Food proteins as potential carriers for phenolics

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ABSTRACT

The development of phenolic-rich functional foods is often limited by the off-tastes of phenolics that might be counteracted by sequestering these compounds using a carrier, thereby preventing them to interact with bitter taste receptors and salivary proteins. A range of common animal food proteins were tested for binding of phenolics. It appeared that a proline-rich open protein structure, as in β-casein, favored binding of phenolics. Globular proteins other than bovine serum albumin showed poor potential for use as carrier. No appropriate carriers for monomeric phenolics were found. β-Casein and Na-caseinate were shown to have good bitter-masking potential for EGCG, as measured by a maximal reduction in bitter receptor activation of ~93% measured *in vitro*. This effective reduction in bitter receptor activation was confirmed by a sensory test. This illustrates the validity of using food proteins with good binding properties as carriers for phenolics.

Different methodologies for probing the interaction between proteins and phenolics were developed: (i) ultrafiltration followed by UV quantification of unbound phenolics in the retentate, (ii) fluorescence quenching, and (iii) ultrafiltration followed by mass spectrometric quantification of unbound phenolics in the retentate. The latter method offered the opportunity to analyze preferential binding to protein of individual phenolics present in a complex mixture. With these methods, it was established that, with respect to phenolics, conformation and flexibility were important drivers of protein-phenolic interaction, besides degree of polymerization and galloylation. With respect to relatively proline-poor unstructured proteins such as α-casein and β-casein, it appeared that there should be other factors, besides proline density, explaining the interaction with phenolics.

TABLE OF CONTENTS

Chapter 1

General introduction

BIOACCESSIBILITY AND ORGANOLEPTIC ASPECTS OF PHENOLICS IN FOODS

Phenolics are widely distributed and abundant in plants and have been associated with multiple benefical effects on human health (e.g. cardioprotective, anticarcinogenic, neuroprotective) (*1,2*). As a consequence, the enrichment of foods with phenolics is seen as a potential tool for dietary-mediated disease prevention (*3*). A simple addition of phenolics to a food product, however, can be limited by the significant contribution of phenolics to the organoleptic properties of foods (*4,5*). A summary of the potential problems encountered in phenolic-rich food products prior and during the first part of digestion is given in **Figure 1**. Amongst those issues, dietary phenolics have been associated with astringency and bitterness of food products, which can be related to the unpalatability of some phenolic-rich foods (*3,6*). Bitterness is the result of the interaction of bitter compounds (e.g. phenolics) with bitter taste receptors located on the tongue (*7,8*). The mechanism underlying the perception of astringency is not yet clearly defined. Nevertheless, it can be partially attributed to interactions of phenolics with salivary proteins causing their precipitation and a loss of lubrication in the mouth (*9,10*).

The bioactivity of dietary phenolics is linked to their bioavailability in the human body, i.e. the rate and extent to which they are absorbed through the gut epithelium and available at the site of action, as such or metabolized (*11*). A major factor determining the absorption of phenolics in the gut is their bioaccessibility, i.e. the amount of compound reaching the intestinal lumen in an absorbable form (*11*). Prior to their delivery to the gut, phenolics can be exposed to various conditions, possibly impacting their bioaccessibility (**Figure 1**). In foods and during food processing, they may be sensitive to oxidation at neutral to basic pH (*12,13*), at increasing temperatures (*14*), or in presence of metal ions (*15*). Oxidation of phenolics can lead to covalent interaction with proteins and free amino acids (*16,17*), thereby affecting the bioaccessibility of phenolics, but also the digestibility and techno-functionality of proteins (*5*). In addition, non-enzymatic and enzymatic oxidation of phenolics can lead to unwanted discoloration of food products (*5,18*). Finally, phenolics can interact non-covalently with food macromolecules, possibly forming unwanted hazes (*5,19*). Bioaccessibility of phenolics is also at stake during the digestion process. The neutral pH of the duodenum and the release of macromolecules and metal ions from the food matrix can be detrimental to the stability of phenolics, and consequently to their bioaccessibility (*13,20*). Furthermore, phenolics, in particular tannins, can inhibit digestive enzymes, thereby reducing the nutritional benefits of the food ingested (*18,21,22*).

Optimal stability, bioaccessibility and palatability are the main challenges for the improvement of existing phenolic-rich foods and the design of functional foods. A tailored food formulation might provide solutions to these challenges.

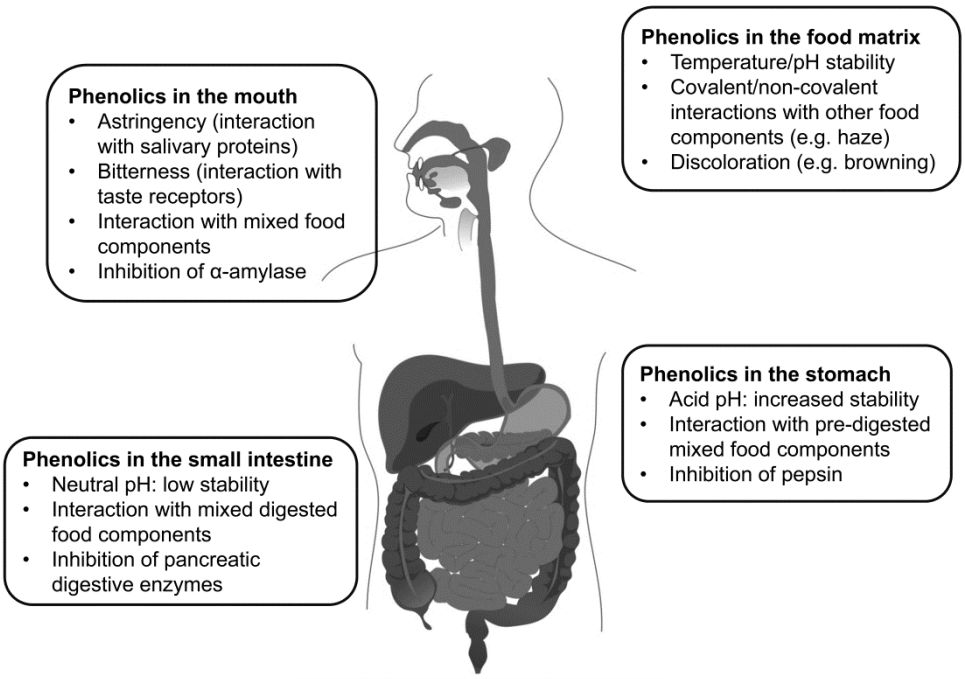

Figure 1. Schematic summary of the various effects of phenolics on the organoleptic properties and digestibility of foods, together with aspects influencing the bioaccessibility of phenolics

STRATEGIES FOR STABILIZATION AND TARGETED DELIVERY OF PHENOLICS IN FOODS

The design of functional foods rich in phenolics requires the design of carriers able to sequester phenolics to bypass potential bitterness/astringency issues and stabilize them from the environment until their release into the intestinal lumen (*23,24*). A range of encapsulation technologies is available from pharmaceutical research, which might be adapted for the delivery of phenolics by foods. Some examples of existing food-based delivery systems are given in **Table 1**. The use of several of these technologies has led to clear improvements in the stability and bioavailability of target phenolics (*25*). Some of them, however, still require an evaluation of their potential toxicity (e.g. nanoemulsions (*26*)) or have limited applications (e.g. protein hydrogels, limited to solid food (*24*)). In addition, the relatively small economic margins in the food industry limit the application of advanced delivery technologies and require the design of easily implementable and cost-efficient delivery systems (*27*).

Delivery systems need to be made of compounds generally recognized as safe (GRAS). In this regard, food proteins from animal or plant sources offer interesting properties (*24,36*). The formation of complexes by mixing the bioactive compound of interest with a

food protein, as exemplified with milk proteins in a recent review (*37*), seems promising. Moreover, the design of phenolic carriers for the food industry requires a fundamental understanding of the interaction at play during the formation of such complexes (*24*).

Table 1. Examples of encapsulation technologies available for the delivery of phenolics

Method	Description	Examples
Nanoemulsion	Clear emulsion (usually oil-in-water) with droplet size between 10-100 nm (controlled by process, surfactant and oil used)	(*26,28*)
Microemulsion	Similar to a nanoemulsion (particle size between 5-100 nm) but thermodynamically stable under a given set of conditions	(*29,30*)
Liposomes	Spherical bilayers of phospholipids (size below 300 nm to avoid visible light scattering)	(*31,32*)
Hydrogel beads	Entrapment of bioactive compound in a gel network by mixing it with a biopolymer and subsequently adjusting the environmental conditions to form and stabilize the beads (e.g. drop-wise addition of an alginate matrix in a $CaCl_2$ solution)	(*33,34*)
Molecular inclusion	Cage-like structure (typically cyclodextrins) interacting with phenolics in its core	(*23,35*)

In industrial applications related to encapsulation of bioactive compounds, animal-based food proteins are widely applied, contrary to plant-based proteins (*36*). Food proteins from animal origin have the advantage of having a relatively bland taste in comparison to plant proteins.

THE DRIVING FORCE IN INTERACTIONS BETWEEN PHENOLICS AND PROTEINS

Non-covalent interactions between proteins and phenolics are mainly driven by hydrophobic interactions and hydrogen bonding (**Figure 2**). The former are related to interactions between the aromatic rings of phenolics and apolar amino acid residues. Hydrogen bonds are formed between the hydroxyl groups of phenolics and the amine or carbonyl group of amino acid residues, and potentially with some of the amino acid side chains (*38*). Electrostatic interactions are not known to contribute directly in protein-phenolic interactions. They have been suggested to play an indirect role as protein-phenolic interaction seemed to be enhanced at pH close to the isoelectric point of the protein (*39,40*). Furthermore, at the acidic or neutral pH in foods, most phenolics are not expected to be charged as the pKa of phenolate groups is higher than 7 which excludes the possibility of ionic interactions (*38*).

Complexation between protein and phenolics has been described in some instances as a surface phenomenon with “hydrophobic effects” as principal driving force, further enhanced by hydrogen bonding (*41,42*). Haslam hypothesized that hydrogen bonding between phenolic and protein would lead to a net gain in entropy because it would increase

the mobility of water molecules initially bound to either the phenolic or the protein (*41*). The predominance of hydrophobic interactions in protein-phenolic interactions is supported by other studies involving proline-rich peptides and proteins, such as gelatin or poly-(L-proline) (*43*). These interactions occur primarily by stacking of the aromatic rings of the phenolics with the prolyl rings of proline residues, although phenylalanine is also described to be involved in π-π stacking (*44-46*). On the contrary, Hagerman and Butler reported a prevalence of hydrogen bonding when screening the precipitation of various proteins by condensed tannins (*40*). This was supported by the interaction of procyanidin B3 with the proline-rich peptide $IB7_{14}$, which was suggested to be mainly driven by hydrogen bonding between the hydroxyl groups of its phenolic rings and the carbonyl groups of proline residues (*47*). In the particular case of globular proteins having a binding cavity, such as bovine serum albumin (BSA), a predominance of hydrogen bonding has also been reported for BSA-epicatechin interaction (*48*).

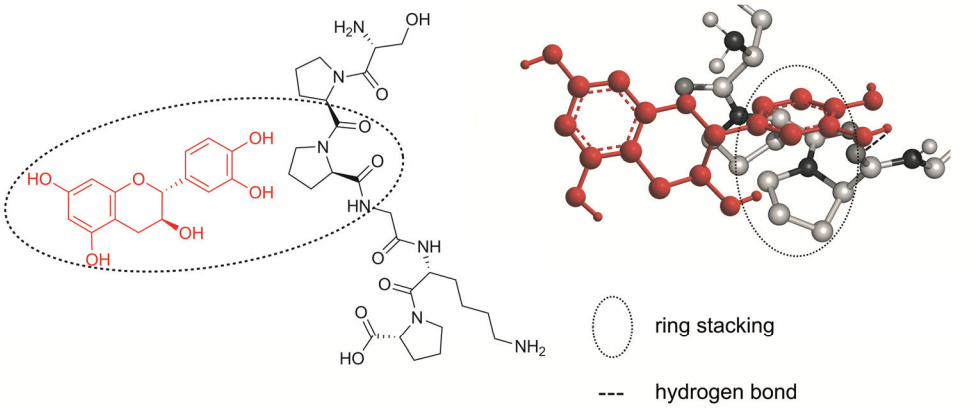

Figure 2. Generally described interactions occurring in protein-phenolic binding (theoretical peptide SPPGKP taken from the salivary proline-rich protein IB7 (*49*))

Typically, proline residues seem to be consistently involved in the interactions between phenolics and proteins. In a peptide sequence, a carbonyl function neighboring a tertiary amide group, as in proline, is a better hydrogen bond acceptor than that neighboring a secondary amide group, as in the other amino acids. Hence, proline's carbonyl function is a favored site for H-bond formation and stabilization of the complexes (*41*). Furthermore, proline has the particularity of being able to accommodate both hydrophobic interactions and hydrogen bonding, which makes it a preferred site of fixation for phenolics.

To date, there is no consensus on whether hydrogen bonding or hydrophobic interaction is the driving force in protein-phenolic interactions, or whether it is actually the concerted action of the two (e.g. (*49*)). It is likely that a generic model for protein-phenolic interaction cannot be established as many other factors influencing these interactions are at

play. The relevance of these factors depends on the structural and chemical properties of the protein and phenolics involved in the interaction, as well as on the experimental conditions employed (e.g. use of organic cosolvent) (*38,39,47,48*).

STRUCTURAL AND CHEMICAL PROPERTIES OF PHENOLICS INFLUENCING INTERACTIONS

Considering the large diversity of phenolic structures, several critical structural features modulating protein-phenolic interactions have been highlighted, although synergy of these features in influencing the interactions should always be kept in mind. Some representative structures of phenolics discussed in this part are given in **Figure 3**. The most frequently reported category of phenolics are called "tannins" and are subdivided into hydrolysable tannins (carbohydrate core esterified with phenolic acids) and condensed tannins (polymers of flavan-3-ols, also called "proanthocyanidins"). Procyanidins belong to the condensed tannins and are polymers of the flavan-3-ols catechin/epicatechin. Flavan-3-ols belong to the flavonoids, a category of phenolics often reported to interact with serum albumins. To date, only scarce information is available on the interactions of monomeric flavonoids, other than flavan-3-ols, with common food proteins, other than BSA.

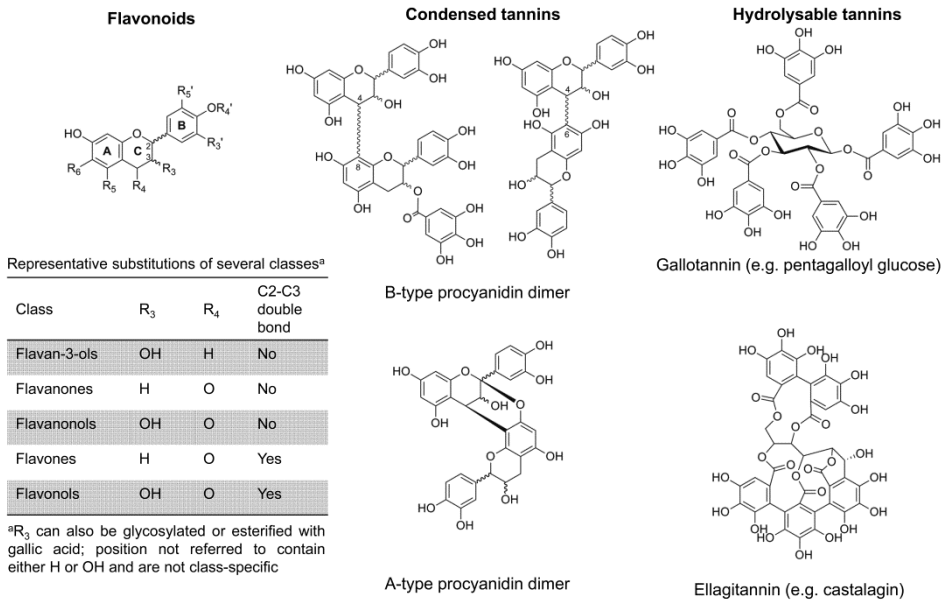

Figure 3. Structure of flavonoids and representative tannins

Degree of polymerization

The affinity of proanthocyanidins for proteins generally increases with increasing degree of polymerization (DP). Fractions with an intermediate average DP (mDP between 5 and 8) were found to better precipitate salivary proline-rich proteins (PRPs) than fractions with a mDP between 2 and 4, which remained in solution (*50*). This was confirmed by studies showing a stronger binding of procyanidin dimer B2 and an oligomeric procyanidin fraction to model proline-rich proteins, in comparison to monomeric flavan-3-ols (*44,51*). The ability of procyanidins to precipitate PRPs, α-amylase or BSA increases with increasing molecular mass, until a mass of 3400 Da, and tends to decrease afterwards. This optimum DP for binding was related to a loss of conformational freedom with increasing molecular size (*52*). The effect of DP on protein binding appeared to be dependent on the protein used (*53,54*).

Conformational flexibility

The flexibility of the molecule and conformational mobility of substituents significantly influence the affinity of different B-type procyanidin dimers, as suggested by the stronger interaction observed for C4-C8-linked procyanidin dimers in comparison to C4-C6-linked procyanidin dimers when binding to proline-rich proteins (*53*). B-type procyanidin dimers exist in solution as compact and extended conformers, and the proportion of both conformers varies for several C4-C8-linked procyanidin dimers (*55*). B-type procyanidin dimers with the largest proportion of extended conformer also displayed a higher affinity for salivary proteins compared to predominantly compact procyanidin dimer conformers (*49*). Procyanidins also exist as A-type, bearing at least one double interflavanic linkage in their structure (*56*). This additional linkage is expected to reduce the conformational flexibility of A-type procyanidin dimers and, hence, their affinity to proteins. A comparison of A and B-type procyanidins for their binding affinity to proteins has never been reported.

Galloylation

In procyanidins and hydrolysable gallotannins, an increase in the number of galloyl groups has been correlated with an increase in binding affinity to proteins. An increase in the number of gallate groups in a galloyl D-glucose series led to an improved binding, which was related to an increased hydrophobicity of the phenolic and to the number of sites available for multiple cooperative interactions (*41,44,45*). In wine fining, a preferential precipitation of polymeric procyanidins containing gallate groups over non-galloylated ones was observed (*57*). This positive influence of gallic acid esters on protein binding was also described for monomeric flavan-3-ols (e.g. epicatechin (EC) and epicatechin gallate (ECG)) (*51,54,58*). Besides offering opportunities for ring stacking and hydrogen bonding, the additional galloyl substituent in ECG or epigallocatechin gallate (EGCG) has greater flexibility than the other phenolic rings. As with the galloyl D-glucose series (*44*), its flexibility allows EGCG to bind in a multidentate fashion (i.e. with multiple anchor points) to neighboring amino acid residues, which explains its higher affinity than that of

non-galloylated flavan-3-ols (*46*). This effect is also highlighted with hydrolysable tannins as a lower binding affinity was measured for ellagitannins than for gallotannins, the latter bearing flexible gallic acid units contrary to the former (*41,42,59*). This effect, however, could also be related to the higher hydrophobicity of the gallotannin pentagalloyl glucose compared to the ellagitannins vescalin and castalagin (*41*).

Degree of hydroxylation of phenolics

Epigallocatechin-rich condensed tannins, which bear three hydroxyl groups on their B-ring, have been shown to be selectively precipitated by a high molecular mass gelatin fining agent (*57*). The positive influence of this additional hydroxyl group in comparison to epicatechin has also been reported for interactions with α-casein and β-casein (*60*). Other flavonoids with variable hydroxylation patterns have mostly been investigated for their interaction with BSA, which can accommodate numerous ligands in its specific binding sites. A systematic comparison of quercetin, quercetin derivatives and similar flavonoids with variation in hydroxylation of their B and C-rings highlighted the importance of the 3' and 4' hydroxyl groups on the B-ring for enhanced binding to BSA (*61*). These findings are difficult to extrapolate to other proteins because of the specific binding mechanism of serum albumins via their binding pockets.

Other structural properties of monomeric flavonoids

The stereochemistry of phenolics might also play a role in their interactions with proteins as a higher haze-forming capacity was reported for (+)-catechin binding with PRPs compared to (-)-epicatechin (*53,62*). Furthermore, the planarity of the C-ring in flavonoids might be a factor of importance in phenolic-food protein interactions. In fact, a non-planar C-ring, as in naringenin (flavanone (**Figure 3**)), was hypothesized to be the reason for a decreased affinity of flavonoids for serum albumins, compared to planar flavonoids, such as quercetin (*61*).

Overall hydrophobicity

As mentioned above, an important chemical property of phenolics for their interaction with proteins is their hydrophobicity. This property is directly linked to the substitution of flavonoids with, for example, gallic acid or a carbohydrate moiety. Hydrophobicity is related to the organization of the water shell around the potential binding sites of the phenolics: if a phenolic is perfectly well soluble in water, i.e. the water molecules are well organized around it, there is very little driving force for its interaction with proteins, because of the unfavorable energy that would result from the reorganization of the water molecules (*41*). This was illustrated, for example, by the correlation between the logPs of monomeric flavan-3-ols and their interaction with poly(L-proline) (*51,58*). One important consequence of this is that the nature of the solution in which the interaction takes place influences the mode of interaction and its strength (*5,47*). Furthermore, glycosylation of quercetin, such as in rutin or isoquercetin, reduces its hydrophobicity, and consequently

decreases its affinity to BSA in comparison to the aglycone. This effect may also be related to steric hindrance limiting the access of the glycosides to the binding pocket of BSA (*39,61,63*).

STRUCTURAL AND SURFACE PROPERTIES OF PROTEINS INFLUENCING INTERACTIONS

The ability of proteins to interact with phenolics depends on their size, secondary and tertiary structure, flexibility, amino acid composition, isoelectric point and glycosylation. As for phenolics, these properties are inter-related, but will be highlighted separately.

Protein tertiary structure

Although only few proteins have been systematically compared in a single study, globular proteins (e.g. BSA) have been consistently reported to have a lower binding affinity for procyanidins and hydrolysable tannins in comparison to unstructured proteins (e.g. gelatin hydrolysate) (*40,64,65*). This was related to the open structure of gelatins, which enhances the accessibility of important amino acid residues potentially involved in binding of phenolics, such as prolines (*40*). In general, the interaction of globular food proteins with phenolics is not extensively reported in literature, but a similar effect of their overall structure is expected (e.g. (*39,66*)). Unstructured proteins are expected to bind more tannin molecules per mole of protein compared to globular proteins, as illustrated with hydrolysable tannins (*59*). Hence, this type of protein might be more appropriate as a carrier for phenolics.

Size of proteins

An increased size of protein has been related to a higher binding affinity when comparing a PRP repeat with a full-length PRP (*67*). This effect cannot be solely explained by a larger number of binding sites per mole in larger unstructured proteins like PRPs, but was also associated to cooperative intramolecular interactions involving folding and wrapping around the ligand (*67,68*). This disorder-to-order transition upon interaction with phenolics was also observed with EGCG binding to the salivary protein IB5 (*69*).

Content and distribution of amino acids in the protein sequence

Proline is a recurring key amino acid involved in the interaction of protein with phenolics throughout literature (*40,42,44,45*). The presence of two consecutive proline residues (so-called 'Pro-Pro repeat') makes the N-terminal proline of the repeat a particularly favored binding site because of the conformation imposed by the following proline (*44*). The proline density in the PRP IB5 causes the occurrence of residual poly-(L-proline) helical structures (PPII helices), which have been described as preferred binding sites for EGCG (*70,71*). Other amino acids are known to be involved in phenolic-protein interaction as well, such as phenylalanine (interaction by π-π stacking), arginine or glycine (interaction

by hydrogen bonding) (*44-47*). Other aromatic amino acids (i.e. tryptophan and tyrosine) may also be involved in π-π stacking, although no reports have stated this. Their limited exposure at the surface of proteins is probably hindering potential interactions. Histidine also appears to participate in protein-phenolic interactions, particularly in the case of histatins, which are histidin-rich salivary proteins not containing any proline. Interaction of EGCG with the imidazole ring of histidine in histatins, and some hydrophobic amino acid side chains, was demonstrated (*72*). However, conflicting data have been reported elsewhere (*45*).

As reported for Pro-Pro repeats, the primary sequence of a protein is of importance for the accessibility of the potential binding sites. The location of aromatic amino acids in the vicinity of proline residues promotes their *cis*-configuration, which has a higher affinity for phenol than the *trans*-configuration, but disfavors the formation of PPII helices (*73,74*). Although not investigated yet, the influence of aromatic amino acids in food proteins on the configuration of proline residues may also impact on their interaction with phenolics. The presence of bulky side chains around a potential binding site can hinder interactions (*42*). Glycine has been linked to an increased flexibility of the protein backbone, thereby improving interactions by the possible adaptation of the protein to the ligand (*47,75*).

Surface properties of proteins

Few reports have evaluated the influence of several surface properties of proteins on protein-phenolic interactions depending on the medium used. Change of pH was reported to affect the interaction of ferulic acid with BSA, lysozyme, whey proteins and gelatin (*39*). However, this effect was not observed for chlorogenic acid binding BSA (*76*) or EGCG interacting with a proline-rich peptide (*45*). The surface exposed hydrophobicity of globular proteins was also related to differences in binding affinity for phenolics (*66,76*).

Protein glycosylation

The effect of protein glycosylation on protein-phenolic interaction is not very well documented. The effect of glycosylation in salivary proteins was mostly reported to be indirect by improving the solubility of colloidal complexes formed upon tannin binding, thereby requiring a higher concentration of tannins for precipitation to occur (*50,77*). In one instance, interactions between procyanidins and a proline-rich glycoprotein were suggested to be stronger than that with the deglycosylated form of the protein, as determined by a competitive binding assay with precipitation of labeled BSA (*78*).

MODEL FOR THE INTERACTION OF PROTEIN WITH PHENOLICS AND THEIR AGGREGATION

Upon interaction, different complexes between proteins and phenolics can be formed, possibly leading to the formation of insoluble aggregates. With the aim of designing

carriers for phenolics, soluble complexes are preferred over insoluble complexes because of their expected broader potential for food application. The interaction of phenolic acids and their derivatives with globular proteins, using BSA, has been described as monodentate (i.e., one anchor point on the protein) and modeled as a progressive surface coating resulting in precipitation once the hydrophobic coating is large enough (*79*). Contradicting results were reported for chlorogenic acid binding to food-related globular proteins, which showed relatively low binding affinities without influence on protein solubility (*76*).

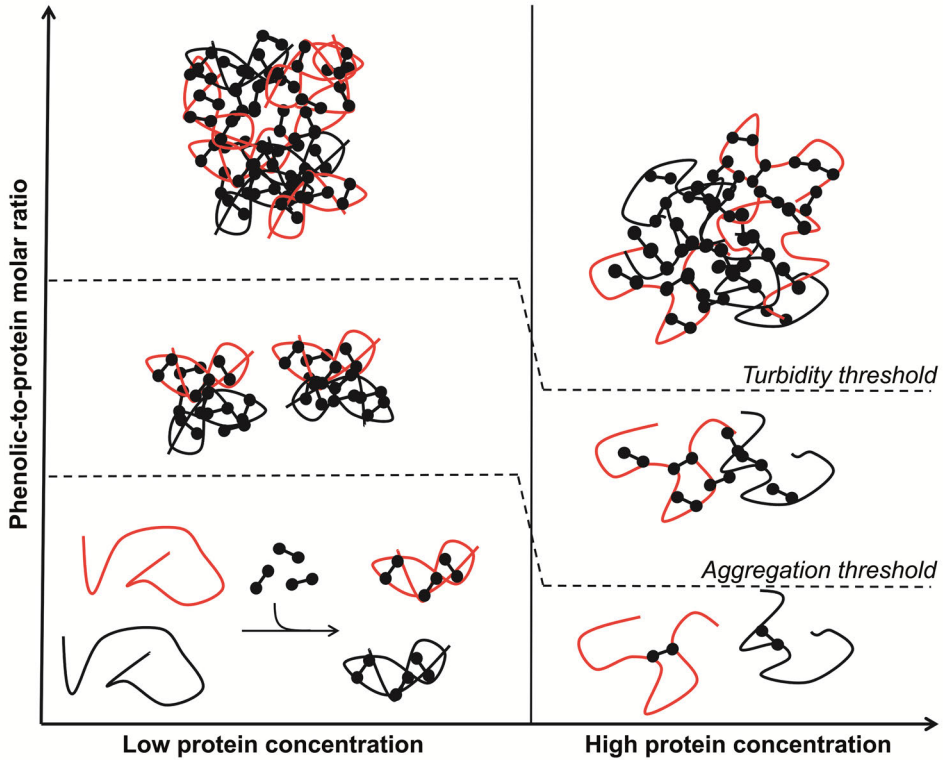

Figure 4. Model for phenolics binding unstructured proteins in a multidentate fashion (adapted from (*69*))

Phenolics bearing several rings can act as multidentate ligands and their interaction with globular proteins can lead to different aggregation behavior depending on the phenolic-to-protein molar ratio and the protein concentration used. At low concentration, globular proteins have been suggested to accommodate phenolics on their surface until the layer formed causes aggregation and precipitation. At high protein concentration, multidentate phenolics can cross-link globular proteins before saturating their surface. This results in the formation of larger aggregates and precipitation at lower phenolic-to-protein molar ratios compared to the previous situation (*79*). A similar model has been developed

to describe the multidentate interaction of phenolics with unstructured proteins. The same effect of protein concentration, as for globular proteins, on the aggregation threshold of unstructured proteins was reported (*45,58,68,69,80*).

In **Figure 4**, the multidentate binding model for unstructured proteins is described. It comprises the same succession of steps at low and high protein concentrations, only they will occur at lower phenolic-to-protein molar ratios at high protein concentration. The steps can be described as follows:

1) The ligand progressively coats the protein, which leads to a compaction of the unstructured protein (so called 'disorder-to-order' transition (*69*)). Assuming that one proline residue is one binding site, a multidentate ligand, such as EGCG, can bind at several neighboring binding sites (*46*), thereby causing the compaction of the unstructured protein (e.g. β-casein (*68*)).
2) With increasing concentration of phenolics, there is saturation of the binding sites and the coating becomes sufficient to cause inter-protein bridging (with possible involvement of EGCG-EGCG stacking interaction causing bridging). The aggregate dimerizes and become visible in solution (onset of precipitation)
3) Further addition of phenolics causes formation of large insoluble complexes with smaller aggregates acting as nuclei.

Furthermore, the tannin-assisted compaction described in step 1 is also valid for β-casein micelles binding EGCG or oligomeric procyanidins (*68,81*), while larger tannins (DP > 10, linear dimension > crown width of micelles) have been shown to cause aggregation of micelles by making bridges between their cores (*81*). Finally, the aggregation and precipitation of the complexes is also dependent on the experimental conditions (e.g. pH close to isoelectric point, high ionic strength (*38*)) as they are likely to affect interactions between protein-phenolic complexes.

AIM AND OUTLINE OF THE THESIS

The binding ability of phenolics and proteins in relation to their structure has mostly been evaluated in the scope of astringency perception (interaction with salivary proteins) and transport through the blood stream (interaction with serum albumins). Both classes of proteins cannot be used as carriers for phenolics in food because of the aggregation behavior and commercial availability for the former, and their presumed low binding capacity at specific binding sites for the latter. A potential protein-based carrier for phenolics should combine a bland taste, a relatively high affinity and binding capacity without precipitation, and a reduction of the bitterness and astringency of phenolics, followed by an effective release of the phenolics in the gut. There are a number of commercially available animal food proteins with a high proline content, which might be suitable carriers for phenolics. With the objective to evaluate the potential of common food proteins as carriers for phenolics, the aim of this thesis is to obtain fundamental

understanding of the structure-binding relationships governing food protein-phenolics interactions, and to evaluate the applicability of promising protein-phenolic pairs for tackling bitterness, a common issue in phenolic-rich food.

