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Correlating *in silico* elucidation of interactions between hydroxybenzoic acids and casein with in vitro release kinetics for designing food packaging

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ABSTRACT

The sustained release of phenolic compounds from packaging films is of prime importance for extending the shelf-life of food products. Thus, understanding the interaction of phenolic compounds with polymeric chains and their influence on release kinetics is of great value since release behavior has a role in controlling the quality of the food product. In this study, the interaction mechanism of a group of hydroxybenzoic acids (gallic, vanillic, and protocatechuic acid) with casein fractions was studied by using molecular docking methods. Sodium caseinate film was developed through the solution casting method. Furthermore, the release kinetics of gallic acid was elucidated into the food simulant (95% ethanol). The interaction of phenolic acids with casein fractions was a spontaneous reaction mainly driven by hydrogen bonding, Van der Waals, and hydrophobic forces. The IC_{50} of gallic acid in terms of DPPH radical scavenging activity was observed to be 30.67 μg/ml, whereas the maximum radical scavenging activity was observed to be ~57%. Furthermore, approximately 26% of the gallic acid was released from the packaging into the simulant. A mathematical model was used to describe the diffusion of gallic acid from the packaging material, which will provide essential information on developing packaging materials based on sodium caseinate to reach the best engineering solution by keeping in view the regulatory constraints on the leeching phenomenon. Finally, the developed film can only be used for packaging purposes of food products with lipophilic surface properties.

1. Introduction

Consumer preferences and a shorter life span of minimally processed fruits and vegetables (F&V) have led the researchers to bring innovation in food packaging (Khan, Di Giuseppe, Torrieri, & Sadiq, 2021). To develop packaging materials with desired properties, it is important to design the packaging system considering the interactions between the food product and the package itself (Khaneghah, Hashemi, & Limbo, 2018; Pinto et al., 2021; Volpe, Mahajan, Rux, Cavella, & Torrieri, 2018). Moreover, the development of an active packaging system requires comprehensive knowledge on the release kinetic parameters (partition coefficient and diffusivity) of bioactive compounds entrapped in polymeric chains (Kurek et al., 2017; Benbettaïeb et al., 2020).

Thus, considering the above facts, a holistic approach for the development of novel packaging systems is required to maintain the quality and extend the shelf-life of F&V. Recently, the researchers have shifted their attention from non-biodegradable packaging derived from petrochemical origin to biodegradable packaging made from agro-livestock resources i.e., biopolymers to pack minimally processed F&V to maintain their nutritional quality index (Motelica et al., 2020). Phenolic compounds are well known for their biological activities i.e., antioxidant, antibacterial, and anticancer effects of phenolic acids have been utilized to develop active packaging for food preservation (Khan, Sadiq, & Mehmood, 2020). Casein is a highly functional rheomorphic milk protein that exhibits flexible and open conformation consisting of four primary protein molecules (α_{S1}, α_{S2}, β, and κ) that exhibit heterogeneous behaviors due to different amino acid sequences and post-translational modifications (Casanova, Nascimento, Silva, de Carvalho, & Gaucheron, 2021). The unique properties of casein i.e., emulsifying capacity, high thermal stability, amphiphilic nature, and strong affinity for small molecules and ions (i.e., especially for phenolic compounds based on its proline content) make it a highly desirable biomaterial for the development of active packaging systems (Ma, Tang, Sun, & Zhang, 2021). The interactions between a group of phenolic acids (i.

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e., hydroxycinnamic acids) with β-casein fraction have been explored in several studies by using molecular docking and spectroscopic analysis indicating the covalent interaction in the protein-phenolic complex (Condict, Kaur, Hung, Ashton, Kasapis, 2019; Kaur, Katopo, Hung, Ashton, & Kasapis, 2018). Condict et al. (2019) reported that heat treatment-induced covalent interactions between phenolic acids and protein, especially the formation of a covalent bond between ferulic acid and glutamine-54 residue of β-casein. Unlike hydroxycinnamic acids, studies are lacking on the interaction between casein and hydroxybenzoic acids, which is another important group of phenolic acids (Özçelik, Kartal, & Orhan, 2011). For instance, gallic acid, protocatechuic acid, and vanillic acids are a type of hydroxybenzoic acids (with a wide range of biological activities) that were utilized in the development of active packaging materials, especially with chitosan as biopolymer; furthermore, their interactions and release kinetics have been widely reported in the literature (Cao, Warner, & Fang, 2019; Liu et al., 2021; Liu, Meng, Liu, Kan, & Jin, 2017; Pacheco et al., 2019; Rezaee, Askari, EmamDjomeh, & Salami, 2018; Yadav, Mehrotra, & Dutta, 2021). However, there are little to no studies on the interaction of phenolic compounds with protein-based cheaper biopolymers (i.e., casein) and the influence of interactions on release kinetic parameters which in turn has a role in controlling the quality of the food product.

