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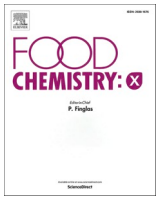

Effect of functional groups in strawberry flavoring on pea protein-flavor interactions: Potential applicable in flavor formulation for plant-based protein aqueous foods

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ABSTRACT

This research aimed to explore binding interactions between pea protein isolate (PPI) and selected strawberry flavorings including vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol within an aqueous system. The results showed that binding affinities of PPI with all various functional group of flavor compounds decreased as temperature increased from 5 °C to 25 °C. Notably, at 25 °C, γ-decalactone displayed the highest binding affinity, followed by vanillin, *(Z)*-3-hexen-1-ol, and furaneol. Lowest binding was observed for furaneol, explained by its greater lipophilicity (lower partition coefficient values or LogP value) and molecular structure in each functional group in the flavor compounds. Thermodynamically, the interaction between PPI and each selected flavor compound was spontaneous, with evidence suggesting primary forces being hydrophobic interactions or hydrogen bonding/van der Waals forces. Computational molecular docking further confirmed these interaction types. This research provides insights into the interactions between PPI and strawberry flavorings, aiding in the selection of optimal flavor compound proportion for protein-rich products.

1. Introduction

One of the main challenges in development of plant-based products is their flavor. Unlike products derived from animal sources, plant-based items often struggle to achieve a desirable flavor. The flavorings added to these products do not always balance well, especially plant-based food sources containing proteins, such as soy, rice, or pea. In the manufacturing process, flavors can be influenced both during production and throughout the shelf-life period. Therefore, it is important to consider how flavors may change over time. When flavorings are incorporated, flavor compounds can interact with the proteins in various ways. These interactions can cause an imbalance in concentration of the flavor compounds in flavorings or even diminish overall flavor intensity (Guo et al., 2024). This challenge highlights the need of flavorings development for plant-based products to meet consumer expectations (Zhang, Kang, Zhang, & Lorenzo, 2021).

The interactions between flavor compounds and proteins are influenced by various parameters such as functional groups of the flavor compounds and the nature of the proteins (Zhang et al., 2021). These interactions can be either reversible (e.g. van der Waals forces and hydrogen bonding) or irreversible (covalent bonding) (Ananthar-amkrishnan, Hoye, & Reineccius, 2020; Suppavorasatit & Cadwallader, 2010). Previous research demonstrated that aldehydes and ketones with the same carbon number (e.g. octanal and 2-octanone) could interact differently with both canola vs pea proteins (Wang & Arntfield, 2015). It was found that both proteins exhibited higher binding capacities for octanal than for 2-octanone because the keto group in the flavors has more steric hindrance, which prevents the flavors from binding and

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LogP values could also play a role in this matrix. Another study focused on soy and dairy proteins interacting with vanillin (Li, Grün, & Fernando, 2000). The binding of vanillin with dairy proteins occurs spontaneously and is enthalpy-driven, likely due to the interactions between the carbonyl and hydroxyl groups of vanillin and the proteins. It also revealed that all proteins could bind with vanillin, and different types of chemical bonding occurred with soy protein as opposed to casein and whey proteins. To visualize these flavor-protein interactions, molecular docking is the alternative method to predict the possible binding sites (Zhang et al., 2021). Previous researchers conducted molecular docking studies to help explain flavor-protein interactions. Various sources of flavorings and proteins were studied, including vanillin, γ- lactones and δ-lactones with bovine serum albumin (Guth & Fritzler, 2004; Siddiqui, Siddiqi, Khan, & Naeem, 2018), or pea protein with ester flavors (Wongprasert et al., 2024). Pea protein, known for its good sources of nutritional value, unique functional properties, and health-enhancing benefits, has emerged as a key protein ingredient, and is increasingly utilized in a variety of meat substituted products (Burger & Zhang, 2019). Pea protein is typically divided into four main groups: globulins make up the majority of the protein content, ranging from 60 to 70%; albumins account for 15–20%; while prolamins and glutelins are found in smaller quantities. Within the globulins, there are two primary types, 11S legumin and 7S vicilin, generally present in a weight ratio of about 2:1 respectively (Tanger, Engel, & Kulozik, 2020). However, pea protein can bind specifically with various flavor groups such as esters, ketones, aldehydes, and alcohols (Bi et al., 2022). Many previous studies have also highlighted interactions between flavors and proteins by using homologous compounds. These compounds differed in chain lengths or have the same carbon chain length, but different functional groups. However, when creating or making flavorings for use in actual foods, homologous compounds are generally not considered. Instead, flavor compounds with non-homologous structures are combined to achieve the desired flavor characteristics (Wright, 2002).

In studies of non-homologous compound systems, the strawberry flavor model has been extensively selected for investigating interactions due to its relatively simple composition of only a small number of odor-active compounds (Martuscelli, Savary, Pittia, & Cayot, 2008). Normally, strawberry flavors are comprised of various functional groups, including esters, aldehydes, ketones, and alcohols. All of these functional groups can represent the characteristic of strawberry flavors. Ester flavors such as ethyl butanoate, ethyl isopentanoate, ethyl hexanoate, or methyl anthranilate primarily impart the sweet and fruity attributes to strawberry flavoring. While ester flavors which mentioned was already studied by the previous reported (Wongprasert et al., 2024), several other important compounds in strawberry flavors model also play an important role including furaneol (burnt-sugar or caramel notes), vanillin (sweet note), γ-decalactone (creamy note), and *(Z)*-3-hexen-1-ol (green or leafy note) (van Ruth, de Witte, & Uriarte, 2004). Previous investigations predominantly aimed to determine the percentage releases of each strawberry flavor compounds in relation to the proteins (Martuscelli et al., 2008; Xu et al., 2017). Very few studies have considered other critical binding parameters, such as number of binding sites, binding affinity, thermodynamic variables, and the characteristics of binding or release of flavor compounds (Zhang et al., 2021).