This was achieved by first screening a range of common animal food proteins for their binding to a selection of flavonoids representative of most classes, as described in **Chapter 2**. The selected range of food proteins was screened using catechin and EGCG as model ligands and a selection of these screened proteins were subsequently tested for binding to a selection of flavonoids from other classes. In **Chapter 3**, interactions between bovine milk caseins and monomeric and dimeric flavan-3-ols were investigated. Dimerization, galloylation and the effect of an extra interflavanic linkage in the procyanidin dimer (A-type versus B-type) were compared for their impact on the binding of flavan-3-ols to caseins. The influence of the structural differences between α-caseins and β-casein was also evaluated. **Chapter 4** describes a method based on mass spectrometry for the evaluation of the protein binding affinity of individual phenolics present in complex mixtures. Using this method, the binding affinity of grape seed procyanidins to β-casein was related to their structural features. In **Chapter 5**, the potential of caseins for reducing the bitterness perception of EGCG is discussed based on the combination of an *in vitro* cell-based bitter receptor assay and binding studies, confirmed with a sensory evaluation of the complexes by a trained panel. Finally, **Chapter 6** addresses the common methods for assessing protein-phenolic interactions regarding their practicality and validity when using food proteins. Moreover, approaches for enhancing the interaction of globular proteins with phenolics are discussed, as well as opportunities of binding monomeric phenolics by proteins. To conclude, perspectives for the application of food proteins as carriers are given.

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Chapter 2

Efficacy of food proteins as carriers for flavonoids

Enrichment of flavonoids in food is often limited by their off-tastes, which might be counteracted by the use of food proteins as carriers of flavonoids. Various milk proteins, egg proteins, and gelatin hydrolysates were compared for their binding characteristics to two flavan-3-ols. Among the proteins tested for their affinities toward epigallocatechin gallate (EGCG), β-casein and gelatin hydrolysates, in particular fish gelatin, were found to be the most promising carriers with an affinity on the order of 10^4 M^{-1}. A flexible open structure of proteins, as present in random coil proteins, was found to be important. The saturation of binding observed at high flavonoid/protein ratios was used to estimate the maximal binding capacity of each protein. To reach a daily intake of EGCG that has been associated with positive health effects, only 519 mg of gelatin B and 787 mg of β-casein were required to complex EGCG on the basis of their maximal binding capacity. When the absence of turbidity is taken into account, β-casein prevails as carrier. Three selected proteins were further investigated for their binding potential of representative flavonoids differing in their C-ring structure. An increase in hydrophobicity of flavonoids was related to a higher affinity for proteins, and the presence of a gallic acid ester on the C-ring showed an overall higher affinity.

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INTRODUCTION

Due to their beneficial health effects, mainly cardioprotective and anticarcinogenic, flavonoids are considered as functional dietary ingredients (*1*). Most of these compounds, however, are bitter and/or astringent. Enhancing their contents in foods may result in off-tastes and, therefore, low consumer acceptance (*2*). Astringency in foods can be reduced via the use of proteins able to complex flavonoids. A typical example is the addition of milk to tea, which has been shown to result in complexation of milk proteins and tea catechins without impairing the bioavailability of the catechins (*3,4*). Milk proteins also have been reported as possible carriers for bioactive compounds (*5*).

Interactions between flavonoids and proteins have been extensively reported, but the emphasis was predominantly on binding of flavonoids to serum albumins (e.g., Dufour and Dangles (*6*), Ishii *et al.* (*7*)). A particular interest has also been on phenolics and proteins involved in haze formation in beverages (e.g. Siebert (*8*)). Interaction of flavonoids with common food proteins, however, is less reported, with mainly a few reports on flavanols interacting with ovalbumin, gelatin, α-lactalbumin, and lysozyme (*9,10*) or milk proteins, such as β-casein (*11,12*) and β-lactoglobulin (*13,14*). To our knowledge, no comparison of common food proteins for their potential binding characteristics with flavonoids has been made within a single study.

Several critical structural features of proteins for binding to flavonoids have been highlighted in the literature. The amino acid composition is a major factor, as prolines are known to be involved in nonspecific interactions primarily via ring stacking with their prolyl residues and with a preference for Pro-Pro repeats (*8,15,16*). Other amino acids, such as phenylalanine, tyrosine, arginine, and histidine, have also been suggested to interact with flavonoids (*15,17,18*). In addition, the presence of bulky amino acid residues close to potential binding sites can reduce their accessibility (*17*). The conformation of the protein is of critical importance as random coil proteins have been shown to display a higher interaction with tannins than globular proteins (*19,20*). Proteins were shown to display an increased binding affinity at pH's close to their p*I* (*10*).

The use of various food proteins as carriers for flavonoids could help the development of functional foods by targeted delivery of bioactive compounds to the gut without sensory defects. The strength of the interaction should be high enough during the residence time in the mouth so as to limit interaction with bitter taste receptors (*21*) and with salivary proteins involved in astringency perception (*22*). The proteins themselves should not impair the taste of foods. Therefore, animal-derived proteins are preferred over plant-derived proteins because of their generally known bland taste. Interaction of proteins with proanthocyanidins and monomeric flavan-3-ols (e.g., catechin, epigallocatechin gallate (EGCG)) is of particular interest because of their relevance for technological and organoleptic properties in beverages (e.g., tea, wine, or beer). In the present study, EGCG and catechin are used as reference ligands to compare a broad range of food proteins for

their binding capacity to flavonoids. The diversity of potential animal-derived food proteins to be used as carriers and the large structural diversity of potential flavonoids to be selected, in addition to the reference ligands, limit the use of laborious methods, such as fluorescence quenching (e.g., Dufour and Dangles (*6*)) or isothermal titration calorimetry (ITC) (e.g., Poncet-Legrand *et al.* (*23*)). Among appropriate methods available, ultrafiltration (UF) seems to be the most versatile and simplest method for the comparison of various animal-derived food proteins binding flavonoids. Our objectives were to first investigate which animal-derived food proteins had the most potential as carriers of EGCG and catechin and, next, to study these most promising proteins for their binding potential to representative compounds from several classes of flavonoids.

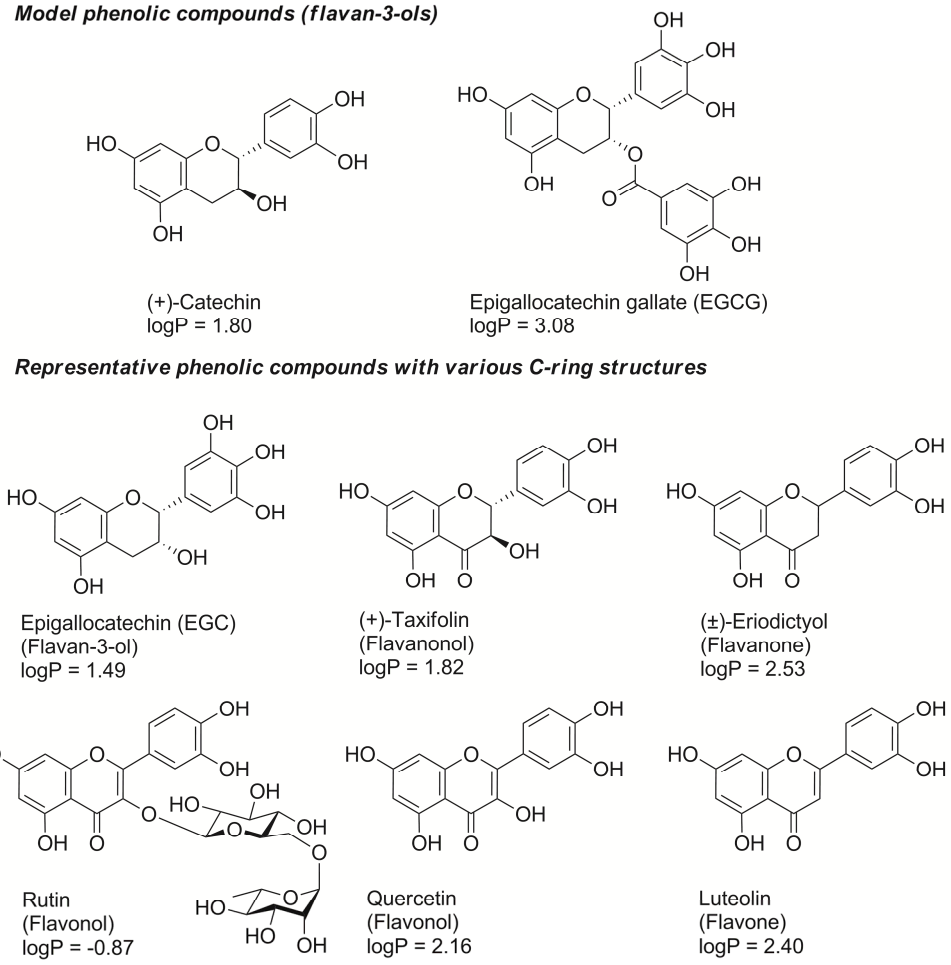

Figure 1. Chemical structure of the flavonoids investigated

MATERIALS AND METHODS

Materials

Phenolics. Rutin hydrate (95%), quercetin dihydrate (99%), and (+)-catechin hydrate (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (+)-Taxifolin (>90%) and (±)-eriodictyol (racemic mixture; >90%) were purchased from Extrasynthèse (Genay, France). Luteolin (99%) was purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). EGCG (Teavigo, ≥85%) was kindly provided by DSM Food Specialties (Delft, The Netherlands). All aforementioned phenolic compounds are detailed in **Figure 1** regarding their structures and log*P* values. Log*P* values were estimated by the software Marvin (Chemaxon).

Proteins. Calcium-depleted type III bovine α-lactalbumin (≥85%), BSA (Cohn V fraction, ≥96%), bovine β-casein (≥98%), bovine β-lactoglobulin (≥90%), solid fish gelatin (gelatin F) from cold water fish skin, gelatin type A (gelatin A) from porcine skin (90-110 bloom), gelatin type B (gelatin B) from bovine skin (75 bloom), lysozyme from chicken egg white (≥90%), ovalbumin from chicken egg white (grade V, ≥98%), and phosvitin from chicken egg yolk were purchased from Sigma-Aldrich. The protein purity of the three gelatins was estimated to be ≥90% on the basis of the sum of amino acid residue weights as detailed later in this section. The protein purity of phosvitin was estimated to be ~75% on the basis of the ratio between the experimental nitrogen content determined by the Dumas method (%N (w/w) = 9.29) and the theoretical nitrogen content (%N (w/w) = 12.43). The latter was calculated from the amino acid composition of phosvitin given in the UniProtKB entry P0245 (uniprot.org), corrected for the phosphorylation and glycosylation of phosvitin.

Other chemicals. Tannase (γ-tannase, Gammazyme) was purchased from Gamma Chemie (Darmstadt, Germany). Methanol (MeOH, analytical grade) was purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Preparation of epigallocatechin (EGC)

EGC was prepared by a tannase treatment of EGCG. EGCG (2 g/L) was dissolved in a 0.1 M sodium acetate buffer, pH 5.0. The tannase was subsequently added (final concentration = 80 mg/L), and the headspace of the flask was flushed with N_2. The reaction flask was incubated in the dark at 30 °C under continuous stirring. After 24 h, a fresh amount of enzyme (final concentration = 80 mg/L) was added. After 48 h, the sample was treated by solid phase extraction (SPE) using a 10 g C18 Sep-Pak column washed and eluted with water and MeOH according to the instructions of the manufacturer (Waters, Milford, MA, USA). The MeOH fraction was evaporated, dissolved in water, and freeze-dried.

The freeze-dried material was dissolved in water/acetonitrile/acetic acid (99:1:0.1, v/v/v) at a concentration of ~50 mg/mL. Next, it was purified by flash chromatography with a 12 g Reveleris C18 column on a Reveleris flash system (Grace, Deerfield, IL, USA) operated at 30 mL/min. The eluents used were water/acetonitrile/acetic acid (99:1:0.1, v/v/v) (eluent A) and acetonitrile/acetic acid (100:0.1, v/v) (eluent B). The elution profile was 0-1 min, 0% B; 1-11 min, 0-35% B; 11-12 min, 35-100% B; 12-15 min, 100% B. Three fractions were generated and analyzed by RP-UHPLC-MS as described elsewhere.(*24*) The fractions contained gallic acid, EGC, and possible oxidation products from the sample treatment. The EGC fraction showed a single peak in UV with a m/z value corresponding to EGC (m/z 305 in MS in negative mode). All along the procedure the sample was kept from light by using aluminum foil.

Amino acid analysis and protein content of gelatins

The amino acid analysis of the gelatin samples (A, B, and F) used in this study was conducted by Ansynth Service BV (Berkel en Roodenrijs, The Netherlands). Most amino acids were analyzed after acid hydrolysis (6 M HCl, 22 h, 110 °C) by classical ion-exchange liquid chromatography with postcolumn ninhydrin derivatization and detection at 440 or 570 nm using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, U.K.). An oxidation step (performic acid, 16 h, 0-5 °C) was included prior to the acid hydrolysis in order to separately quantify cysteine and methionine. Tryptophan was analyzed after alkaline hydrolysis (4.2 M NaOH, 22 h, 110 °C) by reversed phase HPLC using a Beckman Gold HPLC system (Beckman Coulter Inc., Brea, CA, USA) with fluorometric detection. The sum of amino acid residue weights (% (w/w)) was calculated to evaluate the protein content of the samples. Amino acid contents reported in the present study were also corrected for the water contents of the gelatin samples estimated by oven-drying (5.1% (w/w) for gelatin A, 5.7% (w/w) for gelatin B and 6.9% (w/w) for gelatin F).

Circular dichroïsm (CD)

Gelatins were analyzed by far-UV CD on a Jasco J-715 spectropolarimeter (Jasco Inc., Easton, MD, USA). A quartz cell (path length = 1 mm) was filled with 0.1 mg/mL solution of each gelatin in 50 mM sodium phosphate buffer, pH 7.0. Spectra were recorded from 190 to 260 nm with a bandwidth of 2 nm, a scanning speed of 100 nm/min, and an accumulation of 10 scans. Each sample was measured at 4 °C, 20 °C and 70 °C considering that gelatin would be fully structured at 4 °C (reference 100% structure) and totally denatured at 70 °C (reference 0% structure) (*25*). Spectra were corrected for buffer signal using Jasco Standard Analysis software. The intensities of the signals at 200 nm for samples measured at 4 °C and 70 °C were used to evaluate the proportion of structure present in gelatins at 20 °C (see the Supporting Information).

Dynamic light scattering (DLS)

β-Casein was analyzed by DLS on a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.) equipped with a 4 mW HeNe laser beam with a wavelength of 633 nm and a back scattering angle of 173°. The protein was dissolved in 50 mM sodium phosphate buffer, pH 7.0, and diluted to a range of concentrations (6.25, 12.5, 25, 50, and 100 μM). Measurements were carried out at 25 °C.

Binding affinity by ultrafiltration

All samples were prepared in a 50 mM sodium phosphate buffer, pH 7.0. Protein stock solutions (50 μM) were prepared freshly before each experiment. Similarly, stock solutions (6 mM, in the same buffer) of catechin and EGCG were used to obtain a range of dilutions between 0 and 6 mM. The stock solution of taxifolin was prepared in MeOH and then diluted to 10% (v/v) MeOH with the above-mentioned buffer to obtain the aforementioned range of concentrations. Other flavonoids tested were dissolved in DMSO prior to use.

Using a microtiter plate, flavonoid solutions were mixed 1:1 with the protein stock solution (final volume = 300 μL) in order to obtain phenolic to protein molar ratios ranging from 0 to 120. In the case of flavonoids dissolved in DMSO, these were directly added to the protein solution in a volume ratio leading to the same aforementioned range of concentrations and with a final cosolvent concentration of maximum 4% (v/v). At their final concentrations, the amounts of cosolvent (10% (v/v) MeOH or 4% (v/v) DMSO) have negligible effects on the structure of the proteins as verified for BSA by fluorometry at 280 nm (emission spectrum 290-500 nm) and circular dichroism for 10% MeOH (data not shown). The concentration of each cosolvent was kept as low as possible, but their possible influence on the binding affinities measured cannot be excluded.

The microtiter plate was incubated in the dark for 10 min at 25 °C under continuous shaking at 300 rpm (Thermomixer comfort, Eppendorf AG, Hamburg, Germany). Next, the samples were pipetted into an ultrafiltration microtitre plate setup (Ultracel 10, Millipore, Cork, Ireland) and centrifuged (30 min, 1425 × g, 25 °C). The filtrates, containing the unbound flavonoid fraction, were subsequently diluted 10 to 20 times with the buffer and measured at 280 nm. For each set of samples, a calibration curve per flavonoid was made using ultrafiltered blanks. In addition, possible contaminations of the filtrate by proteins were systematically checked with protein controls (no flavonoid added) measured at 280 nm after ultrafiltration.

The protein-bound and free fractions of each flavonoid at each concentration tested were calculated, and plots of the bound fraction against the free fraction were made. For each binding curve obtained, a linear regression was used on the initial linear increase (R^2>0.8) to estimate the binding affinity (K) of the compounds. A maximal binding capacity (R_{max}) was derived from the plateau value or the highest bound fraction observed at high phenolic compound/protein molar ratios.

Isothermal Titration Calorimetry (ITC)

Several samples analyzed by the ultrafiltration assay were further investigated by ITC. All measurements were conducted in duplicate on a MicroCal ITC_{200} microcalorimeter (GE Healthcare, Piscataway, NJ, USA) at 25 °C. EGCG or catechin (8 and 14 mM, respectively) was titrated into the measurement cell (V = 200.1 µL) containing the protein (0.025 mM). Each titration consisted of up to two series of 49 injections of 0.8 µL. The time between injections was set at 4 min, and the sample was continuously stirred at 600 rpm.

Raw data were integrated peak-by-peak to obtain a plot of observed enthalpy change (ΔH) versus the phenolic to protein molar ratio. Control titrations of EGCG or catechin into buffer were performed for data correction. Experimental data were fitted using the "one set of sites" and "two sets of sites" models provided by the equipment supplier in Origin 7.0 (OriginLab, Northampton, MA, USA). The fitting provided one or two sets of parameters depending on the model used: n (the number of binding sites), K (the binding constant (in M^{-1})), and ΔH (the change in enthalpy (in $kJ.mol^{-1}$)). Changes in Gibbs free energy (ΔG) and entropy (ΔS) were calculated for each set of fitting parameters using the standard equation $\Delta G = -RT\ln K = \Delta H - T\Delta S$ where T is the temperature in Kelvin and $R = 8.32\ J.mol^{-1}.K^{-1}$.

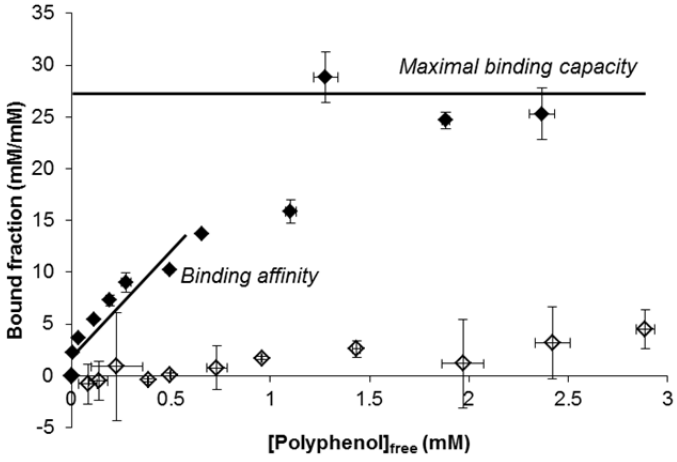

Figure 2. Typical binding curve obtained by ultrafiltration, BSA-EGCG (◆) and BSA-catechin (◇) in 50 mM sodium phosphate buffer pH 7.0

RESULTS

Binding characteristics of common animal-derived food proteins with catechin and EGCG

A range of animal-derived food proteins commonly used as food ingredients was screened for their binding potential to catechin and EGCG. Typical binding curves obtained by ultrafiltration are shown in **Figure 2** and the corresponding affinity values are presented in **Figure 3A** and **Table 3**. It is clear that the affinities of most proteins toward EGCG exceeded those toward catechin. Only in the case of lysozyme could no clear difference in affinity be observed. Hence, the comparison was further made based on data obtained with EGCG. Proteins displaying the highest affinities were gelatin F and β-casein, followed by gelatin A and gelatin B (**Figure 3A**).

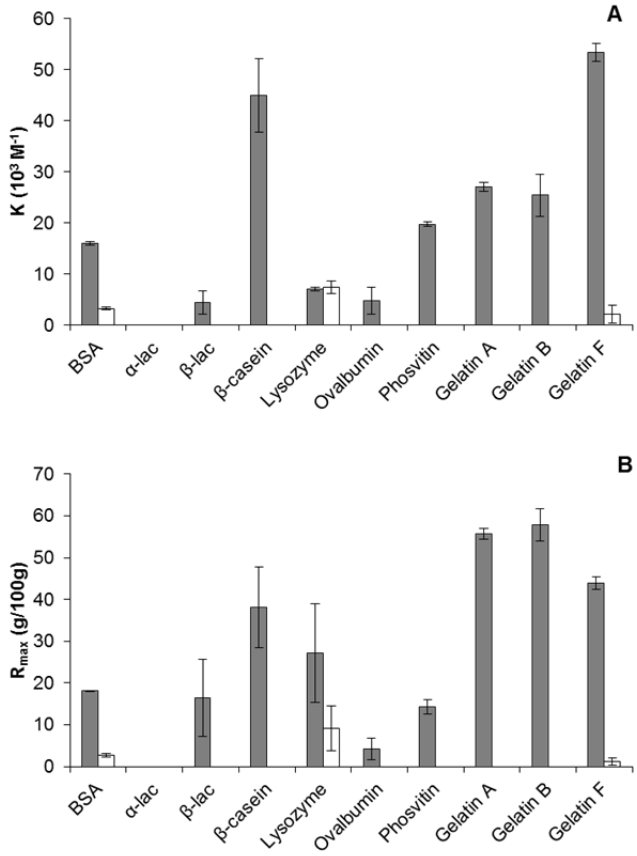

Figure 3. Binding affinity (**A**) and maximal binding capacity (**B**) for the interaction of EGCG (closed bar) and catechin (open bar) with various animal-derived food proteins at pH 7.0 (α-lac, α-lactalbumin; β-lac, β-lactoglobulin)

Table 1. Interaction of EGCG and catechin with selected food proteins by ITC at pH 7.0, 25°C

	BSA		gelatin B		β-casein		β-lactoglobulin	
	EGCG[a]	Catechin[b]	EGCG[a]	Catechin	EGCG[a]	Catechin	EGCG	Catechin
n_1	2.9(±0.4)	6.6(±1.5)	24.5(±4.7)	n.d.	2.4(±0.8)	n.d.	n.d.	n.d.
K_1 (10^3 M^{-1})	29.5(±14.1)	0.5(±0.1)	68.6(±10.1)	n.d.	0.3(±0.1)	n.d.	n.d.	n.d.
ΔG_1 (kJ.mol^{-1})	-25.3(±1.2)	-15.5(±0.4)	-27.5(±0.4)	n.d.	-13.8(±1.1)	n.d.	n.d.	n.d.
ΔH_1 (kJ.mol^{-1})	-26.6(±1.1)	-18.1(±1.2)	-1.8(±0.2)	n.d.	-461.2(±92.1)	n.d.	n.d.	n.d.
$-T\Delta S_1$ (kJ.mol^{-1})	1.3(±0.1)	2.6(±0.8)	-25.8(±0.2)	n.d.	447.4(±91.0)	n.d.	n.d.	n.d.
n_2	107(±7.1)	n.a.	55.4(±18.0)	n.d.	19.1(±0.4)	n.d.	n.d.	n.d.
K_2 (10^3 M^{-1})	0.8(±0.3)	n.a.	0.8(±0.2)	n.d.	8.9(±4.3)	n.d.	n.d.	n.d.
ΔG_2 (kJ.mol^{-1})	-16.6(±0.8)	n.a.	-16.5(±0.7)	n.d.	-22.4(±1.2)	n.d.	n.d.	n.d.
ΔH_2 (kJ.mol^{-1})	-4.4(±0.5)	n.a.	-22.2(±14.8)	n.d.	-24.7(±4.1)	n.d.	n.d.	n.d.
$-T\Delta S_2$ (kJ.mol^{-1})	-12.2(±1.4)	n.a.	-5.7(±14.1)	n.d.	2.4(±5.4)	n.d.	n.d.	n.d.

ITC data derived from: [a]two sets of sites model, [b]one set of sites model; n.d.: not detectable; n.a.: not applicable

n_i: stoichiometry; K_i: binding constant; ΔG_i: Gibbs free energy; ΔH_i: enthalpy; $-T\Delta S_i$: entropic contribution

Table 2. Summary of protein properties[a]

Protein (UniprotKB entry)	Source	General structure	Molecular mass (kDa) (# amino acid residues)	pI	S-S bridges	% Proline[b] (no.)	% OH-Pro[b] (no.)	% Histidine[b] (no.)	% Aromatic amino acids[b,c] (no.)
α-lactalbumin (P00711)	Bovine	Globular	14.2 (123)	4.80	4	1.6 (2)	-	2.4 (3)	9.8 (12)
β-casein (P02666)	Bovine	Random	23.6 (209)	5.13	-	16.7 (35)	-	2.4 (5)	6.7 (14)
β-lactoglobulin (P02754)	Bovine	Globular	18.3 (162)	4.83	2	4.9 (8)	-	1.2 (2)	6.2 (10)
BSA (P02769)	Bovine	Globular	66.4 (583)	5.60	17	4.8 (28)	-	2.9 (17)	8.4 (49)
Gelatin type A (acid-cured)[d]	Porcine	Random /helical	20-25 (283)	7-9	-	11.4 (32)	9.5 (27)	0.6 (2)	1.6 (5)
Gelatin type B (lime-cured)[d]	Bovine	Random /helical	20-25 (295)	4.7-5.2	-	11.4 (33)	9.5 (28)	0.4 (1)	1.4 (4)
Gelatin F[d]	Fish	Random /helical	60 (720)	6	-	9.4 (68)	5.6 (40)	0.7 (5)	1.5 (11)
Lysozyme (P00698)	Chicken	Globular	14.3 (129)	9.32	4	1.6 (2)	-	0.8 (1)	9.3 (12)
Ovalbumin (P01012)	Chicken	Globular	44.5 (385)	5.19	1	3.6 (14)	-	1.8 (7)	8.6 (33)
Phosvitin (P02845)	Chicken	Globular /Random[e]	34.0 (217)	~4[e]	-	1.4 (3)	-	6.0 (13)	1.4 (3)

[a] Data are derived from UniprotKB database (www.uniprot.org)
[b] % amino acid residues is given as amino acid residues per 100 residues
[c] Sum of phenylalanine, tryptophan and tyrosine
[d] Molecular weight and pI based on commercial data available at Sigma-Aldrich; amino acid composition determined experimentally
[e] Structure of phosvitin is pH-dependent (*34*); pI derived from (*35*)

Four of the proteins tested covering a range of affinities to EGCG were also measured with ITC as summarized in **Table 1**. β-Lactoglobulin was chosen as a representative of the proteins displaying low binding affinity to EGCG and compared to BSA, gelatin B and β-casein. The ITC data confirmed the outcome of the UF assay. At the protein concentration used, no clear heat signal related to binding could be observed for all proteins titrated with catechin, which indicates a low binding affinity. A similar observation was made for β-lactoglobulin titrated with EGCG. The calculated binding affinities by ITC were generally on the same order of magnitude as those obtained by ultrafiltration for BSA and gelatin B. For β-casein, a lower binding affinity to EGCG by ITC was calculated. In terms of stoichiometry, the UF assay (**Table 3**) and ITC were in good agreement for BSA and β-casein, although different for gelatin B. It is noteworthy that, except for β-lactoglobulin, all binding isotherms for proteins interacting with EGCG could be fitted with a two sets of site binding model. This model was found to give the best fit, although it was designed for pharmaceutical compounds with a more specific binding in simpler systems. It does not take into account the possibility of nonspecific phenolic-phenolic interactions on the surface of the protein or complex structural arrangements of proteins, such as micelles of β-casein. The latter might explain the discrepancy between ITC and the UF assay found in the present study. More complex models (e.g. Buurma and Haq (*26*)) should be considered for further detailed analysis of ITC data in protein-phenolic interaction. The thermodynamic parameters calculated for BSA and β-casein indicated a predominance of hydrogen bonding (ΔH_1) in the first binding event, whereas hydrophobic interactions ($-T\Delta S_1$) dominated in the case of the first binding event of gelatin B.

Phosvitin had an affinity for EGCG comparable to that of BSA (**Figure 3A**). This protein contains around 50% serine in its sequence, of which most, if not all, are phosphorylated. These phosphoserines are organized by blocks of up to 14 residues and make phosvitin a strong metal ion chelating agent, especially for iron (*27*). Dialysis of this protein against subsequently EDTA and water was performed to remove the iron. This treatment resulted in a large decrease in binding affinity of EGCG to phosvitin, as observed by UF (from $(19.7 \pm 0.4) \times 10^3$ M^{-1} to $(5.6 \pm 0.9) \times 10^3$ M^{-1}) and ITC (data not shown). The presence of iron on the surface of the protein seemed to be critical to the binding of EGCG, which is considered to be related to the metal ion binding capacity of flavonoids (*28*).

Proteins showing the highest affinity did not necessarily bind the most EGCG on a weight basis as can be seen in **Figure 3B** and **Table 3**. In this case, gelatin B and gelatin A had the highest binding capacity followed by gelatin F, β-casein, and lysozyme. With the aim to use proteins as carriers for flavan-3-ols, proteins combining a high binding affinity with a high binding capacity seem to be most appropriate, as is the case for β-casein and gelatin F, followed by gelatin A and gelatin B. Turbidity could be clearly observed for the three gelatins at phenolic/protein molar ratios higher than 20, whereas no precipitation occurred with β-casein. β-Casein was selected as the best carrier for further study and compared with gelatin B, which had a similar average molecular mass and a higher

maximal binding capacity despite its lower binding affinity. BSA was used for further studies as a representative globular protein as it had one of the highest affinities and binding capacities among all globular food proteins tested.

Influence of C-ring structure of flavonoids on their interaction with selected proteins

The three proteins selected were tested for their affinities toward various flavonoids (**Figure 1**). These compounds were selected because they had structural variations only on their C-ring compared to catechin and their A- and B-rings carried the same number of hydroxyl groups.

The binding constants for BSA, β-casein, and gelatin B of the flavonoids selected are summarized in **Figure 4** and **Table 3**. Only BSA showed binding curves with low standard deviations for all compounds. Contrary to what was observed for EGCG, none of the proteins tested displayed a clearly measurable interaction with epigallocatechin (EGC), catechin, and taxifolin. This emphasizes the importance of the extra gallic acid group in EGCG to enhance the affinity to proteins. In addition, a higher binding affinity was measured in the case of eriodictyol binding to BSA, whereas no interaction was detected for the same compound with β-casein and gelatin B. No binding curve could be obtained by ultrafiltration for quercetin and luteolin because of their low solubility in an aqueous environment despite the use of up to 5% (v/v) DMSO as a cosolvent. Rutin was found to have an affinity similar to that of catechin and taxifolin for BSA and β-casein.

