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ABSTRACT: Many drugs and phytochemicals are bitter, leading to non-compliance with prescriptions and avoidance of healthy foods and a need to suppress their taste. The goal of this study was to investigate the binding of bitterants (quinine and caffeine) by whey protein isolate (WPI) and the effect on perceived bitterness. Caffeine interacted minimally with WPI, while the proportion of unbound quinine decreased exponentially with protein concentration. Molecular modeling was used to show the energy of the quinine-B-lactoglobulin interaction was an order of magnitude greater than the caffeine–B-lactoglobulin interaction. Untrained assessors were used to assess the bitterness of caffeine (1.8, 5.7 and 18 mM) and quinine (0.056, 0.10 and 0.18 mM) solutions with 0% or 1% WPI. There was no significant effect of protein on the bitterness of caffeine solutions, but WPI decreased the bitterness of quinine relative to the same concentration in water. This is generally consistent with our hypothesis that higher binding results in lower bitterness; however the magnitude of reduction was not large and the bitterness of the protein-quinine solutions was greater than would be expected for the unbound quinine present.

Keywords: bitter, protein, binding, sensory, taste-masking, computer modeling

Practical Application: Masking bitter taste is of great interest in the food and pharmaceutical industries; however, the mechanisms underlying some current approaches is poorly understood. In this work, the effect of whey protein on bitterant binding and bitterness perception was assessed using hydrophobic quinine and hydrophilic caffeine. Quinine was strongly bound and suppressed in bitterness by protein though not as much as expected; this
shows that measures of physical binding can be used to predict taste-masking effects but only qualitatively.
Introduction. Bitterness is one of a small set of commonly recognized prototypical tastes and tends to be innately aversive. Bitter-tasting drugs and phytochemicals have been shown to reduce compliance with a treatment regimen (Shahiwala 2011), or the selection of certain healthy foods in a diet respectively (Drewnowski and Gomez-Carneros 2000). This is particularly true in children, who are less able to weigh the long-term benefits over the short-term discomfort (Negri et al. 2012). In fact, this problem is so persistent, the European Medicines Agency will require a pediatric development plan to control the bitter/unpleasant tastes caused by the active ingredients in oral medications in coming years (Davies and Tuleu 2008; Mennella and Beauchamp 2008). There are similar challenges in the formulation of functional foods enriched with bitter plant compounds. Because these pharmaceuticals or phytochemicals are essential to the function of the medication or food product, their removal is not possible and suppression of the bitter taste should therefore be a focus. One strategy to reduce bitterness is the prevention of interactions between bitterants and taste receptors via physical means, such as encapsulation or molecular binding (Coupland and Hayes 2014). Proteins can be useful in bitterness-masking in foods because they have the ability to bind small molecules and have demonstrated capacity to alter taste and aroma perception (Bohin et al. 2013).

Whey proteins were chosen for this study because of their good solubility and wide range of applications as food ingredients (de Wit et al. 1988). Whey proteins make up 18-20% of the protein in milk (Morr and Foegeding 1990; Jovanović et al. 2005). They are a mixture of proteins including β-lactoglobulin (BLG) (60%), bovine serum albumin (BSA, 10%), α-lactalbumin (20%), and immunoglobulins (5%) (Morr and Foegeding 1990). Whey protein isolate (WPI) is a powdered food ingredient made from the whey fraction of milk containing about 90% protein.
BLG is known to bind small molecules predominantly via hydrophobic interactions. Ketones (O’Neill and Kinsella 1987), esters (Pelletier et al. 1998), fatty acids (Wu et al. 1999), aldehydes, alcohols, and lactones (Guichard and Langourieux 2000) showed greater binding with BLG the greater their hydrocarbon chain lengths. BLG complexes (i.e., nanoparticles) created by thermal denaturation showed high binding of epigallocatechin gallate (EGCG) and a subsequent reduction of bitterness and astringency (Shpigelman et al. 2010; Shpigelman et al. 2012). While most binding studies have been done with BLG, it is worth remembering that WPI is actually a mixture of proteins, and BSA has also shown hydrophobic binding behavior (Mudgal et al. 2016). Studies on the effect of protein on aroma are also relevant to the present work, as greater binding would be expected to depress the free-aqueous and headspace volatile concentration. For example, whey protein concentrate (0.5%), a less purified whey protein fraction, decreased the flavor intensity of vanillin, benzaldehyde, and d-limonene (Hansen, 1997).

