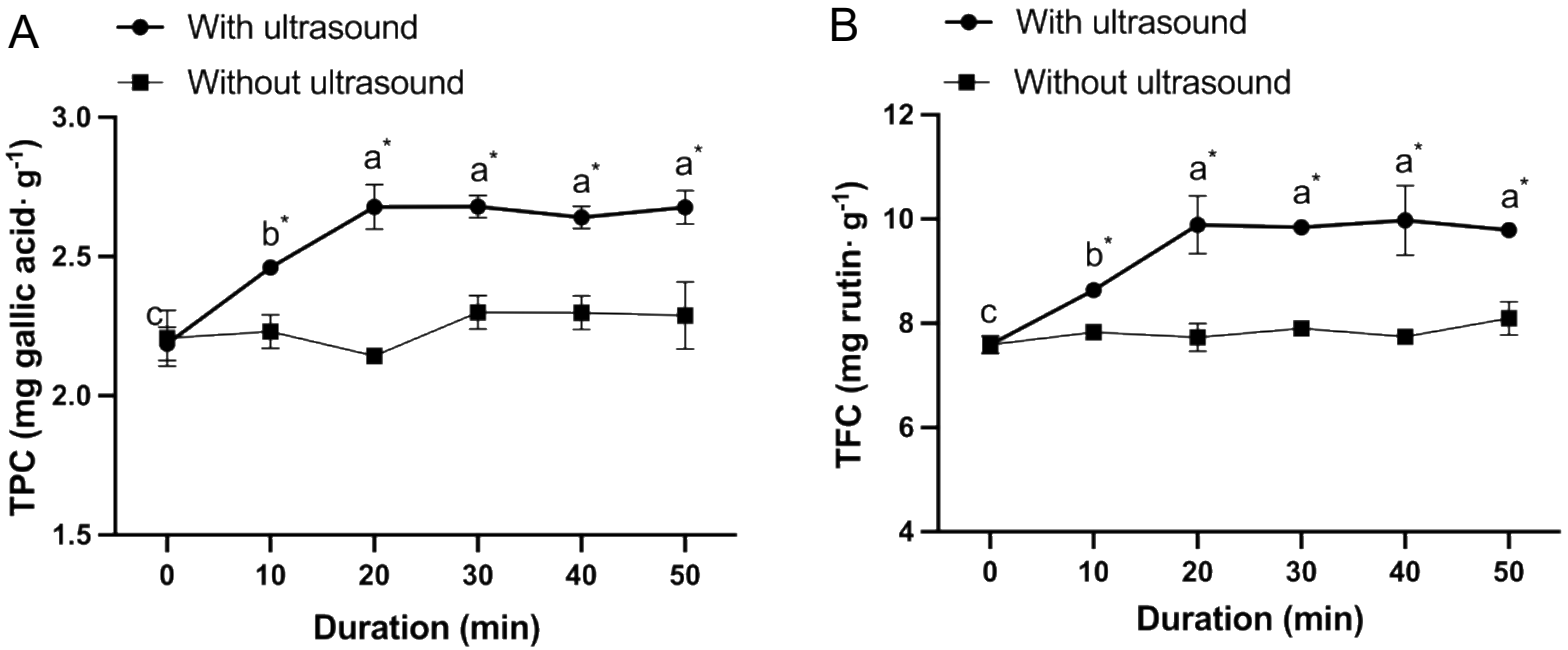
- As ultrasound power was increased from 0 W to 270 W, the TPC and TFC of WPI-propolis conjugates were significantly increased
- Increasing the ultrasound power from 270 W to 450 W did not result in further increases in either TPC or TFC



The TPC and TFC of WPI-propolis conjugates prepared by free radical method and ultrasound treatment at different ultrasound powers for 20 min

Effects of ultrasound conditions on the TPC and TFC

- As ultrasound time was increased from 0 to 20 min, the TPC and TFC of WPI-propolis conjugates were significantly increased
- Extending the ultrasound time from 20 to 50 min did not lead to further increases in TPC and TFC of WPI-propolis conjugates