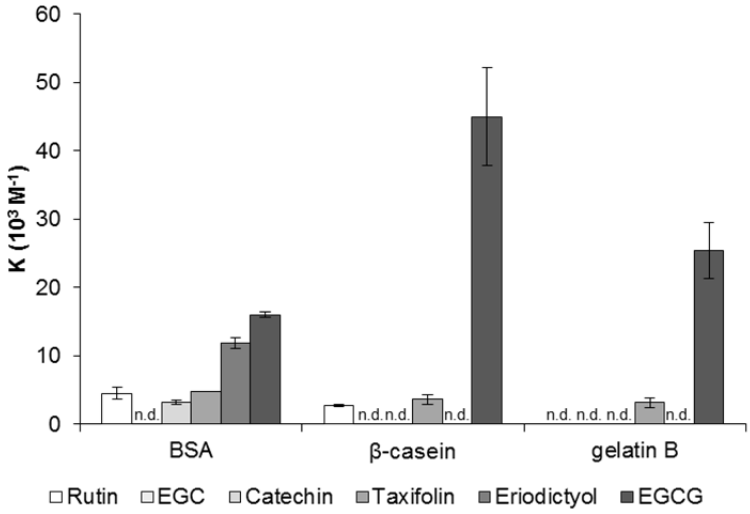

Figure 4. Interaction of flavonoids with various C-ring substitutions with BSA, β-casein and gelatin B at pH 7.0 measured by ultrafiltration (n.d.: not detected)

Table 3. Summary of interactions between tested flavonoids and various food proteins by ultrafiltration at pH 7.0

Flavonoid		BSA	α-lac	β-lac	β-casein	Lysozyme	Ovalbumin	Phosvitin	Gelatin A	Gelatin B	Gelatin F
EGCG	K (mM^{-1})	16.0(±0.4)	n.d.	4.5(±2.3)	45.0(±7.2)	7.1(±0.3)	4.7(±2.6)	19.7(±0.4)	27.1(±0.8)	25.4(±4.1)	53.3(±1.8)
	R_{max} (mol/mol)	26.3(±0.2)	n.d.	6.6(±3.7)	19.6(±4.9)	8.5(±3.7)	4.1(±2.5)	10.7(±1.3)	30.4(±0.7)	31.6(±2.1)	57.5(±1.8)
	R_{max} (g/100g)	18.1(±0.1)	n.d.	16.5(±9.2)	38.1(±9.6)	27.2(±11.8)	4.2(±2.6)	14.4(±1.7)	55.7(±1.3)	57.8(±3.8)	43.9(±1.4)
EGC	K (mM^{-1})	n.d.			n.d.					n.d.	
	R_{max} (mol/mol)	n.d.			n.d.					n.d.	
	R_{max} (g/100g)	n.d.			n.d.					n.d.	
Catechin	K (mM^{-1})	3.2(±0.3)	n.d.	n.d.	negligible	7.4(±1.2)	n.d.	negligible	n.d.	n.d.	2.1(±1.7)
	R_{max} (mol/mol)	6.4(±1.0)	n.d.	n.d.	negligible	4.6(±2.6)	n.d.	negligible	n.d.	n.d.	2.7(±1.8)
	R_{max} (g/100g)	2.8(±0.4)	n.d.	n.d.	negligible	9.2(±5.3)	n.d.	negligible	n.d.	n.d.	1.3(±0.9)
Taxifolin	K (mM^{-1})	4.8(±0.0)	2.3(±0.8)	n.d.	3.7(±0.7)	4.0(±0.4)	2.4(±1.1)	negligible	1.4(±0.2)	3.1(±0.8)	3.4(±0.3)
	R_{max} (mol/mol)	12.6(±3.2)	5.2(±0.7)	n.d.	7.3(±1.0)	7.3(±0.8)	5.6(±2.8)	negligible	3.8(±0.4)	7.8(±3.3)	7.7(±0.5)
	R_{max} (g/100g)	5.8(±1.5)	11.1(±1.5)	n.d.	9.4(±1.3)	15.4(±1.7)	3.8(±1.9)	negligible	4.6(±0.5)	9.5(±4.0)	3.9(±0.3)
Eriodictyol	K (mM^{-1})	11.8(±0.7)			n.d.					n.d.	
	R_{max} (mol/mol)	8.8(±0.3)			n.d.					n.d.	
	R_{max} (g/100g)	3.8(±0.1)			n.d.					n.d.	
Rutin	K (mM^{-1})	4.5(±0.9)			2.7(±0.1)					n.d.	
	R_{max} (mol/mol)	6.3(±1.1)			4.3(±0.3)					n.d.	
	R_{max} (g/100g)	5.8(±1.0)			11.1(±0.7)					n.d.	

Data are the mean ± SD values of two replicates; n.d.: not detectable; α-lac, α-lactalbumin; β-lac, β-lactoglobulin

DISCUSSION

Influence of amino acid composition on the potential of food proteins as carriers

The most potent proteins as carriers for EGCG are also the ones that have the highest content in proline (**Table 2**). It is commonly accepted that proline residues play a key role in protein-phenolic interactions (*8, 15*). However, gelatins A and B have higher total proline contents (sum of proline and hydroxyproline) than β-casein, but displayed a lower affinity to EGCG than the latter. Aromatic amino acid residues have also been found to interact by hydrophobic interaction with phenolic compounds (*15*). Histidine residues have also been considered as possible binding sites (*18*) although contradictory results were later found (*15*). β-Casein has a higher content in aromatic amino acids and histidine than the gelatins (**Table 2**), which might explain our observation. For random coil proteins (i.e., β-casein, phosvitin and gelatins A, B, and F), only weak correlations were calculated in an attempt to estimate the linear relationship between their binding affinities for EGCG and their relative contents (% mol/mol) in hydrophobic amino acids and prolines. The differences in binding affinities for EGCG of random coil proteins cannot be solely explained by their content in these amino acids. For globular proteins, this analysis was not done, as many amino acids are less freely accessible.

Influence of protein structure on its potential as flavonoid carrier

Among the proteins tested, it appeared that the most potent proteins are the ones having a random coil or random/helical structures (**Table 2**). This correlates with studies investigating the binding of random coil and/or globular proteins to tannins, also in terms of order of magnitude of the binding affinity (*11, 19, 20*). In globular proteins, the reduced accessibility of amino acid residues often implicated in binding phenolics is suggested to be responsible for their overall lower affinity observed compared to that of random coil proteins. BSA is an exception in this respect as it is known to have specific phenolic binding cavities (e.g., Dufour and Dangles (*6*)).

Although the proline content is frequently reported as a key factor in protein-phenolic interactions, gelatins A and B displayed a lower binding affinity to EGCG compared to β-casein and gelatin F, despite their higher total proline contents (sum of proline and hydroxyproline). Proline repeats induce a helical structure in the gelatin monomer (polyproline II helix) (*16*). This structure reduces the flexibility in the protein backbone and, thereby, its capacity to interact with ligands (*17*). In addition, hydroxylation of prolines enhances both the formation and the stability of triple-helix structures by hydrogen bonding, thereby possibly lowering ligand binding (*19, 29*). CD spectra of the gelatin samples showed more residual triple-helix structures at room temperature for gelatins A and B (100 and 68%, respectively) compared to gelatin F (56%, see the Supporting Information), thereby possibly explaining the higher binding affinity observed for gelatin F. This structural difference can be explained by the different critical helix-to-coil

temperatures of the gelatins, which are ~36 °C for mammalian gelatins and between 15 and 20 °C for cold water fish gelatin (*30*). The reduced flexibility of gelatins A and B is thought to hinder the availability of binding sites for EGCG compared to gelatin F. In the case of β-casein, prolines are scattered along its chain and probably do not influence its flexibility.

β-Casein is known to form micelles (hydrodynamic radius ~12 nm) by reversible association at low concentration (critical micelle concentration of ~21 μM) (*31*). In the present study, micelles (hydrodynamic radius ~13 nm) were detected with DLS at concentrations exceeding 25 μM. A shift to monomers with remaining micelles was observed at a concentration of 25 μM, although that concentration was too low for an accurate measurement of the particle sizes (data not shown). The involvement of part of the β-casein in micelles is thought to influence its interaction with EGCG. In fact, it could reduce the accessibility of its amino acid residues, thereby reducing the interaction. On the contrary, it could provide a hydrophobic environment inside the micelles, which could also positively influence the interaction. Nevertheless, β-casein displayed one of the highest affinities to EGCG as measured by the UF assay.

Influence of the C-ring structure of flavonoids on their binding to selected proteins

The hydrophobicity of the compounds tested (**Figure 1**) could be related to their binding affinity to BSA (**Figure 4**), which is in line with observations on binding of various flavan-3-ols to poly(L-proline) (*23*). Small variations in hydrophobicity resulting from minor changes in the C-ring structure of the phenolics could neither be linked to changes in binding affinity to BSA nor to β-casein and gelatin B. Therefore, at this stage, animal-derived food proteins do not appear to be suitable carriers for monomeric flavonoids.

Variations in the C-ring of flavonoids involve substitutions on the C(3) position, for example, glycosylation or galloylation. Rutin (glycosylated quercetin) is more hydrophilic than its aglycone and displayed a low binding affinity to BSA, consistent with Dufour and Dangles (*6*). On the contrary, C(3) galloylation of EGCG increased both the hydrophobicity of the flavonoid and its ability to form hydrogen bonds, which resulted in an overall higher binding affinity to the proteins tested, consistent with previous studies (*11, 13, 23*). Under the experimental conditions of this study, only galloylation seems to be a major factor for enhancing binding affinity of monomeric flavonoids to food proteins.

Use of animal-derived food proteins as flavan-3-ol carriers in relation with dietary intake

With gelatin B and β-casein as examples, these proteins were found to be able to bind a maximum of 57.8 and 38.1 g EGCG/100 g protein, respectively, with affinities higher than 2×10^4 M^{-1}. Several intervention studies have shown beneficial health effects after daily consumption of green tea supplements containing 200-300 mg of EGCG (*32*). If the highest value is taken as a recommended daily dietary intake, this would mean that 519 mg of gelatin B or 787 mg of β-casein saturated with EGCG would be needed in food to meet this

intake. Obviously, other factors must be taken into account when this estimate is extrapolated to a food system, such as the stability of these complexes in a food matrix and in the mouth and their fate during digestion for targeted release. Nevertheless, it appears that the use of animal-derived food proteins as carriers of flavonoids in food is feasible, as supplementation in food would require only gram quantities of proteins as ingredients.

In terms of application in food systems, β-casein and gelatin F seemed to be the most promising carriers as they displayed the highest affinities for EGCG with good maximal binding capacity for EGCG, which was related to their random coil character. Among these two proteins, β-casein is the most suitable as it did not show any turbidity in the concentration range of EGCG used in this study, contrary to gelatin F (also observed for gelatins A and B). A relatively higher affinity is linked to a better stability of the complexes for targeted delivery. Formation of insoluble aggregates could be undesirable in food formulation and could also lower the bioaccessibility of flavonoids. Thus, β-casein seems to be the most promising flavonoid carrier among all food proteins tested in the present study. This conclusion is supported by publications on the use of this protein as a carrier for drugs (*33*).

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SUPPORTING INFORMATION

Circular dichroism of the various gelatins at different temperature

Table S1. Proportion of structure in gelatin A, B and F based on the intensity of CD signal at 200 nm

Temperature	Gelatin A		Gelatin B		Gelatin F	
	CD (mdeg)	%structure	CD (mdeg)	%structure	CD (mdeg)	%structure
4°C	-16.5	100%	-18.7	100%	-15.3	100%
20°C	-16.5	100%	-16.3	68%	-14.2	56%
70°C	-8.0	0%	-11.2	0%	-12.8	0%

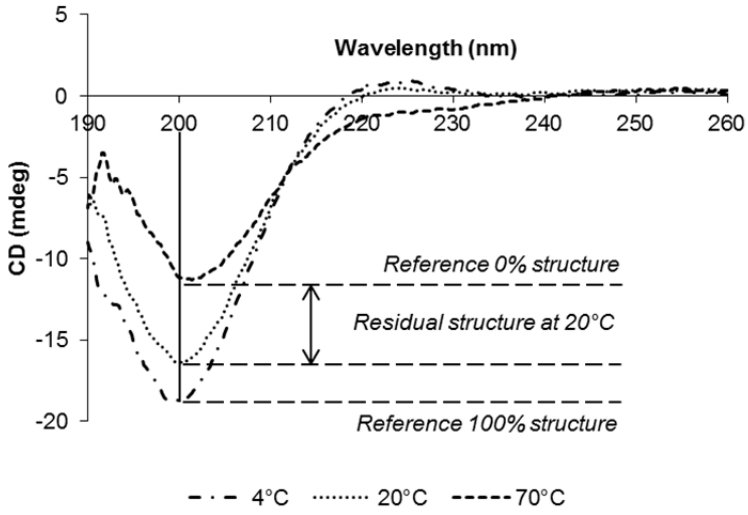

Figure S1. CD spectra of gelatin B at various temperatures. Calculation of the proportion of residual structure at 20 °C is indicated

Chapter 3

Interaction of dimeric flavan-3-ols, with varying flexibility, and different caseins is determined by more than proline content and number of proline repeats

Caseins have potential as carriers for food bioactives. The interaction of A-type and B-type procyanidin dimers with α-casein and β-casein was investigated in comparison with epigallocatechin gallate (EGCG) and epigallocatechin (EGC), based on quenching of tryptophan fluorescence. Esterification of flavan-3-ols with gallic acid, as in EGCG, has a five to ten times greater effect on the binding affinity to caseins compared to the addition of a (+)-catechin or (-)-epicatechin unit, as in B-type procyanidin dimers. This might be related to a larger number of rotatable bonds in EGCG leading to a higher degree of rotational freedom of this ligand compared to the other ligands investigated. Procyanidin dimer A1 displays a higher affinity to α-casein compared to B-type dimers despite its additional interflavanic bond, which possibly reduces its conformational freedom but exposes the B-rings of both monomeric units. Surprisingly, no interaction of procyanidin A1 with β-casein was detected, despite the fact that β-casein showed the highest affinity for the other flavan-3-ols, and the fact that β-casein contained more proline and proline repeats, known to drive the interaction, than α-casein. These results suggest that the interaction between phenolics and caseins is governed by more than proline content and number of proline repeats.

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INTRODUCTION

The interaction of bioactive compounds, especially phenolics, with milk proteins has been used to develop carriers for their targeted delivery (e.g. (*1,2*)). A comparative study of several food proteins binding flavan-3-ols showed the prevalence of β-casein as carrier for epigallocatechin gallate (EGCG) over other common food proteins (*3*). In addition, it was shown that inclusion of EGCG in such complexes might counteract its bitter off-taste in foods (*4*).

The structural features of flavan-3-ols influencing their interaction with proteins have been mainly highlighted with proline-rich proteins (PRP) from saliva or with poly-(L-proline) but much less with common food proteins (e.g. (*5*)). The binding affinity of flavan-3-ols has been reported to increase by addition of a gallic acid ester at the 3-OH position of the molecule and with increasing degree of polymerization (DP), as in procyanidins (*6,7*). The effects of galloylation and degree of polymerization have only been rarely reported in comparative studies. A stronger interaction of epicatechin gallate (ECG) with salivary PRPs compared to procyanidin dimer B2 was reported by nephelometry (*8*), which was contradictory to another comparative study by mass spectrometry with the salivary PRP IB5 (*9*). Additionally, differences inconformational flexibility of procyanidins can significantly impact their affinity for salivary proteins (*7*). Procyanidin dimer B1 (**Figure 1**) has a lower binding affinity to the proline-rich peptide IB7 compared to procyanidin dimer B2, which is related to a preferred compact conformer for procyanidin B1 (95% compact) compared to procyanidin B2 (55% compact) (*10*). Oligomeric procyanidins can be differentiated into A-type or B-type procyanidins depending on their interflavanic linkages, the former differing from the latter by at least one double linkage consisting of a C-C bond and an additional ether bond (**Figure 1**). This additional bond in A-type procyanidins appears as a constraint for the flexibility of the molecule. Its impact on their binding affinity to proteins has, however, never been investigated in detail.

In an attempt to use milk caseins as carriers for flavan-3-ols, a systematic comparison of the various structural features of these ligands for understanding of their interactions with caseins is required. Caseins are subdivided in four different types with varying hydrophobicity, charge, proline content and sensitivity to calcium ions: α_{S1}, α_{S2}, β, and κ caseins. All four types are present in casein-based ingredients. They are described as unstructured proteins with relatively high proline content and the ability to self-associate or associate with other caseins to form micelles (*11*). Relevant properties of the various caseins are summarized in **Table 1**. Proline residues, as well as the number of proline repeats, are often reported as being predominant in protein-phenolic interactions (*7,12,13*). Hence, based on its proline content β-casein is expected to show a higher affinity to phenolics than α-casein, which was confirmed when comparing both caseins for their binding to tea catechins (*14*).

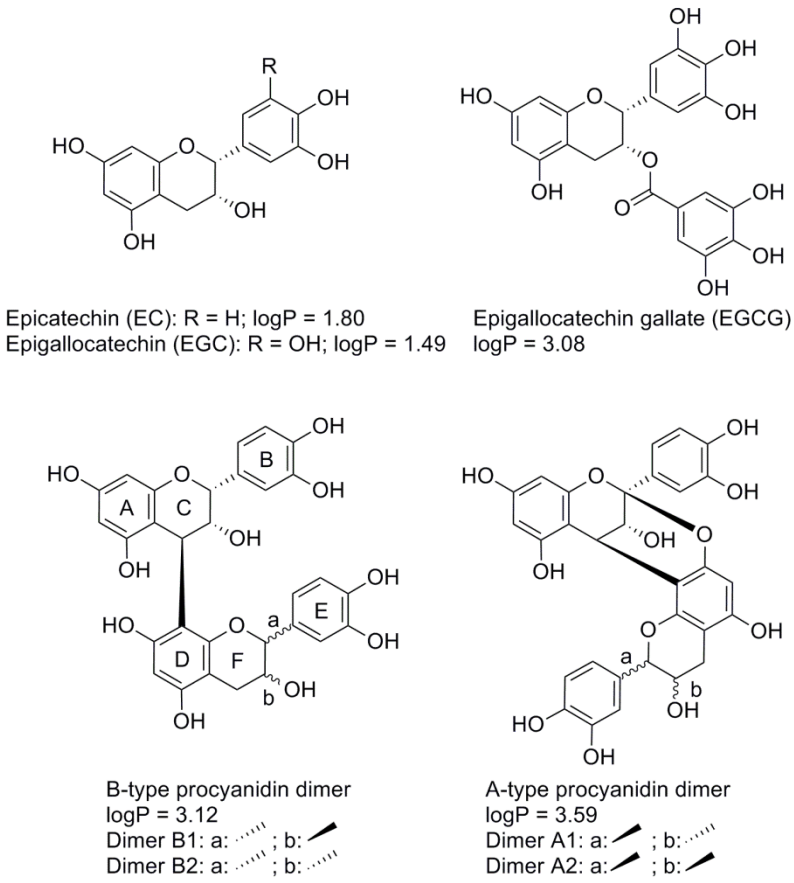

Figure 1. Chemical structures of the ligands investigated

The interaction of flavan-3-ols with caseins has only rarely been reported (e.g. (*14,15*)). To our knowledge, the interaction of procyanidins with caseins has never been reported in detail before. Amongst various methods to assess protein-phenolics interactions, fluorescence quenching has been proven useful by evaluating the decrease in fluorescence of tryptophan residues in proteins while being titrated with a phenolic ligand (*16*). This method can be used with any protein containing at least one tryptophan residue, such as caseins, and has the advantage of requiring low amounts of sample. The aim of the present study is to compare various caseins for binding of EGCG and to investigate the effect of some structural features typically found with flavan-3-ols (i.e. galloylation, DP, and interflavanic linkage) on their ability to bind caseins. This was achieved by using a combination of a fluorescence quenching assay and an ultrafiltration assay described earlier (*3*).

Table 1. Summary of various properties of individual caseins investigated[a]

Protein (UniprotKB entry)	Molecular mass (kDa) (no. amino acid residues)	p*I*	% Pro[b] (no.)	% Phe[b] (no.)	% Tyr[b] (no.)	% Trp[b] (no.)	% His[b] (no.)	PR[c] (no.)
α_{S1}-casein (P02662)	23.0 (199)	4.9	8.5 (17)	4.0 (8)	5.0 (10)	1.0 (2)	2.5 (5)	0
α_{S2}-casein (P02663)	24.3 (207)	8.3	4.8 (10)	2.9 (6)	5.8 (12)	1.0 (2)	1.4 (3)	0
β-casein (P02666)	23.6 (209)	5.1	16.7 (35)	4.3 (9)	1.9 (4)	0.5 (1)	2.4 (5)	4
κ-casein (P02668)	19.0 (169)	5.9	11.8 (20)	2.4 (4)	5.3 (9)	0.6 (1)	1.8 (3)	2

[a]Data derived from the UniprotKB database (www.uniprot.org)
[b]Molar percentage of amino acid residues
[c]Pro-Pro repeats

MATERIALS AND METHODS

Materials

Bovine α-casein (>70% (w/w) of total protein) and β-casein (≥98% (w/w) of total protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-tryptophan (≥99% (w/w)) was purchased from Fluka (Buchs, Switzerland). Procyanidin dimer B2 (≥90% (w/w)) was purchased from Extrasynthèse (Genay, France). Epigallocatechin gallate (EGCG, ≥90% (w/w)) was kindly provided by DSM Food Specialties (Delft, The Netherlands). Sources to isolate procyanidin dimers A1 and B1 were, respectively, peanut skins (kindly provided by Imko The Nut Company BV (Doetinchem, The Netherlands)) and a procyanidin extract from grape seeds (GSE) (Vitaflavan®, DRT, kindly provided by Levita Chemicals International, Antwerpen, Belgium). Fat-free quartz sand was purchased from Büchi Labortechnik AG (Flawil, Switzerland).

Organic solvents used in this study were UHPLC-MS grade and purchased from Biosolve BV (Valkenswaard, The Netherlands), except for n-hexane, which was of technical grade and purchased from VWR International S.A.S. (Fontenay-sous-Bois, France). Water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals were of analytical grade and purchased from Sigma-Aldrich or Merck (Darmstadt, Germany).

Determination of molar absorption coefficients

Molar absorption coefficients for EGCG (at 273 nm), α-casein and β-casein (at 280 nm) were determined in 10 mM sodium phosphate buffer pH 7.0 using a UV-1800 spectrophotometer equipped with a CPS-controller for temperature control (Shimadzu, Kyoto, Japan). A concentration range of each compound (16 to 160 μM for EGCG, 2 to

50 µM for both caseins) was prepared gravimetrically freshly before use. Concentrations were corrected for the purity of each compound provided by their respective supplier. Molar absorption coefficients and purities of samples are summarized in **Table 2**.

Table 2. Sample purity and molar absorption coefficient of EGCG, α-casein and β-casein in 10 mM sodium phosphate buffer pH 7.0 (n.a.: not applicable)

Compound	Purity (% (w/w))[a]	ε_λ ($M^{-1}.cm^{-1}$)	
		Current study	Expasy database[b]
EGCG	94.2	10655 ±149 (273 nm)	n.a.
α-casein	79.2	26747 ±65 (280 nm)	25621 (280 nm)[c]
β-casein	82.3	11443 ±289 (280 nm)	11460 (280 nm)

[a]based on certificate of analysis provided by the supplier
[b]data obtained from www.uniprot.org
[c]calculated assuming 80% (w/w) α_{S1}-casein and 20% (w/w) α_{S2}-casein

Molecular modeling and prediction of some chemical properties of flavan-3-ols
Molecular structures were evaluated using the software Marvin (version 5.12, 2013, Chemaxon, http://www.chemaxon.com). The logP values were predicted using a combination of the three algorithms available, with an equal weight given to each. The minimal energy conformation and the diversity of conformers for each flavan-3-ol were calculated using MMFF94 force field with a strict optimization limit and a diversity limit of 0.5 below which conformers would be considered as identical and deleted. A maximum of 60 conformers were generated and compared with reported structures for validation (*17,18*).

Extraction of procyanidins from peanut skins by pressurized liquid extraction
Peanut skins (150 g) were milled using a 2 mm sieve (ZM 200 mill, Retsch GmbH & Co KG, Haan, Germany) and divided into samples of 10 g. Each sample was defatted with hexane using soxhlet extraction. Procyanidins were extracted from the defatted samples by pressurized liquid extraction (Speed extractor E-916, Büchi Labortechnik) using 70% (v/v) methanol (MeOH) at 40 °C and with a pressure of 100 bar in one cycle with a holding time of 15 min. Each extraction vial (V = 40 mL) was loaded with 3 g of peanut skins homogeneously mixed with 40 g of fat-free quartz sand. The extracts from all sample vials were pooled, evaporated under vacuum, resuspended in water and freeze-dried. The lyophilized extract was purified further by solvent partitioning with water and ethyl acetate as reported elsewhere (*19*), resulting in an extract referred to as 'peanut skin extract' (PSE). Throughout the procedure, the sample was kept from light using aluminum foil.

Fractionation of A-type and B-type procyanidin dimers by Flash chromatography
Separation of procyanidins by degree of polymerization (DP) was achieved using a diol stationary phase based on a method described elsewhere with modifications (*20*). A Reveleris® Flash system (Grace, Deerfield, IL, USA) equipped with ELSD and UV

detection was used. In one run, the sample (GSE or PSE) was dissolved in 90% (v/v) ACN (100 mg/mL) and 5 mL was loaded onto a 40 g diol Flash cartridge with a 40 µm particle size (Grace). The binary mobile phase consisted of acetonitrile (ACN) with 0.1% (v/v) acetic acid (HAc) (A) and MeOH with 1% (v/v) ACN and 0.1% (v/v) HAc (B). The elution profile for separation of PSE procyanidins was as follows: 0-3 min, isocratic at 0% (v/v) B; 3-14 min, linear gradient of 0-40% (v/v) B; 14-16 min, linear gradient of 40-100% (v/v) B; 16-19 min, isocratic at 100% (v/v) B, followed by reconditioning of the column. For GSE procyanidins, the gradient was modified as follows: 0-7 min, linear gradient of 0-40% (v/v) B; 7-9 min, isocratic at 40% (v/v) B; 9-11 min, linear gradient of 40-100% (v/v) B; 11-14 min, isocratic at 100% (v/v) B, followed by reconditioning of the column. The flow rate was 40 mL/min and the eluate was monitored by ELSD and UV at 280 nm. Fractions (10 mL) were collected in glass tubes during the run. The fraction of A-type procyanidin dimers (from PSE) was collected from 3.0-5.5 min and the fraction of B-type procyanidin dimers (from GSE) from 3.2-4.0 min. Fractions of several consecutive runs were pooled, evaporated under vacuum, resuspended in water and freeze-dried.

Purification of procyanidin dimer A1 and B1 by preparative RP-HPLC

The dimer fractions obtained by Flash chromatography were dissolved in MeOH (20 mg/mL) and filtered over a Spartan® 0.45 µm filter (Schleicher & Schuell, Dassel, Germany). Procyanidins A1 and B1 were purified from their respective dimer fractions as described elsewhere (*19*). The purity of each procyanidin dimer was determined by RP-UHPLC using a Waters BEH C18 column (2.1 × 150 mm, particle size of 1.7 µm) on an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, an autosampler and a photodiode array (PDA) detector. The mobile phase consisted of water with 1% (v/v) ACN and 0.1% (v/v) HAc (A) and of ACN with 0.1% (v/v) HAc (B). The flow rate was 300 µL/min and the column oven temperature was controlled at 30 °C. The elution profile used was as follows: 0-1 min, isocratic at 9% (v/v) B; 1-19 min, linear gradient of 9-32.5% (v/v) B; 19-22 min, linear gradient of 32.5-100% (v/v) B; 22-25 min, isocratic at 100% (v/v) B, followed by reconditioning of the column. The eluate was monitored at 280 nm and the purity of each procyanidin dimer was determined based on the ratio of the peak area of the compound to the total peak area of the chromatogram. The peak assignment was confirmed by electrospray ionization-mass spectrometry (ESI-MS) using a LTQ-XL mass spectrometer (Thermo Scientific) equipped with an ESI probe coupled to the RP-UHPLC system. Mass spectrometric analysis was conducted using nitrogen as sheath gas and auxiliary gas. Data were collected in negative ionization mode over the *m/z* range of 200-2000, and most settings were optimized via automatic tuning using "Tune Plus" (Xcalibur 2.07, Thermo Scientific) and (+)-catechin as standard. The transfer tube temperature was set at 270 °C and the source voltage at 4.2 kV. Data acquisition and reprocessing were performed using Xcalibur 2.07 (Thermo Scientific). The

purity of procyanidin A1 was 99% (w/w) and the purity of procyanidin B1 was 87% (w/w) based on UV, which was in accordance with their MS base peak chromatograms.

Binding affinities by ultrafiltration (UF assay)

All samples were prepared in a 50 mM sodium phosphate buffer, pH 7.0. Protein stock solutions (0.05 mM) were prepared freshly before each experiment. For α-casein, an average molecular mass of 23.3 kDa was calculated assuming it contained 80% (w/w) α_{S1}-casein and 20% (w/w) α_{S2}-casein based on the protein composition in bovine milk. A stock solution of EGCG (6 mM) was used to obtain a range of dilutions between 0 and 6 mM. EGCG-protein mixtures were prepared and the binding affinities of each protein towards EGCG were measured using an ultrafiltration microtiter plate setup (Ultracel 10, Millipore, Cork, Ireland) as described previously (*3*).

The protein-bound and free fractions of EGCG at each concentration tested were calculated and plots of the bound fraction versus the concentration of free EGCG were used to determine the binding parameters. For each binding curve obtained, a linear regression was used on the initial linear increase (R^2>0.8) in order to estimate the binding affinity (K) of the compounds. Binding affinities were reported as mean ± standard deviation (SD) of three replicates.

Fluorescence quenching

All samples were prepared in a 10 mM sodium phosphate buffer, pH 7.0. Proteins (5 μM) and phenolic compounds were dissolved freshly before use and kept on ice to limit potential degradation. The protein concentration was determined by the fluorescence intensity of the sample at λ_{em} = 340 nm (λ_{ex} = 295 nm) prior to titration. A calibration curve was established using a series of protein concentrations quantified by their light absorption at 280 nm using the molar absorption coefficients reported in **Table 2**. Fluorimetric experiments were carried out at 25 °C on a Cary Eclipse fluorescence spectrometer (Varian, Victoria, Australia) equipped with a xenon flash lamp and a Peltier thermostated multicell holder allowing for a constant stirring of the sample in the cuvette. Unless stated otherwise, fluorescence emission spectra were recorded from 310 to 450 nm (slit width set to a corresponding bandpass of 5 nm) at an excitation wavelength of 295 nm (slit width set to a corresponding bandpass of 5 nm), specific for the excitation of tryptophan residues. This excitation wavelength was preferred over that of 280 nm to limit the strong background fluorescence of procyanidin dimers (data not shown) and the inner filter effects related to the absorption spectra of phenolic compounds in the UV range. The average of 5 spectra was taken per measurement. The protein sample (1.2 mL) was loaded in a 1 cm quartz cuvette cell (1.5 mL available volume) and equilibrated to temperature in the sample holder for 10 min. Samples were titrated with 10 μL aliquots of ligand solutions to reach a final concentration of ligand between 95 and 117 μM. For each titration point, samples were incubated in the sample holder for 10 min prior to measuring their fluorescence spectra. As

a control, the ligand was titrated into buffer and measured in the same way. Data were collected at least in duplicate on two different days. The Förster radius (R_0) from tryptophan to bound EGCG was calculated for FRET in both caseins, using a value of 2/3 for the squared dipole moment orientation factor (κ^2), 1.4 for the refractive index, 0.12 for the quantum yield of tryptophan and 10655 $M^{-1}.cm^{-1}$ for the extinction coefficient of EGCG at 273 nm.

Data correction and analysis for fluorescence quenching

The use of fluorescence quenching of tryptophan for studying protein-ligand binding requires correction of the fluorescence signal measured for inner-filter effects related to light absorption by the solutes at the excitation and/or emission wavelengths (i.e. primary and/or secondary inner filter effects) and for auto-fluorescence of the ligand. In addition, the occurrence of collisional quenching must be checked (*21*).

To correct for inner-filter effects, another titration experiment, similar to the protocol used for fluorescence quenching, was conducted using 1-cm quartz cuvettes. Absorbance spectra from 200 to 800 nm were recorded using a UV-1800 spectrophotometer equipped with a CPS-controller for temperature control (Shimadzu, Kyoto, Japan). Each fluorescence data point was corrected for inner-filter effects using the following equation adapted from Kubista *et al.* (*22*):

$$F_{corr} = F_{obs} \times 10^{(A_{ex} \times d_{ex} + A_{em} \times d_{em})}$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} is the measured fluorescence intensity, A_{ex} and A_{em} are the absorbances measured at the excitation and emission wavelength, respectively, and d_{ex} and d_{em} are the relative path lengths in the excitation and emission directions, respectively. The path length in the excitation direction was determined as described elsewhere with modifications (*22*). A series of concentrations was measured for absorption and fluorescence at fixed wavelengths. The increase in absorption at λ = 280 nm (A_{280}) and fluorescence signal at λ_{ex} = 280 nm and λ_{em} = 320 nm (F_{320}) were measured with concentrations of procyanidin B1 or B2 ranging from 13 to 117 µM. The d_{em} was calculated as the slope of the linear plot of $\log(A_{280}/F_{320})$ as a function of A_{280} for each ligand and the average value was d_{em} = 0.43 (Supporting information: **Figure S1**). There is no practical method to determine the path length in the emission direction. Considering that we have used 0.4 cm-wide quartz cuvettes in the emission direction, d_{em} was assumed to be 0.172, based on the total path length factor. Corrections for secondary inner filter effect were only applied for samples titrated with EGCG, as procyanidin dimers and EGC did not show absorbance in the emission wavelength range.