The phenomenon of bitterness-masking by proteins has been investigated, but the mechanism is unclear as many studies use complex food matrices (Keast 2008; Bennett et al. 2012; Homma et al. 2012) that make it hard to identify the type of binding as well as failing to adequately combine physicochemical measurements of binding with appropriate sensory techniques (Metcalf and Vickers 2001; Mattes 2007; Keast 2008; Thurgood and Martini 2010).

In this work we selected caffeine and quinine as model bitterants as they are commonly used in sensory studies as well as in real foods yet have very different chemical structures. We measured binding and perceived bitterness of caffeine and quinine to WPI to test the hypothesis that the unbound (aqueous) bitterant (not the total concentration) is responsible for
the perceived bitterness. The nature of the binding was investigated by molecular modelling using the Autodock molecular docking program. This program is able to identify likely binding sites of ligand (caffeine or quinine) with a protein (BLG) and make an estimate of the strength of the binding energy. This information will facilitate the interpretation of the experimental binding data by providing information on the relative binding propensity of the two ligands.

Materials and Methods

Materials. Quinine hydrochloride (food grade), caffeine (food grade), methanol (HPLC grade), and triethylamine (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetonitrile (HPLC grade), phosphoric acid (HPLC grade), and glacial acetic acid (HPLC grade) were purchased from VWR International (Radnor, PA, USA). BiPRO whey protein isolate was donated by Davisco Food International, Inc. (Eden Prairie, MN, USA). Millipore water was used throughout the experiments.

Nitrogen Analysis Testing. Protein content of the WPI was analyzed via a LECO FP-528 Nitrogen analyzer calibrated with EDTA standards. WPI was measured in solid form with samples measuring 0.2026 ± 0.0002 g.

Protein Binding by WPI. Protein binding assessed for quinine HCL (0.13 mM) or caffeine (0.51 mM) with WPI (0-4.5%). The protein was separated with EMD Millipore Amicon Ultra-4 centrifugal filter units with a size cutoff of 3 kDa and centrifuged in a Fisher Scientific Centrifuge
model 228 at 3400 RPM for 30 minutes. The filtrate was collected and measured via HPLC. The experiments were conducted in triplicate.

**High Performance Liquid Chromatography.** HPLC was used to measure the amount of bitterant in the aqueous phase of all of the experiments using an Agilent 1220 Affinity LC Manual Injection instrument from Agilent Instruments with a Zorbax SB-C18 4.6X50mm separation column. A UV-Vis detector set to 254 nm was used for quantification with a mobile phase flow rate of 1.5 ml/min. For caffeine, a mobile phase of 94% water, 5.5% acetonitrile, 0.2% triethylamine, and 0.2% glacial acetic acid at pH = 5 was used for analysis. The mobile phase for quinine was 50% water and 50% methanol adjusted to pH = 2.5 with phosphoric acid for better peak resolution. Prior to analysis, all samples were filtered with a PTFE membrane syringe filter from VWR International with a pore size of 0.45 μm.

**Molecular Modeling.** The docking program AUTODOCK 4 (Morris et al., 1998) was used to probe the potential binding sites for caffeine and quinine on the bovine whey protein β-lactoglobulin. AUTODOCK searches conformation space, using genetic algorithms, for a protein-ligand pair and identifies potential binding sites based on an estimation of the free energy of binding (Huey et al., 2007). This involves estimation of the various interactions between ligand and protein groups, of the desolvation of protein groups that would occur if the ligand were bound, the change in torsional energy (an entropic term) within the ligand between the bound and unbound states. The full equation for free energy estimation is,

\[
\Delta G_{\text{binding}} = \Delta G_{\text{vdW}} + \Delta G_{\text{H-bond}} + \Delta G_{\text{elec}} + \Delta G_{\text{desolv}} + \Delta G_{\text{tors}}
\]
The contributions to $\Delta G_{\text{binding}}$ from specific interactions between the atoms of the protein and ligand are from van der Waals interactions ($\Delta G_{\text{vdW}}$), H-bonding ($\Delta G_{\text{H-bond}}$), and electrostatic interactions ($\Delta G_{\text{elec}}$). The equations used to sum the contributions from these interactions can be found in Huey et al. (2007). The desolvation free energy ($\Delta G_{\text{desolv}}$) estimates the change in energy when a ligand binds on to a protein and water is displaced from the surface of the protein (and ligand). Finally, the change in $\Delta G_{\text{tors}}$ represents the change in energy due to the loss of conformational entropy of the ligand on binding. Changes in conformational entropy occur due to loss of torsional flexibility around some bonds in the ligand.