The purpose of this research was to investigate the interactions of selected strawberry flavor compounds including furaneol, vanillin, γ-decalactone, and *(Z)*-3-hexen-1-ol, with pea protein isolate (PPI) in an aqueous model system. By employing thermodynamic and computational molecular docking methods, this research clarified the interaction mechanisms between these flavor compounds and the PPI. Additionally, it is speculated that the binding affinity between selected strawberry flavors and pea protein isolate (PPI) significantly differs depending on the functional groups and molecular properties of each flavor compound. Moreover, it is hypothesized that the binding capacities of all flavor compounds can be predicted by using partition coefficient values ($LogP_{ow}$). This will in turn will assist flavorists in anticipating the problem of flavor binding and aid in determining optimal rations and concentrations of specific compounds during the development of flavorings for PPI-containing foods, particularly in high-protein products.

2. Materials and methods

2.1. Materials and reagents

Analytical grade (≥ 98% purity) of furaneol, γ-decalactone, *(Z)*-3-hexen-1-ol, and vanillin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals were analytical reagent grades and supplied from Fluka Analyticals (Germany), and Ajax Finechem (NSW, Australia). n-hexanol-d_{11} (99.1% purity) was obtained from CDN Isotopes (Canada). Vanillin-d_3 (98.51% purity) was synthesized according to the methods of Schneider and Rolando (1992). γ-decalactone-d_4 (98.21% purity) was synthesized according to the methods of Cooke, Capone, van Leeuwen, Elsey, and Sefton (2009) with some modifications (Supplementary materials).

2.2. Pea protein isolate (PPI) preparation

Upon its arrival, pea protein powder (Nutralys S85F, Roquette Co. Ltd., Paris, France) was directly vacuum-sealed. The powder, on a dry basis, contained 83.1% protein, 8.7% fat, 3.7% carbohydrates, and 4.6% ash. To produce pea protein isolate (PPI), the powder was put through a lipid removal process that included a hexane washed and followed by Soxhlet extraction (AOAC, 2000). The resulting PPI, which had a protein content of about 93.4 g/100 g, was then kept in vacuum-sealed packaging and stored at −18 °C for use in subsequent experiments.

2.3. Synthesis of maltol-d_3

Maltol (253 mg) was added to a solution of 2% trifluoroacetic acid in D_2O and reacted in a hydrothermal autoclave reactor at 230 °C for 1 h. After cooling to room temperature, the crude reaction mixture was extracted with diethyl ether (Et_2O) and brine and dried over Na_2SO_4. The crude product was then purified using silica gel column chromatography with a MeOH:CH_2Cl_2 eluent. This process yielded CD_3-maltol as a white solid, with a 32% yield and 99.8% purity.

2.4. Flavor compounds solution preparation

Vanillin (11.90 mg/mL), γ-decalactone (10.43 mg/mL), furaneol (10.20 mg/mL), and *(Z)*-3-hexen-1-ol (10.58 mg/mL) were prepared in phosphate buffer (pH 7.0) by using odorless distilled water. Vanillin-d_3 (9.60 mg/mL), d_4-γ-decalactone (1.43 mg/mL), maltol-d_3 (1.01 mg/mL), and n-hexanol-d_{11} (1.09 mg/mL) stock solutions were prepared by using methanol as a solvent. All solutions were stored in amber glass vials and maintained at a temperature of −40 °C.

2.5. Free (unbound) flavor isolation and quantification

The isolation and quantification of free flavor compounds including vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol, was conducted by the method of Temthawee, Panya, Cadwallader, and Suppavorasatit (2020) and Wongprasert et al. (2024). Protein solutions (3 g per 100 mL of PPI) were prepared using a 0.05 mol/L phosphate buffer at pH 7.0. Both proteins and each flavor compound were mixed into 3 mL reaction mixtures, which was then transferred into an Amicon® Ultra-4 centrifugal filter tube with a 3 K molecular weight cut-off (Ultra-4, MilliporeSigma, MA, USA). The mixture was centrifuged for 40 min at 4500 ×*g* using a refrigerated centrifuge (Hettich Universal 320R, Tuttlingen, Germany). All procedures were carried out at a constant temperature of 5, 15, or 25 °C. Subsequently, the filtered liquid was collected, to which a 10 μL internal standard solution was added and mixed entirely. From this mixture, the sample (0.5 mL) was added to a 2 mL amber glass-vial,

and 0.3 mL of methylene chloride was added to perform the extraction. The extracted solution was subsequently analyzed using gas chromatography–mass spectrometry (GC–MS).

Flavor quantification was performed using a 7890B GC/7000B MS Single Quadrupole System (Agilent Technologies, Inc., Palo Alto, CA, USA). Samples, 2 μL, were introduced into the system through hot splitless mode at a temperature of 250 °C. Separation of compounds occurred on a DB-Wax column (30 m × 250 μm internal diameter × 0.25 μm film thickness, Agilent J&W, CA, USA). For vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol, the temperature started at 40 °C and was ramped up at a rate of 10 °C /min until 220 °C, with a final hold time of 5 min. Helium was utilized as the carrier gas at a steady flow of 2.0 mL/min. The settings for the mass spectrometer were as follows: transfer line temperature at 250 °C; ionization voltage at 70 eV; scanning mass range from 35 to 400 amu; and a scan rate of 4.2 Hz.

The quantitation of target flavor compounds was performed using the method of Mathatheeranan et al. (2024) and employing a MS response factor (f_i) for each compound relative to an isotope-labelled internal standard. Isotope-labelled internal standards are chemically analogous to the analytes, allowing for highly accurate quantification, even at trace levels. This is because the labelled and unlabeled compounds have essentially the same physical and chemical properties, which can reduce the quantification error more effectively than using a normal (unlabeled) internal standard. The respective f_i values for vanillin, γ-decalactone, furaneol (vs d_3-maltol), and *(Z)*-3-hexen-1-ol were 0.84, 0.60, 1.46, and 1.22 based on ion mass chromatography peak areas for ion pairs 151/154, 85/87, 128/129, 67/64, respectively. The mass of each flavor compound was calculated using Eq. (1).

$$\text{mass of flavor compounds} = \text{mass of i.s.} \times f_i \times \frac{\text{peak area of flavor compounds}}{\text{peak area of i.s.}} \quad (1)$$