The interactions between casein and phenolic acids at the molecular level can help to understand the conformational changes in protein structure bound to ligands and help to understand the relationship between structure and function of the protein. Additionally, knowledge of binding sites and modes can help packaging manufacturers to employ effective concentration of ligand to obtain significant level of interaction with target protein as well as a better understanding of the development of effective active packaging (Allahdad, Varidi, Zadmard, & Saboury, 2018; Khan et al., 2021). Thus, this study aimed to investigate the interactions between casein fractions and phenolic acids (gallic, vanillic, and protocatechuic acids) by using *in-silico* analysis. Probable binding sites of phenolic acids in each casein fraction by using molecular docking were explored. Furthermore, after the development of casein films, release kinetics of bioactive from sodium caseinate film was also investigated and correlated with predicted data using Fick's Model for appropriate designing of active antioxidant package.

2. Materials and methods

2.1. Computational methodology

The three-dimensional (3D) structure of all casein fractions was predicted by using the I-TASSER protein server, which utilizes a repetitive implementation of the Threading Assembly Refinement (TASSER) program and a secondary enhanced profile-profile threading alignment (PPA) (Yang et al., 2015). The amino acid sequence (FASTA sequence) of all protein fractions was acquired from NCBI (https://www.ncbi.nlm.nih.gov/) which was used for generating protein structure for caseins obtained from *Bos taurus*. I-TASSER generated all possible models for all casein fractions, the models with the highest confidence scores (C-score) (ranging between 2 and −5) were selected (Fig. S1) to carry out molecular docking analysis (Table S1). The 3D conformers of all three phenolic acids (gallic, vanillic, and protocatechuic acids) used in this study were acquired from PubChem databank (https://pubchem.ncbi.nlm.nih.gov/). Proteins and ligands were prepared by using Autodock Tools 1.5.6 from MGL Tools (The Scripps Research Institute). For protein preparation, polar hydrogens were added, and water molecules were deleted. On the other hand, optimized ligand structures were converted into PDB format. Additionally, proteins were assumed to be rigid while all rotatable torsions were activated for ligands.

The ligand and protein in PDB formats were then converted into extended PDBQT (Protein Data Bank, Partial Charge, and Atom Type) format for docking analysis. Since no existing information was available for casein-hydroxybenzoic acids binding pockets, blind dockings were performed. The docking was performed by using grid box dimensions as follows: x-size= 40 Å, y-size= 40 Å, and z-size= 40 Å and spacing of 1 Å. The docking analysis provided a log.PDBQT and output.PDBQT files. The log files have essential information related to binding energies. The results related to interactions were analyzed by using a free version of Discovery Studio 2021 Client (BIOVIA Discovery Studio, Dassault Systèmes). Briefly, The output.PDBQT and protein.PDBQT files obtained during the docking process were submitted to Discovery Studio and assigned as ligand and receptor respectively. The detailed interactions (bond distances and bond types with receptor surface etc.) were studied by using the ligand interactions tab.

2.2. In-vitro studies

Based on computational analysis, gallic acid was selected because of its binding affinity with alpha casein fractions and based on toxicity evaluation using the online pharmacological tool "pkCSM" (Table.S2) for the development of packaging film using sodium caseinate as a biopolymer which is a mixture of α_{S1}, α_{S2}, β and κ-casein fractions.

2.2.1. Film formulation

Caseinate films were prepared by solution casting method (Valentino, Volpe, Di Giuseppe, Cavella, & Torrieri, 2020; Zarandona, Puertas, Dueñas, Guerrero, de la Caba, 2020) with modifications. Initially, 0.25 g of gallic acid (Sigma-Aldrich, Milan, Italy) was dissolved in freshly prepared 50 ml Tris buffer (0.02 M, pH: 8) at room temperature for 45 min under continuous stirring by using a magnetic stirrer (C-MAG, HS-4, IKA, Germany). On the other hand, 10 g of sodium caseinate (Sigma-Aldrich, Milan, Italy) and glycerol (10% w/w of caseinate) was dissolved in Tris buffer (75 ml) at 68 ± 2 °C under stirring for 2 h. After the caseinate was completely dissolved in the Tris buffer, the film-forming solution (FFS) was allowed to cool at room temperature, followed by the incorporation of gallic acid solution and stirring to obtain the final FFS. FFS was cast onto the Petri plates (120 ×120 mm) and allowed to dry for 24 h in the climatic chamber (MMM Medcenter Einrichtungen, GmbH, Germany) at 57 ± 2% relative humidity and 30 °C.

2.3. Migration analysis

For the elucidation of release kinetic parameters of gallic acid from the caseinate film, a specific migration test into 95% ethanol solution as food simulant was performed according to Luzi et al. (2019) with modifications in accordance with European Standard EN 13130–2005 and European Commission Regulation 10/2011. Briefly, film samples (10 cm^2) were immersed completely in 20 ml of simulant in four replicates at 30 °C in the incubator (Memmert, Model: 30–1060, Germany). Samples were diluted 20 times and evaluated at 1, 3, 6, 24, 48, 120, 216, and 288 h; additionally, a blank test was also included for the simulant. The absorbance values for gallic acid were measured at 270 nm by UV–vis spectrophotometer (JASCO, V-550). A gallic acid calibration curve (from 1 to 25 μg/ml) was used to estimate the concentration of gallic acid released from the film into the food simulant. The results were expressed as μg/ml of the food simulant.