For the analysis the X-ray structure of $\beta$-lactoglobulin was downloaded from the RCSB protein database as file 3BLG.pdb (Qin et al., 1998). Quinine and caffeine pdb files were generated using the Automated Topology Builder (ATB) (Malde et al., 2011).

Autodock Tools (Morris et al., 2009) was used to prepare the $\beta$-lactoglobulin and ligands for conformational searching. The caffeine molecule was defined as a rigid molecule with no torsional terms included, since it is comprised of a planar fused six and five membered ring. Quinine has greater flexibility, particularly around the secondary alcohol group that joins the quinoline rings and the quinuclidine bicyclic group. Therefore, some torsional rotations were included in the quinine ligand model. Since the caffeine was defined as a rigid molecule, the contribution to the binding free energy from loss of conformational entropy ($\Delta G_{\text{tors}}$) was zero and is not quoted in the binding free energy results. Similarly, although some torsion rotations
were included in the quinine molecule, the flexibility of the molecule was limited and $\Delta G_{\text{tors}}$ was found to be negligible compared to the total $\Delta G_{\text{binding}}$.

AUTODOCK was used to identify the 50 lowest energy binding conformations for each of the ligands. These conformations were then clustered using the mean root mean square deviation (RMSD) of the conformations to calculate conformations within 0.2 nm RMSD of each other. In this way distinct binding sites can be identified based on sites where clusters of bound ligands are observed. Each member of a cluster is considered to represent different binding orientation to the same binding site. Each binding orientation in a cluster is different and has a different binding energy, but they are close enough in energy and conformation to be considered bound to the same site. The binding energy and dissociation constant data are presented only for the lowest energy conformation of each separate cluster. By clustering the ligands bound to the binding sites in this way, the probability of finding the ligand in a particular binding site can be estimated as the fraction of the total number of searched conformations (50 in this case) that are found in a particular cluster. The most likely binding conformation is usually considered to be the lowest energy conformation (lowest $\Delta G_{\text{binding}}$) from the cluster with the highest probability (fractional number of conformations). For caffeine three binding sites were accessed with similar probability, whereas for quinine one binding site was identified with a high probability, and three of the identified clusters were, on visual inspection, binding at or close to the same binding site.

**Sensory Analysis.** Sensory testing was conducted with two levels of WPI (0% and 1%) and three levels of bitterant (low, medium, and high concentration). Because quinine is a much more
powerful bitterant than caffeine it was necessary to use different concentrations of each. In preliminary studies we tasted the concentrations used by Keast and Roper (2007) but made small adjustments to find a set that were above threshold and acceptable. The final bitterant levels were: quinine HCL = 0.056, 0.10, 0.18 mM; caffeine = 1.8, 5.7, 18 mM. The different bitterants were not formally matched in bitterness but no comparisons are made between the tastes of quinine and caffeine samples.

Untrained assessors were recruited from an opt-in participant database maintained by the Sensory Evaluation Center at Penn State. Potential participants were screened for and excluded based on contraindications related to dairy protein, caffeine, and quinine. Pregnant women, smokers, and individuals with tongue piercings were excluded. Written consent for the sensory test was obtained before tasting, and participants were compensated with a small cash incentive for their time. Tests were conducted on two separate days (quinine on one day, caffeine on the second) with two separate sets of panelists in parallel. Data were collected using Compusense Cloud software (Compusense Inc. ON, Canada). In each test session, the low, medium, and high bitterant levels were tested with and without protein. Controls of water and a WPI blank (to assess the effect of protein on the sample rating) were also included. All 8 samples were equilibrated to room temperature and presented under red light in 1 oz clear, plastic sample cups in a William’s design to reduce position, order, and carryover effects. Randomly generated three-digit blinding codes were used for sample identification. Prior to rating any samples in isolated sensory testing booths, a brief orientation to familiarize the panelists was conducted in
a common area (e.g., Antenucci and Hayes 2015); no more than 4 panelists participated at a time. The orientation sample used for this warm up task was the low concentration of the opposing bitterant (e.g. when quinine was to be tested, caffeine was used in the orientation). All participants were orientated by the lead researcher. A 10 mL orientation sample was taken into the panelist’s mouth, swished for 10 seconds, and expectorated. After orientation was complete, participants entered in isolated testing booths where they evaluated the test samples. They were asked to take a 10 mL solution in their mouth and swish for 10 seconds. They then rated the bitterness intensity on an unstructured line scale (0 = low -100 = high) and liking on a nine-point hedonic scale with the anchors of 1 = Dislike Extremely, 9 = Like Extremely while the sample was swished in the mouth. Participants then expectorated the sample and rinsed with filtered water as needed. An interstimulus interval (ISI) of 2 minutes between samples was enforced via software. 105 participants provided ratings for caffeine and 119 provided ratings for quinine. A participant could only participate in one of the bitterness tests to avoid learning / practice effects.