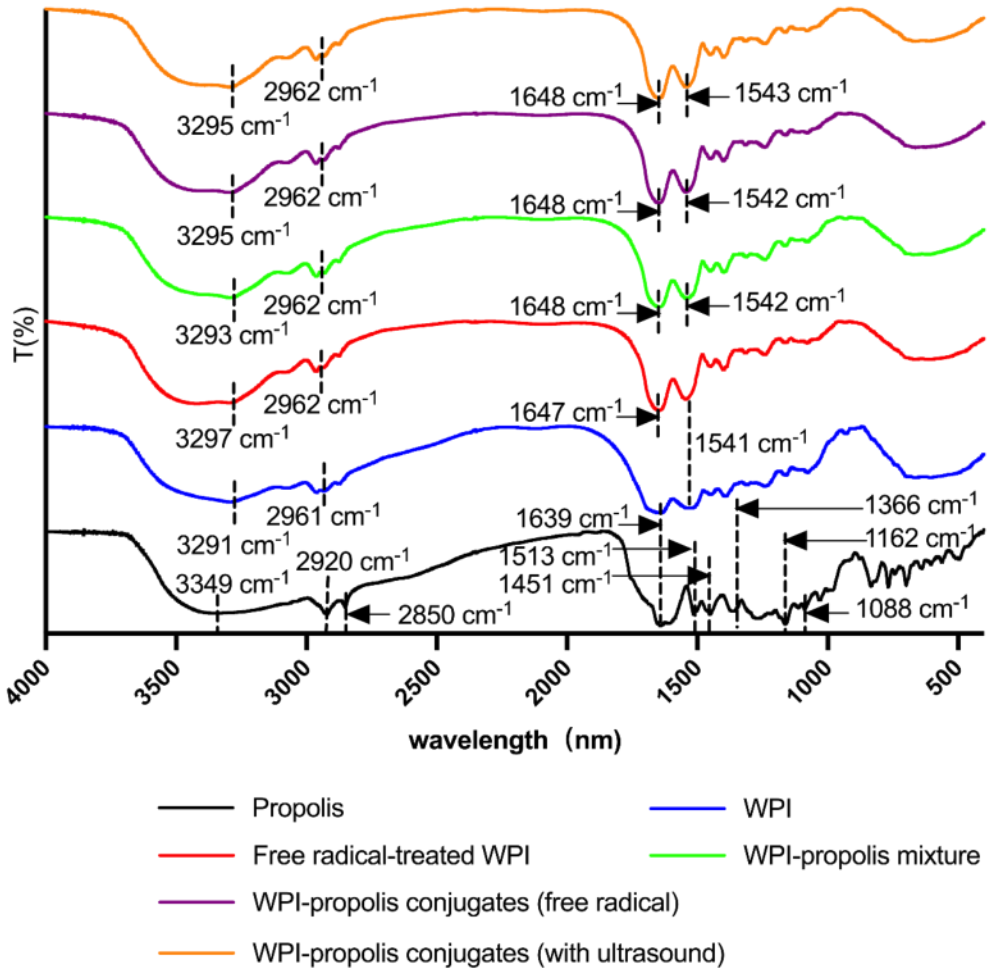
The TPC and TFC of WPI-propolis conjugates prepared by the traditional free radical method and ultrasound treatment at different times

Structural Insights and Functional Enhancements

- Compared to the mixture：

  --the absorption band of amide A (3293 cm^{-1}) in the conjugates showed a 2 cm^{-1} redshift

  --ultrasound treatment induced a further redshift of amide II from 1542 to 1543 cm^{-1}