2.4. DPPH radical scavenging activity of food simulant solutions

The radical scavenging activity of the food simulant solutions recovered (and diluted) after each interval of time was determined by using DPPH assay according to Ruan et al. (2019) with modifications. Initially, 1.5 ml of DPPH solution (25 ppm) was mixed with 1 ml of recovered samples. The mixture was incubated for 30 min in the dark at room temperature. Finally, the absorbance was read at 517 nm by using a spectrophotometer. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH\%inhibition} = \frac{AC - AS}{AC} \times 100 \quad (1)$$

Whereas AC is the absorbance of control and AS is the absorbance of the sample.

2.5. Mathematical modelling

It is useful to estimate the performance of an active package by understanding the information about diffusion coefficients as the performance of antioxidant packaging depends on the release kinetics of antioxidant grafted in the package (Ramos, Beltrán, Peltzer, Valente, & del Carmen Garrigós, 2014). Since migration analysis is costly and time consuming, predictive models by using mathematical modelling can be used to describe migration in terms of release kinetics by applying Fick's Second Law (Tampu, González-Martínez, & Chiralt, 2018). Considering that a limited migration of active compound occurs from a packaging film with limited volume into a limited volume of food simulant, the diffusion coefficient of antioxidants can be expressed in terms of Fick's Second Law (Eq. 2) (Lee, Yam, & Piergiovanni, 2008). By considering that the diffusion occurs from both side of the materials, the Fick's second law was numerically solved based on finite differences methodology. The Fick's second law, the boundary condition and the order differential equation used are reported in Eqs. 2, 3 and 4, respectively:

$$\frac{\partial y(x,t)}{\partial t} = D\left(\frac{\partial^2 y(x,t)}{\partial x^2}\right) \quad (2)$$

$$\begin{cases} \left.\dfrac{dy_i(t)}{dt}\right|_{i=1} = 0 \\ \left.\dfrac{dy_i(t)}{dt}\right|_{i=n} = 0 \end{cases} \quad (3)$$

$$\frac{dy_i(t)}{dt} = D\frac{(y_{i-1}(t) - 2y_i(t) + y_{i+1}(t))}{x^2} \quad (4)$$

where y is the ratio between the concentration of the migrant at time (t) and its concentration after infinite time (M_∞) (5), D is the diffusion coefficient and x is the thickness of the ith layer estimated starting by the thickness of the material (e) divided the total number of layer (5).

$$y = \frac{M_t}{M_\infty} \quad (5)$$

$$x = \frac{e}{n} \quad (6)$$

To determine numerical validity of the mathematical model; comparison between experimental and predicted models was done by using root mean square error (RMSE). RMSE of the observed and predicted residual values was computed by using the following equation to predict the quality of fit (MATLAB version R2021a):

$$RMSE = \sqrt{\frac{\sum_{i=1}^{N}(\hat{y}_i - y_i)^2}{N}} \quad (7)$$

Where $\hat{y}_i$ and y_i is the residual value of observed and predicted values respectively while N is the number of observations.

Furthermore, the partition coefficient (K) can be described as the ratio of bioactive in the food simulant ($C_{s,\infty}$) to the migrant in the polymeric film ($C_{F,\infty}$) at equilibrium (Marvdashti, Yavarmanesh, & Koocheki, 2019):

$$K = \frac{C_{S,\infty}}{C_{F,\infty}} \quad (8)$$

2.6. Statistical analysis

The SPSS software (SPSS version 23.0, IL, USA) was used to perform one-way analysis of variance (ANOVA) to estimate significant differences ($p < 0.05$) among mean observations.

3. Results and discussion

3.1. Binding energy

The basis of designing an active packaging system must start from utilizing the knowledge of biopolymer-ligand interaction, structural data, and binding mechanisms to explore the potential of packaging components in developing effective packaging forms. So, a detailed understanding about molecular interactions involved is of great value in providing insights about designing and developing active packaging (Benbettaïeb et al., 2020; Ma et al., 2021). Molecular docking is a widely utilized computational tool in protein-ligand interaction for predicting binding modes (Sousa et al., 2013). In this study, all three ligands were explicitly docked against all casein fractions. Table 1 highlights the binding affinity of the ligands with all casein fractions. The binding energy of ligands ranged from − 6.0 to − 6.3 kcal/mol for α_{S1} casein, from − 4.9 to − 5.0 kcal/mol for α_{S2} casein, from − 5.5 to − 5.8 kcal/mol for β-casein, and from − 5.0 to − 5.1 kcal/mol for K-casein. Gallic acid showed highest binding affinities for α-caseins because of hydrophilic nature of both the gallic acid (because it has a log P value of 0.6; log P is a constant of lipophilicity, a more polar hydrophilic compound thus has a lower value of log P) and casein fractions (Valderrain-Rodríguez et al., 2018), furthermore, it can also be due to the hydrophilic phosphate centers of α-caseins (Elzoghby, El-Fotoh, & Elgindy, 2011). On the other hand, protocatechuic acid showed better binding ability against β- (due to presence of hydrophilic and hydrophobic domains) and κ-casein because of its slight hydrophobic character (MQG de Faria et al., 2012). Similarly, Kaur et al. (2018) docked *p*-coumaric acid against β-casein and observed a minimal binding energy of − 6.80 kcal/mol, indicating bond formation between hydroxyl groups of the ligand within the core of casein molecule.