**Ethics Statement.** Testing was performed in two sessions in the Sensory Evaluation Center in the Department of Food Science at The Pennsylvania State University. Procedures were exempted from Institutional Review Board review by professional staff in the Penn State University Office of Research Protections under the wholesome foods/approved food additives exemption 6 in the 45 CFR 46. 101(b).
Statistical Analysis. Statistical analysis was performed with Minitab Software (Minitab Inc. PA, USA) and Compusense Cloud (Compusense Inc. ON, Canada) Software with a significance of 0.05. Initial differences among sensory samples were determined using Two Way ANOVA and Tukey’s HSD. Subsequent model development and analysis was performed on Minitab Software. All other benchtop testing differences (two sample t-test) and summary data (mean, standard error, sample distribution) were analyzed via Minitab as well.

Results and Discussion

Protein Binding Study. The WPI used throughout these tests was 14.106% nitrogen, or, using the dairy conversion factor of 6.38, 90.0% protein, which is typical. Figure 1 shows the binding behavior of caffeine and quinine in WPI solutions. Caffeine interacted minimally with WPI (e.g., 88.5% of caffeine remained unbound in 1% WPI) while quinine interacted strongly (e.g., 21.8% of quinine remained unbound in 1% WPI). A logarithmic function was used to model the binding behavior of bitterants with WPI. Because there was only very limited interaction between WPI and caffeine, the model did not fit well (p = 0.236), with an $R^2$ value of 0.4124. Quinine interacted strongly with WPI and the model gave a good fit (p < 0.001), with an $R^2$ value of 0.9707. The data support the hypothesis that WPI binds hydrophobic quinine (Log P ≥ 3) much more strongly than hydrophilic caffeine (Log P ≤ -1) (Klebanov et al. 1967; Barzanti et al. 2007). This observation is consistent with previous work showing a linear relationship between log P and log Kb (a measure of protein binding) for a wide range of compounds (Guichard and Langourieux 2000). Computational modeling was used to further classify the binding measured in this work for caffeine and quinine.
Computational Studies of Caffeine and Quinine Docking with BLG. As mentioned previously, BLG is a large percentage of the proteins present in WPI, and it is often used as the principal protein for modeling small molecule binding to whey proteins (Morr and Foegeding 1990; Kontopidis et al. 2004). AUTODOCK software was used to model the binding conformations and statistical probability of caffeine and quinine binding with BLG. Data for the cluster analysis of the AUTODOCK results for caffeine and quinine binding to BLG are presented in Figure 2. The caffeine molecule binds to a smaller number of unique binding sites (four) compared to nine sites for quinine. Of the nine sites for quinine, only six have more than one conformation as a member of the cluster and only these are considered as sensible potential binding sites. For the four potential binding sites for caffeine and the nine for quinine, the binding free energy and dissociation constant for the lowest energy conformations are show in Table 1, with a breakdown of the components of the binding energy. The binding sites of caffeine and quinine to BLG are illustrated in Figure 3. It is clear that both molecules share similar binding sites, but that the relative affinity for each site differs between the molecules. The most likely binding site is different between the two molecules and is defined by cluster 2 in caffeine and cluster 5 in quinine, although neither are the lowest energy conformations identified. The most likely quinine binding site (56% of conformations) is in a very open disordered region of the BLG molecule, whereas caffeine binds at the opening to the lipid binding beta barrel structure close to the EF loop (i.e. the amino acid loop between beta strands E and F in the BLG structure). Quinine also binds to this site (cluster 2, 3 and 6, with a combined 28% of conformations).
Quinine binds to BLG with a µM dissociation constant that is on average an order of magnitude lower (stronger) than the binding of caffeine. The stronger binding of quinine appears to be driven by both a larger contribution from van der Waals, H-bonds and desolvation of the protein, but also a stronger electrostatic interaction component which is largely missing from the caffeine docked conformations.