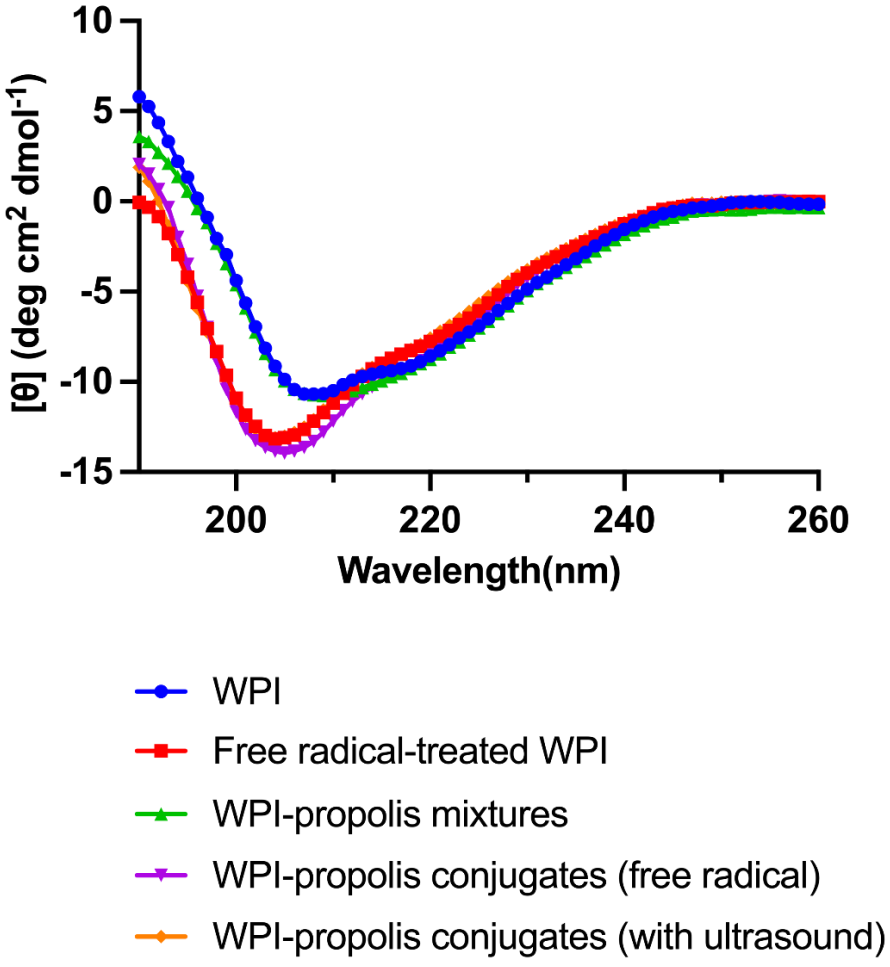
The FTIR spectra of different samples

Structural Insights and Functional Enhancements

- Compared to the free-radical treated WPI, WPI-propolis conjugation resulted in a slight decrease in β-sheet content, and a slight increase in β-turn and random coil contents
- Ultrasound further decreased the α-helix of conjugates, while increase the contents of β-turn and random coil

	α-helix (%)	β-strand (%)	Turns (%)	Unordered (%)
WPI	55	21	10	15
Free radical-treated WPI	26	29	18	25
WPI-propolis mixtures	55	19	9	15
WPI-propolis conjugates (free radical)	26	27	21	26
WPI-propolis conjugates (with ultrasound)	12	28	31	30