3.2. Polymer-ligand interactions

For the interaction analysis of best conformation, Discovery Studio Visualizer was used. In this study, top molecular docking outcome suggested the formation of hydrogen bonds (Fig. 1) between hydrogen of the hydroxyl group of gallic acid with the respective amino acids of α_{S1} casein: ILE 142, GLN 145, TYR 109 and LEU 164 (Fig. 1A) with bond distances of 2.64, 2.45, 2.19, 1.91 Å respectively (Table 2), additionally TYR 109 also formed hydrogen bond with oxygen of the hydroxyl group of gallic acid, other noticeable interactions are with TYR 180, PHE 165 forming π-π stacked and π-alkyl hydrophobic interactions of ILE 142 and LEU 164 with benzene ring of gallic acid. Van der Waals interactions with TRP 179, GLU 163, TYR 161, GLN 146, and HIS 143 were also observed. Protocatechuic acid shares hydrogen bonds with GLN 146 and LEU 164. Van der Waals can also be observed with TRP 179, TYR 180, TYR 109, HIS 143, and GLN 145 (Fig. 1B). Other notable interactions involve π-π stacked with PHE 165 and π-alkyl LEU 164, and ILE 142 with the benzene ring. The docking of vanillic acid with the αS1 revealed that it forms three hydrogen bonds with LEU 164, TRP 179, and GLN 145; one π-donor hydrogen bond and one π-π T-shaped bond with TYR 180; two π-alkyl interactions with TYR 109 (with the longest bond distance of 5.35 Å) and PHE 165; one π-sigma interaction of benzene ring with ILE 142 and several van der Waals interactions with GLN 112, 146 and HIS 143 (Fig. 1C). Similarly, molecular docking of gallic acid with α_{S2} depicted formation of two conventional hydrogen bonds with amino acids: HIS 92 and VAL 198 with bond distances 2.29 and 2.50 Å respectively. The hydrophobic π-alkyl interactions were observed between benzene ring of gallic acid and VAL 88 and ALA 204 of the protein (Fig. 1D). Other notable interactions involved Van der Waals. When protocatechuic acid was docked against αS2 casein it displayed three hydrogen bonds with THR 197, HIS 92 and HIS 201. Several Van der

Table 1
Binding affinity of ligands for casein fractions.

Compounds	Protein	Binding energy (kcal/mol)	Protein	Binding energy (kcal/mol)	Protein	Binding energy (kcal/mol)	Protein	Binding energy (kcal/mol)
Gallic acid	α_{S1}-casein	-6.3	α_{S2}-casein	-5.0	β-casein	-5.7	κ-casein	-5.0
Vanillic acid		-6.0		-4.9		-5.5		-5.0
Protocatechuic acid		-6.1		-4.9		-5.8		-5.1

