Binding of olive oil phenolics to food proteins

Are Hugo Pripp,* Rob Vreeker and John van Duynhoven
Unilever Research & Development Vlaardingen, PO Box 114, 3130 AC Vlaardingen, The Netherlands

Abstract: In this paper we investigate the interaction of phenolics extracted from olive oil with different food proteins (sodium caseinate, bovine serum albumin, β-lactoglobulin and gelatin). Binding parameters are estimated using different experimental techniques: gel filtration, HPLC, isothermal titration calorimetry and NMR diffusion measurements. For comparison, the binding properties of gallic acid and tannic acid are also studied. The affinity of olive oil phenolics for the different food proteins is found to be relatively weak (compared with tannic acid). Binding constants are measured for the different phenolics in the extract: tyrosol and hydroxytyrosol do not (or very weakly) bind to the proteins, whereas other phenolics in the extract had binding constants of the order 10^2 – 10^4 M^-1. The binding parameters determined have been discussed in relation to the possible effect of proteins on sensory properties (bitterness) of food emulsions containing olive oil.

Keywords: olive oil phenolics; polyphenol; protein binding; binding constant; bitterness

INTRODUCTION

Phenolic compounds are synthesized in plants. They have important functions in fruit and floral pigmentation and as chemical defence against predators. The chemical composition of phenolic compounds varies among different plants and the phenolics have been extensively characterised and classified.1 In food products, the phenolics influence quality characteristics such as taste, appearance and nutritional value. Even though taste (bitterness or astringency) and colour of many phenolics are the most obvious food technological effects, their nutritional value has received increased interest in relation to functional and healthy foods. The nutritional interest of phenolics is linked to their antioxidant activity.2 They are the most abundant antioxidants in our diets and some (poly) phenolics are believed to protect the body’s tissue against oxidative stress, which may contribute to cardiovascular diseases, cancer and inflammations.3

Most of the studies on interaction between phenolic compounds and proteins and other macromolecules have focused on larger polyphenols with protein precipitating properties, often designated as tannins.1 Much research on tannin–protein interactions has been linked to its role in astringency perception. Tannins in foodstuffs interact with proline-rich proteins in the saliva and cause them to precipitate, giving a dry mouth sensation that is referred to as astringency.4 Much of the work in this area has been on tea polyphenols and the influence of phenol–protein interaction on taste perception. It is well known, for example, that milk added to a tea fusion produces a milder and less astringent tea. It is generally believed that casein micelles bind tea polyphenols and hinder them in interacting with bitter receptors and saliva proteins, giving a reduced bitterness perception and astringency.5 Another food technological effect of phenolic–protein interactions is haze formation in beer, fruit juice and wine. Tannin–protein haze is unwanted in these products, but the affinity of tannins for proteins can also be used as a mechanism to reduce the content of phenolics during wine making. Relative concentrations of tannin and protein have a large influence on the amount of haze formed.6

Phenolic compounds in olive oil contribute to the characteristic bitter taste of olive oil and are believed to play a role in the health effects associated with Mediterranean diets. Olive oil phenolics may inhibit oxidation of low-density lipoproteins (the most atherogenic ones) and, in combination with other reported biological activities such as inhibition of platelet aggregation, this could partly account for the observed health effects of diets including high-quality olive oil.7 The phenolic compounds in olive oil are hydrolysis products of oleuropein and ligstroside and at least 30 different compounds have been identified.8 These phenolics are mainly monomeric compounds and have a chemical structure that is different from the typical tannins. It is, therefore, likely that they have different protein interaction characteristics from tannins.