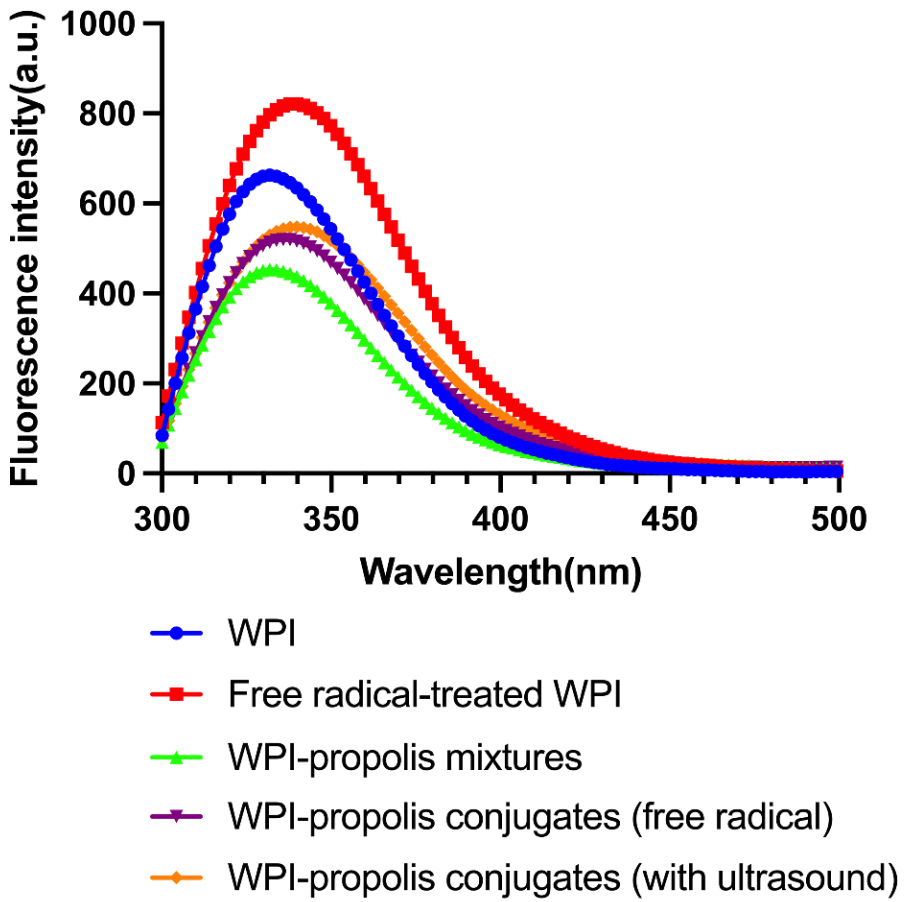
The corresponding secondary structures



The CD spectra of samples

Structural Insights and Functional Enhancements

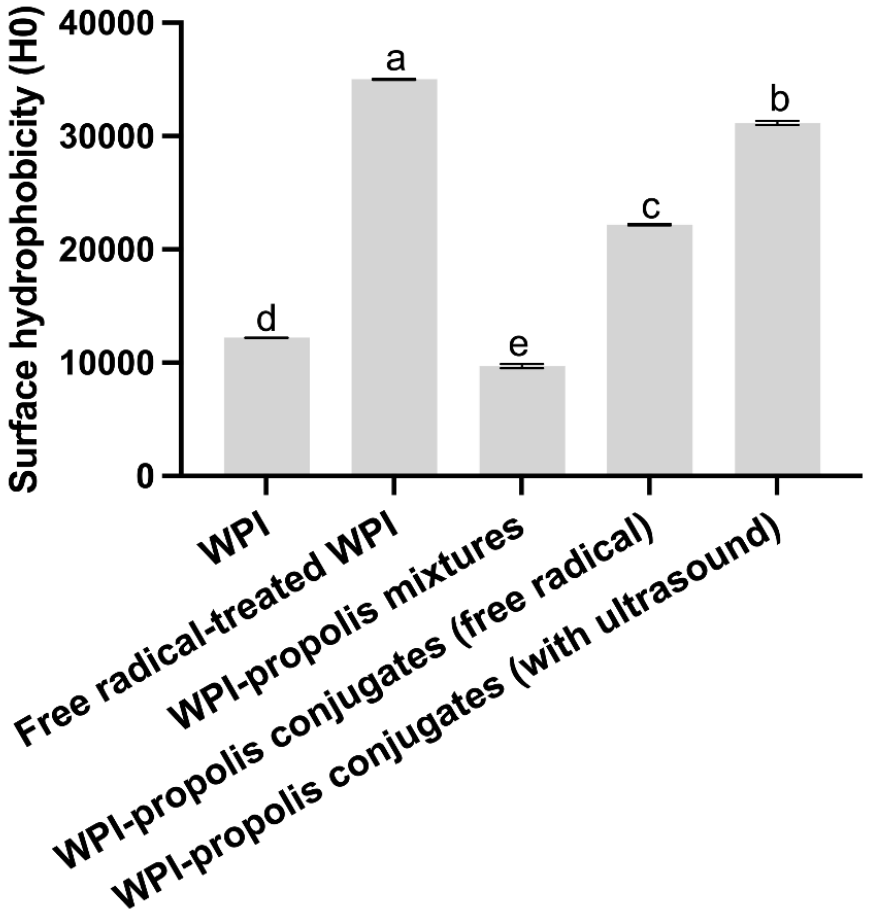
- The maximum fluorescence emission (λ_{max}) of native WPI occurred at 332 nm and remained unchanged for the WPI-propolis mixture
- WPI-propolis conjugation via the traditional method significantly lowered the fluorescence intensity, accompanied by a redshift in λmax from 332 to 335 nm
- Ultrasound treatment increased the fluorescence intensity with a further redshift to 340 nm compared to conjugates