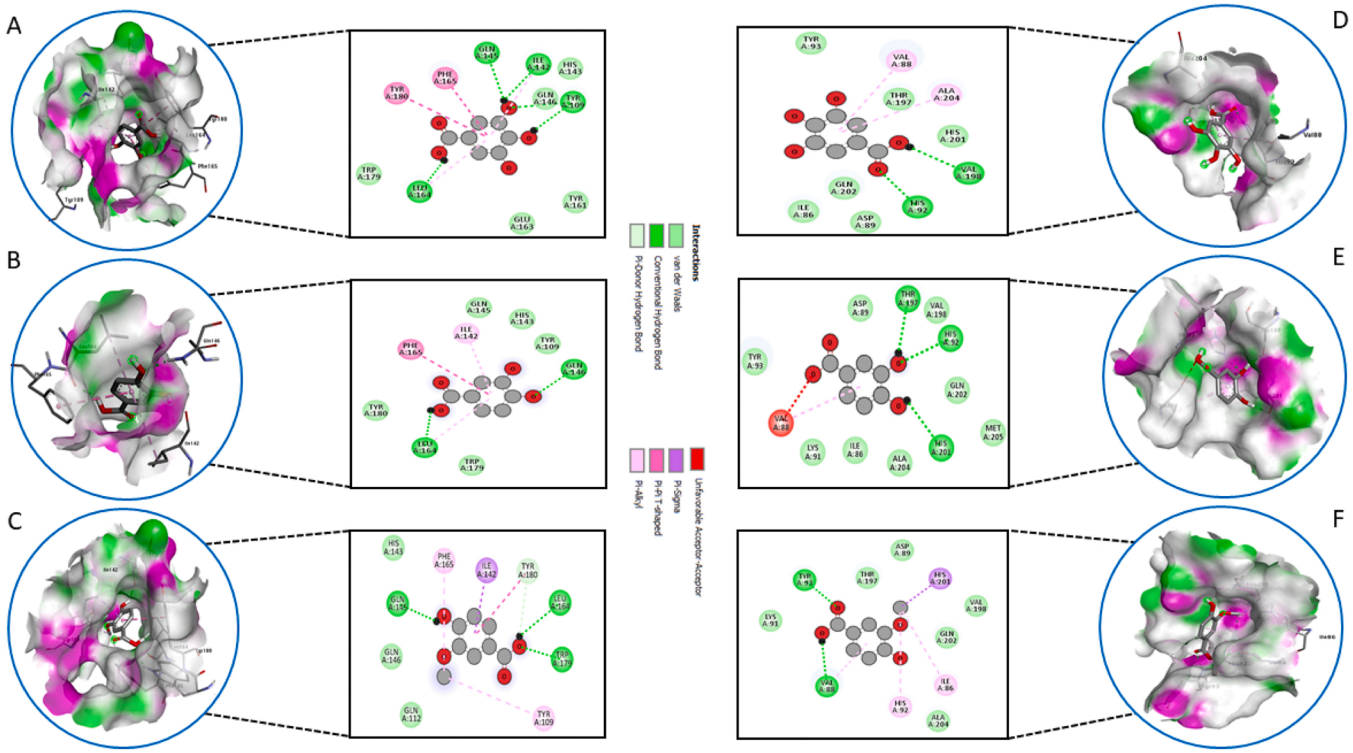

Fig. 1. Interactions of α_{S1} casein with phenolic acids (A) gallic acid, (B) protocatechuic acid, and (C) vanillic acid; and interactions of α_{S2} casein with phenolic acids (D) gallic acid, (E) protocatechuic acid, and (F) vanillic acid.

Waals interactions were observed with GLN 202, MET 205, ALA 204, ILE 86, LYS 92, TYR 93, ASP 89, and VAL 198. Furthermore, a π-alkyl interaction was also observed with VAL 88 (which also formed an unfavorable bond with the oxygen) (Fig. 1E). Vanillic acid formed two hydrogen bonds with TYR 93 and VAL 88, three π-alkyl interactions with ILE 86, HIS 92 (through carbon atom) and VAL 88 (through benzene ring) and several Van der Waals (Fig. 1F) Similarly, Lang et al. (2019) docked α-casein with malvidin-3-o-galactoside (an anthocyanin) and observed the formation of conventional hydrogen bonds between PRO 133, LYS 128, and TYR 180 and hydroxyl group of C ring (carbon site 5), furthermore an interaction of casein with anthocyanin rings was also observed (similar to our findings); thus, protecting the antioxidants against cleavage and improving their stability during processing. In our study, phenolic acids were embedded into the hydrophilic domains of α-caseins, while Allahdad, Varidi, Zadmard, and Saboury (2018) observed superficial interaction of β-carotene with α-casein in a shallow recess on the surface due to its hydrophobic nature.

When gallic acid was docked against β-casein it displayed three hydrogen bonds with SER 139, ASN 147 and LYS 120 and one π-alkyl bond with LUE 140 (Fig. 2A). Similarly, docking of protocatechuic acid revealed four hydrogen bonds with ASP 62, GLN 61, LYS 63 and ASP 144 indicating strong interactions as already evident from binding energy (Fig. 2B). Other notable interactions involved π-alkyl and Van der Waals. Best binding energy and strong interactions of β-casein with protocatechuic acid were observed due to two possible reasons, a) due to hydrophobic nature of protocatechuic acid and b) binding of protocatechuic acid with hydrophobic C-terminal of β-casein (Chao & Yin, 2009). Least number of hydrogen bonds were observed when interactions between vanillic acid and β-casein (amino acids: GLN 61 and ASP 62 with bond distances of 2.63 and 2.08 Å respectively) were studied, which can be directly correlated with its least binding energy among all phenolic acids (Fig. 2C). Similarly, Kaur et al. (2018) suggested formation of hydrogen bonds between side chain amide group of GLU 54 and carbonyl group of GLA 51 with para-hydroxyl group of ferulic acid.

Molecular docking studies between gallic acid and κ-casein revealed three hydrogen bonds (with ASN 74, GLN 112, and ALA 189), one π-π stacked bond with TYR 81 (with highest bond distance of 5.08 Å), and one hydrophobic π-alkyl bond with ALA 111 (Fig. 2D). While protocatechuic acid displayed three hydrogen bonds with LEU 77, GLN 112, and ASN 74 (Fig. 2E). Furthermore, one π-sigma (with ALA 111) and one π-alkyl hydrophobic interaction (with PRO 78) were also observed. Contrarily, vanillic acid displayed only one hydrogen bond with ALA 189, and three alkyl hydrophobic interactions with ALA 111, PRO 78, and LEU 77, thus the reason for low binding affinity (Fig. 2F). Similarly, Allahdad et al. (2018) reported the binding of hydrophobic β-carotene into the hydrophobic core of κ-casein and formed the most stable complex with a binding energy of − 8.5 kcal/mol.

Table 2
Bond distances between protein amino acids and phenolic acids.