2.6. Determination of equilibration time

Equilibrium time for the interactions between each flavor, including vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol, and PPI, were determined using the equilibrium dialysis approach as outlined by previous literatures with some modifications (Suppavorasatit & Cadwallader, 2012; Temthawee et al., 2020; Wongprasert et al., 2024). Before analysis, all pieces of glassware were treated to increase its surface hydrophobicity through silanization. This enhancement was achieved by treating with a 10% solution of dimethyl dichlorosilane in toluene. (MilliporeSigma, MA, USA) (Tsutsumi, Nishikawa, Katagi, & Tsuchihashi, 2003). Protein mixtures were prepared at a concentration of 3 g/100 mL of PPI in a 0.05 mol/L phosphate buffer (pH 7.0) and were left to hydrate completely by storing at 4 °C overnight. Subsequently, 10 mL of these solutions were transferred into 20 mL vials that had been silanized. Each of the specified flavor compounds were then added to achieve a final concentration of 50 μg/mL and closed the bottle with PTFE-lined cap. Each sample was continuously stirred using a magnetic stirrer equipped with a water bath in a low-form jacketed beaker (VR Glasstrade, Thailand) set to specific temperatures (5, 15, or 25 °C). Afterward, sample of the combined flavor compound and PPI was taken to determine the concentration of the unbound flavor compound. Due to the lower stability of furaneol in neutral pH, the total experimental time for furaneol did not exceed 50 h at every temperature and furaneol solutions were freshly prepared prior to quantitative determinations (Reiners, Nicklaus, & Guichard, 2000; Roscher, Schwab, & Schreier, 1997). Graphs showing the incubation time versus the concentrations of each free (unbound) flavor compound were utilized to establish the necessary period for each sample to reach equilibrium (equilibration time) at various temperatures.

2.7. Determination of binding properties

The determination of binding characteristics was conducted by referencing prior literatures and incorporating some modifications (Li et al., 2000; Temthawee et al., 2020; Wongprasert et al., 2024). The mixture was consisted of 3 g/100 mL PPI in a 0.05 mol/L phosphate buffer (pH of 7.0), and then left overnight at 4 °C. Protein suspension (5 mL) was transferred into a 20 mL silanized glass vial equipped with PTFE lined cap. Different flavor compounds were added to individual vials to reach concentrations of 10, 20, 40, 60, 80, to 100 μg/mL. To ensure the PPI concentration remains consistent across all samples after adding each flavor compound to achieve final concentrations, the volume of added flavor compounds (from stock solution at different concentration) were controlled at the same amount. The set of samples included a control sample (50 μg/mL of each flavor in a 0.05 mol/L phosphate buffer at pH 7.0) and other samples containing a full concentration range of each flavor. These were continuously stirred at constant temperatures of 5, 15, or 25 °C until the equilibrium was reached or surpassed. Following this, the sample was taken to measure the concentration of free flavor compounds.

To ascertain binding parameters, specifically total binding sites (n) and binding constant or affinity (K), double reciprocal graphs (Klotz plots) of $1/\nu$ against 1/[L] were employed as per the designated formula (Eq. 2), where ν denotes the number of moles of ligand (flavor compounds) bound per mole total protein, and [L] symbolizes the concentration of free ligand (or free flavors). The slope of graph represents 1/Kn, while the intersection point on the y-axis correlates to 1/n.

$$\frac{1}{\nu} = \frac{1}{n} + \frac{1}{Kn[L]} \quad (2)$$

To determine binding parameters, Gibb's free energy of binding (ΔG°), enthalpy of binding (ΔH°), and entropy of binding (ΔS°), were determined. The ΔG° was calculated by the value of K, derived from the Klotz equation referred to as Eq. (3). In this context, R represents for gas constant (8.314 J K^{-1} mol^{-1}), while T denotes the absolute temperature, measured in degrees Kelvin.

$$\Delta G^{\circ} = -RTlnK \quad (3)$$

The binding enthalpy (ΔH°) was calculated using the Van't Hoff equation, denoted as Eq. (4) and (5). In this context, K_1 and K_2 are the binding constants at temperatures of 5 °C and 25 °C, respectively. T_1 and T_2 are the absolute temperatures expressed in degree Kelvin, while R is defined as the gas constant.

$$ln\left(\frac{K_2}{K_1}\right) = \frac{\Delta H^{\circ}/R}{\left(\frac{1}{T_2} - \frac{1}{T_1}\right)} \quad (4)$$

$$\Delta H^{\circ} = \frac{-R \bullet dlnK}{d\left(\frac{1}{T}\right)} \quad (5)$$

The ΔS° was stated using Eq. (5).

$$\Delta S = \frac{\Delta H^{\circ} - \Delta G^{\circ}}{T} \quad (6)$$

2.8. Molecular docking

Molecular docking was performed by following the methods from Wongprasert et al. (2024). The crystallographic structure in three dimensions of the main pea protein (*Pisum sativum* L., pea prolegumin 11 s seed globulin) was acquired from the RCSB Protein Data Bank (PDB ID: 3KSC). The absent residues (7–89, 105–175, 187–238, 316–489) in the 3KSC model were reconstructed through homology modeling on the SWISS-MODEL server (Waterhouse et al., 2018). The reconstructed structure was then confirmed by generating a Ramachandran diagram using a PROCHECK web interface (Laskowski, MacArthur, Moss, & Thornton, 1993). Additionally, the protonation states of all ionizable amino acids were predicted using the PROPKA 3.0 based on their pKa values (Olsson, Søndergaard, Rostkowski, & Jensen, 2011). Apart from protein preparation, the compounds as ligands, including vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol, were sourced from PubChem (Pubchem CID 1183, 12,813, 19,309, and 10,993, respectively (https://pubchem.ncbi.nlm.nih.gov). Their structure and geometry were then optimized by the steepest descent methodology with the universal force field implemented in the Avogadro 1.2.0 software (https://avogadro.openmolecules.net).