A preliminary study using Klotz analysis of binding data has shown that the interaction between extracted olive oil phenolics and sodium caseinate is relatively weak. Further studies are needed to determine the binding properties of these phenolics and to evaluate their possible effect on sensory properties of olive oil-based products.
weak and that olive oil phenolics do not have properties typical for tannins (ie strong interactions and protein precipitation). In this paper we extend our earlier study on olive oil phenolics–protein interactions. Different proteins were selected on the basis of their physico-chemical characteristics or practical relevance: sodium caseinate (an important ingredient in many fabricated foods), bovine serum albumin (BSA) and β-lactoglobulin (both globular proteins and often used in binding studies) and gelatin (a proline-rich protein). The olive oil extract used was a heterogeneous mixture of different phenolics, including tyrosol, hydroxytyrosol and various ‘aglycones’ (see Fig 3). Isolation of individual phenolics from the mixture in sufficiently large quantities for binding experiments was a very tedious process; therefore no binding experiments were performed on pure (ie isolated) olive oil phenolics. For comparison, some commercially available pure phenolics were also used in this study: gallic acid (a simple phenolic acid found in many plants, including olives) and tannic acid (a member of the tannin family). A variety of experimental techniques was used in this study: gel filtration, HPLC, isothermal titration calorimetry and pulsed-field gradient NMR. All techniques were found to have specific advantages and limitations in studying the binding properties of olive oil phenolics. By combining the results obtained from the various techniques it was possible to estimate relevant binding parameters (ie binding constants and number of binding sites per protein molecule). The results obtained have been discussed in relation to the possible consequences of phenolic–protein interactions for the sensory properties (bitterness) of food products containing olive oil.

METHODS AND MATERIALS

Extraction of phenolic compounds from olive oil

Phenolic compounds were extracted according to a procedure by Montedoro et al10 by dissolving 50 g of high quality extra virgin olive oil (supplied from Unilever R&D Vlaardingen, The Netherlands) in 100 ml of hexane (Merck, Darmstadt, Germany) and shaking with 100 ml of water/methanol (Merck) (40/60) for 2 min. The water/methanol phase was washed with 70 ml of hexane, evaporated to dryness in a rotating evaporator at 45 °C, redissolved in 5 ml of water/methanol (50/50) and then analysed directly by HPLC or in the appropriate buffer when used in protein binding studies.

HPLC analysis of olive oil phenolics extract

The HPLC analysis was performed on a Waters 600 S liquid chromatograph equipped with a Waters 616 pump and a Waters 490 UV multiwavelength detector. Before HPLC injection, samples were filtered using a 0.2-µm syringe filter. Injection of the samples was carried out using a 10-µl Rheodyne sample loop. A Varian Chrompack 25 cm × 4.6 mm Intersil5 ODS2 column (Varian, Middelburg, The Netherlands) was applied using a gradient at a flow rate of 1 ml min⁻¹. The elution system consisted of solvent A (2% acetic acid in water) and solvent B (methanol); gradient: 0–20 min 15% B; 20–50 min 15–75% B; 50–55 min 75% B; 55–65 min 75–100% B; 56–65 min 100% B. UV absorption was measured at λ = 280 nm. A typical chromatogram from HPLC analysis of olive oil phenolics is shown in Fig 3. Several of the phenolics have been identified in earlier work in our laboratory (personal communication, T de Joode and AJ Knoops, Unilever Research & Development, Vlaardingen, The Netherlands).

Gel filtration

Gel filtration is a method to separate molecules based on their size difference. The method makes use of the fact that proteins (with or without bound phenolics) elute before free phenolics because of their larger size. Both proteins and phenolics can be detected by absorbance at 280 nm. If interaction occurs the protein peak absorbance increases and the free phenolic peak absorbance decreases. Prepacked PD-10 columns containing Sephadex G-25 M (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM sodium phosphate buffer, pH 6.5, were used in the gelfiltration experiments. Mixtures of extracted olive oil phenolics and proteins (2.5 ml) were injected, eluted with equilibration buffer and fractions (2.5 ml) collected. The absorbance of fractions at 280 nm was measured.