Casein type	Amino acid	Ligand	Bond distance (Å)
α_{S1}	GLN 145, ILE 142, TYR 109, TYR 109, LEU 164, PHE 165, TYR 180, ILE 142, and LEU 164	Gallic acid	2.45, 2.64, 2.19, 2.75, 4.69, 5.05, 5.77, 5.19, 2.64 and 1.91
	GLN 146, LEU 164, LEU 164, PHE 165, and ILE 142	Protocatechuic acid	2.02, 1.89, 4.79, 5.18, and 4.99
	GLN 145, TRP 179, LEU 164, ILE 142, TYR 109, PHE 165, TYR 180, and TYR 180	Vanillic acid	1.88, 2.10, 2.66, 3.65, 5.35, 4.73, 4.81, and 3.16
α_{S2}	HIS 92, VAL 198, ALA 204, and VAL 88	Gallic acid	2.29, 2.50, 5.48, and 5.40
	HIS 92, HIS 201, THR 197, VAL 88, and VAL 88	Protocatechuic acid	2.41, 2.48, 2.11, 2.81 and 5.37
	TYR 93, VAL 88, VAL 88, HIS 201, HIS 92, and ILE 86	Vanillic acid	2.06, 2.38, 5.43, 3.88, 4.71, and 4.86
β	LYS 120, ASN 147, SER 139, and LEU 140	Gallic acid	1.83, 2.35, 2.43, and 5.06
	ASP 62, GLN 61, LYS 63, ASP 144, and PRO 119	Protocatechuic acid	2.18, 2.96, 2.84, 2.18 and 4.96
	GLN 61, ASP 62, and PRO 119	Vanillic acid	2.63, 2.08, and 5.09
κ	ASN 74, GLN 112, ALA 189, TYR 81, TYR 81, and ALA 111	Gallic acid	2.11, 2.63, 2.03, 2.89, 5.08, and 4.88
	LEU 77, GLN 112, ASN 74, PRO 78, and ALA 111	Protocatechuic acid	2.14, 2.06, 1.90, 4.92, and 3.90
	ALA 189, LEU 77, ALA 111, PRO 78	Vanillic acid	1.82, 4.97, 4.08, and 4.23

3.3. In-vitro studies

3.3.1. Migration analysis

The release test was performed to elucidate the release of gallic acid from the packaging film according to European Standard EN 13130–200522 (Luzi et al., 2019). Fig. 3 illustrates the release behavior of the bioactive compound from the film into the food simulant (95% ethanol) during contact time. Since casein film is highly hydrophilic and easily disintegrates when comes into contact with food simulants with less than 95% ethanol, thus only 95% ethanol was selected as a food simulant. The quantification of the gallic acid was done by using spectrophotometry. During the first 6 h of incubation, the concentration of released gallic acid was 171.76 ± 18.21 μg/ml, with a threefold increase (~624 μg/ml) in concentration after 48 h. However, after 120 h, a non-significant increase in concentration was observed indicating towards equilibrium stage, which can be better defined in terms of the "swelling-controlled" model: that when the simulant enters the film matrix, it dissolves the active compound causing its release from the film matrix leading towards polymer swelling, until a plateau is reached, followed by a time-dependent relaxation (Suppakul, Sonneveld, Bigger, & Miltz, 2011). Luzi et al. (2019) similarly observed the release of gallic acid (at 5% and 10% wt) from PVA (Polyvinyl alcohol)-based system with concentration ranging between 296.76 and 701.54 μg/ml in 50% ethanol. In our study, ~26% of the gallic acid leached out into the food simulant, which could be due to migrant polarity similar to that of the food simulant and swelling of the polymer in the presence of the simulant (Ramos et al., 2014); furthermore, retention of gallic acid in caseinate film indicates strong conventional hydrogen interactions between casein polymeric chains and gallic acid. Schreiber (2012) observed a slow release of gallic acid from multifunctional chitosan films with leaching of only 13%, with a large proportion of gallic acid, was retained by the polymeric film. In our case, a better leaching percentage was observed because gallic acid has a high affinity for ethanol; in fact, it is 30 times more soluble in ethanol than water, thus with increasing ethanol concentration, an increase in the gallic acid release can be expected (Daneshfar, Ghaziaskar, & Homayoun, 2008; Noubigh, Mgaidi, & Abderrabba, 2012). The maximum Admissible Daily Intake (ADI) has not been established for gallic acid yet, however, for propyl gallate (an ester of gallic acid) it is 0.2 mg/kg (EFSA, 2014), corresponding to