We should bear in mind that the BLG conformation used in the docking studies is treated as a rigid molecule, i.e. its conformation is fixed during the docking search. It is believed that many hydrophobic ligands bind to BLG in the lipid binding pocket defined by the β-strands A-H. The entry to this pocket is controlled by the flexibility of the EF loop between the E and F β-strands. The EF loop is believed to form a ‘gate’ that controls ligand entry to the lipid binding site (Sawyer and Kontopidis 2000). In our rigid BLG model used in these docking studies, the EF loop will be fixed in position, and therefore the quinine and caffeine ligands may not be able to fully explore binding in the lipid binding pocket. The dissociation constant for both caffeine and quinine are comparable to those determined experimentally for a range of saturated fatty acid ligands (C12-C18) and other molecules (SDS, β-ionone, retinol, fatty acid lactones) (Muresan et al., 2001; Bello et al., 2011; Loch et al., 2012). These compounds all bind with dissociation constants in the range 10^{-2}-10^{-8} M, with the more hydrophobic compounds having the strongest binding affinity. It is clear from the Autodock results that the binding of quinine to BLG is significantly stronger than for caffeine. Whilst Autodock detects an interaction between caffeine and BLG, this is weak compared to quinine binding. Chowdhry & Harding (2001) state that dissociation constants less than 5 µM are considered strong, whilst those greater than 50
µM are termed weak. On this basis caffeine has a weak interaction with BLG, whilst the interaction with quinine is intermediate between weak and strong.

Effect of Protein on Bitterness. Differences in binding are useful for this study in order to provide a point of contrast between results. It is generally understood that in order for something to reach the taste receptor and be perceived, it must first dissolve in saliva (Matsuo 2000; Coupland and Hayes 2014). We hypothesized that by preventing the bitterant from accessing the saliva in the mouth, binding by WPI would cause a reduction of bitterness. Specifically, added protein would suppress the bitterness of quinine but not caffeine.

Here, human psychophysical testing was used to determine how WPI (0% or 1%) influences bitterness of several concentrations of caffeine and quinine (Figures 4 and 5). Liking scores were inversely correlated with perceived bitterness (r=0.945 and 0.845 respectively, data not reported). The aqueous concentrations of all samples were measured, and there was no significant difference in the pattern of binding from the data collected in the WPI-binding study (p > 0.05).

For both bitterants in 0% WPI (water), bitterness intensity increased with increasing concentration (Figures 4 and 5), as would be expected. The observed dose-response relationships also correspond well with previously reported data (Keast and Roper 2007). The 1% WPI blank (i.e., no caffeine or quinine present) did not differ significantly in bitterness from water, suggesting there is no contribution to perceived bitterness from the WPI, at least at the concentration used here (Figures 4 and 5). There was no significant change in caffeine bitterness across all concentrations with the addition of 1% WPI (p = 0.508), illustrated in Figure
4. This was expected, because there was little chemical interaction between caffeine and WPI, as shown in Figure 1, and the measured aqueous concentration of caffeine did not substantially change with the addition of WPI.

   In contrast, WPI reduced the bitterness of quinine solutions (Figure 5). The perceived bitterness of both the low and medium quinine concentrations were significantly lower in 1% WPI compared to the same concentrations in water (p < 0.05). However, WPI did not cause a significant reduction in bitterness for the highest concentration of quinine (p = 0.0622), although the pattern was in the same direction of the two lower concentrations. Consistent with the binding study, there was a substantial reduction in aqueous concentration of quinine in the WPI samples which supports the hypothesis that strong protein binding is involved in the reduced bitterness observed for quinine.