The intrinsic fluorescence spectra of samples

Structural Insights and Functional Enhancements

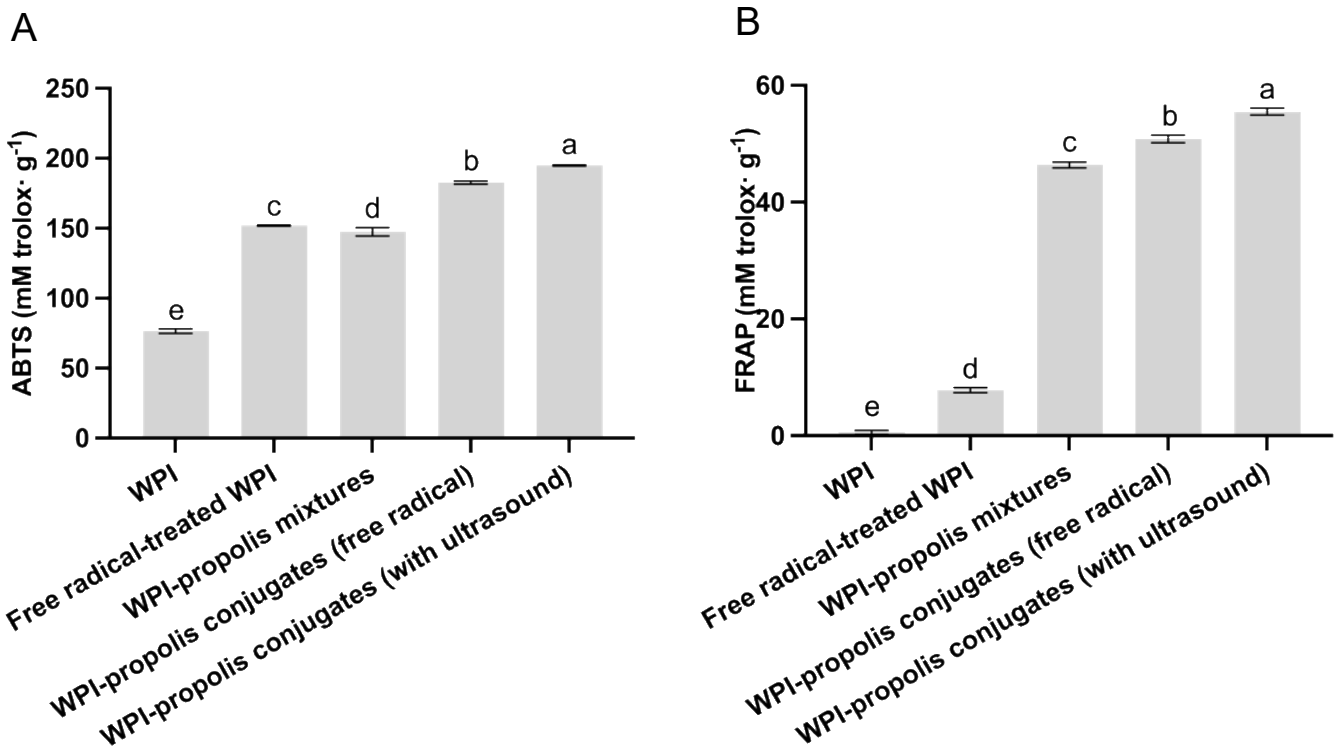
- The WPI-propolis conjugates exhibited increased surface hydrophobicity compared to the physical mixture
- Ultrasound treatment resulted in a significantly higher surface hydrophobicity in the conjugates



The surface hydrophobicity of samples

Structural Insights and Functional Enhancements

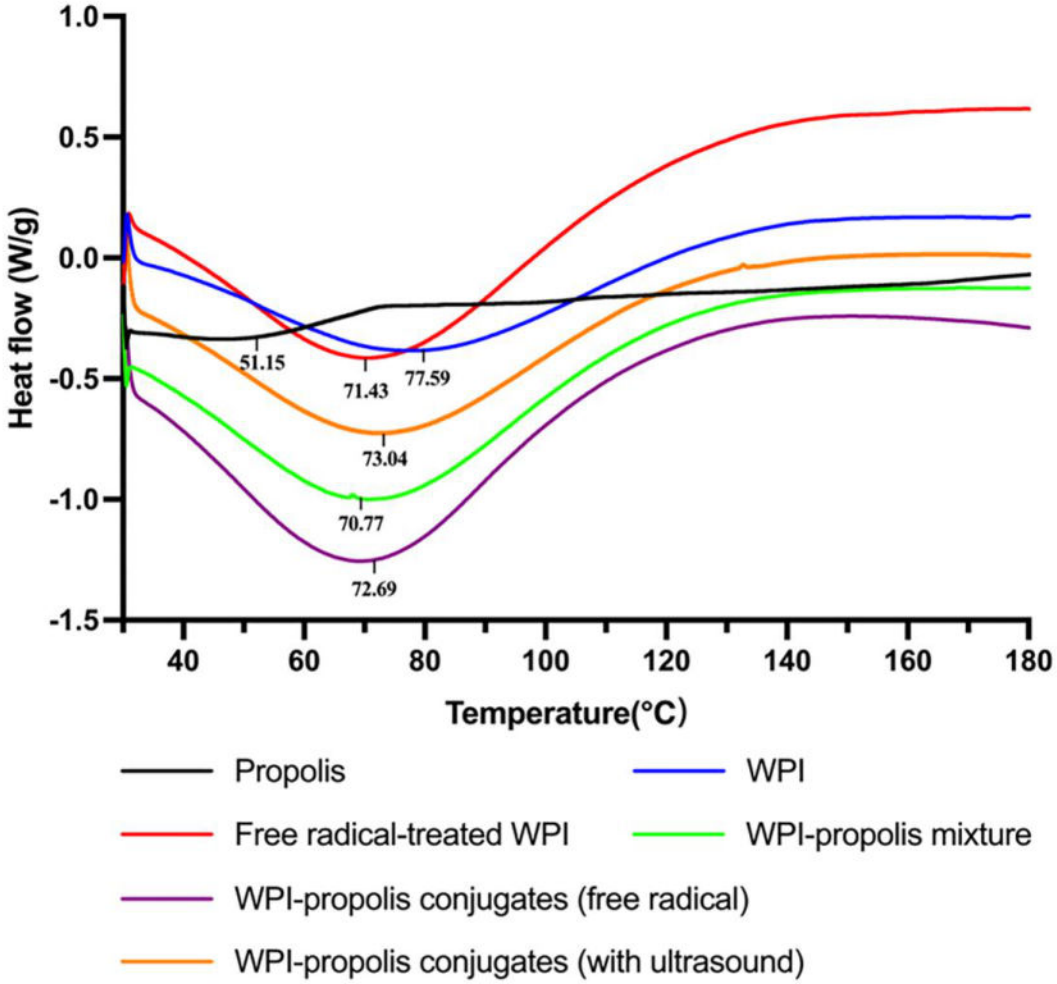
- Compared to the physical mixture, the ABTS and FRAP activities for conjugates prepared by the traditional method demonstrated a significant increase
- Ultrasound treatment at 270 W for 20 min, the ABTS and FRAP activities of the conjugate were further increased