Binding properties of olive oil phenolics using HPLC analysis

An attempt was made to study the binding properties of the different phenolics in the olive oil extract using HPLC analysis. Olive oil phenolics were dissolved in an aqueous protein solution and left overnight at room temperature to establish equilibrium. The concentration of free phenolics in the solution was determined by HPLC using the conditions described above. An experimental complication was the sensitivity of the HPLC column to (high molecular weight) proteins. Small amounts of proteins easily blocked the column and special care was taken to separate the proteins (and protein-bound phenolics) from the free phenolics prior to the HPLC analysis. This was done using a microfiltration procedure, which has also been used in other binding studies.n Microfiltration with a cut-off weight of 3 kDa was performed using Microsep 3 K Omega centrifugal devices (Filtron Technology Corporation, Northborough, MA) and centrifugation at 5000 × g for 1 h at 20 °C. Measurements were performed on bovine serum albumin, sodium caseinate and (native) β-lactoglobulin (all from Sigma Chemicals, St Louis, MO). The protein concentration was varied between 0 and 0.5 wt%; pH was varied from 4.0 to 5.5 (using a sodium acetate buffer) or 5.0 to 7.0 (using a sodium phosphate buffer).
buffer). The phenolics concentration was 3.75 or 4.2 mM caffeic acid (Sigma Chemicals) equivalents (determined with Folin–Ciocalteu’s phenol reagent, F-9252, Sigma Chemicals). In some experiments gallic acid was added to the mixture of olive oil phenolics for comparison.

**Isothermal titration calorimetry**

Isothermal titration calorimetry (ITC) is a technique frequently used in binding studies. An aqueous solution of olive oil phenolics or tannic acid was added stepwise to a protein solution in the sample cell of the instrument. The sample cell is contained inside an adiabatic jacket together with a reference cell (filled with water or buffer). Injection of a small amount of phenolics into the protein solution results in the production (or consumption) of a small amount of heat in the sample cell. The heat effects arising from an injection are actively balanced by the calorimeter feedback keeping the temperature difference between the sample cell and reference cell constant. The heat flow (µcal s⁻¹) required to keep the temperature difference constant is measured and recorded as a function of time. Integration of the heat flow yields the reaction enthalpy for every injection. A typical experiment consists of a series of 20–40 injections. Subsequent injections result in an increase of the phenolics concentration in solution. The reaction enthalpy for each injection is plotted as a function of time. Integration of the heat flow yields a binding curve. In a typical experiment the diffusion coefficient of the ligand is the average of the diffusion coefficients in the free and bound forms, weighted by the molar fractions of the two forms:

\[
D_{\text{obs}} = x_{\text{free}} D_{\text{free}} + x_{\text{bound}} D_{\text{bound}}, \tag{1}
\]

where \(x_{\text{free}}\) and \(x_{\text{bound}}\) are molar fractions and \(D_{\text{free}}\) and \(D_{\text{bound}}\) are diffusion coefficients of free and bound ligands, respectively. Equation (1) can be rewritten as:

\[
x_{\text{bound}} = (D_{\text{obs}} - D_{\text{bound}})/(D_{\text{free}} - D_{\text{bound}}). \tag{2}
\]

In a typical experiment the diffusion coefficient of the ligand is measured at a series of protein concentrations. The mole fraction of the ligands bound to the protein is then calculated (using eqn (2)) and plotted as a function of protein concentration in the sample cell to yield a binding curve. Diffusion coefficients were measured using the pulsed field gradient (PFG) bipolar-stimulated echo technique. A first series of measurements was made on mixtures of gallic acid (20 mM) and BSA (0, 0.1, 0.25, 0.4, 0.6, 0.8, 1, 1.3, 1.6 and 2 mM) in 200 mM sodium phosphate buffer pH 6.8 in D₂O. A blank sample with 2 mM BSA and no gallic acid was also analysed. A Varian Unity Plus 400 spectrometer operating at 400 MHz was used for the measurements. The diffusion time (\(\Delta\)) and maximum gradient strength (\(\delta\)) were set to 400 ms and 30 G cm⁻¹, respectively. A second series of measurements was made on mixtures of olive oil phenolics (3.8 mM caffeic acid equivalents) and BSA (0, 0.03, 0.07, 0.15, 0.23, 0.30 and 0.38 mM) in the same buffer. This time a Bruker DMX 600 spectrometer operating at 600 MHz
was used, with a diffusion time and maximum gradient strength set to 400 ms and 50 G cm\(^{-1}\), respectively.