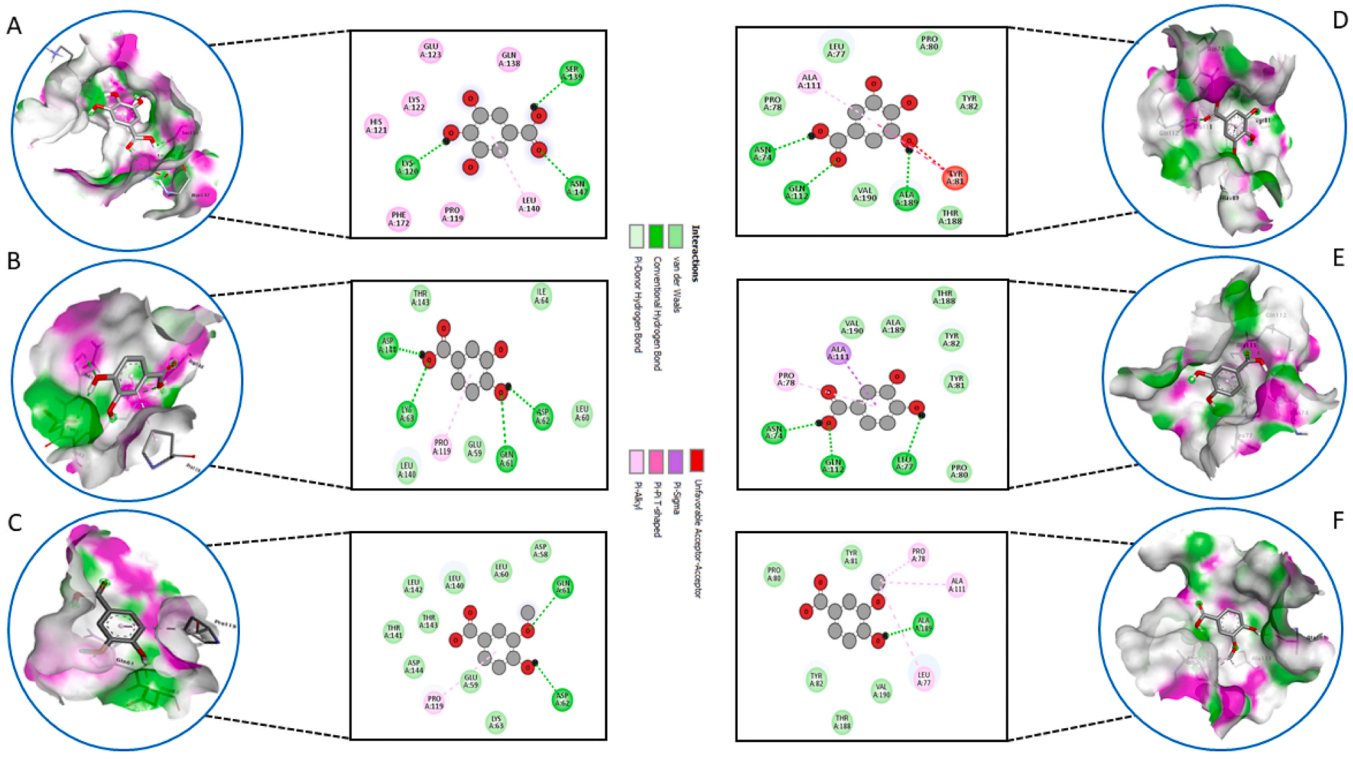

Fig. 2. Interactions of β casein with phenolic acids (A) gallic acid, (B) protocatechuic acid, and (C) vanillic acid; and interactions of κ casein with phenolic acids (D) gallic acid, (E) protocatechuic acid, and (F) vanillic acid.

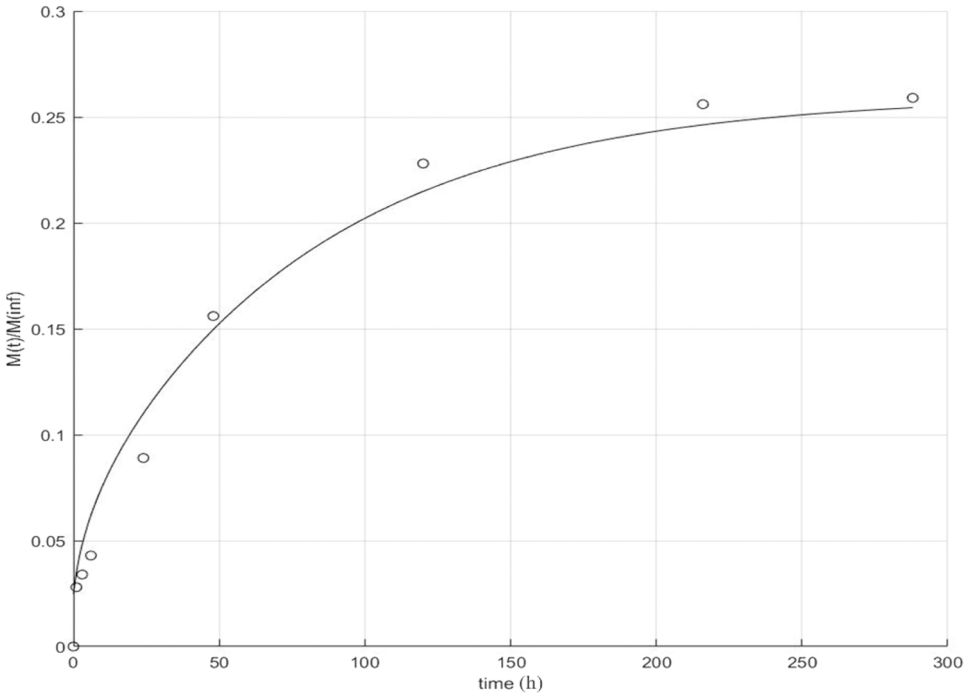

Fig. 3. Correlation between experimental and predicted release kinetics data from Fick's model (where "o" is experimental, and "black line (-)" is predicted data).

12 mg for an adult having a bodyweight of 60 kg. If we assume that an adult person eats the food from a conventional packaging (with a packaging film as lid weighting 1 g), considering the released amount of gallic acid from the film (which in this case is 26%) having 0.08 g of weight (dimension: 10 cm^2) with a gallic acid concentration of 4000 μg, only 11.8 mg of gallic acid will leach out which is lower than ADI (that too only if the food is still in contact with the film even after 216 h), furthermore, the limit set for specific migration is 30 mg/kg in foodstuff for all gallates (EFSA, 2014), thus the food will be safe to consume. Contrarily, it must be considered that gallic acid intake also comes from various food sources. Hence, such calculations should include consideration of the exposure assessment for gallic acid (which is the total quantity consumed with the whole diet) for more accurate estimation.