The antioxidant activity

Structural Insights and Functional Enhancements

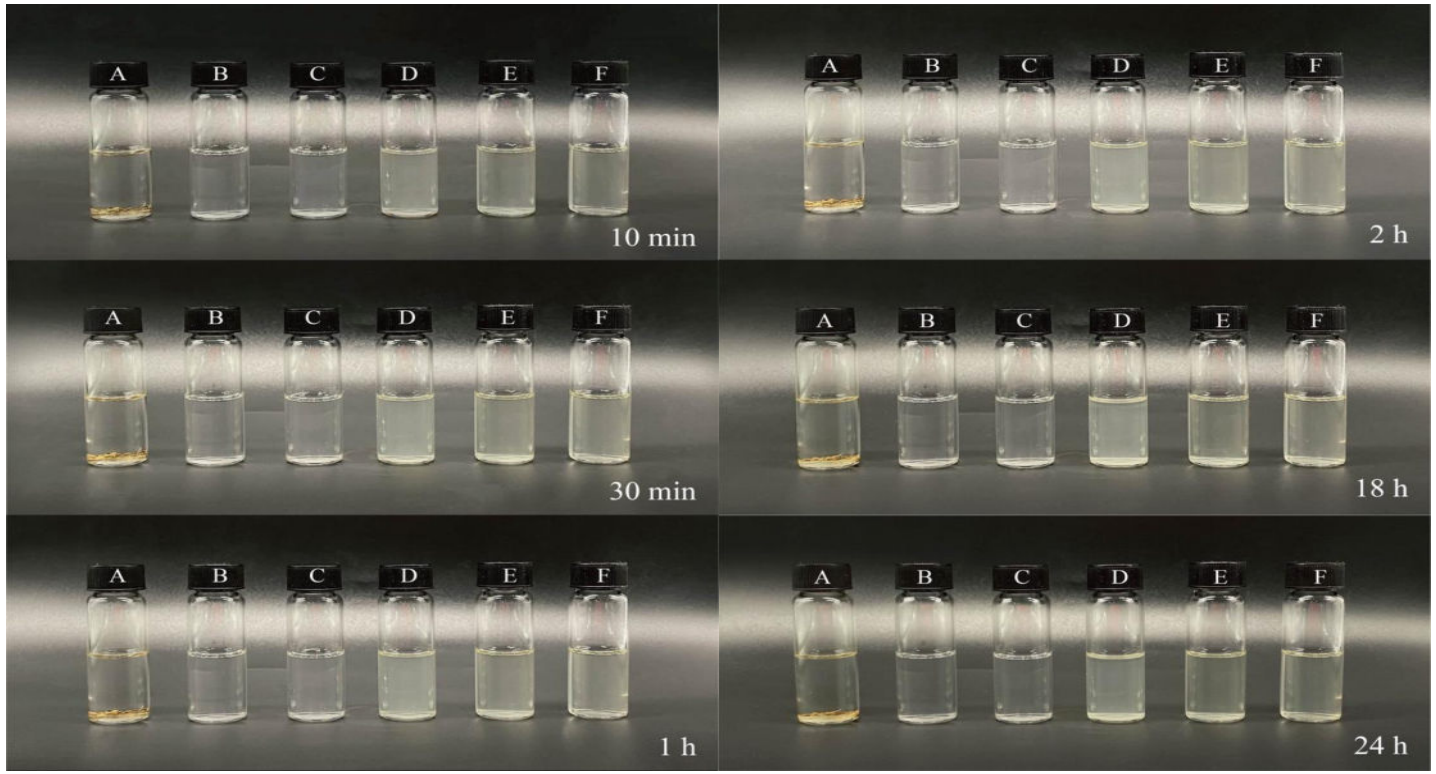
- Compared to the mixture and free radical-treated WPI, WPI-propolis conjugation samples showed higher Tp
- Compared to the conjugate prepared using the traditional method, ultrasound-treated conjugate resulted in an even higher Tp