   Linear modeling of the effects of WPI on the perception of bitterness from caffeine was not conducted given the absence of an effect in the ANOVA model (Figure 4). The effect of WPI on quinine bitterness was modeled via regression using Minitab 17 to determine which parameters were significant. The participant effect was significant (p < 0.001) as expected due to person-to-person variation in scale usage and individual sensitivity to the bitternants. Protein level was also a significant predictor (p < 0.001), indicating the importance of protein on bitterness perception. While aqueous concentration was not significant (p = 0.099), the interaction between protein and aqueous concentration was significant (p = 0.024). This suggests the aqueous concentration, as influenced by WPI, significantly affects the bitterness of quinine.
While it was expected WPI would decrease the bitterness of quinine due to its strong binding behavior, the decrease in perceived bitterness was rather modest compared to the large reduction observed in the aqueous phase concentration. This point is illustrated in Figure 6, where the open points are the dose-response functions for quinine in water, and the filled points are the aqueous concentrations in the presence of 1% WPI (i.e., the amount of bitterant not bound to the protein and not the total concentration of bitterant present). The protein samples are much more bitter than would be expected for a given aqueous concentration (i.e., the curve is shifted left on the x-axis while the slope of the lines is not changed). For example, the 0.1 mM quinine sample in 1% protein solution is almost entirely bound by the protein (6 μM aqueous concentration) yet has the same bitterness intensity as the 56 μM quinine sample in water. If the aqueous quinine concentration was solely responsible for bitter taste, then both sets of data—the response curves in water and in WPI solution—should fit on the same trend line. While there was a suppression of bitterness caused by the protein binding, this suppression was much smaller than would be expected by the reduction in aqueous concentration. This suggests the protein-bound fraction must still somehow contribute to taste and contradicts our hypothesis that the aqueous concentration of bitterant (i.e., the amount that remains unbound) predicts perceived bitterness.

Similar discrepancies have been noted elsewhere. For example, Bohin et al. (2012, 2013) evaluated the masking of bitterness of EGCG by different proteins. EGCG is typically considered to be a ligand for the bitter receptor hTAS2R39. This receptor was used in an in vitro assay to evaluate receptor activation, and these results were compared to in vivo sensory tests. Casein had the strongest binding behavior with EGCG, reduced the activation of hTAS2R39 the
most, and was rated as the least bitter by panelists. There was good agreement between the
reduction in in vitro receptor activation in the presence of casein and reduction in perceived
bitterness (38.5% and 34.3% respectively). However, predictions from the binding curve (i.e.,
based on the free, non-casein bound, EGCG concentration) suggested bitter receptor activation
should have been reduced by even more (51.9%) in the presence of protein (casein). This
observation is consistent with present data: bound bitterant still has the ability to interact with
the receptors in some capacity. However, the magnitude of the effect reported by Bohin and
colleagues (2013) was much smaller than seen here.

Two possible explanations for this discrepancy are that either a) the bound bitterant can
still be tasted or that b) the in vitro measurement of binding is not representative of the
situation in the mouth.

However, the first of these (the bitterant-protein complex can stimulate the bitter
receptor on the tongue) seems improbable as our modeling data shows strong binding affinity
between quinine and BLG (Figure 3b). Additionally, Kontopidis et al. (2004) showed that when
hydrophobic small molecules are bound by BLG, they insert deeply into the β-barrel structure
where they would be unavailable for the delicate docking required with the receptor.

Furthermore, a computer model for the binding of phenylthiocarbamide (PTC) to its principle
receptor, which also shows strong interaction with caffeine and quinine, shows that a small
molecule must be deeply inserted into the transmembrane protein receptor before the bitter
taste is triggered (Floriano et al. 2006). It seems unlikely that a small molecule would be able to
do both. The alternative explanation may therefore involve some changes to the complex in the
mouth.
When the WPI-bitterant solution is taken into the mouth, it is diluted somewhat by saliva. Assuming the saliva behaves simply as water, then changing the relative phase volumes would alter the proportion of bitterant bound. For example, if the sample was diluted in, say an equal volume of saliva, then a 1% WPI solution would become a 0.5% WPI solution. According to the binding curve (Figure 1) this would increase the proportion of free quinine from 24% to 34%. However, that addition of saliva would increase the volume of water diluting the concentration of quinine in the solution. The theoretical concentration of aqueous quinine would then become 0.022 mM as compared to the 0.13 mM original concentration in the binding studies—an equivalent measure to approximately 17% free quinine. This, in turn, cancels out any effect coming from the reduced protein binding and therefore cannot explain the large discrepancy between aqueous concentration and bitterness perception.