Because the ligand binding site was not previously identified, we initially pinpointed the most possible binding pocket by the blind docking approach with SwissDock® software (Grosdidier, Zoete, & Michielin, 2011). Default settings were used, and cluster analysis was conducted to determine the predominant binding location. To estimating the binding affinity, molecular docking was performed using the fitness score implemented in the Gold 5.3.0 docking program (Jones, Willett, Glen, Leach, & Taylor, 1997). The spherical grid was set as 6 Å at the X, Y, and Z coordinates of 12.95, 256.67, and 55.85, respectively, and 100 genetic algorithm (GA) trials were executed to predict ligand conformations. Subsequently, the conformation that achieved highest fitness score was selected to represent the molecular recognition in terms of non-covalent intermolecular interactions, visualized by the Discovery Studio 2.0 software (Dassault Systemes BIOVIA Ltd., France).

2.9. Statistical analysis

The differences between treatments were assessed using Analysis of Variance (ANOVA) and further examined with Duncan's New Multiple Range Test (DNMRT) at a significance level of $p < 0.05$ by using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Equilibration time determination for binding of selected flavor compounds to PPI

For flavor-binding studies using the modified equilibrium dialysis method, determining the appropriate incubation duration to achieve equilibrium under varying conditions is necessary. To determine the equilibration duration, the concentration of free flavor compounds consisting of vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol were plotted against incubation time at temperatures of 5, 15, and 25 °C. Examples of typical equilibration curves for the binding interactions of PPI to vanillin and γ-decalactone at 5 and 25 °C are shown in Figs. S2. In the context of this study, the equilibration time indicated the shortest duration needed for the free flavor compounds to stabilize at their lowest concentration. Table 1 presents the equilibration periods for binding of all flavor compounds PPI across 3 different temperatures tested. Notably, as the temperature increased from 5 to 25 °C, the time required for each flavor to equilibrate was reduced. This could be explained that higher temperatures expedited reaction rates. In addition, the binding of PPI to flavor compounds was accelerated at increased temperatures, resulting in a faster establishment of equilibrium. This observation aligns with previous literature reporting a reduced equilibrium time for soy protein combined with vanillin and maltol as the temperature was increased (Suppavorasatit & Cadwallader, 2012). Similarly, a contraction in the equilibrium duration with rising temperature was also observed in the coconut protein-vanillin matrix (Temthawee et al., 2020).

Table 1
Equilibration time for the binding of selected flavor compounds to PPI at different temperatures.

T (°C)	Minimum to reach equilibrium (h)			
	vanillin	γ-decalactone	furaneol	*(Z)*-3-hexen-1-ol
5	48	48	48	48
15	36	36	24	12
25	24	24	12	9

3.2. Binding affinity of selected flavor compounds to PPI

Fig. 1 illustrates double-reciprocal Klotz plots that depict the binding patterns for vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol to PPI at temperatures of 5, 15, and 25 °C. The linearity of these plots suggests that these compounds have a proportional, noncooperative binding relationship with PPI across the examined temperature interval. This finding is consistent with prior studies, for instance, Li et al. (2000) also found that vanillin had noncooperative interactions with soy and dairy proteins at 4 and 12 °C. In a related study, Druaux, Lubbers, Charpentier, and Voilley (1995) documented similar linearity plots between bovine serum albumin (BSA) and γ-decalactone in a wine model system. The equations of linear regression derived from the Klotz plots are presented in Table 2. These equations provided insights into the binding interactions between the specified compounds and pea proteins. Importantly, the coefficients of determination (r^2) for these equations exceed 0.97, signifying that over 97% of the total data variability is accounted for.

The binding and thermodynamic parameters for the interaction of vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol with PPI are presented in Table 3. As previously mentioned, the Klotz plots enable the determination of the number of binding site (n) and the binding constant (K). These values are derived from the y-intercepts (1/n) and the slopes of the plots (1/Kn), respectively. Table 3 revealed that the n values of vanillin, γ-decalactone, and furaneol decreased as the temperature increased from 5 to 25 °C, while the n values of *(Z)*-3-hexen-1-ol increased. The decrease in n values could be explained by the alterations in protein structure, particularly the unfolding pattern, due to increased temperatures. Furthermore, interactions between proteins might have occurred, potentially leading to a reduction in the protein binding sites (Guo, He, Wu, Zeng, & Chen, 2019). Only a few studies have reported on the n values related to PPI binding with flavor compounds. Interactions of pea protein with flavor compounds, including *(Z)*-2-penten-1-ol, hexanal, and *(E)*-2-octenal were examined and the n values at 37 °C were 2.55, 4.58, and 26.95, respectively (Bi et al., 2022). Considering the K values in this study, there was a significantly increase in the K values between vanillin and γ-decalactone towards PPI, increasing from $147.48 \times 10^5\ M^{-1}$ at 5 °C to $230.35 \times 10^5\ M^{-1}$ at 25 °C for vanillin and $113.22 \times 10^5\ M^{-1}$ at 5 °C to $210.17 \times 10^5\ M^{-1}$ at 25 °C for γ-decalactone. This trend was inconsistent with previous studies in which the K values for the binding of vanillin to soy protein were found to increase from $5.16 \times 10^4\ M^{-1}$ at 5 °C to $186 \times 10^4\ M^{-1}$ at 25 °C (Suppavorasatit & Cadwallader, 2012). Moreover, Zhang, Ma, Wang, Zhang, and Zhou (2012), examined the interaction between BSA and maltol, revealing that the K values for the binding of maltol to BSA increased from $0.39 \times 10^5\ M^{-1}$ at 25 °C to $8.69 \times 10^5\ M^{-1}$ at 37 °C. Conversely, the K values for furaneol and *(Z)*-3-hexen-1-ol declined respectively from 24.56×10^5