**RESULTS AND DISCUSSION**

**Gel filtration measurements**

Figure 1A shows a chromatograph for a mixture of olive oil phenolics and sodium caseinate at pH 6.5. As a reference, the chromatographs of aqueous solutions containing sodium caseinate or phenolics only are also shown. The chromatograph of the mixture shows two peaks, detected at 5 ml and 12.5 ml, respectively. The peak detected at 5 ml is attributed to UV absorption by aromatic groups in the protein or by phenolics attached to the protein. The peak observed at 12.5 ml is attributed to absorption by unbound (free) phenolics. The decrease of the peak at 12.5 ml and the simultaneous increase of the peak at 5 ml is an indication for binding. A series of gel filtration experiments at increasing sodium caseinate and BSA concentrations were performed. Figure 1B shows the reduction of the free phenolics concentration (expressed as decrease of peak area at 12.5 ml elution volume) in the mixture with increasing protein concentration. The experiments indicate that olive oil phenolics have affinity for both sodium caseinate and BSA (at pH 6.5).

Gel filtration is an efficient method to screen for binding. However, a major objective of this study was to estimate binding constants for olive oil phenolics. Dilution with eluents and also interaction of phenolics with the gel filtration material, as reported by Bartolome et al.\(^\text{15}\), make this technique less suited for estimating binding constants.

**HPLC measurements**

The binding properties of the different phenolics in an olive oil extract were studied using HPLC to quantify the unbound phenolics. BSA and sodium caseinate were used as proteins. Figure 3 shows how the concentration of free phenolics (after equilibration) changes with the protein concentration in solution at pH 6.75 (the different peak numbers correspond with different phenolics as shown in Fig 3). Figure 2 also shows results for gallic acid, which was added to the olive oil extract to allow comparison of binding properties. The influence of proteins on the

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**Figure 1.** A) Gel filtration chromatograms showing interaction between olive oil phenolics and sodium caseinate at pH = 6.5. B) Percentage unbound olive oil phenolics at different concentrations of bovine serum albumin or sodium caseinate at pH = 6.5.

**Figure 2.** Effect of increasing bovine serum albumin and sodium caseinate concentrations on percentage of free (unbound) phenolics. Free phenolics were separated by microfiltration from phenolics bound to proteins and analysed by HPLC. Drawn lines through the data points corresponding to peak 3 were calculated assuming a reversible binding mechanism (see text) with a binding constant \(K = 10000\) M\(^{-1}\) and number of binding sites \(n = 20\) for bovine serum albumin and \(K = 11000\) M\(^{-1}\) and \(n = 9\) for sodium caseinate.
concentration of free phenolics was clearly different for the different olive oil phenolics in the extract. Tyrosol did not show any sign of binding to BSA or sodium caseinate. This was confirmed by measurements using pure tyrosol (results not shown). It was therefore used in the measurements as an internal standard to reduce the noise in the data. In case of hydroxytyrosol, no sign of binding could be detected. The olive oil phenolics corresponding to peaks 3 and 6 showed significantly stronger binding to BSA and sodium caseinate than the other phenolics. An attempt was made to estimate the binding constant of phenolics corresponding to peak 3 to BSA and sodium caseinate. A simple reversible binding mechanism was assumed, where all binding sites are identical and independent and have the same (apparent) binding constant $K$. The binding constant is defined as $K = [\text{LS}] / [\text{L}] \cdot [\text{S}]$, where $[\text{L}]$ and $[\text{S}]$ denote the equilibrium concentrations of free phenolics and free binding sites, respectively, and $[\text{LS}]$ denotes the concentration bound phenolics. The total concentration of binding sites is $n[P] = [S] + [\text{LS}]$, where $n$ represents the number of binding sites per protein molecule and $[P]$ is the concentration of protein in solution (in mol l$^{-1}$). Competition for binding sites between different phenolics in the extract was ignored, which obviously is a drastic simplification. The lines drawn in Fig 2 are results from calculations based on the simple binding model, with $K = 10\,000\,\text{M}^{-1}$ and $n = 20$ (BSA) and $K = 11\,000\,\text{M}^{-1}$ and $n = 9$ (caseinate). In the calculations, the concentration of phenols (corresponding to peak 3) was assumed to be $0.16\,\text{mM}$. The calculated curves fit the experimental data reasonably well, but acceptable fits were also obtained with other combinations of $K$ and $n$. The values for $K$ and $n$ should, therefore, be considered as rough indications only (and obviously are model dependent). It is not clear whether the relatively high number of binding sites for BSA ($n = 20$) is a realistic value or whether the high number resulted from the simplifications in the model. A high number of sites could indicate that binding is not very specific and that phenolics form a ‘shell’ around the protein.$^4$ Lower values ($n = 6–8$) have been reported in the literature for binding of alkanones to BSA at pH 7.2.$^{16}$ It is conceivable that the different phenolics in the mixture are competing for binding sites (in particular the phenolics corresponding to peaks 3 and 6 of the HPLC chromatogram). No attempt was made to incorporate competitive binding in the binding model in view of the large number of different phenolics present in the olive oil extract.