After correction for inner-filter effect, the fluorescence of the control sample (ie. ligand titrated into buffer) was subtracted from the measured fluorescence, assuming that the quantum yield of the bound ligand is identical to that of the free ligand.

The occurrence of collisional quenching was checked by titrating each ligand into a solution of L-tryptophan and assessing potential changes in the fluorescence spectrum of tryptophan as described above. No significant changes were observed for EGC and procyanidin dimers (data not shown) and therefore no collisional quenching is expected to occur (*21*). The small decrease in tryptophan fluorescence observed with EGCG is attributed to possible stacking interactions, as the concentrations of ligand used are assumed to be too low to observe a significant amount of collisional quenching.

After correction, data were analyzed using least square regression analysis with a 1:1 binding model assuming the formation of a non-fluorescent complex:

$$\frac{F_0 - F}{F_0} = \frac{(K_d + [L]_t + [P]_t) - \sqrt{(K_d + [L]_t + [P]_t)^2 - 4[L]_t[P]_t}}{2[P]_t}$$

where F_0 is the initial fluorescence of the protein; F is the fluorescence of the protein after ligand addition; K_d is the dissociation constant (in M); $[L]_t$ is the concentration of ligand added (in M); and $[P]_t$ is the concentration of protein corrected for dilution (in M). The derivation of this model is given in the supplementary information.

RESULTS

Comparison of α-casein and β-casein for their affinity to EGCG

The affinity of α-casein and β-casein for EGCG was assessed by ultrafiltration and fluorescence quenching (FQ). Typical fluorescence emission spectra for α-casein and β-casein are given in **Figure 2**, and binding affinities are summarized in **Table 3** and **Figure 3A**. Fluorescence quenching showed a ~2-fold higher binding affinity of β-casein for EGCG compared to α-casein. The difference between the two caseins observed by FQ was in line with previously reported values, although no details were reported about fluorescence data corrections for inner-effects (*14*). Plotting our data for β-casein in the modified Stern-Volmer plots, as reported by Hasni *et al.* (*14*), provided an average binding affinity for EGCG of 1.64 (± 0.22) $\times 10^4$ M^{-1}. In the present study, the β-casein concentration is well below its critical micelle concentration (CMC of 21 µM (*23*)), whereas Hasni *et al.* used a concentration equivalent to 4 times its CMC. Provided that the proper fluorescence data corrections were performed, the similarity in binding affinities found in both studies suggests that β-casein has the same affinity for EGCG, independently from its colloidal state. This observation correlates with data reported in our previous work (*4*).

Table 3. Summary of the interactions of EGCG to the various caseins and mixtures measured by ultrafiltration (UF) and fluorescence quenching (FQ) at pH 7.0

Ligand	Method	K (10^3 M^{-1})	
		α-casein	β-casein
EGCG	UF	13.9 (±2.8)	38.5 (±10.8)
EGCG	FQ	6.51 (±0.69)	13.9 (±2.48)
EGC	FQ	1.66 (±0.17)	1.35 (±0.16)
Procyanidin A1	FQ	2.15 (±0.14)	n.d.
Procyanidin B1	FQ	1.81 (±0.17)	2.11 (±0.12)
Procyanidin B2	FQ	1.64 (±0.07)	1.56 (±0.14)

n.d.: not detectable

The higher binding affinity of β-casein for EGCG than that of α-casein is confirmed by the UF assay. A ~3-fold difference was found by the UF assay, which agrees with the 2-fold difference measured by fluorescence quenching. Binding affinities from the UF assay are overall higher than those found by FQ. A major difference between the two assays is that the FQ experiments consisted of a slow addition of aliquots of ligand to the same protein sample, whereas in the UF assay one concentration of ligand was incubated with a fixed protein concentration to generate independent data points in adsorption isotherms. This might result in different colloidal complexes formed in solution, as shown with poly(L-proline) binding flavan-3-ols at various polyphenol-to-protein ratios (*24*). In addition, FQ is measured at the binding equilibrium whereas in the UF assay, the ultrafiltration process concentrates the protein on the membrane, which might influence the binding equilibrium. When measuring binding affinities of monomeric proteins to EGCG, the indirect approach by FQ can provide a more sensitive measurement than the UF assay, requiring lower concentrations of ligand and protein.

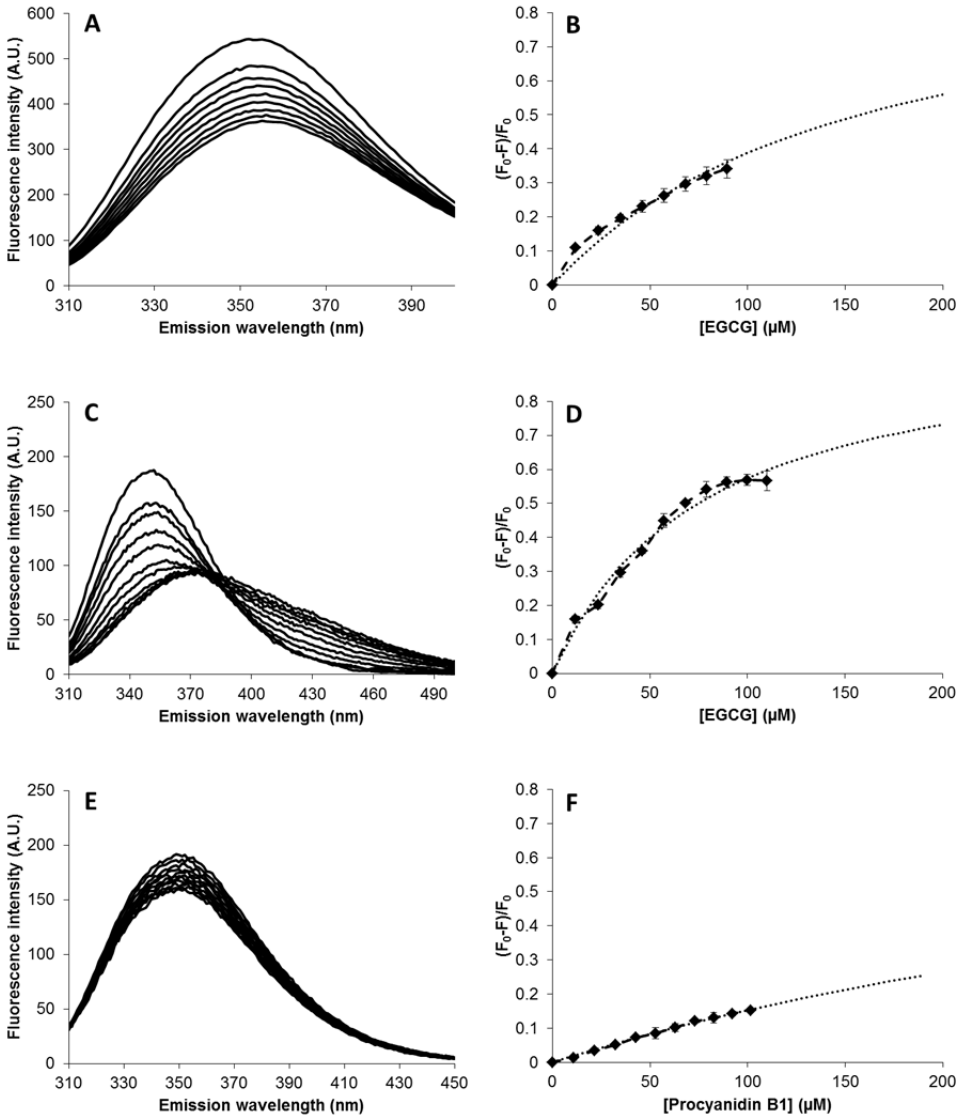

Figure 2. Typical fluorescence spectra measured upon increasing concentration of ligand after data correction and related Stern-Volmer plots: α-casein – EGCG (**A-B**), β-casein – EGCG (**C-D**) and β-casein – procyanidin B1 (**E-F**)

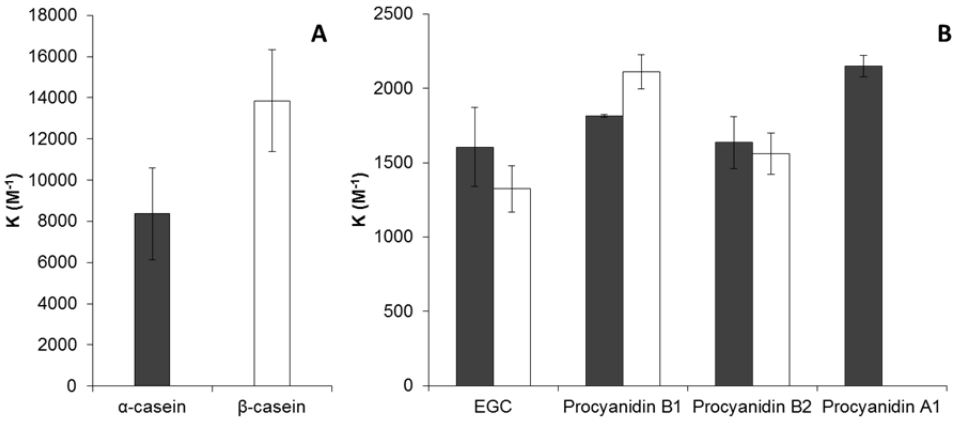

Figure 3. Binding affinity of EGCG to bovine milk α-casein and β-casein, evaluated by fluorescence quenching (**A**); Comparison of the binding affinities of A-type and B-type procyanidin dimers to α-casein and β-casein evaluated by fluorescence quenching (**B**)

The fluorescence spectrum of β-casein displayed a strong red shift in its maximum during titration and the fluorescence at 352 nm leveled off and started to increase at the end of the titration (**Figure 2C**). This might have resulted either from an increased fluorescence of EGCG once bound, which would not be corrected for by our buffer control, or from fluorescence resonance energy transfer (FRET) between the tryptophan and EGCG. The fluorescence of EGCG bound to β-casein, when excited directly at 325 nm, is slightly higher (10 fluorescence intensity units) than that of its free form (Supplementary information **Figure S2**). This small increase is possibly due to a more hydrophobic environment of the bound EGCG. The total increase in fluorescence emission observed above 380 nm upon excitation of the β-casein-EGCG complex at 295 nm has a magnitude of about 50 units. Hence, the small change in the fluorescence quantum yield of EGCG upon binding is unlikely to be the only contribution to the increased fluorescence above 380 nm. Quenching of fluorescence by EGCG (and, thus, the derived binding constant) as a consequence of FRET between the tryptophan residue and EGCG is very plausible because the absorption spectrum of EGCG overlaps with the fluorescence emission spectrum of β-casein. The concomitant Förster radius yields a value of 2.5 nm. Hence, there is a possibility of FRET occurring between the tryptophan of β-casein and EGCG, provided that the two species are within this radius. Likely, FRET has generated some additional fluorescence in the emission range of EGCG, but this is not taken into account in our data analysis. The same spectral overlap exist between α-casein and EGCG, hence, FRET may also occur between the tryptophans of α-caseins and EGCG. Nevertheless, no visible increase in the fluorescence spectra above 380 nm was observed, which suggests a different binding mode.

Effect of dimerization and galloylation of flavan-3-ols on their interaction with caseins

EGC, and procyanidin dimers B1 and B2 were assessed for their affinity with α-casein and β-casein by fluorescence quenching as summarized in **Table 3** and **Figure 3B**. A typical set of fluorescence spectra observed upon titration of a procyanidin dimer to β-casein is given in **Figure 2E**. The absence of the galloyl group at the 3-OH position in EGC, compared to EGCG, resulted in a decrease of its binding affinity for α-casein and β-casein by 4-fold and 10-fold, respectively. This finding is in accordance with previous data (*3,6*).

Procyanidin dimers B1 and B2 displayed affinities for β-casein only slightly higher compared to EGC. No difference is observed when comparing the binding of these ligands to α-casein. The binding affinity of EGCG for both caseins is 4 to 10 times higher compared to procyanidins B1 and B2.

Effect of an extra interflavanic linkage on binding of procyanidin dimers to caseins

Taken independently, procyanidins B1 or B2 do not show a marked difference in binding affinity for either β-casein or α-casein, especially in the case of procyanidin B2 (**Figure 3B**). Procyanidin B1 displays a higher affinity for β-casein (K_a = 2111 (±115) M^{-1}) compared to procyanidin B2 (K_a = 1561 (±141) M^{-1}), whereas differences in affinity observed with α-casein are within the experimental error. This suggests different modes of interaction between ligands and caseins, which may be caused by structural differences between procyanidins B1 and B2.

The binding affinities of procyanidin dimers B1 and B2 to caseins were compared to that of procyanidin A1, which contains an additional interflavanic bond. Procyanidin A1 displays the highest affinity for α-casein compared to the other procyanidins tested (**Figure 3B**). On the contrary, no significant change in fluorescence could be measured for A1 mixed with β-casein, which means either no interaction or an interaction not influencing the fluorescence of tryptophan.

DISCUSSION

Flavan-3-ols' flexibility as a driver for their binding to unstructured proteins

Comparing EGC and procyanidins B1 and B2 shows that the binding affinities of procyanidins for caseins are only slightly different between monomeric gallocatechins and dimers of catechin/epicatechin. EGC has been reported to have a slightly higher binding affinity to caseins compared to (-)-epicatechin or (+)-catechin (*14*), and therefore it is not unexpected that it has an affinity for β-casein intermediate between catechin and procyanidin dimers. Our observations with β-casein are in accordance with previous studies, as, for example, procyanidin dimer B3 (catechin 4-8 catechin) was found to have better binding, and precipitating capacity, with salivary PRPs compared to (+)-catechin (*8*). The calculated hydrophobicities (logP) of procyanidins B1 and B2 are about 2 times higher compared to EGC (**Figure 1**), which is a known positive property for protein-phenolic

interactions (*7*). Assuming that both hydrogen bonding and hydrophobic interactions occur, the aforementioned procyanidins contain 10 hydroxyl groups and two rotatable rings (i.e. rings B and E), compared to EGC which contains only 6 hydroxyl groups and one rotatable ring (i.e. ring B) (**Figure 4**). Rings B and E in procyanidin B1 and B2 might act as adaptable anchor points promoting the interaction to proline-rich proteins (*12,25*).

Hydrophobicity and hydroxylation of flavan-3-ols do not seem to be consistently a driver for their interaction with caseins. In fact, EGCG and procyanidins B1 and B2 have similar logP values and about equivalent side groups available for hydrogen bonding (**Figure 1**). Their affinities for caseins, however, are clearly different. As summarized in **Figure 4**, EGCG has 4 rotatable bonds and a stereochemistry not allowing π-π stacking of the B and D rings in its lowest energy conformer. EGCG was already proven to interact with neighboring proline residues with its A and D-rings, and the B-ring stabilizing the complex with a different residue (*26*). Procyanidin dimers B1 and B2 have 3 rotatable bonds and preferentially adopt a compact conformation in water (*17*). It is hypothesized that EGCG benefits from a higher rotational freedom in solution compared to the procyanidin dimers. Hence, addition of another flavan-3-ol unit to a flavan-3-ol has less impact on binding affinity to caseins compared to esterification of its 3-OH position with gallic acid. This finding highlights the importance of galloylation in polyphenol-protein interactions.

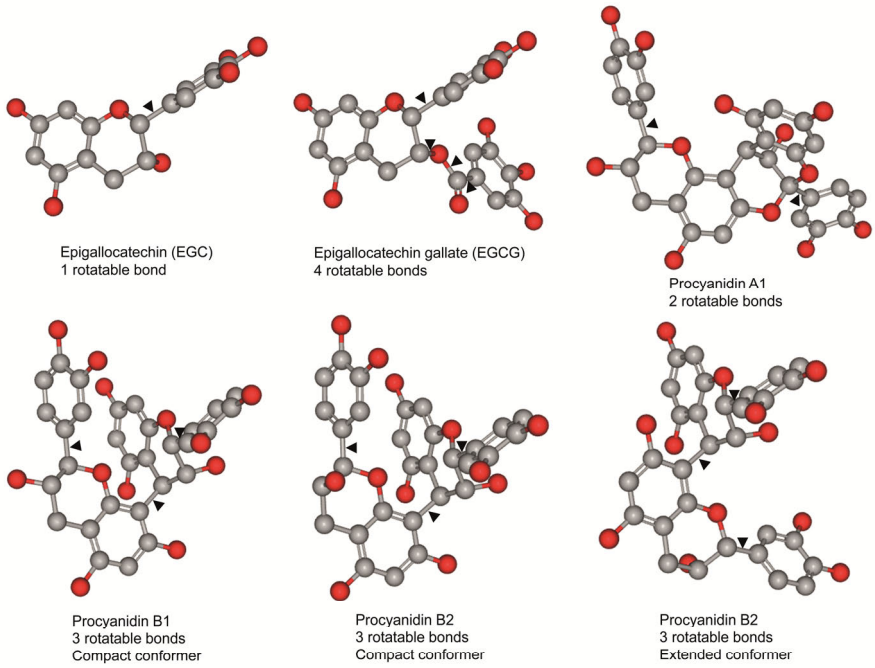

Figure 4. 3D representations of ligands investigated. Rotatable bonds are indicated with arrows

Comparing specifically the procyanidins B1 and B2 binding β-casein, procyanidin B1 has a higher affinity than procyanidin B2. Procyanidin B1 is known to be preferentially in its compact conformer state (ratio compact-to-extended 95:5), whereas procyanidin B2 has a more equal distribution of its conformers (ratio compact-to-extended 55:45) (*17*). Based on the higher accessibility of the rotatable rings in the more extended procyanidin B2, we had expected better binding of β-casein with procyanidin B2 than with procyanidin B1, but our results indicate the opposite. Conflicting data on the binding affinity of procyanidins B1 and B2 to proteins have been reported. Consistent with our results, a higher interaction for procyanidin B1 compared to procyanidin B2 has been suggested based on their ability to precipitate a mixture of salivary proline-rich proteins (*8*). In contrast with our results, a higher binding affinity of procyanidin B2 to the proline-rich peptide $IB7_{14}$ was found compared to procyanidin B1 (K_{B2} = 910 M^{-1} and K_{B1} = 344 M^{-1}), which was related to their respective ratios of conformers (*10*).

As shown in **Figure 4**, the addition of an ether bond seems to force the procyanidin dimer into an extended conformation with the two rotatable catechol rings exposed. This observation, together with the higher hydrophobicity of procyanidin A1 (**Figure 1**), might explain the higher binding affinity for α-casein observed for procyanidin A1 compared to B-type procyanidin dimers. Others have hypothesized that A-type interflavanic bonds would result in an increased hydrophobicity, which should have a positive effect on the binding affinity, although this effect might be counteracted by the loss of flexibility within the phenolic compound, reducing its adaptability to binding sites on the protein (*27*). Our results show that the loss of flexibility in procyanidin A1 does not impact its affinity for α-casein.

Distribution of prolines and hydrophobic amino acids in caseins as driver for binding of phenolics?

β-Casein shows a higher binding affinity for EGCG compared to α-casein, which might be related to its overall higher hydrophobicity, proline content, and number of proline repeats (**Table 1**). For other phenolics tested (i.e. EGC, procyanidin B1, and procyanidin B2), the expected higher binding affinity of β-casein compared to α-casein was less clear. Interestingly, α-casein interacted with procyanidin A1, whereas no interaction was detected in the case of β-casein. This observation is contrary to the expected preferential binding of procyanidins to Pro-Pro repeats by hydrophobic ring stacking, as described for procyanidin B2 with a PRP fragment (*12*). Nevertheless, the binding mechanism of procyanidins is not clearly defined, as others have described a predominance of hydrogen bonding involving proline residues (*25*).

The binding affinities of α-casein for the investigated procyanidins can be ranked as follows: $K_{A1} > K_{B2} \approx K_{B1}$; whereas the binding affinities of β-casein for the same ligands can be ranked as follows: $K_{B1} > K_{B2} > K_{A1}$. According to **Table 1**, α-casein and β-casein do not differ greatly in their contents in aromatic amino acids (except for tyrosine) and

histidine, which may be directly involved in the bindings sites of the proteins (*7,13*). Moreover, it has been suggested that the presence of aromatic amino acids in the vicinity of proline favors the *cis* conformation of proline, which is positively associated to its binding affinity for phenolics (*28,29*). The occurrence of aromatic-proline pairs is similar in the sequences of α_{S1}-casein, α_{S2}-casein and β-casein; several prolines with neighboring aromatic residues could be found in each. We speculate that factors other than proline content, and distribution of proline and aromatic amino acids in the protein might be of importance, particularly when the proteins are less proline-rich (e.g. caseins) than the more commonly described salivary proteins.

ACKNOWLEDGEMENT

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SUPPORTING INFORMATION

Figure S1. Determination of d_{ex} at λ_{ex} = 280 nm and λ_{em} = 320 nm using procyanidins B1 and B2 at pH 7.0

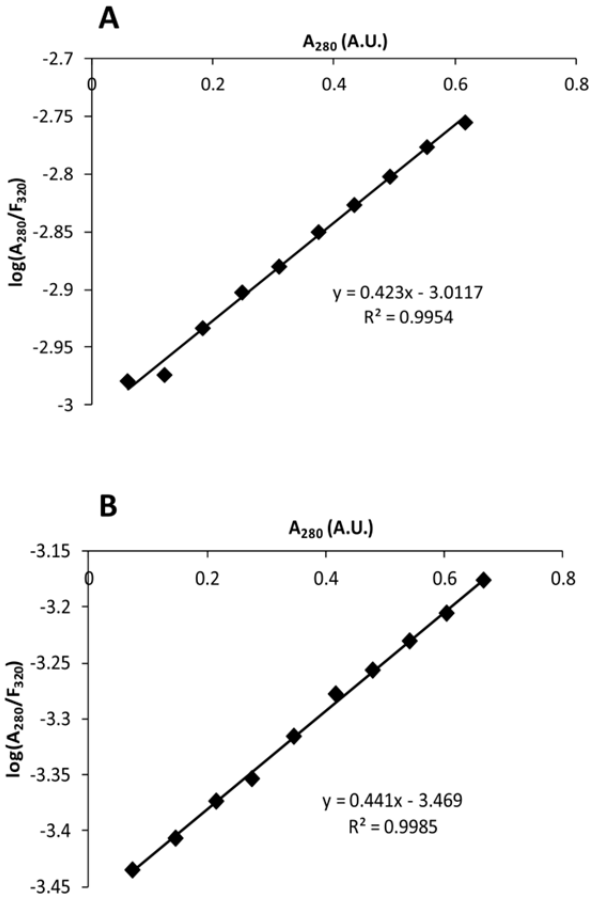
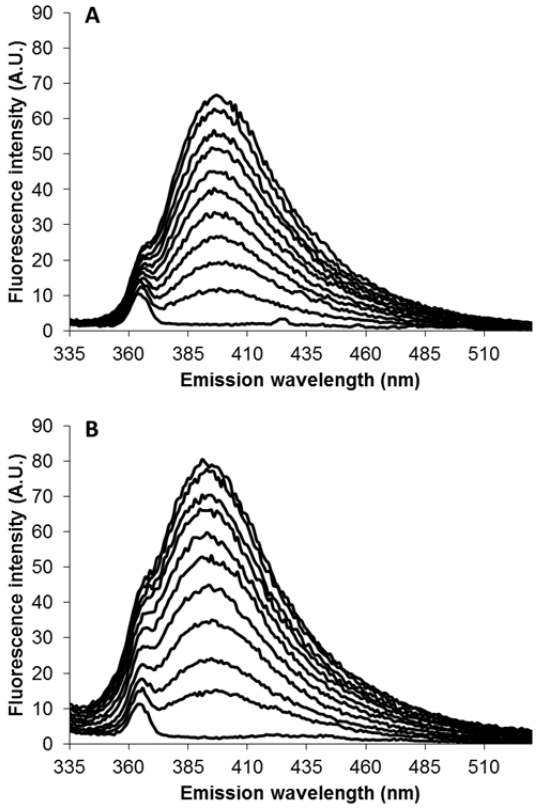


Figure S2. Fluorescence spectra of EGCG in buffer only (A) and mixed with β-casein (B) in 10 mM sodium phosphate buffer pH 7.0 at 25 °C. Spectra were corrected for inner filter effects. λ_{ex} = 325 nm; λ_{em} = 335 – 530 nm



Supplementary information S3. Derivation of the model used for the fitting of the fluorescence quenching data by least square regression analysis (all concentrations are expressed in M)

When considering a protein-ligand interaction with a 1:1 stoichiometry, the dissociation constant (K_d (M)) can be expressed as follows (equation 1):

$$K_d = \frac{[P]_f \times [L]_f}{[PL]}$$

with $[P]_f$, the concentration of free protein at equilibrium; $[L]_f$, the concentration of free ligand at equilibrium; and [PL] the concentration of protein-ligand complex at equilibrium.

The concentration of free ligand and free protein can be expressed as follows:

$[L]_f = [L]_t - [PL]$ and $[P]_f = [P]_t - [PL]$

with $[L]_t$, the concentration of ligand added, and $[P]_t$ the total concentration of protein in the sample.

Incorporating these expressions in equation 1 gives the following quadratic equation (equation 2):

$$[PL]^2 - [PL]([P]_t + [L]_t + K_d) + [P]_t[L]_t = 0$$

Only one of the two solutions to equation 2 gives acceptable values and is given by equation 3:

$$[PL] = \frac{(K_d + [L]_t + [P]_t) - \sqrt{(K_d + [L]_t + [P]_t)^2 - 4[L]_t[P]_t}}{2}$$

The fluorescence measured at equilibrium (F), assuming that the complex formed is not fluorescent, is a function of the fraction of protein bound (x_b) and the initial fluorescence of the protein (F_0), and can be expressed as follows (equation 4):

$$F = (1 - x_{b)}.F_0 = \left(1 - \frac{[PL]}{[P]_t}\right) \times F_0$$

Rearranging equation 4 and incorporating it in equation 3 gives:

$$\frac{F_0 - F}{F_0} = \frac{[PL]}{[P]_t} = \frac{(K_d + [L]_t + [P]_t) - \sqrt{(K_d + [L]_t + [P]_t)^2 - 4[L]_t[P]_t}}{2[P]_t}$$

Chapter 4

Quantifying the preferential binding of individual phenolics in complex mixtures to β-casein using UHPLC-MS-MS

The interaction of phenolics with proteins is commonly evaluated using purified or synthesized phenolic standards. The current study aimed at evaluating the binding affinity of individual phenolics in a complex mixture to β-casein by applying an ultrafiltration assay coupled to UHPLC-MS-MS analysis. A procyanidin trimer fraction from grape seed was used as a representative mixture containing phenolics with structural features known to influence binding to proteins. Targeted free phenolics were analyzed by selected reaction monitoring in a linear ion-trap MS and quantified relative to their initial amount in the extract used. This method allowed the rapid and sensitive monitoring of 4 compounds simultaneously, and provided data in a linear analytical range up to 0.13 mg/mL of extract. The comparison of the binding characteristics of several individual procyanidins in the mixture showed that galloylation enhanced the binding affinity of procyanidins to β-casein. Degree of polymerization and galloylation of procyanidins were shown to affect synergistically protein-phenolic interactions. In a black tea extract, galloylation of theasinensins was also found important in binding. Moreover, oligomeric phenolics appeared to prime the binding of monomeric phenolics from the same mixture. The UF-MS assay presented in this study was found to be a valuable tool for quantifying the binding to casein of many phenolics from a complex mixture simultaneously.

Bohin, M.C., Verloop, A.J.W., Gruppen, H., van Erven, G., Dekker P., van der Hijden H.T.W.M., Vincken, J.-P. (2013) -*To be submitted*

INTRODUCTION

Phenolic–rich food can have unpleasant organoleptic properties as a result of the interaction of phenolics with food macromolecules, salivary proteins or bitter taste receptors (*1,2*). Food proteins, in particular milk proteins, have been proposed for the development of delivery systems for phenolics with effective reduction in taste perception (*3-5*). The binding affinity of phenolics to proteins is known to be high when the protein has an open and flexible structure rich in proline residues, as found in caseins (*1,6*). The molecular structure of phenolics is also known to have an impact on their interaction with proteins. Degree of polymerization (DP), conformational freedom, hydrophobicity and galloylation are amongst the main structural characteristics positively influencing the binding affinity (*1,6-8*). These features have mostly been highlighted with monomeric and oligomeric flavan-3-ols, the latter being often referred to as proanthocyanidins or condensed tannins.

Phenolics-protein interactions can be studied through a variety of methods, including isothermal titration calorimetry (ITC) (*8*), dynamic light scattering (DLS) (*9*), small angle X-ray scattering (*10*), fluorescence quenching (*11*), ultrafiltration (*6*), nuclear magnetic resonance spectroscopy (NMR) (*12*), or mass spectrometry (MS) (*13*). These methods usually require the use of relatively pure phenolics in order to establish structure-affinity relationships. Commercially unavailable phenolics may require tedious purification from complex extracts due to the occurrence of many isomers or other compounds with similar polarity. For the evaluation of the binding of individual phenolics present in complex mixtures to target food proteins, few tools are available.

In analogy to pulsed ultrafiltration-MS used in pharmacological studies for receptor-ligand screening (*14*), MS might be applied as a tool for the selective identification and/or quantification of phenolics bound to food proteins. The application of MS for the assessment of food protein-phenolics interactions has only been rarely reported. For example, matrix-assisted laser desorption ionization – time of flight – MS (MALDI-TOF-MS) has been used to qualify unbound proanthocyanidins (*15*), and selected reaction monitoring (SRM) in LC-MS to quantify unbound phenolics (*16*). In the latter case, mixtures of commercially available phenolic standards were used. SRM has initially been developed for accurate quantification of selected compounds in complex mixtures, such as plasma samples or protein extracts from cells. This method consists of the monitoring of a specific fragment ion from a selected precursor ion and is traditionally applied using triple-quadrupole mass spectrometers, although linear ion traps with rapid analysis capacity can also be used (*17,18*). This method seems particularly applicable to mixtures of phenolics with similar hydrophobicity and multiple isomers. Extracts containing procyanidins (flavan-3-ol oligomers of catechin/epicatechin, possibly galloylated) or processed tea phenolics (monomeric flavan-3-ols and oxidation products (e.g. theasinensin)) are typical examples of such mixtures. A representative compound from both extracts is depicted in **Figure 1**.

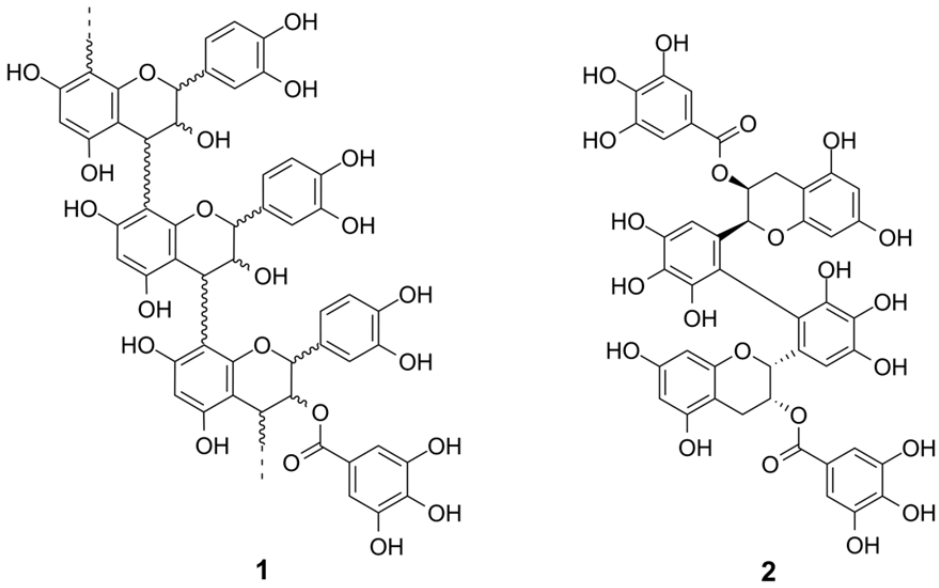


Figure 1. Representative structures of the ligands tested (1 = B-type galloylated procyanidin oligomer (GSE); 2 = digalloylated theasinensin (black tea))

The aim of the present study was to employ ultrahigh-pressure liquid chromatography-MS-MS (UHPLC-MS-MS) combined with ultrafiltration to evaluate the affinity of target phenolics present in complex mixtures to β-casein. Comparing ligands in mixtures for their affinity to a protein may help highlighting structural features of phenolics not available as standards that positively influence binding to proteins. This method was developed using a B-type procyanidin fraction containing molecules with a variety of structural features known to impact procyanidin-protein interactions. Subsequently, its potential to characterize the binding of rarely studied phenolics from black tea to protein was evaluated.