However, saliva is more than just water, and other components have been implicated in taste perception (Matsuo 2000; Humphrey and Williamson 2001; Dsamou et al. 2012; Melis et al. 2013). For example, saliva contains approximately 0.5-0.9% protein (Dsamou et al. 2012), and it is possible these proteins competitively bind tastants and affect taste perceptions (Fábián et al. 2015). Additionally, it has been proposed that several salivary proteins are important in delivering tastants to the taste receptors (Melis et al. 2013; Tucker et al. 2014). If this is the case, then the delivery system (i.e. water versus WPI solution) may not be as important for perception as we have hypothesized. Evolutionarily, reduced bitterness of toxins due to complexation in the food matrix would presumably reduce the protective aversive effect of bitter taste. Thus, it is not unreasonable to speculate that salivary proteins may conceivably bind and release bitterants as a means to recover function that would otherwise be lost.
Conclusion

The key conclusions of this work are (i) WPI binds quinine but not caffeine, (ii) WPI suppresses the bitterness of quinine but not caffeine, (iii) the degree of bitterness suppression of quinine by WPI is less than expected given the degree of binding. While to our knowledge, there are no published binding isotherms of caffeine or quinine to WPI, the first conclusion is not surprising given the difference in hydrophobicity of the two bitterants. Similarly, while a new observation, the second conclusion is consistent with the commonly accepted perspective in the literature. The third conclusion however, was not expected. Saliva could play a role in taste that is not accounted for in the design of this study which may explain the discrepancy observed in the third conclusion.

Acknowledgments

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Author Contributions
K. Tenney designed the study, conducted the experiments, collected data, and drafted the manuscript. J. Coupland assisted with the design of the study, interpreted the data and edited the manuscript. J. Hayes assisted with the design of the sensory experiments and edited the manuscript. S. Euston performed the computational modeling and analysis thereof.

References


Bohin MC, Vincken JP, Van Der Hijden HTWM, Gruppen H (2012) Efficacy of food proteins as


## Tables

Table 1. Binding energy components of caffeine and quinine lowest energy conformations to BLG.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Dissociation Constant (μM)</th>
<th>Binding energy (kJ/mol)</th>
<th>vdW+H-bond+ de-solvation energy (kJ/mol)</th>
<th>Electrostatic (kJ/mol)</th>
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<tbody>
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* indicates the most likely binding site
**Figures** (graphs, charts, line drawings, photographs)

Figure 1. Proportion of caffeine (0.51 mM, solid line) and quinine (0.13 mM, dashed line) bound as a function of native WPI solution concentration. Error bars indicate standard error. A logarithmic model is shown alongside each data set.
Figure 2. Cluster analysis for the bound conformations of caffeine (black bars) and quinine (white bars) bound to BLG. Autodock clusters bound conformations based on binding energy and root mean square displacement (RMSD) of the atomic coordinates. The lowest energy conformations of clusters 1-4 for caffeine, and 1-6 for quinine are shown in Figure 3.
Figure 3. a) Caffeine binding sites on BLG. Caffeine cluster 1 H-bonds with TRP19, TYR20, GLU157, GLU158. Caffeine cluster 2 H-bonds with THR4, GLN5, ALA139, LYS141, ALA142. Caffeine cluster 3 H-bonds with ARG124, THR125, GLU127. Caffeine cluster 4 H-bonds with SER36, LYS60, TRP61, GLU62, ASN63. b) Quinine binding sites on BLG. Quinine cluster 1 H-bonds with THR18, GLU44, GLU 157.
Figure 4. Mean protein sensory test results for caffeine (n=105). Bitterness intensity is plotted on the y-axis. White bars represent the samples presented in 0% WPI and black bars represent the samples presented in 1% WPI. Different letters indicate significant differences in bitterness rating (p<0.05). Error bars indicate standard error.
Figure 5. Mean protein sensory test results for quinine (n=119). Bitterness intensity is plotted on the y-axis. White bars represent the samples presented in 0% WPI and black bars represent the samples presented in 1% WPI. Different letters indicate significant differences in bitterness rating (p<0.05). Error bars indicate standard error.
Figure 6. Relationship between aqueous (unbound) concentration of quinine, plotted on a logarithmic axis, and perceived bitterness in water (○) and in 1% WPI (●). Logarithmic fit shown alongside the data. Error bars indicate standard error. The added protein bound the quinine and reduced the aqueous concentration by about an order of magnitude. However, the reduction in bitterness is less than would be expected given that change in aqueous concentration.