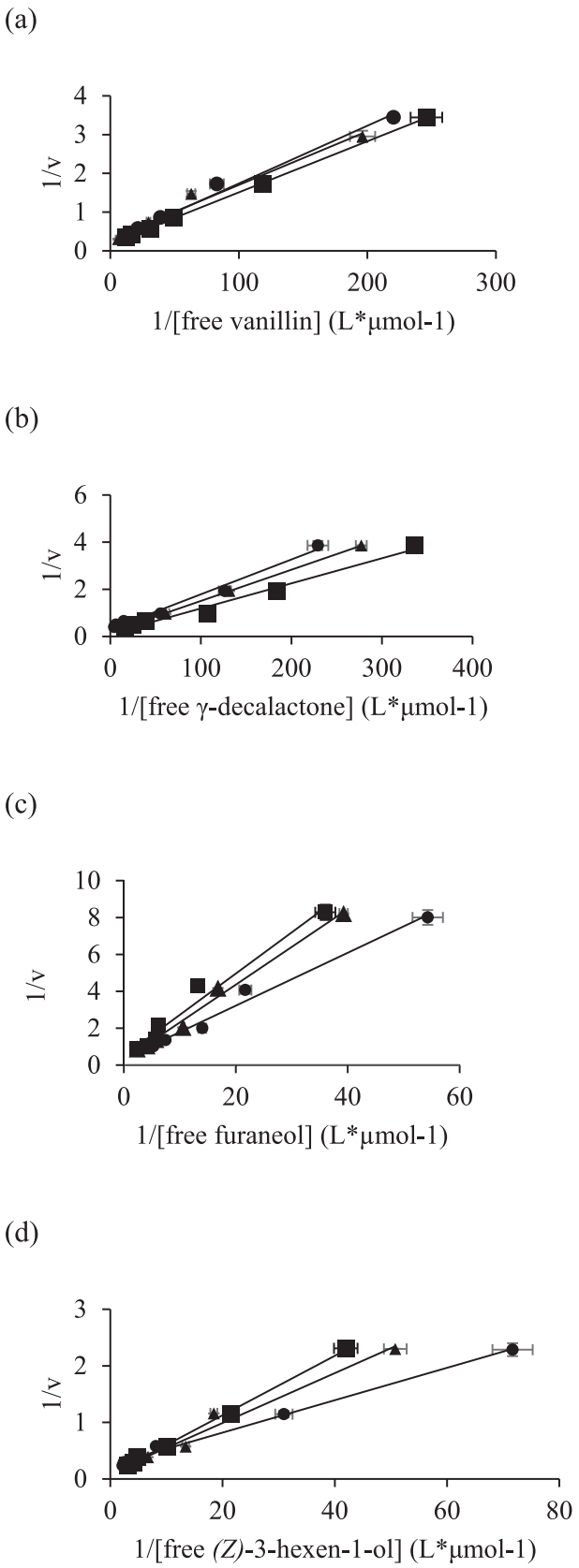


Fig. 1. Klotz plots for binding of vanillin (a), γ-decalactone (b), furaneol (c), and *(Z)*-3-hexen-1-ol (d) with pea protein isolate (PPI) at 5 °C (■), 15 °C (▲), and 25 °C (●). Plots represent an average of two complete replications.

Table 2
Linear equations from Klotz plots for the binding of selected flavor compounds to PPI obtained from two replications.

Flavor compounds	T (°C)	Replication 1	r^2	Replication 2	r^2
vanillin	5	y = 0.0129× + 0.2105	0.9979	y = 0.0134× + 0.1766	0.9952
	15	y = 0.0149× + 0.2449	0.9819	y = 0.0149× + 0.2495	0.9912
	25	y = 0.0161× + 0.3804	0.9788	y = 0.0163× + 0.3658	0.9792
γ-decalactone	5	y = 0.0106× + 0.1227	0.9952	y = 0.0102× + 0.1129	0.9814
	15	y = 0.0131× + 0.1819	0.9927	y = 0.0134× + 0.1812	0.9777
	25	y = 0.0154× + 0.3192	0.9885	y = 0.0150× + 0.3003	0.9798
furaneol	5	y = 0.0764× + 0.3547	0.9849	y = 0.0756× + 0.3005	0.9871
	15	y = 0.1112× + 0.1745	0.9898	y = 0.0849× + 0.5324	0.9888
	25	y = 0.2188× + 0.0924	0.9729	y = 0.2284× + 0.0865	0.9685
(Z)-3-hexen-1-ol	5	y = 0.0300× + 0.5416	0.9950	y = 0.0274× + 0.2510	0.9943
	15	y = 0.0455× + 0.0970	0.9824	y = 0.0427× + 0.1199	0.9960
	25	y = 0.0530× + 0.0617	0.9973	y = 0.0521× + 0.0894	0.9966

M^{-1} and 86.07 × 10^5 M^{-1} at 5 °C to 21.82 × 10^5 M^{-1} and 14.40 × 10^5 M^{-1} at 25 °C. This observation aligns with the findings of other studies, such as the interaction of soy protein with 2-nonanone (Damodaran & Kinsella, 1981).

The K values from our study differed from the previous findings which were around 438.68, 684.46, and 8207.72 M-1 for *(Z)*-2-penten-1-ol, hexanal, and *(E)*-2-octenal with pea protein, respectively (Bi et al., 2022). These could be attributed to the different protein sources and preparation techniques, coupled with the different methodologies used to derive these values. Additionally, for all flavor compounds, different n and K values were noted, especially at temperatures of 5 and 25 °C (Table 3). As the temperature increased, the arrangement of the protein subunits might be changed, leading to the reduction of interaction strengths among protein molecules (Damodaran & Kinsella, 1981). As a result, both n and K values of binding could be changed significantly. This phenomenon could also be influenced by changes in the protein structure or the emergence of more hydrophobic areas on the exterior of the protein, potentially impacting the specific binding sites (Guo et al., 2019; Temthawee et al., 2020).

Suppavorasatit and Cadwallader (2012) earlier indicated that solely relying on either n or K might not accurately depict the binding affinity of a flavor compound to a protein. Instead, the combined value of nK provides a more accurate representation. When observing the nK values at temperatures of 5, 15, and 25 °C, it became obvious that the binding affinity between the chosen flavor compounds and PPI weakened while the temperature increased (as shown in Table 3). At 25 °C, γ-decalactone showed the strongest binding affinity, with an nK value of 678.16 × 10^5 M^{-1}, followed by vanillin (617.31 × 10^5 M^{-1}), *(Z)*-3-hexen-1-ol (190.31 × 10^5 M^{-1}) and furaneol (44.74 × 10^5 M^{-1}), respectively. This decreasing trend of nK values with rising temperature aligns with findings from previous studies (Suppavorasatit & Cadwallader, 2012; Temthawee et al., 2020).