MATERIALS AND METHODS

Materials

Bovine β-casein (≥98% of total protein) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A commercially available procyanidin extract from grape seeds (Vitaflavan®, kindly provided by Levita Chemicals International NV, Antwerpen, Belgium) was used as a source to generate fractions of B-type procyanidins. This extract will be referred to as 'grape seed extract' (GSE). Black tea was obtained from a local store and originated from the Uva Highlands (Sri Lanka). 2,5-Dihydroxybenzoic acid (DHB) was purchased from Bruker Daltonics (Bremen, Germany). Maldodextrin MD20 (DP1 to 20) was obtained from

Avebe (Veendam, The Netherlands). Organic solvents used were ULC-MS grade and purchased from Biosolve BV (Valkenswaard, The Netherlands). Water (MQ) was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Fractionation of Vitaflavan® by flash chromatography

Separation of procyanidins by degree of polymerization (DP) was achieved using a diol stationary phase based on a method described elsewhere with modifications (*19*). A Reveleris® flash system (Grace, Deerfield, IL, USA) equipped with an ELSD and a UV detector was used. For each run, the sample (100 mg/mL) was dissolved in 90% (v/v) acetonitrile (ACN) and 5 mL was loaded onto a 40 g diol flash cartridge with a 40 µm particle size (Grace). The binary mobile phase consisted of ACN containing 0.1% (v/v) acetic acid (HAc) (A) and methanol (MeOH) containing 10% (v/v) MQ, 1% (v/v) ACN and 0.1% (v/v) HAc (B). The elution profile applied was as follows: 0-3 min, isocratic at 0% (v/v) B; 3-14 min, linear gradient of 0-40% (v/v) B; 14-16 min, linear gradient of 40-100% (v/v) B; 16-19 min, isocratic at 100% (v/v) B, followed by reconditioning of the column. The flow rate was 40 mL/min and the eluate was monitored by UV at 280 nm. Fractions (10 mL) were collected in glass tubes. The fractions were pooled based on UV (280 nm) and named after the predominant DP expected as follows: 'monomer fraction', 1.7-2.8 min; 'dimer fraction', 5.4-7.1 min; 'trimer fraction', 8-8.8 min; 'tetramer fraction', 9.3-10.1 min; 'higher DP fraction', 10.1-15.3 min. Fractions of several consecutive runs were pooled, evaporated under vacuum, resuspended in water and freeze-dried. Throughout the procedure, samples were kept from light by using aluminum foil.

Characterization of grape seed procyanidin fractions by MALDI-TOF-MS

Samples were prepared in water (2 mg/mL) and desalted by addition of Dowex 50WX8 resin (ratio of 60 (w/v), Bio-Rad, Hercules, CA, USA), followed by incubation at room temperature under constant stirring for 30 min, and subsequent centrifugation (5 min, 21380×g, room temperature). A solution of 10 mg/mL of DHB in water was used as matrix. Spots were prepared using the sandwich method on a stainless steel sample plate (Bruker Daltonics): 1 µL of DHB solution was applied and air dried, then 1 µL of sample was applied on top and air dried; finally, the spot was covered by 1 µL of DHB solution and air dried. Samples were analyzed on an Ultraflextreme workstation equipped with a N_2 laser of 337 nm, operated in positive mode and controlled by FlexControl 3.3 software (Bruker Daltonics). Full MS spectra were collected in reflector mode with voltages of 25.00 and 22.35 kV. For each sample, the laser intensity was optimized for maximum signal intensity. Calibration was performed using maltodextrins MD20 and a mass scan range from 500 to 3500 Da was used. Data analysis was conducted using FlexAnalysis v3.3 software (Bruker Daltonics).

Characterization of grape seed procyanidin fractions by RP-UHPLC-MS-MS

The fractions isolated by flash chromatography were dissolved in water (0.65 mg/mL) and characterized by RP-UHPLC-MS-MS using a Thermo Hypersyl Gold aQ column (2.1 × 150 mm, particle size of 1.7 µm) on an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, an autosampler, and a photodiode array (PDA) detector. 5 µL of sample was injected (full loop injection) and 30% (v/v) ACN containing 0.2% (v/v) formic acid (FA) was used as wash solvent. The mobile phase consisted of water containing 1% (v/v) ACN and 0.2% (v/v) FA (A) and of ACN containing 0.2% (v/v) FA (B). The flow rate was 350 µL/min, and the column oven temperature was set at 15 °C. The elution profile used was as follows: 0-2 min, isocratic at 5% (v/v) B; 2-3 min, linear gradient of 5-12% (v/v) B; 3-10 min, isocratic at 12% (v/v) B; 10-22 min, linear gradient of 12-60% (v/v) B; 22-24 min, linear gradient of 60-100% (v/v) B; 24-27 min, isocratic at 100% (v/v) B; followed by reconditioning of the column for 13 min. The eluate was monitored at 280 nm and the peak assignment was based on electrospray ionization-mass spectrometry (ESI-MS) using a LTQ-Velos Pro (Thermo Scientific) equipped with a heated ESI probe coupled to the RP-UHPLC system. Mass spectrometric analysis was conducted using nitrogen as sheath gas and auxiliary gas. Data were collected in negative ionization mode over the *m/z* range of 280-2000, and most settings were optimized via automatic tuning using "Tune Plus" (Xcalibur 2.07, Thermo Scientific) and the trimer fraction of the grape seed extract as standard (*m/z* 865). The ESI probe temperature was set at 150 °C, the transfer tube temperature was set at 370 °C and the source voltage was set at 3.7 kV. Data dependent MS^2 analysis was performed on the most intense ion in the full MS spectrum with a normalized collision induced energy of 35%. A dynamic mass exclusion list was used for MS^2, in which a compound detected twice as most intense was subsequently excluded for the next 5 s, allowing data dependent MS^2 of less intense co-eluting compounds. Data acquisition and reprocessing were performed using Xcalibur 2.1 (Thermo Scientific).

Fractions were also characterized by in-flow direct injection in the MS using a flow of 390 µL/min of 30% B from the UHPLC system and a flow of 10 µL/min from the syringe pump of the MS. Compounds to be analyzed for their binding to β-casein (see below) were selected based on the intensity of the MS signal at their respective *m/z* values.

Extraction and characterization of black tea extract

Dried black tea leaves were bead-milled prior to extraction. The resulting powder was extracted twice in boiling water (1 % (w/v)) for 10 min under constant stirring. After each extraction cycle, the extract was filtered through a 595 cellulose filter (Whatman, Dassel, Germany) by Büchner filtration. Both filtrates were combined and freeze dried. The extraction yield was 46.2 % (w/w) and the resulting extract was referred to as "black tea extract". Aluminum foil was used throughout the procedure to protect the extract from light.

The extract was characterized using the same UHPLC system described above using a Thermo Hypersyl Gold column (2.1 × 150 mm, particle size of 1.7 µm). One µL of sample was injected and MeOH was used as wash solvent. The mobile phase consisted of water containing 1% (v/v) ACN and 0.1 % (v/v) HAc (A) and of ACN containing 0.1 % (v/v) HAc (B). The flow rate was 400 µL/min, and the column oven temperature was set at 30 °C. The elution profile used was as follows: 0-1.5 min, isocratic at 2% (v/v) B; 1.5-21 min, linear gradient of 2-100% (v/v) B; 21-25 min, isocratic at 100% (v/v) B; followed by reconditioning of the column for 6 min. The eluate was monitored at 280 nm and the peak assignment was based on ESI-MS, as mentioned above. Data were collected in negative ionization mode over the *m/z* range of 250-2000, and most settings were optimized as described above using the black tea extract with the *m/z* value of theaflavin (*m/z* 563). The ESI probe temperature was set at 150 °C, the transfer tube temperature was set at 350 °C and the source voltage was set at 4 kV. Data dependent MS^2 analysis and data acquisition were performed as described above.

Binding of selected phenolic mixtures to β-casein and separation of the unbound fraction by ultrafiltration

All samples were prepared in a 10 mM sodium phosphate buffer, pH 7.0. Protein stock solutions (2 to 100 µM) were prepared freshly before each experiment. A stock solution of the mixture of phenolics of interest (2.1 mg/mL) was used to obtain a range of concentrations between 97 µg/mL and 2.1 mg/mL. For experiments at constant phenolic concentration, a stock solution of 1.30 mg/mL was prepared. Phenolic-protein mixtures were prepared and the unbound phenolic fraction collected using an ultrafiltration microtiter plate setup (Ultracel 10, Millipore, Cork, Ireland) as described previously (*6*). The centrifugation time for ultrafiltration was extended to 45 min in order to maximize the volume of the unbound fraction.

Quantification of binding of individual unbound phenolics by Selective Reaction Monitoring (SRM)

Unbound fractions obtained by ultrafiltration were stabilized against oxidation by acidification to pH~4 by mixing 2 µL of 4% (v/v) FA with 200 µL of filtrate. Samples were characterized by RP-UHPLC-MS-MS using a Thermo Hypersyl Gold column (2.1 × 50 mm, particle size of 1.9 µm) on the same UHPLC system as described above with the same mobile phase, injection volume and wash solvent. The flow rate was 400 µL/min, and the column oven temperature was set at 30 °C. The elution profile used was as follows: 0-1.5 min, isocratic at 2% (v/v) B; 1.5-5 min, linear gradient of 2-100% (v/v) B; 5-7 min, isocratic at 100% (v/v) B; followed by reconditioning of the column for 6 min. The eluate was monitored at 280 nm and a maximum of 4 target compounds of interest per run were detected using SRM in a LTQ-Velos Pro (Thermo Scientific) equipped with a heated ESI probe coupled to the RP-UHPLC system. Settings for mass spectrometric analysis were the

same as described above in the characterization section. The microscan count was set to 1 and the maximum ion injection time to 20 ms. Normalized collision energy was optimized for each target compound and the two major fragment ions were used for monitoring. Settings per compound are summarized in **Table 3**. Data acquisition and reprocessing were performed as described above.

A range of concentrations of the untreated mixture of phenolics was first analyzed in order to determine the linear range for the peak area as a function of total extract concentration (in mg/mL). Samples from the binding experiment were diluted with water, such that the expected concentration of phenolics was within the linear measuring range. For each compound, the area under the curve (AUC) was determined from the total ion count (TIC) signal for increased sensitivity at low concentrations. If required, the AUC was then corrected for the dilution applied to each sample. For experiments with constant ligand concentration (0.65 mg/mL) and increasing protein concentrations (7.5 to 50 μM), binding was expressed as percentages of the area under the curve (AUC) of the unbound compound relative to the area of the control sample without protein (also ultrafiltered, therefore including possible non-specific binding to the filter's membrane). For experiments with increasing ligand concentrations (0.049 to 1.04 mg/mL) and constant protein concentration (12.5 μM), the protein-bound fraction (in AUC/mol of protein) was plotted against the concentration of unbound ligand. For each binding curve obtained, a linear regression was used on the initial linear increase (R^2>0.8) and the slope was used as a representative of the binding affinity although the absolute concentrations of each individual compounds were not determined. This binding parameter was reported as mean ±standard deviation (SD) of two replicates conducted on different days.

RESULTS

Characterization of GSE and GSE 'trimer fraction' by MALDI-TOF-MS and UHPLC-MS-MS and selection of target ligands

As summarized in **Table 1**, MALDI-TOF-MS spectra of GSE showed that the extract contained up to heptameric non-galloylated procyanidins and galloylated procyanidin oligomers containing generally one or two gallic acids. LC-MS analysis allowed the detection of monomers and galloylated monomers, and confirmed the presence of oligomeric procyanidin, including galloylated ones, up to DP5. The identification of procyanidins was confirmed by their major fragments in MS^2 (**Table 2**). The smaller diversity of compounds observed by LC-MS compared to MALDI-TOF-MS was attributed to the chromatographic separation of isomers, which lowered their individual MS signal below the detection limit. Nonetheless, the diversity of compounds reported was in accordance with previous studies using MALDI-TOF-MS and LC-MS (*15,20*). The *m/z* values corresponding to A-type procyanidins were also observed by MALDI-TOF-MS, as

already reported (*21*). They could not be confirmed in LC-MS, most likely due to their low abundance. A-type procyanidins were not further considered in this study.

Table 1. Diversity of B-type procyanidins in the grape seed extract by MALDI-TOF-MS

DP[a]	DG[b]	MALDI-TOF-MS		
		$[M-H]^+$	$[M-H]^+$+Na	$[M-H]^+$+K
1	0	n.a.	n.a.	n.a.
1	1	n.a.	n.a.	n.a.
2[c]	0	579	601	617
2[c]	1	n.d.	753	769
2[c]	2	n.d.	905	921
3[c]	0	867	889	905
3[c]	1	n.d.	1041	1057
3[c]	2	n.d.	1193	n.d.
3	3	n.d.	1345	n.d.
4[c]	0	n.d.	1177	1193
4[c]	1	n.d.	1329	1345
4[c]	2	n.d.	1481	1497
4	3	n.d.	1633	n.d.
4	4	n.d.	1785	n.d.
5[c]	0	n.d.	1465	1481
5	1	n.d.	1617	1633
5	2	n.d.	1769	1785
5	3	n.d.	1921	n.d.
5	4	n.d.	2073	n.d.
6	0	n.d.	1753	1769
6	1	n.d.	1905	1921
6	2	n.d.	2056	2073
6	3	n.d.	2209	n.d.
7	0	n.d.	2041	2056
7	1	n.d.	2193	2209

[a]degree of polymerization; [b]degree of galloylation; [c]also detected in the 'trimer fraction' of GSE
n.a.: not applicable; n.d.: not detected

Amongst the fractions of GSE obtained by flash chromatography, the 'trimer fraction' was chosen as the most representative of the common structural variations reported for B-type procyanidins. As summarized in **Table 1** and **Table 2**, this fraction contained procyanidins up to DP4 with one or two gallic acids. Traces of procyanidin pentamers and hexamers were detected by MALDI-TOF-MS, but not by LC-MS. Thus, we focused on the binding of the following phenolics to β-casein: monomeric flavan-3-ols, procyanidin dimers, mono/digalloylated procyanidin dimers, procyanidin trimers, monogalloylated procyanidin trimers, and procyanidin tetramers.

Table 2. B-type procyanidins and their main fragment ions detected in GSE and its 'trimer fraction' by UHPLC-MS-MS

DP	DG	$[M-H]^-$	Main fragment ions[a]	Detected in GSE	Detected in 'trimer fraction'
1	0	289	245, 205, 179	Yes	Yes
1	1	441	289, 169, 331, 271	Yes	Low[b]
2	0	577	425, 407, 289, 451	Yes	Yes
2	1	729	577, 559, 407, 425	Yes	Yes
2	2	881	729, 711, 559, 407, 577	Yes	Yes
3	0	865	695, 577, 739, 713, 407	Yes	Yes
3	1	1017	729, 847, 891, 865, 999	Yes	Yes
3	2	1169	1017, 729, 999, 891, 847	Yes	Low[b]
3	3	1321	n.a.	Low[b]	Low[b]
4	0	1153	983, 865, 1027, 575, 577	Yes	Low[b]
4	1	1305	n.a.	Yes	No
4	2	1457	n.a.	Low[b]	No
5	0	1441	n.a.	Yes	No
5	1	1593	n.a.	Low[b]	No

n.a.: not available

[a]Fragment ions with a relative abundance higher than 10% and listed in order of relative abundance (a maximum of 5 fragments was selected)

[b]Level of detection was 'low' when no peak could be defined and the base peak signal intensity was lower than 10^3

Preferential binding of some procyanidins to β-casein

As depicted in **Figure 2**, the procyanidin 'trimer fraction' was characterized by LC-MS after ultrafiltration with or without added β-casein (12 μM). The UV chromatograms were rather similar with the exception of one region corresponding to hydrophobic compounds (13-17 min). Procyanidins eluting in this time slot were mostly digalloylated procyanidin dimer and monogalloylated procyanidin trimer, as characterized by MS^2. Their clear disappearance from the mixture incubated with β-casein indicated preferential binding of these molecules to the protein, which might be related to their galloylation, known to enhance interactions of phenolics with proteins (*1,8*). The total peak area of the rest of the chromatogram also decreased after addition of β-casein, but to a much lesser extent. The co-elution and multiplicity of peaks did not allow any proper quantification by UV for individual compounds. In this respect, a targeted monitoring using MS seemed more suitable.

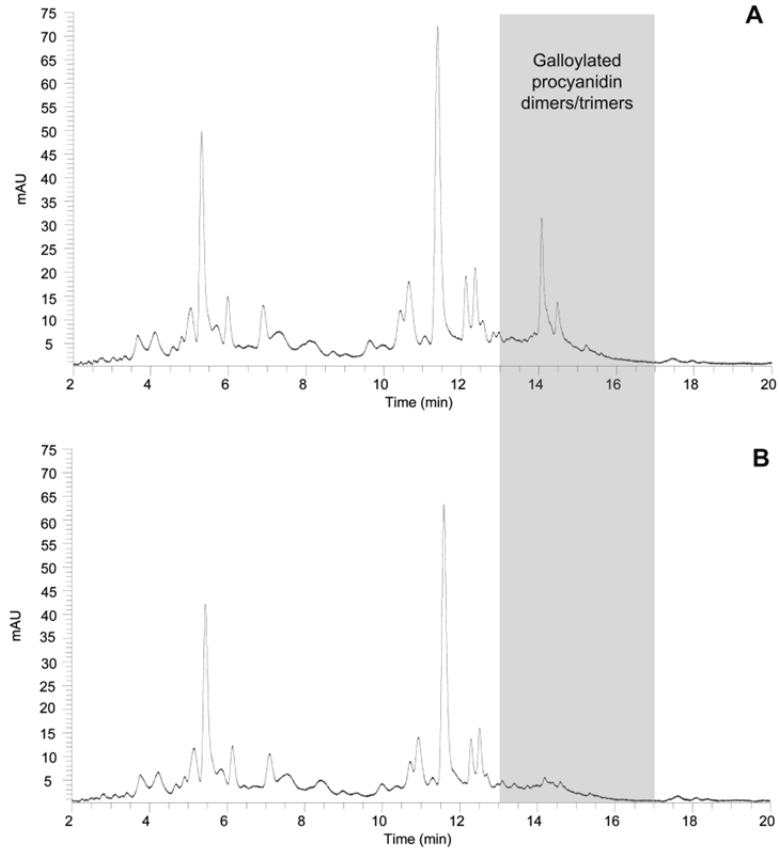


Figure 2. UV chromatogram of ultrafiltered procyanidin trimer fraction from GSE without addition of β-casein (**A**) and after addition of 12 μM β-casein (**B**)

Selection of transitions for SRM and determination of analytical linearity

The two most abundant fragment ions of each ligand selected after characterization were confirmed by in-flow direct injection into the MS. Normalized CID for each compound was optimized and found to be similar for all, hence, it was set at one value. MS monitoring parameters for each ligand are summarized in **Table 3**.

The linearity of the assay was evaluated by measuring a dilution series of the two extracts. The quantity of each compound was monitored by the peak area from the total ion count (TIC) corresponding to their respective two major fragments. As illustrated for non-galloylated trimers (*m/z* 865) in **Figure 3**, the peak area deviates rapidly from linearity with increasing concentration of the fraction. Extract concentrations above 0.13 mg/mL were outside the linearity of the assay for all compounds measured and were, therefore, diluted two to ten times in order to fall within this linear range. The lowest extract

concentration measured (49 µg/mL) allowed an accurate detection of each target compound. Only procyanidin tetramers could not be detected well and thus were not considered for further quantification.

Table 3. SRM transitions and collision induced dissociation (CID) for selected B-type procyanidins from the 'trimer fraction' of GSE

DP	DG	$[M-H]^-$	Isolation width (*m/z*)	Monitored product ions[b]	Isolation width (*m/z*)	Normalized CID (%)
1	0	289	2	205, 245	5	37
2	0	577	2	407, 425	5	25
2	1	729	2	559, 577	5	28
2	2	881	2	711, 729	5	25
3	0	865	2	577, 695	5	35
3	1	1017	2	729, 847	5	27
4	0	1153	2	983, 1001	5	35
4	1	1169	2	999, 1017	5	35

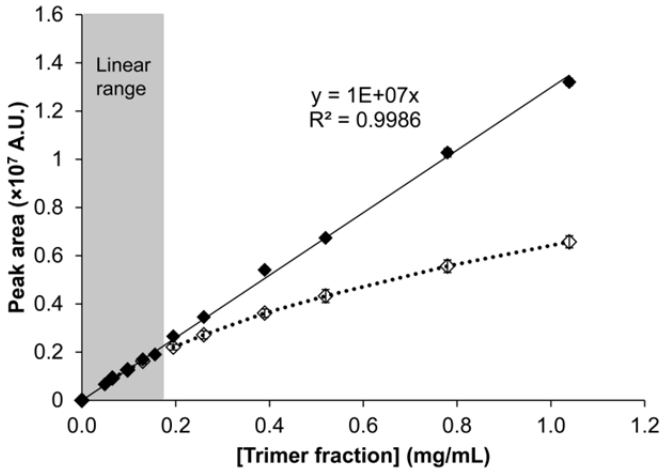


Figure 3. Typical calibration curve obtained for the phenolics investigated (example given procyanidin trimer (*m/z* 865)) and determination of linear concentration range for SRM. ◇ = undiluted concentration range; ◆ = concentration range measured in the linear range by dilution and recalculated to its initial concentration

Binding of target ligands with increasing protein concentration

Percentages of the individual bound procyanidins from the 'trimer fraction' with increasing β-casein concentration are reported in **Figure 4A**. The amounts of bound procyanidins were expressed relatively to the peak area corresponding to the initial amount without protein added. Digalloylated procyanidin dimers and monogalloylated procyanidin trimers bound

well to β-casein, whereas the monogalloylated procyanidin dimer showed an intermediate binding. The non-galloylated species showed the weakest binding to β-casein. It was noticed that monomeric flavan-3-ols were bound at lower concentrations of β-casein compared to procyanidin dimers and trimers, although the affinity of procyanidins for proteins is usually increasing with increasing DP (*1,22*). The sigmoid shape of the curves for non-galloylated procyanidin dimers and trimers and for monogalloylated procyanidin dimers was attributed to preferential binding to β-casein of high-affinity procyanidins, occupying the majority of binding sites on the protein at low protein concentrations.

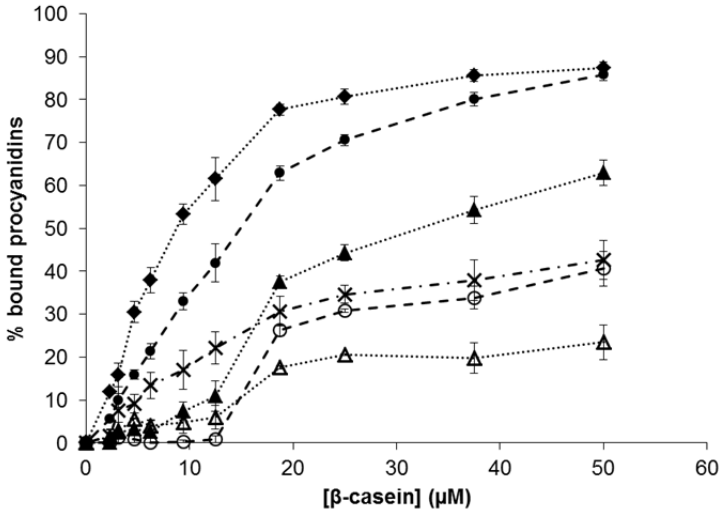


Figure 4. Percentages of bound phenolics from the GSE trimer fraction as a function of β-casein concentration. Compounds represented are: monomeric flavan-3-ols (×), procyanidin dimers (△), monogalloylated procyanidin dimers (▲), digalloylated procyanidin dimers (◆), procyanidin trimers (○), and monogalloylated procyanidin trimers (●).

Apparent binding affinity of target ligands to β-casein

Selected procyanidins were evaluated for their binding affinity to β-casein using binding isotherms established at constant protein concentration. As the exact concentrations of individual ligands (in M) were unknown, the concentrations of bound and free ligand were expressed as peak area. Typical binding curves are depicted in **Figure 5**, and the apparent binding affinities are summarized in **Table 4**. These apparent affinities confirmed the trends observed in **Figure 4**. The non-galloylated procyanidin dimers and trimers showed half of the affinity to β-casein compared to that of the monomeric flavan-3-ols. Monogalloylation of procyanidin dimers resulted in a 4-fold increase in their affinity to β-casein compared to the affinity of the non-galloylated procyanidin dimers. The addition of a second gallic acid to procyanidin dimers increased their affinity to β-casein by another 5-fold. The effect of the size of procyanidins on their binding affinity to protein was relatively small as

non-galloylated procyanidin trimers had less than 2 times higher binding affinity compared to non-galloylated procyanidin dimers. On the other hand, the addition of one gallic acid on procyanidin trimers increased their binding affinity by about 13-fold. This illustrated the strong influence of galloylation on procyanidin-protein interactions in complex mixtures and suggested a synergy between DP and galloylation, as the binding affinity of monogalloylated trimers were 5 times higher compared to that of monogalloylated dimers.

Table 4. Summary of binding affinity of procyandins to β-casein at pH 7

Compound	Binding affinity ($\times 10^4$ M^{-1})	Affinity relative to monomer
Epicatechin/catechin	3.2 (±0.3)	-
Dimer	1.3 (±0.1)	0.4
Monogalloylated dimer	5.2 (±0.4)	1.6
Digalloylated dimer	26.9 (±0.4)	8.4
Trimer	1.6 (±0.5)	0.5
Monogalloylated trimer	24.7 (±0.5)	7.7

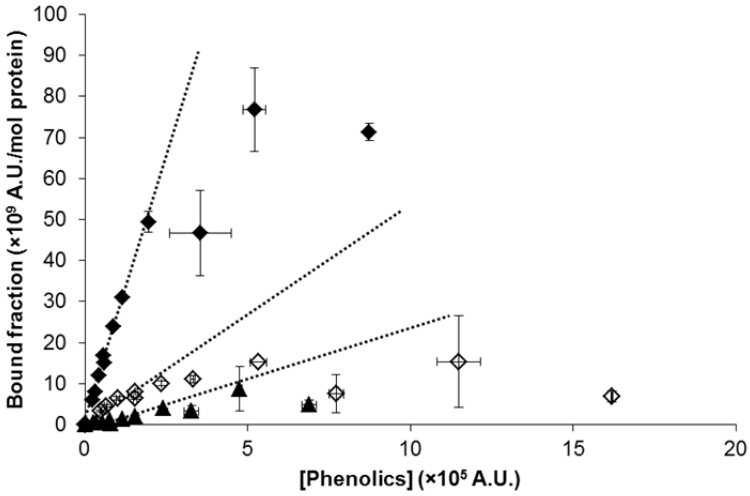


Figure 5. Typical binding curves obtained by ultrafiltration-MS for individual compounds of the GSE trimer fraction. Compounds represented are: procyanidin dimer (▲), monogalloylated procyanidin dimer (◇), and digalloylated procyanidin dimer (◆)

DISCUSSION

Applicability of linear ion trap SRM for the assessment of phenolic-protein interactions

In ligand-protein interactions, MS has mostly been used to evaluate complexes directly in the apparatus (*23-25*). To our knowledge, only one report used SRM with a triple quadrupole MS to quantify the amount of bound flavonoids to milk proteins using commercially available standards (*16*). Here, we report for the first time the use of SRM in a linear ion-trap MS for the evaluation of the binding affinity of individual phenolics in a complex mixture to β-casein. Linear ion-trap MS are not traditionally applied for SRM because of their lower performance in analysis speed compared to triple-quadrupole MS. In the current study, we found that this type of MS could be reliably used to evaluate the binding capacity of phenolics with the simultaneous monitoring of four compounds per analysis cycle.

In comparison with a previous report of a similar UF assay based on UV (*6*), the targeted analysis by SRM of selected compounds in a mixture was more sensitive. Phenolics with a low affinity for β-casein showed larger variations between replicates as the absolute amounts bound per mole of protein were small over the concentration range studied. Compared to a method like fluorescence quenching (*7*), which measures the protein's intrinsic fluorescence at equilibrium, the ultrafiltration process may influence the binding equilibrium due to the increasing concentration of the protein in the retentate. This phenomenon might cause the higher variability between replicates in UF-MS compared to a method like fluorescence quenching. The UF-MS assay appears to be applicable for the comparative study of structurally different phenolics in a mixture binding to a protein.

Structural features of phenolics predominantly enhancing their interaction with proteins

According to **Figure 4** and **Table 4**, the binding affinity of B-type procyanidins to β-casein, when binding as a mixture, can be ranked as follows: digalloylated procyanidin dimer ≈ monogalloylated procyanidin trimer > monogalloylated procyanidin dimer > epicatechin/catechin > procyanidin trimers > procyanidin dimers. Thus, it can be concluded that the addition of an extra galloyl group to the monogalloylated dimer had as much an effect on its binding affinity to β-casein as the addition of a monomeric flavan-3-ol unit. Furthermore, an extra galloyl group to a phenolic is not simply additive (i.e. sum of aromatic rings present) with respect to protein binding, but rather synergistic. The stronger binding of galloylated procyanidins to proteins than that of non-galloylated equivalents is in accordance with previous reports, where these ligands were tested individually with milk caseins (*7*) and salivary proteins (*9*). As reported for epigallocatechin gallate (EGCG), the addition of a gallic acid to epigallocatechin adds a flexible group, which might enable the phenolic to adapt to the protein (*7,26*).

Canon *et al.* has ranked the chemical characteristics of procyanidins, measured individually with a salivary protein, based on their impact on the stability of the complexes in a mass spectrometer (MS), and reported the following: DP > galloylation > B-ring hydroxylation (*13*). This different ranking compared to the current study might be related to the fact that the stability of complexes in the gas phase of the MS may not be representative of the affinity of procyanidins for proteins in solution as hydrophobic forces are destabilized in a vacuum (*27*).

In the current study, the effect of the degree of polymerization of phenolics with respect to protein binding is not as clear as reported before. The increasing number of aromatic and pyranic rings with increasing size of procyanidins is usually linked to an increased interaction with proteins (*28*). Here, we showed that epicatechin/catechin displayed a higher affinity to β-casein compared to procyanidin oligomers. The main difference between our study and others is that in our study the phenolics were present in a mixture. It might be that the high-affinity phenolics in the mixture, e.g. galloylated procyanidin oligomers, bind preferentially to the protein and create a nucleation site, favorable for binding smaller phenolics, e.g. catechin/epicatechin. We speculate that the unexpected relatively high binding affinity measured for epicatechin/catechin to β-casein might be an indication for such cooperative binding, possibly determined by the mutual affinity of phenolics.

Our results indicate that the UF-MS method developed is particularly powerful for identifying the best interactors with protein in mixtures of phenolics. In a preliminary experiment, the binding of phenolics to β-casein from a black tea extract, one of the most complex mixtures of phenolics known (*29*), was tested to investigate whether our protocol could also be extrapolated to the analysis of other samples. As illustrated in **Figure 6**, MS base peaks corresponding to galloylated theasinensins (TSG and TSGG) and monomeric flavan-3-ols with a gallic acid substituent ((E)CG and (E)GCG) display a visible decrease after mixing with β-casein. Other unidentified peaks also seem to decrease and could potentially be monitored by the UF-MS assay. This demonstrated that our protocol can be easily adapted to the analysis of preferential binding with other plant extracts.