Additional binding studies were performed at different values of pH (4.0–7.0). No significant effect of pH was found on the binding properties of tyrosol and hydroxytyrosol, but the binding of the olive oil phenolics corresponding to peaks 3–13 showed a slight pH dependence (Fig 4). The figure shows the total amount of these olive oil phenolics bound to proteins (calculated from the reduction of the total area under peaks 3–13) as a function of pH. Apart from data for BSA and sodium caseinate, the figure also shows data for $\beta$-lactoglobulin. The lowest binding was observed around the isoelectric point of the proteins. Binding of olive oil phenolics to sodium caseinate at pH values below 5.5 was not measured, because protein precipitation made it difficult to obtain efficient separation of free phenolics by microfiltration. The results were somewhat surprising, as an increase in phenolics–protein binding was anticipated around the iso-electric point. To explain the results, it is
speculated that protein–protein interactions increase at the isoelectric point, leading to association and causing a reduction of the number of available binding sites and thus a reduction of the amount of bound phenolics.

**Isothermal titration calorimetry**

Isothermal titration calorimetry was used as an alternative approach to determine binding properties for olive oil phenolics and tannic acid. The proteins used in this study were BSA, β-lactoglobulin, sodium caseinate and gelatin. A characteristic ITC binding curve for tannic acid titrated to an aqueous gelatin solution is shown in Fig 5. Binding constants, number of binding sites and reaction enthalpy were estimated from the ITC binding curves using the instrument’s software (assuming n independent and identical binding sites on the protein molecule). Results are shown in Table 1. Estimation of the binding parameters using ITC depends on the assumed molecular weight of the protein. For proteins with a defined structure and molecular weight such as β-lactoglobulin and BSA, this caused no uncertainty. However, for proteins with a large size distribution, such as gelatin, it may involve some uncertainty. Based on earlier analysis of the gelatin a (number-average) molecular weight of \( M_n = 71 \, 000 \, \text{g mole}^{-1} \) was used in the calculations. Results from ITC showed that the binding between tannic acid and proteins is relatively strong under the conditions used.

Extracted olive oil phenolics produced smaller heat effects than tannic acid. Small heat effects of interaction were measurable during titration of an aqueous solution of BSA and gelatin, but not in case of β-lactoglobulin (no measurements were made for sodium caseinate). As an example, Fig 5D shows the ITC curve for titration of extracted olive oil phenols to a BSA solution at pH 4.0. Heat effects were found to be less than 1 kcal mole\(^{-1}\) of injectant (after correction for dilution of olive oil phenolics in the buffer solution). Unfortunately, it was not possible to estimate the number of binding sites or the binding constant from the ITC data. However, the data clearly

<table>
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<th>Protein</th>
<th>BSA</th>
<th>Gelatin</th>
<th>β-Lactoglobulin</th>
<th>Caseinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K (\text{M}^{-1}) )</td>
<td>2.7 \times 10^4</td>
<td>3.6 \times 10^4</td>
<td>5.9 \times 10^3</td>
<td>2.6 \times 10^5</td>
</tr>
<tr>
<td>( n )</td>
<td>14</td>
<td>48.5</td>
<td>6.4</td>
<td>5.3</td>
</tr>
<tr>
<td>( H (\text{kJ mol}^{-1}) )</td>
<td>-21.7</td>
<td>-66</td>
<td>-32.4</td>
<td>-31.5</td>
</tr>
</tbody>
</table>
indicate that heat effects associated with binding of olive oil phenolics to the various food proteins are much smaller than those of tannic acid (under the conditions used).