In general, the interaction between proteins and flavor compounds can be influenced by the chain length, structure, and functional groups of the compounds. Moreover, another important parameter is partition

Table 3
Binding and thermodynamic parameters for the binding of selected flavors to PPI.

parameter	T (°C)	vanillin	γ-decalactone	furaneol	*(Z)*-3-hexen-1-ol
n	5	5.21 ±0.64 aB	8.50 ±0.50 aA	2.86 ±0.34 aD	4.06 ±0.11 aC
	15	4.05 ±0.05 aC	5.51 ±0.02 bB	4.35 ±1.11 bC	9.32 ±1.39 abA
	25	2.68 ±0.07 bB	3.23 ±0.14 cB	2.05 ±0.07 aB	13.70 ±3.55 bA
K (x10^5) (M^{-1})	5	147.48 ±22.19 bA	113.22 ±3.58 bAB	24.56 ±2.70 bC	86.07 ±7.83 aB
	15	165.91 ±2.18 bA	137.04 ±2.57 bB	11.53 ±3.37 aD	24.70 ±4.78 bC
	25	230.35 ±8.38 aA	210.17 ±14.10 aB	21.82 ±1.40 bCD	14.40 ±3.90 bD
nK (x10^5) (M^{-1})	5	760.73 ±20.45 aB	961.89 ±26.16 aA	69.74 ±0.55 aD	349.15 ±22.37 aC
	15	671.14 ±0.01 bB	754.81 ±12.08 bA	48.34 ±1.85 bD	226.99 ±10.19 bC
	25	617.31 ± 5.39 cB	678.16 ± 16.26 cA	44.74 ± 1.36 cD	190.31 ±2.30 bC
ΔG° (kcal/mol)	5	−9.11 ±0.08 aD	−8.97 ±0.02 aC	−8.12 ±0.06 aA	−8.81 ±0.05 bB
	15	−9.51 ± 0.01 bC	−9.40 ± 0.01 bC	−7.97 ± 0.17 aA	−8.41 ± 0.11 aB
	25	−10.03 ± 0.02 cC	−9.98 ± 0.04 cC	−8.64 ± 0.04 bB	−8.38 ± 0.16 aA
ΔH° (kcal/mol)	5–25	3.71 ±0.94 B	5.08 ±0.29 A	−0.96 ±0.38C	−14.84 ±1.51 D
ΔS° (cal K^{-1} mol^{-1})	5	13.38 ±3.39 nsA	18.31 ±1.05 nsA	−3.41 ±1.36 nsB	−53.36 ±5.42 nsC
	15	12.92 ±3.27 nsA	17.68 ±1.01 nsA	−3.29 ±1.31 nsB	−51.51 ±5.23 nsC
	25	12.49 ±3.16 nsA	17.08 ±0.98 nsA	−3.18 ±1.26 nsB	−49.78 ±5.06 nsC

a,b,… Within columns, values with the same lower case letters are not significantly different at $p > 0.05$. ns = not significant
A,B,… Within rows, values with the same upper case letters are not significantly different at $p > 0.05$.
c Average ± standard deviation ($n = 2$).

coefficient between octanol and water, known as $LogP_{ow}$. Variations in LogP values significantly influence the strength of the bond between proteins and flavor compounds. For instance, the LogP values of vanillin (1.21), γ-decalactone (2.72), furaneol (0.95), and *(Z)*-3-hexen-1-ol (1.61) provide valuable insights (Sayers et al., 2023). These values not only denote the hydrophobic nature of these compounds but also suggest potential interactions within the system. As expected, compounds with higher LogP values tended to form stronger interactions. The strength of this engagement contributed favorably to the enthalpic (ΔH°) aspect of the interaction (Guth & Fritzler, 2004). In this context, γ-decalactone exhibited the highest nK values, reflecting its hydrophobic nature of the compounds which supported by higher LogP values. In contrast, furaneol has a higher solubility in water (18.5 g/L) compared to the other flavor compounds (ex. solubility of γ-decalactone; 0.3 g/L), which intensifies its interaction with water over proteins (Guo et al., 2019; Sayers et al., 2023). Consequently, furaneol displays lower nK values relative to the other flavor compounds. The findings from our study aligned with previous literature on the binding of β-lactoglobulin (BLG) with the furanones, including furaneol. Reiners et al. (2000) demonstrated that furaneol exhibited minimal interactions with β-lactoglobulin, as indicated by the lowest binding constants among the analyzed substances, which could be related to its lowest LogP values. Furthermore, Anantharamkrishnan et al. (2020) stated that furaneol did not form covalent bonds with BLG.

While LogP values are substantial in understanding these phenomena, vanillin did not strictly conform to these observed trends. Vanillin is frequently used as a reference compound in studies investigating flavor-protein interactions. A large number of studies have elucidated the binding characteristics of vanillin with various sources of proteins, for example BLG, soy protein, faba bean protein, coconut protein, or canola protein. The aldehyde group of vanillin can bind with proteins through covalent bonds via Schiff base formation (Suppavorasatit & Cadwallader, 2012; Anantharamkrishnan et al., 2020; Temthawee et al., 2020; Zhang et al., 2021; Guichard, 2002). Moreover, noncovalent interactions might occur as well in the system, which can be determined by thermodynamics parameters. This complexity in interactions might be the reason why vanillin was not included in these trends.

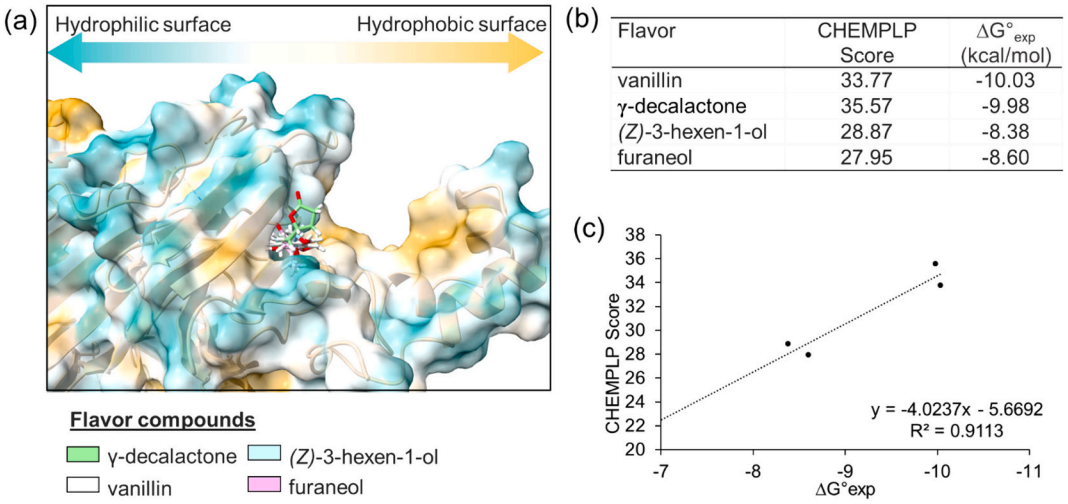
3.3. Thermodynamics of binding between selected flavor compounds to PPI