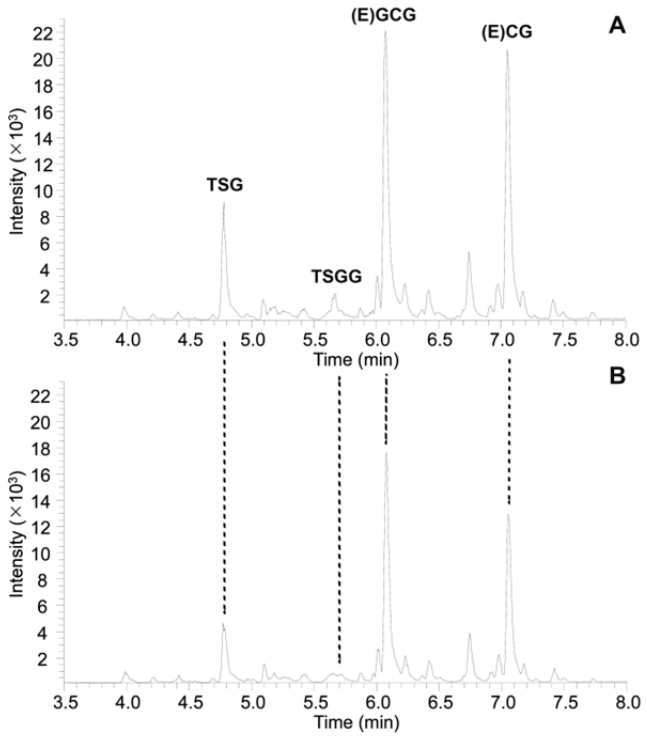


Figure 6. MS base peak chromatogram of ultrafiltered black tea extract without addition of β-casein (**A**) and after addition of 12 μM β-casein (**B**). TSG = monogalloylated theasinensin; TSGG = digalloylated theasinensin; (E)GCG = (epi)gallocatechin gallate; (E)CG = (epi)catechin gallate

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Chapter 5

Evaluation of the bitter-masking potential of food proteins for EGCG by a cell-based human bitter taste receptor assay and binding studies

Epigallocatechin gallate (EGCG) has been ascribed to several health benefits, but its astringent and bitter taste influences the liking of products with high concentrations of this compound. In an earlier study, β-casein, and several gelatins were identified as strong binders of EGCG, contrary to β-lactoglobulin. The current study aimed at relating the EGCG-binding characteristics of those proteins, and their food-grade equivalents, to their effects on reducing bitter receptor activation by EGCG *in vitro* and their bitter-masking potential *in vivo*. β-Casein had a high affinity for EGCG ($K = 45.0 (\pm 7.2) \times 10^3$ M^{-1}) and showed the strongest effect in a bitter receptor assay, with a maximum reduction of hTAS2R39 activation of about 93%. A similar potency was observed for Na-caseinate. β-Lactoglobulin had little effect on bitter receptor activation, as expected based on its low binding affinity for EGCG. The bitter-masking potential of Na-caseinate was confirmed *in vivo* using a trained sensory panel. β-Lactoglobulin also significantly reduced EGCG bitter perception, although to a lesser extent, which could not be directly related to its binding capacity. The bitter receptor assay appeared to be a valid tool to evaluate *in vitro* the efficacy of food proteins as bitter-masking agents.

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INTRODUCTION

Epigallocatechin gallate (EGCG) is known to be the most abundant catechin in green tea (ca. 60 % of the total catechins) and has been ascribed to several beneficial health effects (e.g. anticarcinogenic and cardioprotective effects) (*1*). With respect to taste, tea catechins are known to be astringent and bitter (*2*). The mechanism of astringency perception is not yet fully defined, but can be partially attributed to the interaction of EGCG with salivary proteins. Astringency seems sensorially coupled with bitterness, although, compared to the latter, it has been usually reported as a secondary attribute in time-intensity experiments (*3, 4*). On the human tongue, bitter compounds are perceived by bitter taste receptors, referred to as hTAS2Rs, which are part of the family of G-protein coupled receptors (*5*). To date, 20 hTAS2Rs out of the 25 known have identified agonists (*6*). Amongst these, hTAS2R39 has been associated with taste perception of green tea catechins (*7*). As evaluated *in vitro* with hTAS2R39*,* EGCG has a two times lower EC_{50} value (181.6 μM) compared to its non-galloylated equivalent epigallocatechin (EGC; EC_{50} = 395.5 μM). This difference was confirmed *in vivo* by a higher perceived bitterness for EGCG (*2,7*).

Effective health benefits against cardiovascular and metabolic diseases have been associated with a daily intake of green tea containing 200-300 mg of EGCG (*8*). Food products with high concentrations of EGCG may have off-tastes and consequently low consumer acceptance (*9*). Various approaches to modulate bitterness of bioactive compounds in functional foods have been described, such as the use of sweeteners, blockers for bitter taste receptors and complexation with other compounds. In the latter approach, cyclodextrins are the most commonly used carriers while other carriers (e.g., proteins) are seldomly reported (*9-12*). A typical example of off-taste reduction in food is the addition of milk to tea, which has been linked to the interaction of tea catechins with milk proteins (*3*) without impairing their bioavailability (*13*). Milk proteins have also been suggested as carriers for bioactive compounds and, in particular, thermally-induced β-lactoglobulin-EGCG complexes (*11,14*).

In our previous work (*15*), we investigated the potential of food proteins as carriers for flavonoids. Based on affinities and binding capacities measured, β-casein and gelatins, in particular fish gelatin, were found to be the most promising carriers for EGCG. One necessary condition for the applicability of those complexes in food is their effective reduction in bitter taste perception of EGCG. Bitter receptor activation by flavonoids can be evaluated *in vitro* by a cell-based receptor assay (*7,16*). To our knowledge, the present study is the first report using such a setup to evaluate the reduction in activation of bitter receptors by EGCG after forming complexes with proteins. This primary approach can help to predict the outcome of sensory panels. The aim of the present study was to correlate EGCG-binding of pure β-casein, food-grade caseinates and several gelatins to the potential of these proteins for reducing bitterness perception of EGCG. This was first tested *in vitro*

using a cell-based bitter receptor assay and then *in vivo* with a trained sensory panel, in order to evaluate the applicability of those complexes in foods.

MATERIALS AND METHODS

Materials

Bovine β-casein (≥98% of total protein), bovine β-lactoglobulin (≥90% of protein), solid fish gelatin (Gelatin F) from cold water fish skin, and gelatin type B (Gelatin B) from bovine skin (75 bloom) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The protein contents of the two gelatins were estimated to be ≥90% as detailed in a previous study (*15*). Food-grade EGCG (≥94%), Sunphenon 90LB (>80% catechins), and food-grade calcium and sodium caseinates (protein content ≥90%, N×6.38) were kindly provided by DSM Food Specialties (Delft, The Netherlands), Taiyo GmbH (Filderstadt, Germany) and DMV International (Veghel, The Netherlands), respectively. Bovine Vinoferm® gelatin powder (≥85% of protein; N×5.55), Vinoferm® gelatin liquid (20% (w/v) gelatin (supplier information)) and Vinoferm® Isinglass (fish gelatin, 2% (w/v) protein (supplier information)) were food-grade and purchased from Brouwland (Beverlo, Belgium). Throughout this study, these gelatins are referred to as gelatin B2, gelatin B3, and gelatin F2, respectively. Food-grade BioPURE - β-lactoglobulin™ (≥90% of total protein) was kindly provided by Davisco Food International (Eden Prairie, MN, USA). Water for *in vitro* tests was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Water (Spa Reine, Spadel Group, Brussels, Belgium) for *in* vivo tests was obtained from a local supermarket. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Assessment of binding of proteins to EGCG by ultrafiltration (UF assay)

Determination of binding parameters of food-grade proteins for EGCG (UF assay – method 1). All samples were prepared in a 50 mM sodium phosphate buffer, pH 7.0. Protein stock solutions (0.2 mM) were prepared freshly before each experiment. For both caseinates, an average molecular mass of 23.3 kDa was calculated from the protein composition in bovine milk (*17*). Gelatins were prepared at a concentration equivalent to 0.2 mM β-casein (i.e. 4.72 mg/mL). Similarly, a stock solution of EGCG (6 mM) was used to obtain a range of dilutions between 0 and 6 mM. EGCG-protein mixtures were prepared and the binding affinities of each protein towards EGCG measured using an ultrafiltration microtiter plate setup (Ultracel 10, Millipore, Cork, Ireland) as described previously (*15*).

The protein-bound and free fractions of EGCG at each concentration tested were calculated and plots of the bound fraction versus the concentration of free EGCG were used to determine the binding parameters. For each binding curve obtained, a linear regression was used on the initial linear increase (R^2>0.8) in order to estimate the binding affinity (K)

of the compounds. A maximal binding capacity (R_{max}) was derived from the plateau value or the highest bound fraction observed at high phenolic compound/protein molar ratios. Binding parameters were reported as mean ±standard deviation (SD) of two replicates.

Determination of $[EGCG]_{free}$ with increasing concentrations of various proteins (UF assay – method 2). An EGCG stock solution (0.5 mM) and solutions of proteins with concentrations ranging from 0.013 to 0.2 mM (EGCG-to-protein molar ratios from 2.5 to 40) were prepared in a similar way as described above. The concentration of free EGCG remaining in the mixtures after incubation was determined using an ultrafiltration microtiter plate setup as described previously (*15*).

In vitro assessment of hTAS2R39 activation by intracellular calcium release

Activation of bitter receptors was investigated by the release of intracellular Ca^{2+}, using a fluorescent calcium dye (*18*). The expression of hTAS2R39 in HEK293 cells and the detailed procedure for monitoring its activation were performed as reported elsewhere (*16*).

All samples were prepared in Tyrode's buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM Hepes, pH7.4). EGCG stock solution (1 mM) was prepared freshly before each experiment. Similarly, stock solutions of proteins (0.8 mM) were used to obtain a concentration range from 0.006 to 0.8 mM. Protein-EGCG complexes were made in a microtiter plate by mixing protein solutions 1:1 with EGCG solutions. Controls were made by mixing EGCG with buffer without proteins. The microtiter plate was incubated at room temperature under constant shaking (300 rpm, 10 min).

Next, the complexes were loaded (ratio 1:1 (v/v)) in a microtiter plate containing the cells (final concentrations of EGCG of 0.25 mM and of protein between 0 and 0.2 mM) and evaluated for their potential to activate bitter receptor hTAS2R39 at 37 °C with a FlexStation II 384 (Molecular Devices Corp., Sunnyvale, CA, USA) for 90 s as described elsewhere (*16*). Prior to the addition of the complexes to the cells, the baseline signal was determined in the first 17 s. Then, fluorescence signals (excitation 485 nm/emission 520 nm) were measured until 90 s. As negative control, non-induced cells, which do not express the hTAS2R receptor, were always measured in parallel. Additionally, a dose-response curve of EGCG was determined in the same way by measuring concentrations of EGCG up to 1 mM without proteins. Measurements were performed at least in duplicates on two or more different days.

Sensory analysis

Panelists. –The panel consisted of 13 persons (10 males and 3 females) selected within Unilever R&D (Vlaardingen, The Netherlands) for their ability to taste and rank bitterness. Panelists could join each session on a voluntary basis and participated in at least one of the sessions described below. Panelists were trained to taste bitterness and rate it on a scale

from 1 to 10 using a known reference (Sunphenon 90 LB). No specific training was conducted to discriminate between astringency and bitterness.

Sample preparation.–Proteins (10 g/L) and EGCG (1 g/L) stock solutions were prepared freshly before each experiment. The compounds were dissolved in water (Spa Reine), known for its neutral pH and low content in minerals. EGCG was mixed 1:1 with each protein solution and incubated at room temperature for at least 10 min. 15 mL of each sample was poured into a yellow cup. The colored cups were used to limit the visual perception of the differences between samples (e.g. slight haze or color) with or without proteins and thus to limit its possible impact on the choice and rating by the panelists. Additionally, three solutions of Sunphenon 90 LB were prepared: Two references known to the panelists (0.3 g/L and 0.8 g/L) and one unknown to the panelists (0.4 or 0.5 g/L). The former were used as a calibration for the panelists prior to and throughout each session. The latter was used by the panelists to check the accuracy of their rating at the beginning of each session.

Selection of suitable food-grade proteins for quantitative sensory analysis.–A preliminary session was organized to select food-grade proteins with the highest potential for bitterness reduction. Volunteers from the panel (n=8) were allowed to taste a known sample with only EGCG (0.5 g/L) and rate it against the references. Then, each protein-EGCG sample was tasted, described individually and subsequently discussed with the other panelists. The group rating and the most recurrent descriptors were used to select the most suitable proteins for further experiments.

2-Alternative Forced Choice (2-AFC) test.–EGCG complexed with Na-caseinate and β-lactoglobulin were evaluated in duplicate against a control EGCG sample without proteins. The experiment was conducted on two different days with 12 panelists and 6 of them were present on both days (n=36 per sample). Pairs of samples were all provided at once to each panelist. In between samples, panelists were instructed to rinse their mouth with water or milk, and to eat a piece of cucumber or plain cracker. For each pair, panelists had to indicate which sample was the most bitter and to rate the two samples on a scale from 0 to 10 as described above.

Ranking test.–EGCG (1 g/L) was mixed 1:1 with 4 different concentrations of Na-caseinate (2, 5, 10, and 15 g/L) and prepared as described above. Panelists were provided with a series of 5 samples in duplicate (13 panelists, n=26 per sample) and were instructed to rank them in order of increasing bitterness and rate them on a scale from 0 to 10 as described above. Series included one control containing only EGCG (0.5 g/L) and no proteins. The instructions given to the panelists for rinsing their mouth between samples were the same as for the 2-AFC test.

Data processing and statistical analysis

For hTAS2R39 activation data, SoftMax Pro 5.4 software (Molecular Devices Corp.) was used to plot fluorescence signals. Data processing for the activation curve of hTAS2R39 by EGCG was performed as described previously (*16*). Similarly, for EGCG-protein complexes, the fluorescence values ($\Delta F/F_0$) were calculated by subtracting the baseline fluorescence (F_0) prior to loading from the maximum fluorescence (F) after addition of the compounds, divided by the signals of the baseline to normalize to background fluorescence (*16*). Besides the response of induced cells, also the response of non-induced cells was measured as negative control for every compound at every concentration on the same plate. In cases that a non-specific signal occurred with $\Delta F/F_0 > 0.25$, the corresponding concentration of the protein was not considered for further calculations. Response of non-induced cells was subtracted from its corresponding response of induced cells at all valid concentrations. The activation was expressed as a percentage of the maximum response measured (i.e. EGCG control) and plotted versus the protein concentration. Data were reported as the mean value of the replicates and error bars represented the standard error of the mean (SEM).

The dose-response curve of hTAS2R39 by EGCG was fitted with nonlinear regression curves in Graph Pad Prism (version 4 for Windows, Graph Pad Software, San Diego, CA, USA). The sigmoidal dose-response curve model with variable slope corresponded to the following equation:

$$\frac{\Delta F}{F_0} = B + \frac{(T-B)}{1+10^{(LogEC_{50}-Log[EGCG])-H}} \quad \text{(equation 1)}$$

with B, the bottom plateau value; T, the top plateau value; $LogEC_{50}$, the Log([EGCG]) value at which the response is halfway between B and T; and H, the Hill slope or steepness of the curve. Best-fit parameters for the activation curve of hTAS2R39 by EGCG were as follows: B = 0.107, T = 1.531, $LogEC_{50}$ = -3.793, and H = 1.976.

The aforementioned best-fit parameters and the dose-response curve equation were used to predict the receptor activation ($\Delta F/F_0$) which should be observed based on the concentration of free EGCG measured in the UF assay – method 2. The theoretical receptor activation was plotted as percentage of reduction of activation versus the EGCG-to-protein molar ratio using the following equation:

$$\%\, reduction\ activation\ = \left(1 - \frac{\left(\Delta F/F_0\right)_i}{\left(\Delta F/F_0\right)_{250}}\right) \times 100 \quad \text{(equation 2)}$$

With $\left(\Delta F/F_0\right)_i$, the theoretical receptor activation at $[EGCG]_{free}$ = i (in µM), and $\left(\Delta F/F_0\right)_{250}$, the theoretical receptor activation at $[EGCG]_{free}$ = 250 µM. The data were reported as the mean value of the replicates and error bars represented the SEM.

Averages and confidence intervals (95%) from the 2-AFC and ranking tests were calculated. Significance ($p<0.05$) for the 2-AFC test was determined based on a minimum number of correct judgments for paired difference using a statistical table reported elsewhere.(*19*) Significance ($p<0.05$) for the ranking test was determined by Kramer's rank sum test.(*20*) For comparison with the data obtained *in vitro*, averages of the percentages of rating differences for each pair of samples and SEM were also calculated.

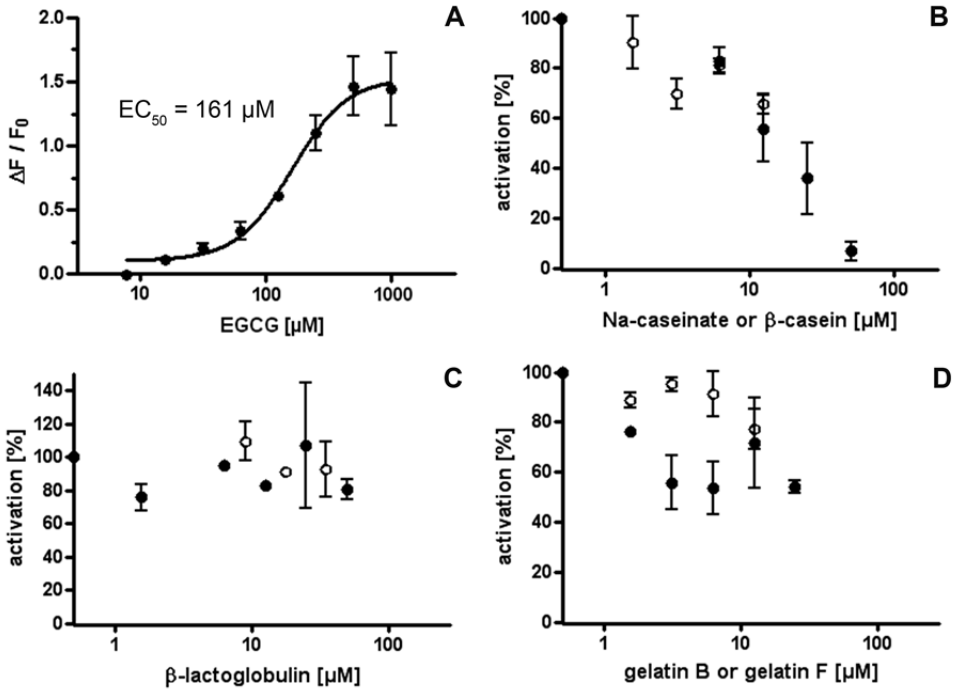

Figure1. Dose-response curve of hTAS2R39 stimulated with EGCG (**A**) and percentages of receptor activation by EGCG (250 µM) complexed with increasing concentrations of β-casein (●) or Na-caseinate (○) (**B**), analytical-grade β-lactoglobulin (●) or food-grade β-lactoglobulin (○) (**C**), or gelatin B (○) or gelatin F (●) (**D**)

RESULTS

Reduction of activation of bitter receptor hTAS2R39 by complexing EGCG to proteins

Based on a previous study on common food proteins binding EGCG(*15*), β-casein, β-lactoglobulin, gelatin B and gelatin F were selected and tested for their potential to reduce the activation of the bitter receptor hTAS2R39 by EGCG in a cell-based assay. The test was conducted at a concentration of EGCG of 250 μM, which was about the EC_{70} value (EC_{50} = 161 μM, determined experimentally) and provided sufficient signal to clearly observe the effect of the proteins (**Figure 1A**). Maximum reductions of receptor activation (as percentages of reduction of activation from control EGCG without protein) for protein-EGCG complexes are summarized in **Table 1**. Dose response curves of hTAS2R39 activation by EGCG with the various proteins tested are reported in **Figure 1B-D**.

Table 1. Comparison of the bitterness reduction potential of proteins evaluated *in vitro* and *in vivo* (based on preliminary experiment) at an EGCG-to-protein molar ratio of 5.

Protein	Grade	% reduction of activation (cell assay)	% reduction of activation (UF assay)[c]	Reduction of rating (*in vivo*)[d]	% reduction of rating (*in vivo*)[d]
β-casein	Analytical	93.3 (±5.3)	72.8 (±2.9)	n.a.	n.a.
Na-caseinate	Food	34.3 (±4.1)[a]	51.9 (±0.9)	3	43
Ca-caseinate	Food	n.d.	49.2 (±0.5)	3.5	50
β-lactoglobulin	Analytical	-[b]	5.8 (±2.9)	n.a.	n.a.
β-lactoglobulin	Food	-[b]	9.0 (±0.5)	1	14
gelatin B	Analytical	23.0 (±8.0)[a]	18.3 (±0.8)	n.a.	n.a.
gelatin B2	Food	n.d.	n.d.	2.5	36
gelatin B3	Food	n.d.	n.d.	1.5	21
gelatin F	Analytical	46.0 (±2.6)[a]	30.6 (±6.5)	n.a.	n.a.
gelatin F2	Food	n.d.	n.d.	n.a.[e]	n.a.[e]

[a]percentage of the highest measurable protein concentration in the bitter receptor assay
[b]no clear trend in reduction of receptor activation detected
[c]percentages calculated based on concentrations of unbound EGCG in UF assay – method 2
[d]reduction of ratings and percentages calculated using a bitterness score of 7 for the EGCG reference
[e]strong sour taste overruled bitter taste
n.a.: not applicable; n.d.: not determined

Amongst the four tested proteins, β-casein showed the strongest reduction of the receptor activation by EGCG, with a decrease of 93.3 (±5.3)% at the highest measurable protein concentration applied (i.e. 50 μM). β-Lactoglobulin had only a limited effect on decreasing the receptor activation, with no clear trend and relatively high variations

between replicates. Gelatin F was found to have the second strongest reduction of the receptor activation by EGCG (maximum receptor activation decrease measured of 46.0 (±2.6)%), whereas its maximum decrease was reached at a lower protein concentration than with β-casein. Gelatin B did not reduce the activation of hTAS2R39 by EGCG by more than 23.0 (±8.0)% in the measurable protein concentration range, indicating that it had low potential for masking bitterness. The lack of a clear trend for gelatins B and F, as observed for β-casein, might be caused by the turbidity of these samples, affecting the accuracy of the measurements. In fact, the presence of insoluble aggregates might have affected the loading volumes on the cells and interfered with fluorescence readings.

Relationship between hTAS2R39 activation and binding characteristics of EGCG to analytical-grade proteins

The binding affinity (K) and maximal binding capacity (R_{max}) of the aforementioned proteins for EGCG were obtained from an earlier study(*15*) and are summarized in **Table 2**. β-Casein and gelatin F had similar affinities for EGCG, both about two times and ten times higher than the ones measured with gelatin B and β-lactoglobulin, respectively. Those affinities were found to be sufficient to have a strong effect on the reduction of activation of hTAS2R39 (**Figures 1B and 1D**). The limited effect of β-lactoglobulin on the reduction of receptor activation in the bitter receptor assay is thought to be linked to its low affinity for EGCG. Even though gelatin B had an intermediate affinity for EGCG, it had a limited effect on the receptor activation by EGCG, suggesting that a minimum affinity is required for a significant reduction of receptor activation. Binding affinity seemed to be a more important factor than R_{max} as gelatin B had a higher R_{max} than β-casein, but only showed a limited effect on decreasing receptor activation.

A second ultrafiltration method (UF assay – method 2) was used to mimic the conditions of the bitter receptor assay (i.e. constant [EGCG] and variable [protein]). The concentrations of free EGCG measured in the UF assay – method 2 were used to predict the percentage of reduction of receptor activation using equations 1 and 2, and compared to the experimental data obtained. β-Casein was used to evaluate the accuracy of this approach (**Figure 2A**). The data derived from the UF assay – method 2 and the bitter receptor assay were in good agreement, with an underestimation of only 10-20% for the theoretical values compared to the experimental values.

In contrast to β-casein, the theoretical and experimental values showed clear discrepancies for gelatin F (**Figure 2C**). As mentioned earlier, interferences due to aggregates could explain this observation. In the case of gelatin B (**Figure 2D**), a limited amount of experimental data points could be matched, but similar trends between the theoretical and experimental values were observed. The theoretical percentages of reduction of receptor activation calculated for gelatin F are two times lower than those for β-casein although their affinities for EGCG were similar (**Table 2**). This shows that affinity is not

the only parameter for an efficient reduction of receptor activation, although it might be a good first indicator. For example, the flexibility of β-casein and its ability to form micelles which could entrap EGCG might be advantageous characteristics compared to the more rigid structure of gelatins (*15,21,22*).

In the present study, β-casein was confirmed as promising carrier for EGCG in food as its high binding affinity and capacity for EGCG resulted in an effective reduction of bitter receptor activation by EGCG.

Efficiency of food-grade protein ingredients to complex EGCG

Highly purified proteins are not commonly used in the food industry. Hence, the analytical-grade proteins were replaced by common food ingredients containing these proteins (e.g. β-casein replaced by caseinates). These food-grade proteins were evaluated for their binding potential for EGCG by the UF assay and their binding parameters were compared to the ones of their equivalent analytical-grade proteins as summarized in **Table 2**. A higher concentration of proteins as used in the current assay (i.e. 100 μM) did not seem to influence the binding affinity as found for β-casein ((45.0 ±7.2) $\times 10^3$ M^{-1} at 25 μM versus (43.3 ±2.2) $\times 10^3$ M^{-1} at 100 μM). Ca-Caseinate and Na-caseinate were considered as an acceptable replacement for β-casein. The lower values obtained for the binding parameters of caseinates compared to β-casein might be related to their more heterogeneous protein composition, with a molar ratio α_{S1}:α_{S2}:β:κ of about 11:3:10:4, on a molar basis (*17*). In fact, it has been shown that α-casein had a two times lower binding affinity for EGCG compared to β-casein (*23*).

Na-Caseinate was tested for its capacity to reduce hTAS2R39 activation by EGCG (**Figure 1B**). Although only a more narrow range of protein concentration could be measured due to non-specific signals, Na-caseinate showed a similar trend in receptor activation decrease compared to β-casein at protein concentrations between 1.5 and 6 μM. Theoretical percentages of reduction of receptor activation were calculated for Na-caseinate using the UF assay – method 2 and were similar to those obtained for β-casein (**Figure2B**), with a maximum reduction of 70.1 (±1.2)% at an EGCG-to-protein molar ratio of 2.5 (85.3 (±0.6)% for β-casein). Theoretical and experimental values at EGCG-to-protein molar ratios of 20 and 40 were in good agreement. Ca-Caseinate showed the same potential of reduction of receptor activation compared to Na-caseinate using the UF assay – method 2, with a maximum reduction of 72.0 (±0.9)% (data not shown). Hence, Na-caseinate and Ca-caseinate were confirmed as acceptable food-grade alternatives to analytical-grade β-casein for reducing hTAS2R39 activation by EGCG.

Table 2. Binding characteristics (affinity, K; maximum binding capacity, R_{max}) of analytical-grade[a] and food-grade proteins with EGCG by UF assay at pH 7.0, 25°C.

Analytical-grade proteins	K (10^3 M^{-1})	R_{max} (mol/mol)	R_{max} (g/100g)	Food-grade protein equivalents	K (10^3 M^{-1})	R_{max} (mol/mol)	R_{max} (g/100g)
β-casein	45.0(±7.2)	19.6(±4.9)	38.1(±9.6)	Na-caseinate	22.8(±0.5)	12.8(±0.4)	24.7(±0.7)
				Ca-caseinate	24.8(±0.5)	12.7(±0.0)	24.7(±0.1)
β-lactoglobulin	4.5(±2.3)	6.6(±3.7)	16.5(±9.2)	β-lactoglobulin	3.6(±0.3)	7.5(±0.3)	14.6(±0.5)
gelatin B	25.4(±4.1)	31.6(±2.1)	57.8(±3.8)	gelatin B2	29.2(±0.6)	16.9(±0.4)	32.8(±0.8)
				gelatin B3	6.9(±1.1)	9.8(±1.0)	19.1(±2.0)
gelatin F	53.3(±1.8)	57.5(±1.8)	43.9(±1.4)	gelatin F2	7.8(±0.8)	10.8(±0.7)	21.1(±1.3)

[a]binding characteristics were derived from previously published data[15]

n.d.: not detectable; n.a.: not applicable

Gelatin B2 showed similar binding potential compared to the model protein gelatin B, whereas gelatins B3 and F2 displayed lower binding affinities compared to their analytical equivalents. This difference compared to the model proteins could be due to variations in the characteristics of the gelatin samples (e.g. amino acid composition, average molecular mass). Food-grade and analytical-grade β-lactoglobulins had similar low binding affinities (**Table 2**). As shown in Figure 1 and Table 1, their weak binding properties to EGCG also did not result in a significant effect in reduction of hTAS2R39 activation. This was also shown by the UF assay – method 2 with a maximum value in reduction of receptor activation below 15% calculated for both proteins (**Figures 2E** and **2F**).

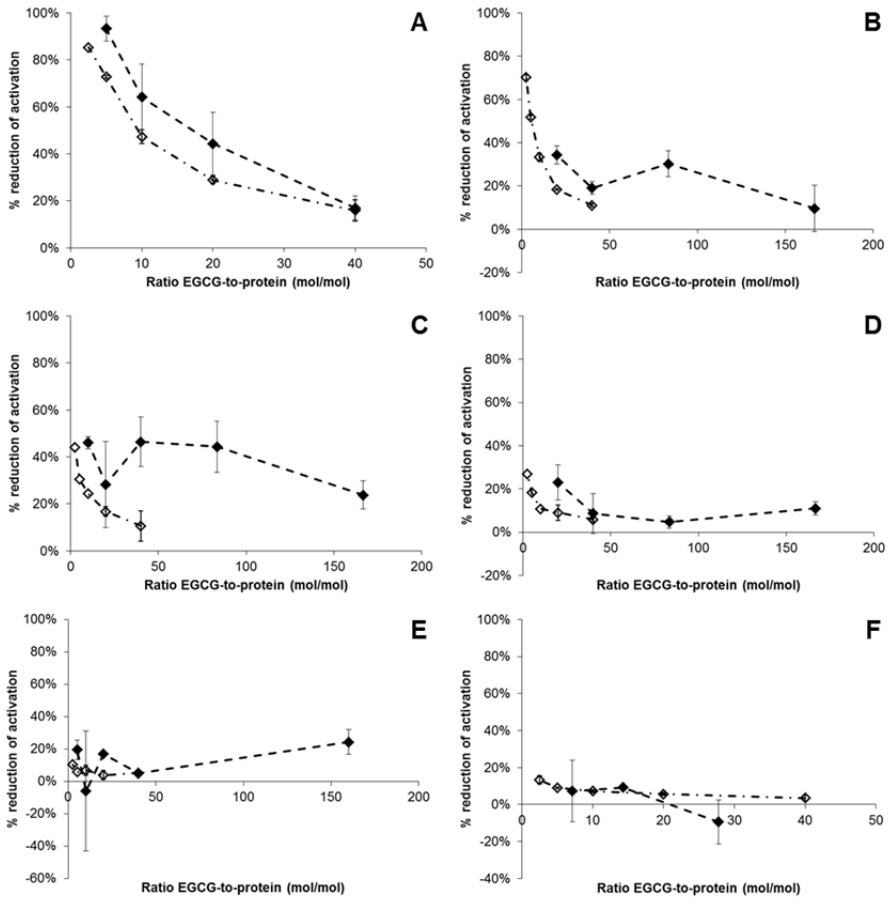

Figure 2. Comparison of percentages of reduction of hTAS2R39 activation by EGCG by various proteins measured experimentally with the bitter receptor assay (◆) and predicted with data from the UF assay – method 2 at constant concentration of EGCG (◇). (**A**) β-casein, (**B**) Na-caseinate, (**C**) gelatin F, (**D**) gelatin B, (**E**) analytical-grade β-lactoglobulin, (**F**) food-grade β-lactoglobulin

Sensory analysis of EGCG complexed with proteins

As summarized in **Table 3**, various food-grade proteins were compared in a preliminary sensory experiment for their potential to reduce bitterness of EGCG at a protein-to-EGCG mass ratio of 10, which was equivalent to an EGCG-to-protein molar ratio of about 5 using the molecular mass of β-casein. Ca-Caseinate and Na-caseinate had similar effects on the taste of EGCG with a reduction of EGCG bitterness rating by 50 and 43% (3.5 and 3 units), respectively. The relatively transparent appearance of Na-caseinate was preferred over the white color of Ca-caseinate in water for further investigation as it offers fewer limitations for applications (e.g. in clear beverages). β-Lactoglobulin had the least effect on the bitterness reduction of EGCG (14% (1 unit)). Therefore, it was selected as a negative control for further experiments. The three gelatin samples generally had unpleasant off-tastes, especially gelatin F2. In addition, gelatins B2 and F2 formed visible aggregates with EGCG at the molar ratio used. Taken together, gelatins were considered as unsuitable for further sensory tests and applications as bitter masking compounds.