**NMR diffusion measurements**

NMR diffusion measurements were performed for mixtures of gallic acid and BSA at different concentrations. The diffusion coefficient of gallic acid decreased from \( D = 8 \times 10^{-10} \text{m}^2 \text{s}^{-1} \) in the absence of BSA to \( D = 3 \times 10^{-10} \text{m}^2 \text{s}^{-1} \) in the presence of 2 mM BSA (Fig 6). The decrease of \( D \) was assumed to result from binding of gallic acid to BSA (the diffusion coefficient of the latter was estimated as \( D = 5.4 \times 10^{-11} \text{m}^2 \text{s}^{-1} \)). The diffusion coefficients were used to calculate the fraction of gallic acid bound to BSA (eqn (2)); in the calculation the diffusion coefficient of gallic acid bound to the protein was assumed to be similar to that of BSA. The results are also plotted in Fig 6.

The data are interpreted using the simple binding model that has also been used to interpret the HPLC data. Again we consider a 1:n binding stoichiometry between the protein and gallic acid and assume that all \( n \) binding sites are independent and identical. The fraction of ligand bound, \( x_{\text{bound}} \), is then given as:\(^{18}\)

\[
x_{\text{bound}} = \frac{n[P_0] + [L_0] - K^{-1}}{2[L_0]}
\]

\[
-\sqrt{\frac{(n[P_0] + [L_0] - K^{-1})^2}{4[L_0]}} - 4n[P_0][L_0]^{-1},
\]

where \([P_0]\) and \([L_0]\) represent the total concentration of protein and phenol, respectively, and \( K \) is the binding constant. The drawn line in Fig 6 is the result of a calculation using \( K = 62.5 \text{M}^{-1} \) and \( n = 30 \). The line fits the experimental results reasonably well, but acceptable fits were also obtained with combinations of other \( K \) and \( n \) values. The values for \( K \) and \( n \) should, therefore, be considered as rough indications only. The low binding strength is in line with the results from HPLC measurements (Fig 2) and a previous analysis of gallic acid–BSA interactions using Klotz plots;\(^{9}\) the number of binding sites is larger than obtained from the HPLC measurements. The results indicate that binding of gallic acid to BSA is weak compared with typical tannins.

NMR measurements were also performed for mixtures of olive oil phenolics and BSA. The \(^1\)H NMR spectra of the mixtures are rather well resolved (Fig 7). Resonance observed at 7.08 and 6.76 ppm could be assigned to tyrosol; no attempt was made to assign the other resonances to the different olive oil phenolics in the extract. The diffusion coefficient of tyrosol was found to decrease slightly with increasing BSA concentration (results not shown). Using the simple binding model described above, a binding constant \( K = 1–10 \text{M}^{-1} \) was estimated (the number of binding sites was arbitrarily set at \( n = 30 \)). This is in line with the results from HPLC measurements, which also showed very weak (or no) binding for tyrosol. It should be noted that the simple model used to interpret the results neglects competition between the different phenolics in the mixture for binding sites. This, obviously, is a drastic simplification and may lead to an underestimation of \( K \). Binding constants were also estimated for the other olive oils phenolics in the mixture using the unidentified resonance peaks in the spectrum. The diffusion coefficients of phenolics corresponding to several of these resonances decreased in the presence of BSA. The binding constants derived from the data (using the simple binding model and neglecting competition effects) are of the order of \( K = 200–300 \text{M}^{-1} \) (using an estimated number of binding sites \( n = 30 \)). For some resonances, the results suggest stronger binding with binding constants \( K > 1000 \text{M}^{-1} \). The binding constants deduced from

\[\text{Figure 6. Diffusion coefficient (A) and fraction of gallic acid bound (B) at different BSA concentrations (pH 6.8). The drawn line represents a best fit of eqn (3) to the data with } K = 62.5 \text{M}^{-1} \text{ and } n = 30.\]

\[\text{Figure 7. NMR spectrum of a mixture of olive oil phenolics and BSA (pH 6.8). In the spectrum A and B are assigned to weakly binding olive oil phenolics with } K_b = 300 \text{M}^{-1} \text{ and } K_b = 200 \text{M}^{-1} \text{, respectively, and C and D are assigned to stronger binding compounds with } K_b > 10^3 \text{M}^{-1} \text{ and } K_b > 10^5 \text{M}^{-1} \text{, respectively.}\]

the NMR diffusion measurements are in line with the results from HPLC and titration measurements.