3.3.2. Antioxidant activity

The antioxidant activity of recovered food simulant solutions varied between 25% and 57.2% (Fig. 4). Initially, an increase in DPPH radical scavenging activity was observed till 120 h. Afterward, a decline in antioxidant activity was observed due to two possible reasons, a) the migrant reached the equilibrium stage between film and the simulant, or b) at high antioxidant concentration, the reaction was impeded (since enough DPPH radicals were not there to interact with bioactive compound) (Schaich, Tian, & Xie, 2015). The IC_{50} value of gallic acid in this study was found to be 30.67 μg/ml, which was slightly higher than reported (23.9 μg/ml) by Valentino, Volpe, Di Giuseppe, Cavella, and Torrieri (2020), which could be due to the interaction of casein with gallic acid or maybe due to difference in protocols. Luzi et al. (2019) similarly reported radical scavenging activity between ~18–62% for food simulants containing gallic acid released from a PVA-based system, supporting our results.

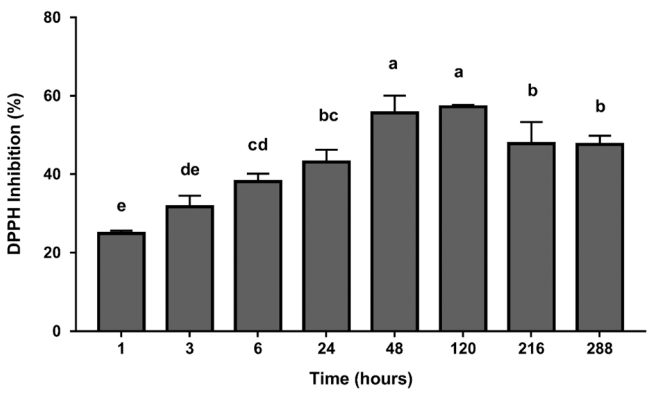

Fig. 4. DPPH radical scavenging activity of the food simulant with gallic acid.

3.4. Mathematical modelling

For predicting the release behavior of bioactive compounds during shelf-life and designing a new packaging system; mathematical modelling can be of great value. In this study, the release kinetics of gallic acid from caseinate film was evaluated and mathematical modelling was used to fit experimental data to estimate the capacity of Fick's model to predict the release of gallic acid from the film. The results obtained for gallic acid release are shown in Fig. 3. The diffusion coefficient found in this study was 5.99×10^{-12} m^2/s with a coefficient of determination (R^2) value of 0.99 (Fig. S2) and the root mean square error (RMSE) of 0.02 mg/L, indicating excellent linearity between the experimental release of bioactive and the data suggested by the model (Since R^2 value should be > 0.94 and RMSE should be < 0.1 mg/L), which indicates that release data is adequately described by Fick's model (Benbettaieb, Cox, Gilbert, & Debeaufort, 2021; Ramos et al., 2014). Rubilar et al. (2017) similarly reported diffusion coefficient to be around 1.9×10^{-13} m^2/s which was slower than observed in our study, which could be due to difference in temperature at which release kinetics was observed, furthermore, a higher diffusion coefficient in our study is also due to higher affinity of gallic acid towards 95% ethanol. Some studies in literature also reported higher diffusion coefficients for other compounds (Desai & Park, 2005; Del Nobile, Conte, Incoronato, & Panza, 2008). The lower magnitude of D value means that film can provide a long-term release of gallic acid with a higher quantity of bioactive compound retained inside the film matrix (Suppakul et al., 2011). A partition coefficient value of 0.25 was observed in this study, which indicates a higher affinity of migrant for polymer rather than food

simulant (Marvdashti et al., 2019). Several factors, for instance, solubility, chemical nature, polarity, and affinity of a diffusing agent towards polymeric matrix have been reported to affect partition coefficient (Franz & Störmer, 2008).

4. Conclusion

Molecular docking was used to elucidate interactions between phenolic acids and casein fractions. The computational study revealed possible binding sites of phenolic acids (gallic, vanillic, and protocatechuic acids) on all casein fractions. Gallic acid demonstrated better binding ability with α-casein subunits due to their hydrophilicity, while protocatechuic acid showed the best binding affinity against β casein due to its lipophilic moiety. The main forces involved in the binding complexes were hydrogen bonding, hydrophobic interactions, and Van der Waals. Molecular interaction studies clarified the release kinetics of active packaging systems (i.e., antioxidant packaging) to develop better systems with sustained release of compounds to ensure that minimal concentration is always present to protect the packaged product from oxidation. Mathematical modelling revealed an excellent correlation between experimental and modelling data with a diffusion coefficient helpful in maintaining sustained release and concentration of gallic acid both in simulant and film, respectively. Furthermore, it is suggested that this packaging material should only be used for fatty food due to the hydrophilic nature of the biopolymer.

Declaration of Competing Interest

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

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Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fpsl.2022.100859.

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