The thermal stability

Structural Insights and Functional Enhancements

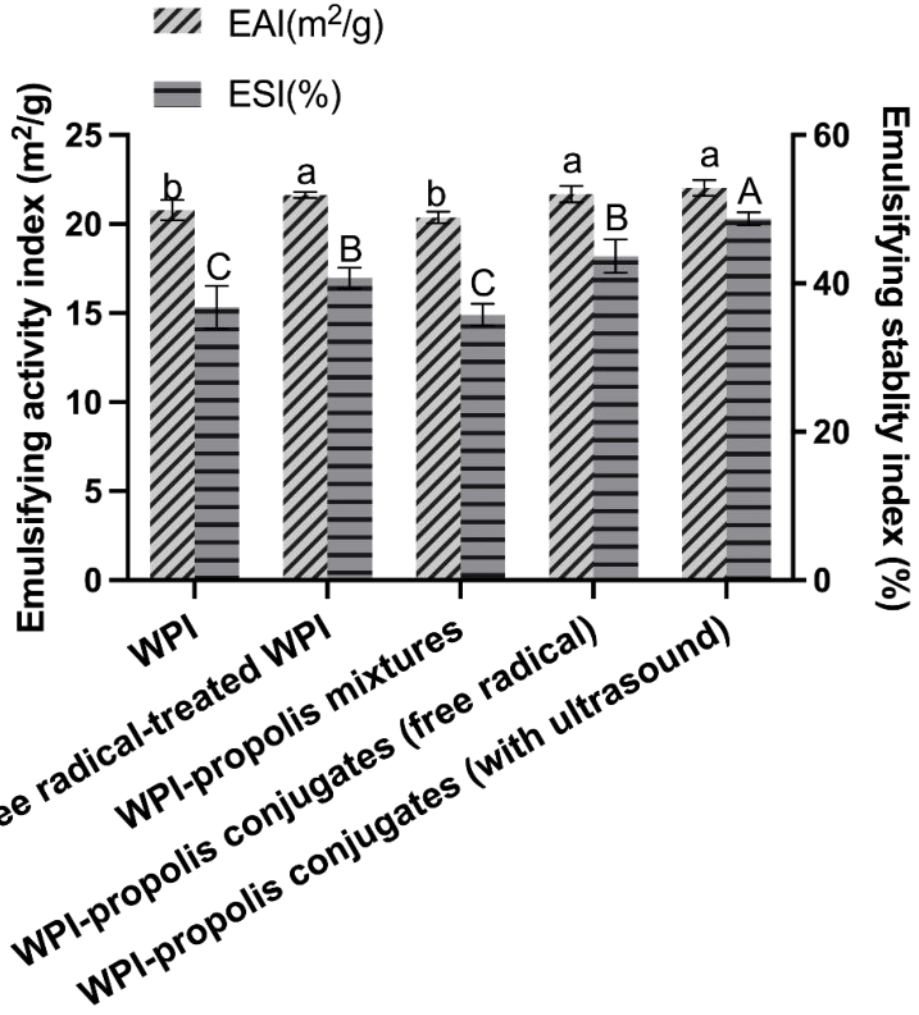
- No significant changes in any of the samples during this storage time
- The WPI-propolis prepared by traditional free radical and ultrasound treatment exhibited excellent water solubility