Table 3. Sensorial comparison of EGCG (0.5 g/L) complexed with various food-grade proteins (5 g/L)

Protein	pH in water[a]	Rating	Aspect	Taste attributes (other than bitter)
EGCG control	6.3	7	Clear	-
Ca-caseinate	6.9	3.5	Turbid (milk-like)	Milky, astringent
Na-caseinate	6.9	4	Slightly turbid	Milky, slightly metallic
gelatin B2	6.0	5.5	Visible aggregates	Astringent, burned, strong off-taste
gelatin B3	4.9	4.5	Slightly turbid	Off-taste
gelatin F2	2.6	n.a.[b]	Visible aggregates	Very sour
β-lactoglobulin	6.3	6	Clear	Slight off-taste

[a]pH of EGCG-protein complexes after incubation; [b]n.a.: not applicable – strong sour taste overruled the bitter taste

In a 2-AFC test, β-lactoglobulin and Na-caseinate significantly reduced the bitter taste of EGCG by 20.5 ±2.8% and 32.8 ±5.8% (1.4 ±0.4 and 2.3 ±0.5 units), respectively (**Figure 3**). The effect of Na-caseinate on EGCG bitterness perception was in accordance with the expectations based on the reduction of hTAS2R39 activation by Na-caseinate (**Figure 2B**). A significant, although lower, effect of β-lactoglobulin on bitterness of EGCG was not expected as only a limited effect was observed in a preliminary sensory session (**Table 3**) and also in the *in vitro* assays (**Figure 2D**).

In a ranking test with increasing concentrations of Na-caseinate, it appeared that the lowest bitterness score (~4) was already reached at a concentration of 0.25 % (w/v) of Na-caseinate (**Figure 4A**). This observation concurs with a sensory study on olive oil phenolics binding Na-caseinate, for which a minimum bitterness score was reached with 1% (w/v) protein and did not decrease further with increasing protein concentration.(*24*) In

the current study, 0.25% (w/v) Na-caseinate resulted in 42% of bitterness reduction compared to the EGCG reference, and percentages of bitterness reduction measured at lower EGCG-to-protein molar ratios remained between 42 and 46% (**Figure 4B**). As shown in **Figure 4B**, the percentages of bitterness reduction measured *in vivo* at EGCG-to-protein molar ratios of 10 and 25 followed the trend of the theoretical values for the reduction of receptor activation calculated from the UF assay – method 2. At molar ratios lower than 10, however, a plateau was observed *in vivo* while *in vitro* theoretical values predicted a continuously increasing percentage of reduction of hTAS2R39 activation.

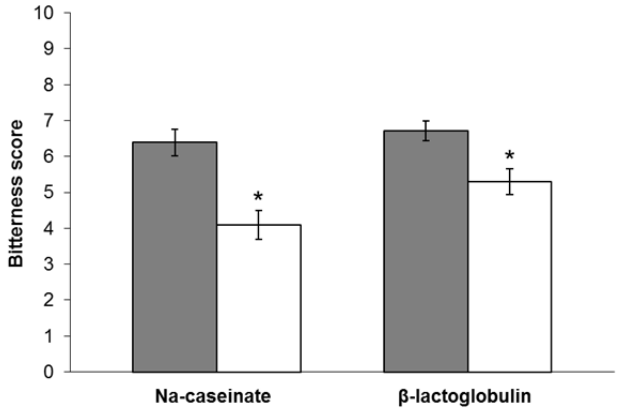

Figure 3. Comparison of perceived bitterness of EGCG, free (plain bar) or complexed with Na-caseinate or β-lactoglobulin (empty bar), in a 2-AFC sensory test. (*) significant difference ($p<0.05$)

DISCUSSION

In vitro prediction of bitterness reduction compared to *in vivo* sensory analysis

According to the *in vitro* assays conducted in this study (**Figure 2**), the efficacy in reducing bitterness of EGCG of the food-grade proteins tested should be ranked as follows: Ca-caseinate/Na-caseinate ≥ gelatins > β-lactoglobulin. This order was in line with our first sensory experiment (**Table 3**), confirming the applicability of our *in vitro* approach for screening the potential of food proteins for bitter masking.

A 2-AFC test demonstrated a decrease of perceived bitterness of EGCG when complexed to Na-caseinate by approximately one third. The effect of Na-caseinate on the intrinsic bitterness of EGCG *in vitro* at the same molar ratio was calculated to be a decrease of 50% based on the concentration of free EGCG after binding measured by ultrafiltration and related to the activation curve of hTAS2R39 by EGCG. Although our *in vivo* and *in vitro* results match well, it should be noted that the *in vitro* assay does not take account of actors other than hTAS2R39 in the mouth environment, such as other hTAS2Rs being

activated by EGCG (although their response will also be modulated by complexation of EGCG to protein) and salivary proteins which might interact with EGCG and disturb the binding equilibrium. The intrinsic bitterness is calculated under the assumptions that hTAS2R39 is the only bitter receptor sensing EGCG, that complexes remain stable in the mouth, and that the slight off-taste of Na-caseinate does not influence bitterness ratings. In addition, a stronger effect of β-lactoglobulin on EGCG bitterness was observed *in vivo* compared to the *in vitro* experiments. This effect is unlikely to have resulted from its binding to EGCG, nor from a direct interaction of β-lactoglobulin with the hTAS2R39 receptor, as suggested for another protein (*25*). An indirect effect due to the interaction of β-lactoglobulin with the buccal environment could have interfered with the bitter perception (e.g. interaction with saliva and buccal cells) (*26,27*).

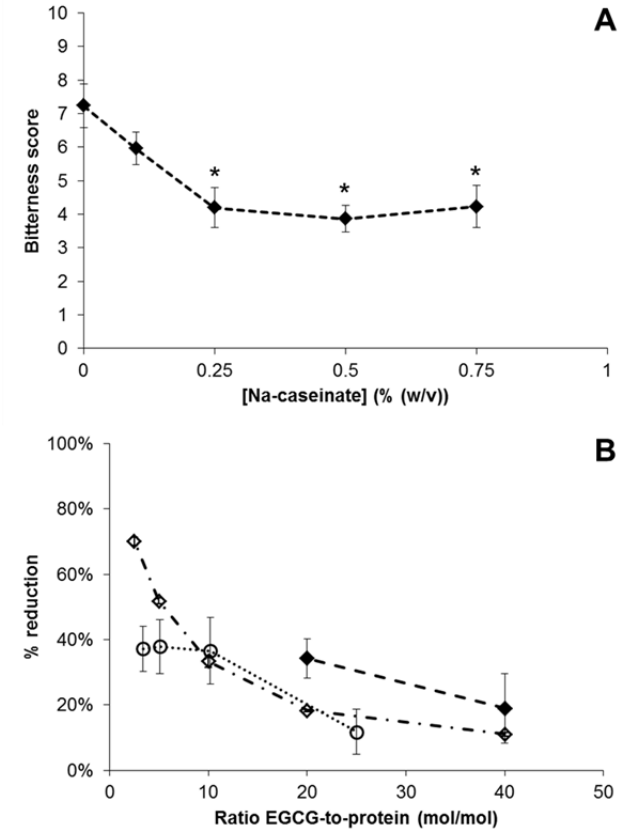

Figure 4. Dose-response curve for perceived bitterness of EGCG (0.5 g/L) with increasing concentration of Na-caseinate ((*) significant difference ($p<0.05$)) (**A**) and comparison of the percentages of bitterness reduction *in vivo* (○) with percentages of reduction of hTAS2R39 activation by EGCG with Na-caseinate measured experimentally with the bitter receptor assay (◆) and predicted with data from the UF assay (◇) (**B**)

In this study, a maximum reduction in bitterness of EGCG was achieved at 0.25% (w/v) of Na-caseinate, although our *in vitro* assay predicted a continuous decrease in receptor activation with increasing protein concentrations (**Figure 4B**). This concurs with a model proposed by Pripp *et al.*, which predicted a minimum bitterness reached at 0.5% (w/v) Na-caseinate for olive oil phenolics, assuming a binding affinity of 10^5 M^{-1} (*24*). The bitter receptor assay complemented with ultrafiltration appears as an appropriate tool to evaluate the efficacy of a given macromolecule as a bitter-masking ingredient, although it tends to overestimate its potential (**Figure 4B**). Discrepancies between *in vitro* and *in vivo* evaluation of bitterness have already been reported. For example, higher threshold concentrations and EC_{50} values for bitter hop compounds were found in a sensory test compared to the taste receptor assay, whereas the ranking in order of potency for the compounds was the same (*28*).

Bitter receptor assay as a tool to screen for bitter-masking agents

It has been shown that a cell-based bitter receptor assay can be a valuable tool to predict the intrinsic bitterness of food-related components (*7, 16, 28*). In the present study, we report for the first time its potential in evaluating the modulation of the intrinsic bitterness of bitter tastants, e.g. EGCG, by combining them with complexing agents, such as proteins. Despite some limitations related to the range of protein concentrations that can be used, or the influence of turbidity on the measurement, challenging hTAS2R39 with a combination of bitter tastant and protein allowed a rapid identification of good candidates for complexing, such as β-casein. Proteins were ranked for their efficacy for reducing receptor activation as follows, β-casein > gelatin F ≈ Na-caseinate > gelatin B > β-lactoglobulin. This ranking was in good agreement with findings from a complementary ultrafiltration assay relating the concentration of free EGCG with increasing protein concentration to hTAS2R39 activation.

Provided that the hTAS2R activated by the bitter compound of interest is known, the bitter receptor assay seems to be promising for the discovery of bitter-masking agents. It has been applied in several instances for high-throughput screening for so-called bitter blockers, i.e. compounds that act antagonistically on the bitter receptor of interest (*29, 30*). These blockers are thought to be rather specific in reducing bitterness, although their suggested promiscuity (i.e. the bitter blocker inhibits several hTAS2Rs) (*30*), or their potential agonistic behavior on other bitter receptors, might compromise this idea (*31*). Besides, some bitter compounds have been described to activate more than one bitter receptor, which might call for more than one blocker for a particular bitter tastant (*6, 16, 28*). Therefore, it might be advantageous to use a more generic approach for masking bitterness, e.g. by applying complexing agents, such as food proteins, which do not act directly at the receptor. We have now shown that the cell-based bitter receptor assay can be used as a tool to study such complexing agents, given that the protein is able to bind a significant amount of the bitter tastant.

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Chapter 6

General Discussion

As described in the General Introduction of this thesis, phenolic-rich food may have strong organoleptic limitations, mostly related to taste. These limitations require the design of carriers for phenolics that would decrease the taste defects, stabilize phenolics in the food matrix and during digestion, and release them in the gut for absorption. The design of such carriers requires the use of GRAS compounds. In this regard, animal food proteins, owing to their generally bland taste, appear as promising candidates. Most structure-binding relationships between phenolics and proteins have been established with salivary PRPs and serum albumins. These proteins are not suitable as carriers in food because of their origin for the former or their low abundance in common food ingredients for the latter (~5% of total proteins as BSA in whey protein concentrates or isolates). The aim of the research presented in this thesis was to extrapolate this knowledge to the interaction of dietary phenolics with food proteins, identify promising carriers and evaluate the applicability of these carriers for efficient off-taste reduction of phenolics.

The structural features of both phenolics and food proteins in relation to their ability to bind each other, as well as methods for assessing protein-phenolic interactions, are discussed in this chapter. Furthermore, this chapter will address alternative carriers for monomeric flavonoids.

METHODS FOR ASSESSING PROTEIN-PHENOLIC INTERACTIONS

Throughout this thesis, several methods have been applied to assess protein-phenolic interactions. Recent reviews have provided an exhaustive overview of the techniques employed in protein-phenolic interactions (*1,2*). A summary of the methods used in this thesis and a few others commonly reported in literature is given in **Table 1**, each with their main advantages and inconveniences. These are discussed below in the light of evaluating food protein-phenolic interactions.

In **Chapter 2**, a binding assay based on ultrafiltration (UF) of protein-phenolic mixtures was developed for screening purposes, combining turbidimetry and UV quantification of the free ligands. Ultrafiltration is traditionally used to determine the amount of a given ligand bound to a protein and is faster than equilibrium dialysis, which serves the same purpose (*3*). Ultrafiltration, however, had not been applied to determine binding affinities (*4*). Determining the slope close to the origin of the binding curve ($[ligand]_{bound}/[protein]$ (in M) as a function of $[ligand]_{free}$ (in M)) gave an estimation of the binding affinity. The UF assay allowed the systematic comparison of a range of food proteins with several representative flavonoids. The non-specific binding of phenolics to the ultrafiltration membrane is a disadvantage, but can possibly be lowered by a membrane pretreatment with detergent (e.g. Tween 80) (*5*). The UF assay has a low sensitivity for phenolics with a low affinity for proteins and is, therefore, not meant to replace other existing methods (e.g. nuclear magnetic resonance (NMR) spectroscopy). It can allow a rapid screening of various protein-phenolic pairs under different environmental conditions

and includes a dimension of 'quantitative binding' or loading efficiency by measuring the absolute amounts of phenolic bound to food proteins, which is an essential information for further application in food. In addition, the UF assay can be combined with other techniques, such as UHPLC-MS-MS (**Chapter 4**), to provide unique information not obtainable with other techniques mentioned in **Table 1**. The UF-MS assay can demonstrate preferential binding to a protein of some phenolics occurring in complex mixtures. The method can give access to the binding characteristics of commercially unavailable compounds present in some plant extracts. For the development of functional ingredients, plant extracts are more likely to be used in the industry and, in this respect, the UF-MS assay can provide valuable information.

ITC (**Chapter 2**) and fluorescence quenching (**Chapter 3**) are amongst the most frequently reported methods to assess protein-ligand binding. Both techniques undeniably provide valuable data and are easy to use, although they can be considered as low throughput methods. The easy data collection is, however, counteracted by often overlooked difficulties in data analysis. ITC and fluorescence quenching are gross measurements of any event occurring during the titration involving heat effects or a change in tryptophan fluorescence, respectively. In the case of ITC, binding isotherms obtained might be difficult to interpret with built-in models provided by the supplier because of their unusual shape (e.g. (*6*)), which often requires the development of tailored models (e.g. (*7*)), if possible at all. The fluorescence quenching data handling requires a careful data correction (e.g. for inner filter effects (**Chapter 3**)) and the accuracy of the binding constant is usually dependent on the model applied and the saturation of the fluorescence signal (*8*). ITC has the advantage of allowing the simultaneous determination of stoichiometry, affinity and thermodynamic parameters in one titration (*9*), but fluorescence quenching requires less sample and is more sensitive than ITC to weak binding (e.g. B-type procyanidin dimers binding β-casein (data not shown)). For the determination of binding constants in food protein-phenolics interactions with phenolics only available in small amounts (or commercially unavailable), fluorescence quenching seems to be more suitable than ITC, provided that the protein studied contains at least one tryptophan residue. ITC gives access to valuable data when dealing with simple and well-defined systems, such as poly(L-proline)-phenolics interaction (e.g. (*10,11*)).

Table 1. Commonly used methods for assessing protein-phenolic interactions

Method	Principle	Main advantages	Main inconveniences	Examples
Ultrafiltration	Separation of bound and free phenolics through a membrane with a specific molecular weight cut-off	Easy to implement, adaptable to different output reading (e.g. UV or MS), determination of the absolute amount of phenolic bound	Non-specific interaction with the ultrafiltration membrane, amounts of phenolics required (limitation for poorly water-soluble phenolics)	(*5*), this thesis
Fluorescence quenching	Measures the decrease of the quantum yield of a fluorophore (e.g. tryptophan) by a quencher (e.g. upon ligand binding)	Suitable for a broad range of protein and phenolics, sensitive at low concentrations	Requires pure compounds, sensitive to precipitation, requires accurate data correction for interferences (e.g. inner filter effect)	(*18*), this thesis
Mass spectrometry	Detection of ionized phenolic-protein complexes in a gas phase and determination of stability with increasing collision induced dissociation	Sensitive at low concentrations of phenolics and protein	Limited to small proteins and phenolics, interactions in gas phase are not always representative of interactions in solution	(*16*), (*19*)
Nuclear magnetic resonance spectroscopy	Detection of variations in chemical shifts of elements involved in phenolic-protein interactions	Sensitive and direct determination of phenolic binding sites	Sensitive to precipitation, requires high concentrations of sample, not amenable to mixtures	(*20*), (*21*)
Dynamic light scattering	Detection of the formation of aggregates by scattering of light	Versatile and describes the colloidal behavior of proteins (size, shape) upon binding of phenolics	Inaccurate if sedimentation occurs, determination of shape is restricted to ideal situations, does not give access to the very initial stage of the interaction	(*6*), (*13*)
Isothermal titration calorimetry	Measures heat effects resulting from phenolic-protein interactions	Simultaneous determination of binding constant and thermodynamic parameters	Sensitive to precipitation, multiple interferences possible besides binding events, requires matching solvents for phenolic and protein (limitation for hydrophobic phenolics)	(*22*), this thesis

Each method summarized in **Table 1** provides meaningful information on protein-phenolic interactions at a variable level, depending on the phenomenon investigated. For interactions at a molecular level, NMR spectroscopy and fluorescence quenching appear to be the most versatile techniques. NMR spectroscopy can be used to localize binding sites on proteins (e.g. (*12*)), although, to our knowledge, its applicability to food proteins has not been reported. Fluorescence quenching was used in this thesis as it is a more accessible method and requires a lesser amount of sample than NMR spectroscopy. Dynamic light scattering (DLS) and small angle X-ray or neutron scattering (SAXS/SANS) allow the observation of molecular assemblies and can help visualize the colloidal behaviors of proteins upon binding of phenolics (*13,14*). Although SAXS/SANS provides a description of the internal structure of the aggregates, its use is limited because it requires a special beam available only in few facilities. DLS is preferred in that regard, although it only provides information about the size and shape of the aggregates, and cannot be used to determine binding affinities. Nevertheless, this information may be valuable for further developments of phenolic carriers. Finally, MS has been used for the direct analysis of peptide-phenolics interaction (e.g. (*15,16*)). This sensitive method provides data about stoichiometry and stability of the complexes, and possibly their binding affinity, provided that stable complexes can be obtained in the gas phase of the mass spectrometer after ionization. Because a mass spectrometer measures molecules in vacuum, hydrophobic forces are destabilized. Therefore, the possibility of directly comparing complexes measured in gas phase and in solution is still debated (*2,17*).

For the characterization of food proteins-phenolic interactions, a combination of ultrafiltration, fluorescence quenching and, at later stage, DLS should provide the necessary set of data for selection of a potential carrier for phenolics.

OPENING GLOBULAR PROTEINS TO IMPROVE THEIR POTENTIAL AS CARRIERS OF PHENOLICS

As described in **Chapter 2**, globular food proteins are poor carriers for phenolics. The amino acid residues potentially involved in binding (e.g. prolines, hydrophobic amino acids) might be less accessible for the ligand in globular protein than in unstructured proteins. Loosening the structure of globular proteins by limited proteolysis or heat denaturation may improve the accessibility of these amino acid residues and, hence, improve the binding affinity of phenolics for globular proteins. Although not dealt with in the previous chapters, this hypothesis was investigated by either limited proteolysis or heat denaturation, and their influence on protein-EGCG interactions.

Improving the affinity of globular proteins for phenolics by proteolysis

Proteolysis using specific proteases was preferred over non-specific proteases in order to obtain limited proteolysis. As a proof of principle, we used a histidin-specific protease

(named 'HSP') and chicken egg ovalbumin as substrate. The selection of ovalbumin was based on its molecular mass and contents in proline and aromatic amino acids (**Chapter 2**). A protein with a molecular mass higher than 20 kDa was preferred in order to obtain fragments large enough to have good binding affinities for EGCG. Based on its three-dimensional structure, ovalbumin contains at least 2 inaccessible proline residues in addition to the buried hydrophobic amino acids. As summarized in **Table 2**, each hydrolysate was centrifuged (20000×g, 10 °C, 5 min) to remove the insoluble aggregates, and the supernatant was diafiltrated twice with 50 mM sodium citrate buffer pH 3.5 on a 3 kDa ultrafiltration Eppendorf filter (Millipore, Cork, Ireland). The soluble material after hydrolysis was characterized by SDS-PAGE and size exclusion chromatography (SEC) to evaluate the hydrolysis (**Figure 1**). A significant part of the peak/band corresponding to the native protein remained (43% AUC left after 17 h), and it was obvious that the enzyme generated fragments more than only loosening the structure of ovalbumin.

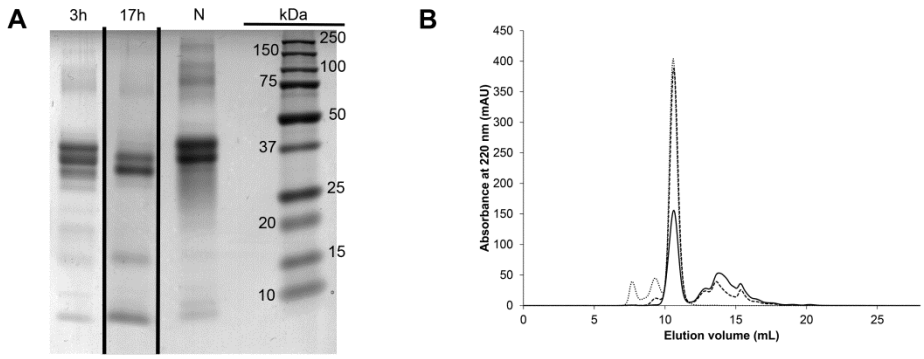

Figure 1. SDS-PAGE (Protean TGX Any kD precast gel (Bio-Rad, Hercules, CA, USA)) of soluble proteinaceous material (**A**) prior to UF (7 µL of sample applied after standardization to a protein concentration 0.1% (w/v)) and size exclusion chromatography (SEC) of soluble proteinaceous material > 3 kDa (**B**) of ovalbumin before and after hydrolysis with HSP in a pH-stat (45 °C, pH 3.5) (N and dotted line: native protein; dashed line: hydrolysate after 3 h; continuous line: hydrolysate after 17 h). For SEC, samples were standardized to a protein concentration of 0.5% (w/v) and applied on Superdex 75 column (GE Healthcare, Uppsala, Sweden) eluted with 50 mM sodium phosphate buffer containing 150 mM NaCl

The binding of the soluble proteinaceous material >3 kDa, with standardized protein concentration, was evaluated using EGCG with a similar setup as described in **Chapter 2**. As illustrated in **Figure 2**, this material did not show an improvement in binding to EGCG compared to the native protein. A similar observation was made with trypsin hydrolysis (data not shown). Also, proteolysis in presence of phenolics yielded more aggregation (data not shown). Although these aggregates of hydrophobic peptides might contain a significant amount of entrapped EGCG, their applicability in food formulations seems to be limited.

Table 2. Protein mass balance for ovalbumin after enzymatic hydrolysis (45 °C, pH 3.5) and processing of the hydrolysates

Duration hydrolysis (h)	% soluble proteinaceous material >3 kDa (mg/100 mg native)[a]	% protein loss (mg/100 mg native)		
		% from precipitation[b]	% from peptides < 3 kDa[a]	% total
3	35	35	30	65
17	17	27	56	83

[a]protein content of retentate and filtrate measured by Dumas (N×6.38)
[b]calculated by deduction from the measured percentages

The loss of protein material as a result of hydrolysis (insoluble proteinaceous material and peptides < 3 kDa), amounted up to 83 % (w/w) (**Table 2**). Moreover, the binding of EGCG by ovalbumin was not improved and was inferior to that by β-casein (**Chapter 2**). Hence, it is concluded that proteolysis does not seem to be a suitable tool for upgrading globular proteins as carriers of phenolics.

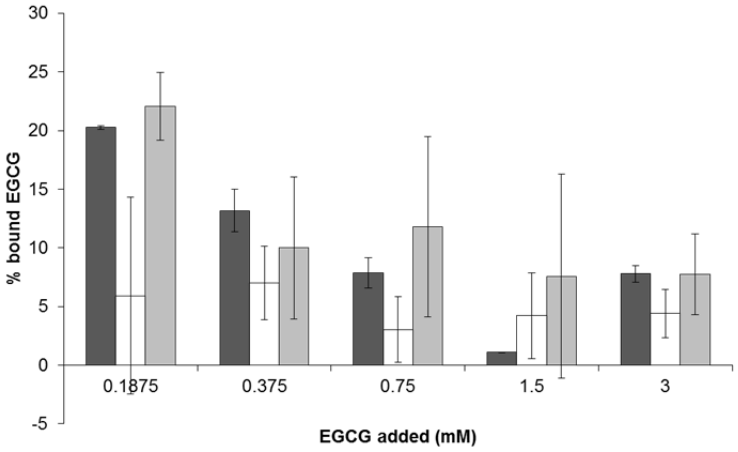

Figure 2. Proportion (mole %) of EGCG bound to 1.1 mg/mL of native or hydrolyzed ovalbumin in 50 mM sodium citrate buffer pH 3.5. Dark grey solid bars: hydrolysate 3 h; open bars: hydrolysate 17 h; light grey solid bars: native protein

Improving the affinity of globular proteins for phenolics by heat denaturation

Unfolding of globular proteins upon heating, thereby exposing their hydrophobic core, is expected to improve their interaction with phenolics. Each protein has a specific denaturation temperature, although various states of unfolding are often occurring over a relatively wide range of temperatures (*18*). In the unfolded state, globular proteins generally aggregate, ultimately leading to their precipitation. Unfolding and aggregation can be controlled by varying experimental factors, e.g. protein concentration, temperature, time and pH. The addition of phenolics just prior to cooling may allow their entrapment within the protein aggregates.

The addition of phenolics to heated β-lactoglobulin (5 °C below and above its denaturation temperature (Td = 80.3 °C (*19*))) directly followed by intense vortexing prior to cooling has been reported to generate EGCG-loaded soluble aggregates able to stabilize EGCG against oxidation (*20*). As ovalbumin contains more prolines and hydrophobic amino acids than β-lactoglobulin, its capacity to bind EGCG upon heat treatment may be higher than that of β-lactoglobulin. Ovalbumin (denaturation temperature 78 °C (*21*)) and β-lactoglobulin were heated to 80 °C prior to adding EGCG.

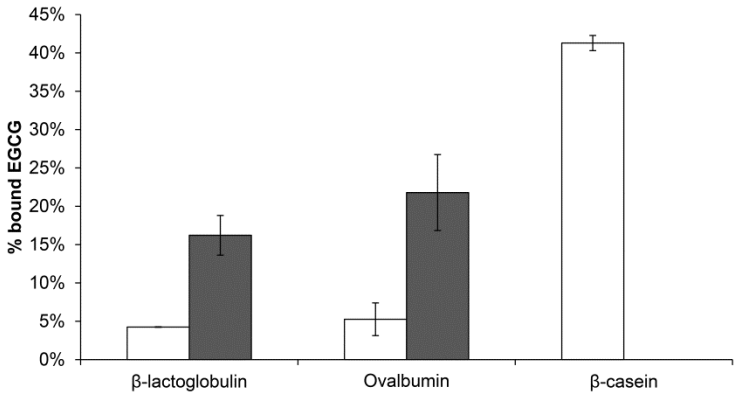

Figure 3. Comparison of the binding of native (open bar) and heat-treated (closed bar) β-lactoglobulin and ovalbumin (25 μM protein, EGCG-to-protein molar ratio of 30) in 50 mM sodium phosphate buffer at pH 7. EGCG was added just prior to intense vortexing (20 s) and cooling on ice. β-casein is given as a reference protein

As shown in **Figure 3**, the binding of EGCG by β-lactoglobulin and ovalbumin increased both ~4-fold. The increased binding of heated β-lactoglobulin compared to the native protein is in accordance with previous reports (*20, 22*). The higher contents in prolines and hydrophobic amino acids of ovalbumin did not result in a larger effect compared to β-lactoglobulin. Although the binding of both globular proteins was increased by the heat treatment compared to their native proteins, it was still ~2-fold lower than that of native β-casein, our reference.

In terms of applicability for delivery of phenolics in food, heat denaturation showed potential in improving globular proteins as carriers for phenolics. β-Casein, however, still prevails as carrier for EGCG compared to heat-treated globular proteins. Heat-induced carriers for phenolics may provide an interesting tool to deliver flavonoids other than EGCG, which could not be efficiently bound to β-casein (**Chapter 2**), but this methodology remains to be tested.

METAL-ION MEDIATED CARRIERS OF MONOMERIC FLAVONOIDS: THE EXAMPLE OF PHOSVITIN

Based on results obtained in **Chapter 2**, it was concluded that monomeric flavonoids did neither bind efficiently to the globular proteins tested nor to the most promising carrier, β-casein. Amongst the proteins tested, chicken egg yolk phosvitin seemed to be able to bind monomeric flavonoids. Most likely, metal ions bound at the surface of phosvitin were involved in the interaction, as loss of EGCG-binding was evidenced with EDTA-treated phosvitin (**Chapter 2**), a treatment known to remove iron from phosvitin (*23*). Flavonoids are known to coordinate iron (II) and iron (III), primarily via their catechol ring, with a higher stability constant for iron (III) than for iron (II) (*24*). Hence, the iron-mediated interaction of flavonoids might provide an opportunity for developing phosvitin into a carrier of monomeric flavonoids.

Phosvitin (34 kDa, 217 amino acid residues (**Chapter 2**)) is an unstructured protein at neutral pH and is primarily composed of phosphorylated serine residues (*25*). These phosphate groups are able to chelate iron (III) atoms in an iron-to-phosphate ratio of 0.5. Iron (II) itself binds weakly to phosvitin, but is readily oxidized into iron (III) in the presence of phosvitin, after which it is chelated (*26, 27*). The binding of EGCG by phosvitin was investigated under conditions reported ideal for iron chelation by phosvitin (i.e. 50 mM MES buffer pH 6.5) (*28*). As shown in **Figure 4**, the binding potential of EDTA-treated phosvitin was restored after addition of iron in the form of $FeCl_2$ (iron-to-phosphate ratio of 0.25 (assuming that all serine residues were phosphorylated)), and even improved compared to the non-treated phosvitin. Thus, the affinity of phosvitin for EGCG appeared to be metal ion-driven. The iron-enriched phosvitin showed an increased blue-purple color intensity compared to the control at the end of the ITC run (**Figure 4**). This color has been ascribed to the formation of EGCG-iron (III) complexes (*29*).