The binding interactions between flavor compounds and proteins, specifically the thermodynamic parameters, play an important role in understanding their stability and spontaneity, which are crucial for the development of protein-based products. This study focused on the thermodynamic aspects of the binding of selected flavors with PPIs, with data presented in Table 3. These parameters include the Gibb's free energy (ΔG°), enthalpy (ΔH°), and entropy (ΔS°) of binding. The observed negative values for ΔG° indicated that the binding process is not only favorable, but also spontaneous, meaning these interactions can occur without additional energy. This is in agreement with results of previous research on the interaction between BSA and methyl anthranilate, which also found that the interaction was a spontaneous reaction, thus supporting the validity and reliability of our findings (Dinu et al., 2022). Moreover, the negative ΔH° values indicate that the binding of PPI with furaneol and *(Z)*-3-hexenol were exothermic and thus favorable. The negative ΔS° values for these compounds, were not significant enough to counteract the ΔG° values, suggesting that these interactions were primarily driven by enthalpy. Based on ΔH° and ΔS° values, the types of binding interactions could be inferred. For instance, when both values are positive, a hydrophobic interaction is suggested. In contrast, the presence of van der Waals forces or hydrogen bonding when both values are negative. In addition, an electrostatic interaction can dominate when ΔH° is approximately zero and ΔS° is positive (Bi et al., 2022; Temthawee et al., 2020; Zhang et al., 2021). From Table 3, given that the ΔH° and ΔS° values for furaneol and *(Z)*-3-hexenol were all negative it is probable that their interactions with PPI involved van der Waals forces or hydrogen bonding, which agrees with the study by Siddiqui et al. (2018). Conversely, both ΔH° and ΔS° for vanillin and γ-decalactone binding to PPI were positive. These indicates the likelihood that a hydrophobic interaction is the primary binding force, which is supported by results of previous studies on the interaction of BLG and BSA with γ-decalactone and interaction of coconut protein with vanillin (Guth & Fritzler, 2004; Temthawee et al., 2020).

3.4 Molecular docking of selected flavor compounds to PPI.

3.4. Prediction of ligand binding sites

A computational study was conducted to explore the potential binding sites between pea protein and selected flavor compounds consisting of vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol using the Swissdock® tool. The objective was to identify possible ligand-binding regions on the pea protein. The findings pointed to multiple potential binding locations (as shown in Fig. S3). Interestingly, these predicted sites align with a prior study that proposes these ligands might associate with the hydrophobic sections present at the terminal end of protein monomers. In addition, previous studies have highlighted that the most extensive hydrophobic zones of pea protein are found in overlapping areas, starting from the amino acid residue 381 in its tertiary structure. These zones are believed to serve as hydrophobic binding regions in the monomeric state of protein (Han et al., 2022). These insights are in line with previous research which analyzed flavor binding



Flavor	CHEMPLP Score	ΔG°_{exp} (kcal/mol)
vanillin	33.77	-10.03
γ-decalactone	35.57	-9.98
(Z)-3-hexen-1-ol	28.87	-8.38
furaneol	27.95	-8.60

Fig. 2. The possible binding sites of vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol with pea protein (a) ChemPLP Score and ΔG° experiment of selected flavors compounds (b), and linear regression between vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol by ChemPLP Score and ΔG° experiment values (c). The part marked in red for the compound indicates the position of the oxygen atom in the molecule. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