The visual representation of the samples' behaviours in water

Structural Insights and Functional Enhancements

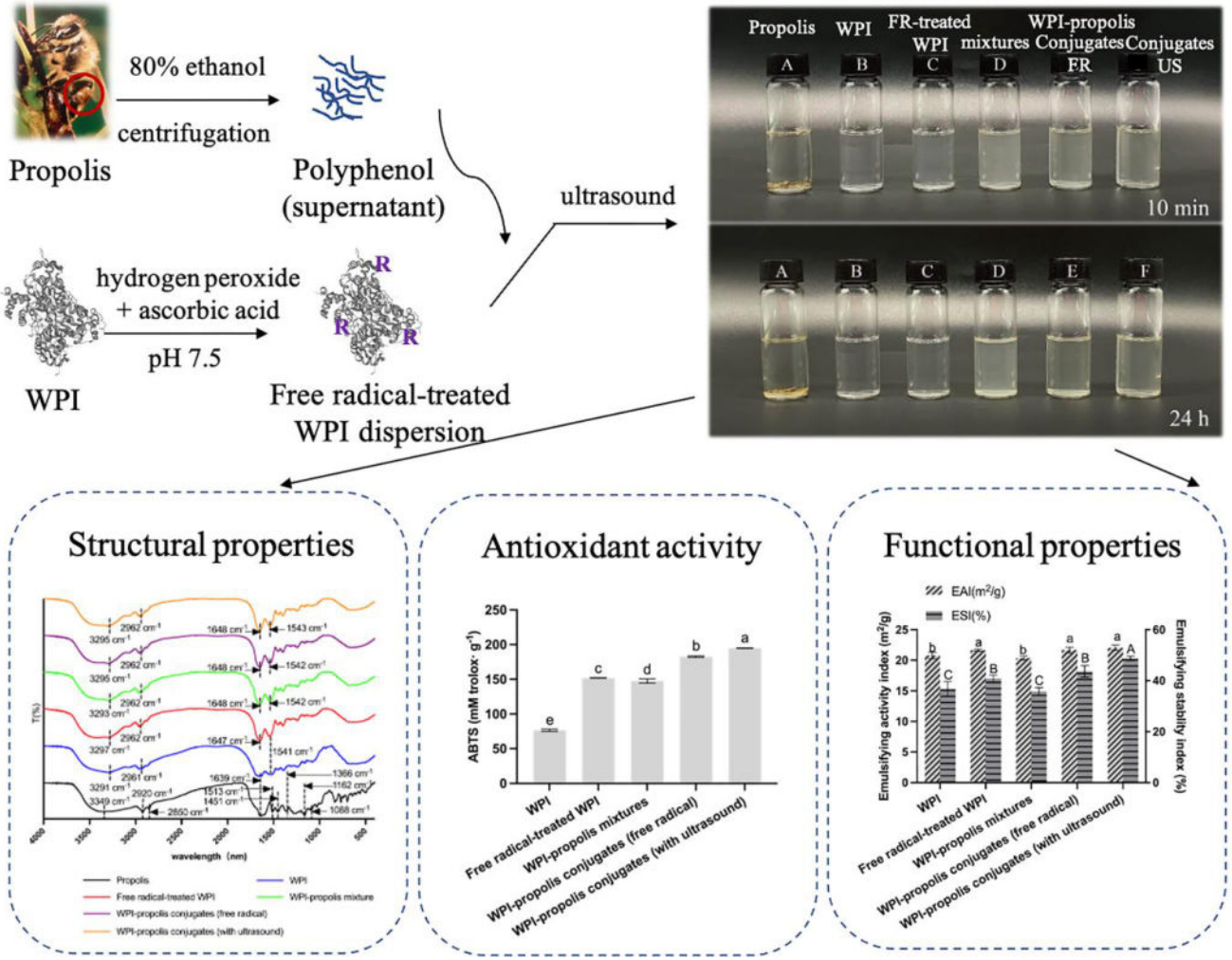
- WPI-propolis conjugation significantly improved emulsifying activity index and emulsifying stability index
- Compared to WPI-propolis conjugates prepared by free radical methods, ultrasound-treated conjugates demonstrated significantly increased emulsifying stability index



The emulsifying properties

Conclusion

- Ultrasound treatment at a power of 270 W for 20 min significantly accelerated the conjugation processes between WPI and propolis
- This might be ascribed to the favourable structural modifications induced by ultrasound treatment
- Both conjugation and ultrasound treatment contributed to higher antioxidant activity, thermostability, water solubility and emulsifying stability



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