Implications for bitterness perception

From a food technological point of view, interactions between phenolics and proteins are of interest as a possible means to reduce bitterness. In this section we use the interaction parameters obtained for olive oil phenolics to discuss the possible effect of proteins on the bitterness of food emulsions containing olive oil. This was done on the basis of calculations using a simple model for bitterness perception, where it is assumed that (olive oil) phenolics bound to proteins are not perceived by bitterness receptors on the tongue. In this simple model, bitterness is related to the concentration of free (unbound) phenolics in the aqueous phase rather than to the overall phenolics concentration in the food. In a realistic taste model, the situation is probably more complex. Here, however, we will accept the simple picture and try to illustrate the relevance of protein–phenolics interactions for bitterness perception by calculating the free phenolics concentration in the presence of proteins. As many food products are oil–water emulsions, we explicitly consider the influence of an oil phase.

In an emulsion system, olive oil phenolics will partition between the aqueous phase and the oil phase, as described by an equilibrium partition coefficient \( P_{o/w} \). The free phenolics concentration in the aqueous phase may then be calculated from the following equations:

\[
K = \frac{S L_{aq}^{\text{free}}}{L_{aq}^{\text{free}} \times S_{aq}} \quad \text{and} \quad P_{o/w} = \frac{L_{oq}^{\text{oil}}}{L_{aq}^{\text{free}}},
\]

where \( L_{aq}^{\text{free}} \) represents the concentration of free phenolics in the aqueous phase, \( S_{aq} \) and \( S L_{aq}^{\text{aq}} \) represent the concentration unreacted and reacted protein binding sites, respectively, and \( L_{oq}^{\text{oil}} \) is the (free) phenolics concentration in the oil phase. A single value of \( P_{o/w} \) will be assumed in the calculations \( (P_{o/w} = 1) \), although different values may be expected for the (hydroxy)tyrosols and aglycones in the olive oil extract. Results of calculations are shown in Fig 8 for two different binding constants: \( K = 500 \text{M}^{-1} \) (representative for weakly binding olive oil phenolics) and \( K = 10\ 000 \text{M}^{-1} \) (representative for the phenolic compound corresponding to peak 3 in Fig 3). The figure shows the concentration of free (unbound) phenols in the aqueous phase as a function of protein concentration (wt%) in the aqueous phase. The protein molecular weight was assumed to be \( 2 \times 10^4 \text{Da} \), and \( n = 10 \), and the amount of phenols was fixed at 0.1 mM (in actual product applications the phenol concentration will vary with the amount of oil). The results in Fig 8 show that the amount of free phenols in the aqueous phase decreases with increasing protein concentration, but the decrease is smaller in emulsion systems than in purely aqueous systems. The results thus indicate that higher protein concentrations in the aqueous phase are required to reduce free phenol concentration (and bitterness) in emulsion systems. For phenols with relatively low binding constants \( (K \approx 500 \text{M}^{-1}) \), the protein concentration required to significantly affect bitterness may be too high to be practically feasible. These simple calculations thus illustrate the practical relevance of the binding parameters as determined in this study: they allow one to make a rough prediction of the amount of protein required to obtain a detectable reduction of bitterness perception in emulsion foods containing olive oil. Some of these predictions have been tested in sensory tests; for a detailed discussion the reader is referred to Reference 20.

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Figure 8. Calculation of concentration free phenolics in the aqueous phase as a function of protein concentration for (A) \( K = 500 \text{M}^{-1} \) (representative for some of the weakly binding olive oil phenols) and (B) \( K = 10\ 000 \text{M}^{-1} \) (representative for relatively strongly binding olive oil phenolics). Total phenolics concentration is 0.1 mmol L\(^{-1}\). The protein in the calculation has molecular weight \( M_w = 20 \text{kDa} \) and number of binding sites \( n = 10 \). The oil–water part it on coefficient was assumed to be \( P_{o/w} = 1 \).
analysis and Anneke Groenewegen and Afranina Leika are acknowledged for the NMR measurements.

REFERENCES