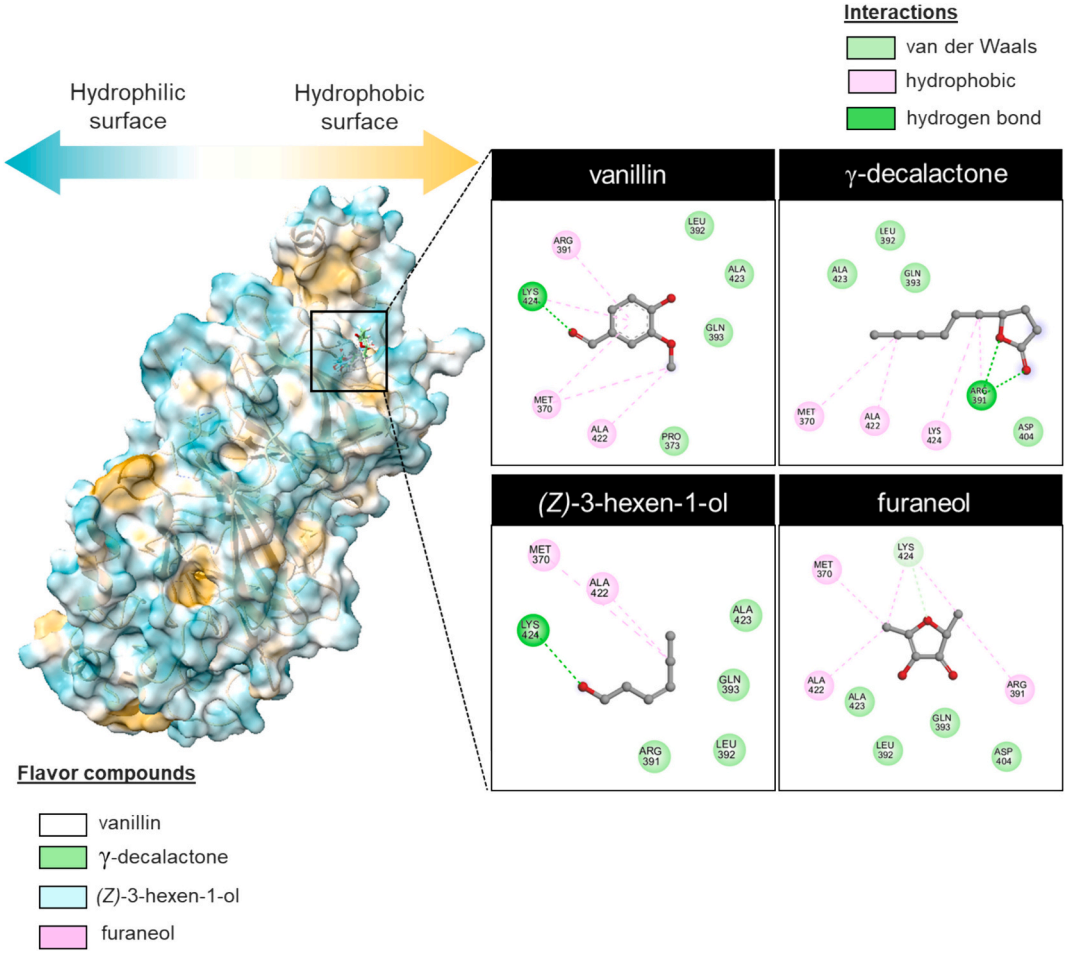


Fig. 3. Computational binding results for pea protein with each flavor compound: vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol. Representative of the best molecular docking model in 3D diagram (b) 2D enlarged view diagrams of pea protein – each selected flavor compounds. The part marked in red for the compound indicates the position of the oxygen atom in the molecule. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in pea protein with compounds such as *(E)*-2-octenal (Bi et al., 2022) and the other strawberry esters consisted of ethyl butanoate, ethyl isopentanoate, ethyl hexanoate, and methyl anthranilate (Wongprasert et al., 2024). Their findings also pinpointed the binding sites at the end of protein monomers.

3.5. Binding affinity and mode of interaction of flavor compounds with pea protein

The interaction strength between pea protein and selected flavor compounds was assessed through in silico molecular docking, employing the Gold docking program with the ChemPLP scoring function. Fig. 2 depicts the predicted conformation and ChemPLP scores of these compounds within the most promising binding site of pea protein. Notably, γ-decalactone exhibited the highest ChemPLP score, followed by vanillin, *(Z)*-3-hexen-1-ol, and furaneol, aligning linearly with experimental ΔG° values. A substantial correlation ($r^2 = 0.91$) emerged between ChemPLP scores and experimental ΔG° values, indicating a good agreement between computational predictions and experimental findings.

Apart from binding affinity, we observed the flavor compounds' mode of binding in terms of non-covalent intermolecular interactions in both 3D and 2D representations (Fig. 3). Notably, the predicted interactions align with the thermodynamic parameters as mentioned earlier (section 3.3), highlighting the roles of van der Waals forces, hydrophobic interactions, and hydrogen bonding mainly responsible for protein-ligand complex formations. Of particular note, γ-decalactone had the most significant binding affinity, resulting from its four van der Waals interactions, four hydrophobic attractions, and two hydrogen bonds. In contrast, lower or loss of hydrogen bonding was found in the rest compounds. Vanillin contains four van der Waals, five hydrophobic interactions, and one hydrogen bond, while *(Z)*-3-hexen-1-ol showed four van der Waals, two hydrophobic interactions, and one hydrogen bond. In addition, furaneol displayed four van der Waals and four hydrophobic interactions. It is also worth noting that these flavors commonly interact with amino acids including Met370, Pro373, Arg391, Leu392, Gln393, Asp404, Ala422, Ala423, and Lys424 which seem to play pivotal roles in driving protein-ligand complex formations (Fig. S4). These results showed that hydrogen bond played a dominant role in flavor-protein interactions because it provides a stronger binding affinities compared to van der Waals or hydrophobic interactions. Therefore, even though vanillin and *(Z)*-3-hexen-1-ol contains the same number of van der Waals forces and different number of hydrophobic interactions, these two compounds can exhibit higher binding affinities compared to the furaneol. The results are consistent with previous literature, which reported that the binding site between flavor compounds and pea protein is located in this mentioned hydrophobic region, such as with *(E)*-2-octenal (Bi et al., 2022) and the other strawberry esters (Wongprasert et al., 2024). Collectively, the findings indicate that pea protein can effectively bind with the investigated flavor compounds including vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol through van der Waals forces, alkyl hydrophobic interactions, and hydrogen bonds.

4. Conclusions

This research offers significant understanding regarding the interactions between PPIs and selected flavor compounds including vanillin, γ-decalactone, furaneol, and *(Z)*-3-hexen-1-ol. All the chosen flavor compounds had the capability to interact with PPIs across different temperature ranges. Among them, furaneol displayed the lowest overall binding affinities, which a characteristic best explained by its partition coefficient. Thermodynamic parameters of the complexes formed between PPIs and flavor compounds showed that the interactions were spontaneous processes, driven by both enthalpy and entropy. Supportive of these findings, computational molecular docking showed the predominant roles of van der Waals forces, hydrogen bonds, and alkyl hydrophobic interactions in these complexes. This knowledge enhanced our comprehension of how PPI interacts with flavor compounds, thereby facilitating more informed choices in flavorings added to protein-containing products, notably high-protein beverages. In addition, the flavor-protein binding problem solving could be achieved by optimization of flavor formula using in the product through flavor compounds adjustment to compensate for the losses due to the binding. This can make the flavor of the final product more acceptable to consumers.

CRediT authorship contribution statement

Thanakorn Wongprasert: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Pakavit Mathatheeranan:** Investigation, Formal analysis. **Panatthida Siripitakpong:** Investigation, Formal analysis. **Tirayut Vilaivan:** Writing – review & editing, Validation, Methodology. **Utid Suriya:** Writing – review & editing, Visualization, Supervision, Methodology. **Thanyada Rungrotmongkol:** Writing – review & editing, Visualization, Supervision, Methodology. **Keith Cadwallader:** Writing – review & editing, Supervision, Methodology. **Inthawoot Suppavorasatit:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101702.

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