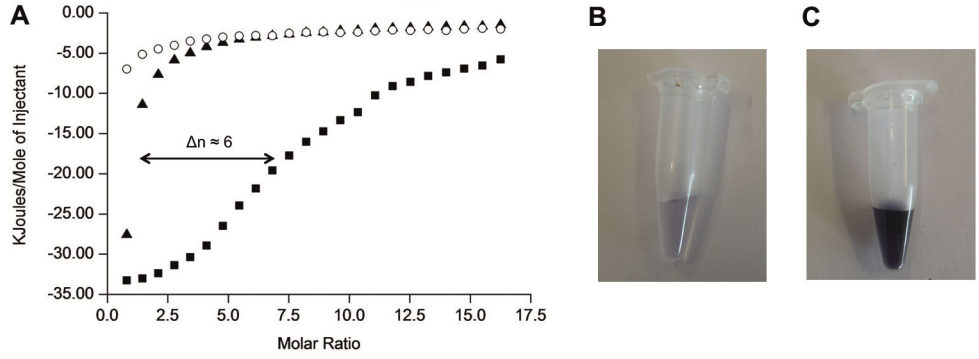

Figure 4. ITC binding isotherm (**A**) (25 °C) of 0.1 mM untreated phosvitin (▲), EDTA-treated phosvitin (○) and iron-enriched phosvitin (■) (iron-to-phosphate ratio = 0.25) binding EGCG in 50 mM MES buffer pH 6.5. Untreated phosvitin-EGCG (**B**) and iron-enriched phosvitin (**C**) colored complexes after ITC

The potential of phosvitin to bind monomeric flavonoids was studied by monitoring color formation upon titration of phosvitin (untreated) with several flavonoids. **Figure 5A** shows the typical bathochromic shift and hyperchromic shift at 550 nm, which were observed for all flavonoids tested, except catechin. The chromic shifts are indicative for interaction of the flavonoids with phosvitin, which is further illustrated by **Figure 5C**. The absence of color formation when titrating catechin in phosvitin is in accordance with the absence of interaction reported in **Chapter 2**. Moreover, glycosylation of quercetin, as in rutin, or removal of one hydroxyl group, as in luteolin or kaempferol, decreased color formation and potentially the interaction between these flavonoids and phosvitin. The less intense color observed with kaempferol compared to quercetin illustrates the importance of hydroxylation of the catechol motif in the flavonoid skeleton might also be involved in iron chelation (*30*). These preliminary observations provide evidence that phosvitin could act as a carrier for a range of monomeric flavonoids, although quantification of the interaction is required to establish this further.

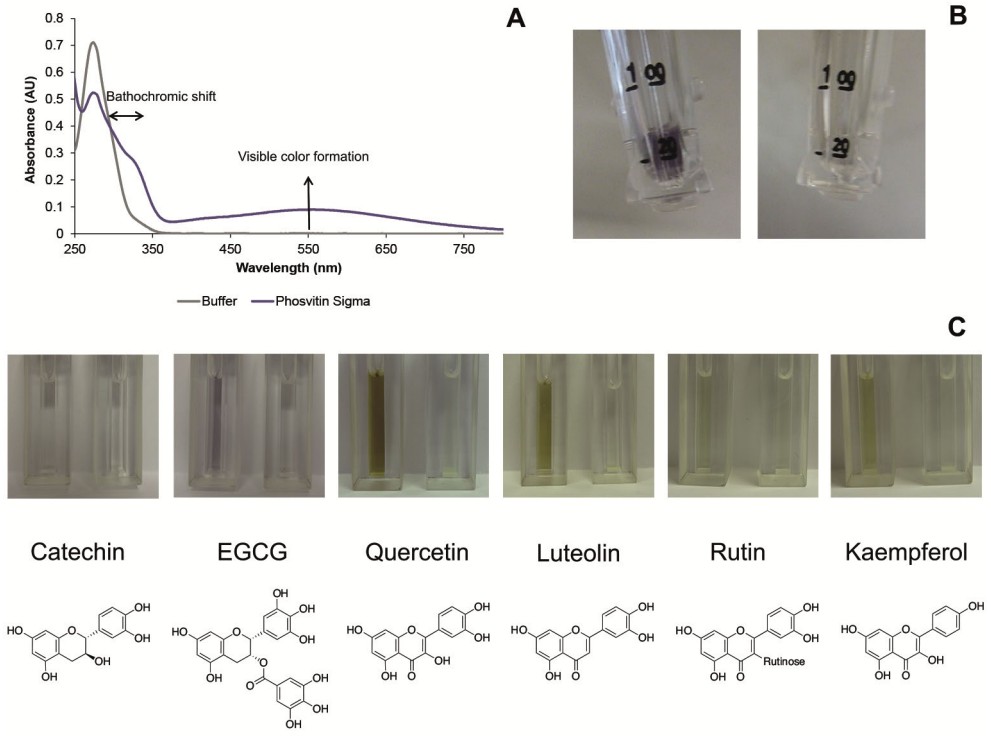

Figure 5. Typical spectral changes (**A**) observed for flavonoids binding to phosvitin (EGCG (0.03 mM) in buffer or mixed with untreated phosvitin (molar ratio 1:1)); (**B**) Stability of EGCG-phosvitin colored complexes against buffer wash (left) and EDTA wash (right); (**C**) Colored complexes with untreated phosvitin in presence of different flavonoids (final flavonoid concentration of 0.15 mM except for quercetin, luteolin and kaempferol (0.09 mM); stock solutions of flavonoids were made in DMSO; left cuvette with 0.05 mM phosvitin; right cuvette without phosvitin)

The colored EGCG-phosvitin complex formed was relatively stable against diafiltration with buffer, but was destabilized by diafiltration with EDTA in water (10 mM) (**Figure 5B**). UHPLC-MS-MS analysis of the permeate after EDTA treatment showed release of intact EGCG, whereas no EGCG could be detected anymore in the permeates after diafiltration with several cycles of MES buffer prior to EDTA (data not shown). Although further confirmation of the stability of phenolics bound to phosvitin is required, it seems as if phosvitin has potential as a carrier for flavonoids. The fact that phosvitin-flavonoid complexes are colored, might be a limitation for food applications.

PROTEIN-PHENOLIC INTERACTIONS REVISITED

As discussed in **Chapter 2**, β-casein was able to bind relevant amounts of EGCG per gram of protein in terms of dietary intake. In addition, no insoluble aggregates were observed at EGCG-to-protein molar ratios up to 120 (**Figure 6**), which was considered as a major advantage of this protein over gelatins for future food applications. The aggregation threshold of the salivary PRP IB5 L interacting with EGCG was reported to be at an EGCG-to-protein molar ratio of about 50 at a protein concentration of 0.2 mg/mL, and at a ratio of 3 at a protein concentration of 1 mg/mL (*6*). The concentration of β-casein used in **Chapter 2** (0.6 mg/mL) and **Chapter 5** (2.4 mg/mL – EGCG-to-protein molar ratio ranging from 0-30) indicates that the aggregation behavior of β-casein might be different from the model reported in the general introduction. The aggregation and precipitation of dephosphorylated β-casein upon EGCG binding observed at molar ratios above 10 (*31*) indicates that the phosphate groups of β-casein might help maintaining the complexes in solution. In comparison to PRP IB5, β-casein has a lower proline density, which might induce less cross-linking between β-casein molecules with increasing EGCG concentrations.

The prevalence of proline residues in protein-phenolic interactions might lead to think that "the more proline, the better" for increased affinity of proteins for phenolics. In **Chapter 3**, we have shown that the interaction of the procyanidin dimer A1 was dependent on the protein structure, with clear binding observed with α-casein (8.5% (mol/mol) proline in α_{S1}-casein) and no significant binding with β-casein (16.7% (mol/mol) proline). Moreover, α-casein had a preference to bind procyanidin dimer B2, a preferentially extended molecule, just like procyanidin A1. In contrast, β-casein seemed to have preference for binding the compact procyanidin dimer B1. Therefore, the choice of an unstructured protein as carrier for phenolics cannot only be made based on its proline content. Other structural factors, such as the distribution of proline and hydrophobic amino acids might also be important. Furthermore, the presence of certain amino acids in the vicinity of proline might have a greater impact on proline-phenolic interaction than previously thought. The presence of bulky amino acid side-chains neighboring potential binding sites might influence the binding by food proteins, which have a larger diversity in

amino acid residues than salivary proline-rich proteins (*32*). Finally, aromatic amino acid side chains have been described to influence the *cis*/*trans* isomerism of prolines in their vicinity via CH-π interactions (*33*), thereby possibly influencing binding as a higher affinity for phenol has been attributed to the *cis* conformation of proline in comparison to its *trans* conformation (*34*). These CH-π interactions might also compete with the phenolics' aromatic rings for binding to the prolyl ring, as both the aromatic rings of amino acids and phenolics can engage in the same type of interactions with proline residues (*33*).

As indicated in **Chapter 3** and **Chapter 4**, the presence of a galloyl group with high rotational freedom, as in EGCG or galloylated procyanidins, has a larger impact on increasing the binding affinity of phenolics to proteins than an extra monomeric flavan-3-ol unit in procyanidins. Procyanidin dimer B1 is known to be present preferentially in a compact conformation, contrary to procyanidin dimer B2, that is present as both compact and extended conformations in a 1:1 ratio (*35*). These characteristics do not mean per se that the extended procyanidin B2 binds better to α- and β-caseins than procyanidin B1 (**Chapter 3**), as reported for their interaction with the salivary proline-rich peptide $IB7_{14}$ (*36*). The conformation of oligomeric flavan-3-ols, in particular, should be better considered in studies of food protein-phenolic interactions.

PERSPECTIVES FOR PROTEIN-BASED CARRIERS OF PHENOLICS

When aiming at using native animal food proteins as carriers for phenolics, unstructured caseins appear to be the best option. As discussed in **Chapter 2**, β-casein binds relevant amounts of EGCG per gram of protein in terms of dietary intake. In addition, no insoluble aggregates were observed at EGCG-to-protein molar ratios up to 120 (data not shown), which is considered a major advantage of this protein over gelatins for future food applications. By replacing β-casein by Na-caseinate, we have shown that casein complexes with EGCG could effectively reduce its bitterness perception (**Chapter 5**), while its effect on astringency was not evaluated. In the mouth, casein-EGCG complexes coexist with proline-rich salivary proteins, which might compete with casein for EGCG, possibly leading to taste defects (*37*). The interaction between these salivary proteins and EGCG has been suggested to underlie astringency. Considering the short residence time of the casein-EGCG complexes in the mouth, it remains to be seen whether such competition with concomitant astringency really occurs. The effective reduction in both bitterness and astringency of EGCG after its binding to heat-treated β-lactoglobulin suggests that it might not be a problem (*22*).

The binding affinity of β-casein for monomeric flavonoids other than EGCG was low in comparison to BSA (**Chapter 2**). This is contrary to findings from a recent study (*38*), which reported affinities of curcumin, genistein and resveratrol to both α- and β-caseins with a binding constant of ~10^4 M^{-1}. Although their fluorescence quenching study design is arguable in terms of data correction (e.g. inner filter effects (see **Chapter 3**) and modeling

(modified Stern-Volmer with possibly high error on the determination of the affinity), the order of magnitude of their binding constant found suggests that caseins might be better carriers of phenolics that indicated by our study. The discrepancy might be explained by the fact that their study was conducted at a ~β-casein concentration of 80 μM. β-Casein was, therefore, present in micellar state (critical micelle concentration = 21 μM (*39*)), whereas our study was done with casein concentrations just at the critical micelle concentration (25 μM). β-Casein micelles have been used as carriers for hydrophobic drugs, and also for curcumin (*40, 41*). This is supported by a recent review on the potential of milk proteins as carriers for bioactive compounds (*42*). Therefore, caseins should not be disqualified as carriers of phenolics (even monomeric), at least when they are used in micellar state.

Table 3. Binding characteristics of several animal and plant food proteins for EGCG at pH 7

Protein	K (10^3 M^{-1})	R_{max} (mol/mol)
soy protein isolate (SPI)[a]	71.1 (±0.4)	75.8 (±4.5)[b]
patatin[a]	8.3 (±1.1)	3.5 (±0.7)[b]
wheat gluten hydrolysate[a]	26.5 (±2.4)	20.7 (±0.7)[b]
β-casein[c]	45.0 (±7.2)	19.6 (±4.9)
fish gelatin[c]	53.3 (±1.8)	57.5 (±1.8)

[a]measurements were conducted under the same conditions as described in **Chapter 2**. Data are given as the average (±standard deviation) of two replicates

[b]Rmax values reported are the maximum bound fraction observed in each binding isotherm

[c]from this thesis (**Chapter 2**)

Plant proteins were not investigated in this thesis, but some of them seem to have useful structural features. For example, soy glycinin has proline residues distributed over its surface and has been shown to bind proanthocyanidins (*43*). There are only few reports studying non-covalent interactions of plant proteins with phenolics, most of them using processed protein (e.g. electrospun zein fibers (*44*)). To our knowledge, no reports are available on a systematic comparison of native plant proteins as performed for animal food proteins in **Chapter 2**. Using a similar setup as described in **Chapter 2**, a soy protein isolate (estimated molecular mass = 295 kDa), a water-soluble fraction of a wheat gluten hydrolysate (Nutralys W®, Roquette, Lestrem, France; average molecular mass = 30 kDa), and a water-soluble fraction of patatin (Laboratory of Food Chemistry, Wageningen, The Netherlands; molecular mass = 40 kDa) were compared for their binding to EGCG (**Table 3** and **Figure 6**). Although the binding characteristics of these plant proteins were in a similar range as the ones determined for animal food proteins, the precipitation observed for all three proteins was seen as a drawback for further food application. The use of

carbohydrates (e.g. pectin, arabic gum) to modulate this precipitating behavior may be helpful, but remains to be investigated for these proteins (*45, 46*).

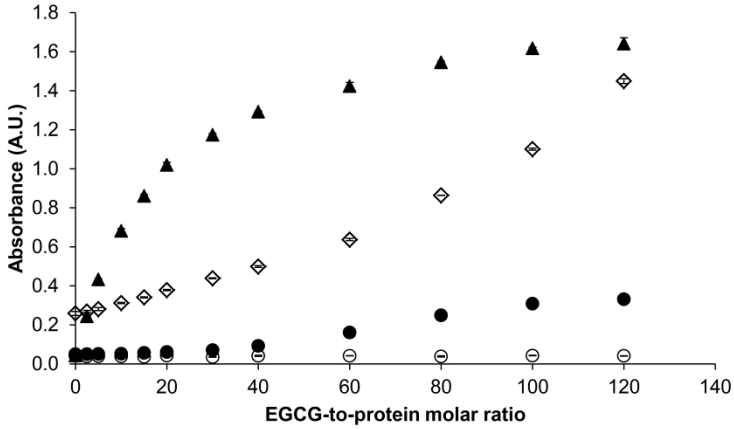

Figure 6. Turbidity of several plant protein solutions upon EGCG binding at pH 7 (patatin (●), SPI (◇), and wheat gluten hydrolysate (▲)). β-Casein (○) is indicated as a control. Turbidity was determined by measuring the light absorption of the complexes at 720 nm after incubation

From the range of proteins tested in this thesis, milk caseins appeared consistently as the most promising carriers for phenolics. These proteins could efficiently bind EGCG and tackle its taste defects, but under their CMC the caseins showed negligible affinity for monomeric phenolics. From the literature, there are indications that β-casein might also sequester monomeric flavonoids. Overall, caseins appear to be the most promising and versatile proteinaceous carrier of phenolics for food applications.

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Summary

Samenvatting

Résumé

Owing to the beneficial effects of phenolics on human health, foods enriched in phenolics are seen as a potential tool for dietary-mediated disease prevention. The enrichment of foods with phenolics, however, is limited due to their impact on the organoleptic properties of food, primarily related to their contribution to bitterness and astringency. At the start of this thesis, it was hypothesized that this might be counteracted by the use of animal food proteins as carriers of phenolics. The aim of this thesis was to obtain an understanding of the structure-binding relationships governing food protein-phenolics interactions. Affinities of a range of animal food proteins for representative phenolics, pure or in mixtures, were investigated with several methods and related to the structural characteristics of both phenolics and proteins. Subsequently, the efficiency of the most promising phenolic carrier for reducing the bitterness perception of phenolics was evaluated.

Chapter 1 provides an overview of the structural characteristics of phenolics and proteins that influence their interaction. Driving forces behind this interaction and concomitant aggregation mechanisms with proteins and phenolics are also presented. Most of the information available is based on studies of the interaction between phenolics and salivary proline-rich proteins or serum albumins. The limited availability of information on the interaction of phenolics with animal food proteins requires a systematic study of these interactions, using representative phenolics and common animal food proteins.

Chapter 2 describes a comparative study of various milk proteins, egg proteins and gelatin hydrolysates for their binding characteristics to two flavan-3-ols: catechin and epigallocatechin gallate (EGCG). For this an assay was employed, in which unbound phenolics were removed from protein-bound phenolics by ultrafiltration, after which they were quantified by UV in the permeate. Amongst the proteins tested, β-casein and gelatin hydrolysates, in particular fish gelatin, were found to be the most promising carriers with an affinity in the order of 10^4 M^{-1}. When the absence of turbidity is taken into account, β-casein prevails as potential carrier. A flexible open structure of proteins, as present in random coil proteins, was found to be important. Monomeric flavonoids, representative of several classes (flavan-3-ols, flavanones, flavanonols, flavones and flavonols), did neither bind efficiently to the globular food proteins tested nor to β-casein. Considering the maximal binding capacity of β-casein for EGCG (i.e. its saturation expressed as mole phenolics/mole protein), it was shown that only gram quantities of protein could bind all EGCG required to meet a daily intake associated with positive health effects.

In **Chapter 3**, the potential of milk caseins as carriers for monomeric flavan-3-ols, and dimeric A and B-type procyanidins was further investigated by fluorescence quenching, and the affinities of the phenolics were related to their three-dimensional structure. It was observed that esterification of flavan-3-ol with gallic acid, as in EGCG, has a five to ten-fold greater effect on binding affinity to caseins compared to extension with a catechin or epicatechin unit, as in B-type procyanidin dimers. The higher degree of rotational freedom of aromatic rings in EGCG, compared to the other phenolics investigated, might explain this difference. Procyanidin dimer A1 displays a higher affinity to α-casein than

B-type procyanidin dimers, despite its additional interflavanic bond, which possibly reduces its conformational freedom, but exposes the B-rings of both monomeric units. Surprisingly, no interaction of procyanidin A1 with β-casein was detected. This is despite the fact that β-casein showed the highest affinity for the other flavan-3-ols and that β-casein contains more proline and proline repeats, known to drive the interaction, than α-casein. These results suggest that the interaction between phenolics and caseins is governed by more than only proline content and number of proline repeats.

Chapter 4 aimed at evaluating the binding affinity of individual phenolics in a complex mixture to β-casein by applying an assay in which ultrafiltration (UF) was coupled to UHPLC-MS-MS analysis, using a procyanidin trimer fraction from grape seed as a representative mixture. Targeted free phenolics were analyzed in the permeate by selected reaction monitoring in a linear ion-trap MS and quantified relative to their initial amounts in the extract used. This method allowed the rapid and sensitive monitoring of four compounds simultaneously. Comparison of the binding characteristics of several individual procyanidins in the mixture showed that galloylation enhanced the binding affinity of procyanidins to β-casein. The degree of polymerization and the galloylation of procyanidins were shown to act synergistically in protein-phenolic interactions. Moreover, oligomeric phenolics appeared to prime the binding of monomeric phenolics in the same mixture. Preliminary data using a black tea extract illustrated the applicability of this UF-MS assay to other plant extracts. Galloylation of theasinensins, oxidation products of flavan-3-ols present in black tea, seems to promote their binding to β-casein. The UF-MS assay presented appears to be a valuable tool for quantifying the binding to casein of different phenolics from a complex mixture simultaneously.

In **Chapter 5**, the EGCG-binding characteristics of several pure milk proteins, and their food-grade equivalents, were related to their effects on reducing bitter receptor activation by EGCG *in vitro* and their bitter-masking potential *in vivo*. β-Casein had a high affinity for EGCG ($K = 45.0\ (\pm 7.2) \times 10^3\ M^{-1}$) and showed the strongest effect in a bitter receptor assay, with a maximum reduction of hTAS2R39 (the bitter receptor sensing EGCG) activation of about 93%. A similar potency was observed for Na-caseinate. β-Lactoglobulin had little effect on bitter receptor activation, as expected based on its low binding affinity for EGCG. The bitter-masking potential of Na-caseinate was confirmed *in vivo* using a trained sensory panel. β-Lactoglobulin also significantly reduced EGCG bitter perception, although to a lesser extent. This could not be directly related to its binding to proteins. It is concluded that the bitter receptor assay applied is a valid tool to evaluate *in vitro* the efficacy of food proteins as bitter-masking agents.

Chapter 6 combines the information described in previous chapters and addresses protein-phenolic interactions in the light of the knowledge acquired with food proteins. In addition, the advantages and inconveniences of commonly applied methods to study protein-phenolic interactions are discussed. The need to develop methods to evaluate mixtures of phenolics binding to proteins is highlighted. Moreover, alternative strategies to

improve the potential of globular food proteins as carriers of phenolics are presented, in order to extend the range of proteins to be used as carriers beyond unstructured proteins. The opening of globular proteins by proteolysis does not provide a valuable alternative for binding phenolics, but heat denaturation followed by refolding in presence of phenolics might offer opportunities in this respect. Good carriers for monomeric flavonoids were not found, except for bovine serum albumin, which is too expensive in food applications. Metal-ion mediated binding, as in phosvitin-phenolics complexes, might provide a means to bind monomeric flavonoids, although the resulting colored complexes might be a limitation for food application. Finally, the perspectives for application of food proteins as carriers for phenolics are given.

Summary

Samenvatting

Résumé

Vanwege de gezondheidsbevorderlijke effecten van fenolen, worden levensmiddelen verrijkt met fenolen beschouwd als potentieel hulpmiddel voor dieet-gestuurde ziektepreventie. De verrijking van levensmiddelen met fenolen wordt echter gelimiteerd door hun invloed op de organoleptische eigenschappen van levensmiddelen, vooral op bitterheid en adstringentie. In het begin van dit proefschrift werd de hypothese gesteld dat dit mogelijk tegen gegaan kan worden door dierlijke voedseleiwitten als drager voor fenolen te gebruiken. Het doel van dit proefschrift was het begrijpen van de structuur-binding relaties die bepalend zijn voor voedseleiwit-fenol interacties. Van een aantal dierlijke voedseleiwitten werd de affiniteit voor representatieve fenolen, zuiver of in mengsels, onderzocht met verschillende methoden en gerelateerd aan de structurele karakteristieken van zowel de fenolen als de eiwitten. Daaropvolgend werd van de meest veelbelovende drager voor fenolen de efficiëntie waarmee hij de perceptie van bitterheid van fenolen verlaagde geëvalueerd.

Hoofdstuk 1 geeft een overzicht van de structurele karakteristieken van fenolen en eiwitten die invloed hebben op hun interactie. De drijvende kracht van deze interactie en de bijbehorende aggregatiemechanismen met eiwitten en fenolen worden ook gegeven. De meeste beschikbare informatie is gebaseerd op studies naar de interactie tussen fenolen en speekseleiwitten rijk aan proline of serum albumines. De gelimiteerde informatie over de interactie van fenolen met dierlijke voedseleiwitten die beschikbaar is maakt een systematische studie naar deze interacties noodzakelijk, gebruik makend van representatieve fenolen en veel voorkomende dierlijke voedseleiwitten.

Hoofdstuk 2 beschrijft een vergelijkende studie van verschillende melkeiwitten, ei-eiwitten en gelatine hydrolysaten voor hun bindingseigenschappen met twee flavan-3-olen: catechine en epigallocatachinegallaat (EGCG). Er werd gebruik gemaakt van een assay waarin ongebonden fenolen van eiwit-gebonden fenolen werden gescheiden door middel van ultrafiltratie, waarna ze in het permeaat werden gekwantificeerd door middel van UV. Van de geteste eiwitten werden β-caseïne en gelatine hydrolysaten, in het bijzonder visgelatine, als meest veelbelovende dragers gevonden, met een affiniteit in de orde van 10^4 M^{-1}. Wanneer de afwezigheid van troebelheid in ogenschouw wordt genomen, blijft β-caseïne als potentiële drager over. Een flexibele open structuur, zoals aanwezig in ongestructureerde eiwitten, werd als belangrijke factor gevonden. Monomere flavonoïden, representatief voor verschillende klassen (flavan-3-olen, flavanonen, flavanonolen, flavonen en flavonolen), bonden niet efficiënt aan de geteste globulaire voedseleiwitten, noch aan β-caseïne. De maximale EGCG bindingscapaciteit van β-caseïne (de verzadiging uitgedrukt als mol fenolen/mol eiwit) toonde aan dat de hoeveelheid eiwit benodigd om een hoeveelheid EGCG overeenkomstig de dagelijks inname die wordt geassocieerd met gezondheidsbevorderende effecten te binden in de orde van grammen ligt.

In **hoofdstuk 3** werd het potentieel van melkcaseïnes als dragers voor monomere flavan-3-olen en dimeren van A- en B-type procyanidinen verder onderzocht met behulp van fluorescentie quenching, en de affiniteiten van de fenolen werden gerelateerd aan hun

driedimensionale structuur. Gevonden werd dat verestering van een flavan-3-ol met galluszuur, zoals in EGCG, een vijf tot tien maal groter effect heeft op de bindingsaffiniteit voor caseïnes dan uitbreiding met een catechine of epicatechine eenheid, zoals in B-type procyanidinen. De grotere rotatievrijheid van aromatische ringen in EGCG, vergeleken met de andere onderzochte fenolen, kan dit verschil mogelijk verklaren. Procyanidine dimeer A1 laat een grotere affiniteit voor α-caseïne zien dan B-type procyanidine dimeren, ondanks de additionele interflavanische binding, die mogelijk de rotatievrijheid vermindert, maar tegelijkertijd de B-ringen van beide monomere eenheden blootgeeft. Verrassend genoeg werd er geen interactie tussen procyanidine A1 en β-caseïne gevonden. Dit ondanks dat β-caseïne de hoogste affiniteit voor de andere flavan-3-olen liet zien en dat β-caseïne meer proline en proline herhalingen dan α-caseïne bevat, welke bekend staan als sturend voor de interactie. Deze resultaten wijzen erop dat de interactie tussen fenolen en caseïnes niet alleen door de hoeveelheid proline en proline herhalingen worden bepaald.

Hoofdstuk 4 richt zich op het evalueren van de bindingsaffiniteit van individuele fenolen in een complex mengsel voor β-caseïne, gebruikmakend van een assay waarin ultrafiltratie (UF) gekoppeld was aan UHPLC-MS-MS analyse. Een procyanidine trimeer fractie uit druivenpitten werd gebruikt als een representatief mengsel. Geselecteerde vrije fenolen in het permeaat werden geanalyseerd met behulp van 'selected reaction monitoring' in een 'linear ion trap MS', en relatief gekwantificeerd ten opzichte van hun initiële concentraties in het gebruikte extract. Met deze methode konden vier componenten tegelijkertijd, op een snelle en gevoelige manier worden gevolgd. Vergelijking van de bindingseigenschappen van verschillende individuele procyanidinen in het mengsel liet zien dat galluszuur additie de bindingsaffiniteit van procyanidinen voor β-caseïne verhoogde. De graad van polymerisatie en galluszuur additie werkten synergistisch in eiwit-fenol interacties. Bovendien leek het er op dat oligomere procyanidinen de binding van monomere fenolen in hetzelfde mengsel op gang brachten. Voorlopige resultaten met een extract van zwarte thee illustreren de toepasbaarheid van deze UF-MS assay op andere plantenextracten. Galluszuur additie aan theasinensinen, oxidatieproducten van flavan-3-olen aanwezig in zwarte thee, lijkt hun binding aan β-caseïne te bevorderen. De hier gepresenteerde UF-MS assay lijkt een waardevolle methode te zijn voor het simultaan kwantificeren van de binding van caseïne en verschillende fenolen in complexe mengsels.

In **hoofdstuk 5** werden de EGCG bindingseigenschappen van verschillende zuivere melkeiwitten, en hun 'food grade' equivalenten, gerelateerd aan hun effecten op bitter receptor activering door EGCG *in vitro* en hun bitter maskerende potentieel *in vivo*. β-Caseïne had een hoge affiniteit voor EGCG ($K = 45.0 (\pm 7.2) \times 10^3\ M^{-1}$) en had het meeste effect in een bitter receptor assay, met een maximale reductie van hTAS2R39 (de bitter receptor voor EGCG) activatie van ongeveer 93%. Een vergelijkbaar effect werd gevonden met Na-caseïnaat. β-Lactoglobuline had weinig effect op bitter receptor activering, zoals verwacht werd op basis van zijn lage bindingsaffiniteit voor EGCG. De bitter maskering door Na-caseïnaat werd bevestigd *in vivo* door gebruik te maken van een getraind

sensorisch panel. β-Lactoglobuline reduceerde de perceptie van bitterheid van EGCG ook significant, hoewel minder dan Na-caseïnaat. Dit kon niet direct gerelateerd worden aan de binding van EGCG aan eiwit. Geconcludeerd kan worden dat de gebruikte bitter receptor assay een goede methode is om de efficiëntie van voedingseiwitten als bitter maskerende verbindingen *in vitro* te bepalen.

Hoofdstuk 6 combineert de informatie beschreven in de voorafgaande hoofdstukken en bespreekt eiwit-fenol interacties in het licht van de verkregen kennis met voedingseiwitten. Daarnaast worden de voor- en nadelen van veelgebruikte methodes om eiwit-fenol interacties te bestuderen besproken. De noodzaak om methoden te ontwikkelen voor de evaluatie van de binding van mengsels van fenolen aan eiwitten wordt benadrukt. Daarnaast worden alternatieve strategieën om het potentieel van globulaire voedseleiwitten als dragers van fenolen te verbeteren gepresenteerd, om de range van eiwitten die als dragers kunnen worden gebruikt uit te breiden met andere dan ongestructureerde eiwitten. Het openen van globulaire eiwitten door proteolyse resulteert niet in een bruikbaar alternatief om fenolen te binden, maar denaturatie door verhitten gevolgd door hervouwing in aanwezigheid van fenolen biedt mogelijk kansen. Goede dragers voor monomere flavonoïden werden niet gevonden, behalve runderalbumine, wat te duur is voor toepassing in levensmiddelen. Metaal ion gemedieerde binding, zoals in phosvitine-fenol complexen, zou een mogelijkheid kunnen bieden om monomere flavonoïden te binden, hoewel de gekleurde complexen die daarvan het resultaat zijn een limitatie voor wat betreft de toepasbaarheid in levensmiddelen kunnen zijn. Tot slot worden de perspectieven voor de toepasbaarheid van voedseleiwitten als dragers van fenolen gegeven.

Summary
Samenvatting
Résumé

Les composés phénoliques sont connus pour leurs effets bénéfiques sur la santé humaine. L'enrichissement en phénoliques de certains aliments peut être un outil potentiel pour la prévention de certaines maladies par l'alimentation. Cependant, l'ajout de composés phénoliques aux aliments est limité par leur impact sur les propriétés organoleptiques de ces derniers, essentiellement lié à leur contribution à l'amertume et l'astringence. L'hypothèse de départ de cette thèse était que cette limitation pouvait être résolue en utilisant des protéines alimentaires d'origine animale en tant que vecteurs des composés phénoliques. L'objectif de cette thèse a été d'obtenir une compréhension globale des relations structure-interactions régissant les interactions entre les protéines alimentaires et les composés phénoliques. Les affinités d'une sélection de protéines alimentaires d'origine animale et de composés phénoliques (purs ou sous forme de mixtures) ont été évaluées par plusieurs méthodes et ont ensuite été reliées aux caractéristiques structurelles des molécules considérées. Enfin, l'efficacité du meilleur vecteur protéique pour réduire la perception de l'amertume des composés phénoliques a été évaluée.

Le **Chapitre 1** fournit une vue d'ensemble des caractéristiques structurelles des composés phénoliques et des protéines influençant leurs interactions l'un avec l'autre. La nature des interactions entre les protéines et les composés phénoliques et le mécanisme d'agrégation pouvant s'ensuivre sont également décris. La plupart des informations disponibles sur ces interactions est basée sur des études des interactions entre les composés phénoliques et les protéines salivaires ou les albumines du sérum. Le manque d'informations sur les interactions entre les protéines alimentaires d'origine animale demande une étude systématique de ces interactions basée sur des composés phénoliques représentatifs et des protéines alimentaires communément utilisées.

Le **Chapitre 2** décrit une étude comparative des caractéristiques de l'interaction de plusieurs protéines laitières, protéines d'œuf et hydrolysats de gélatines avec deux flavan-3-ols : la catéchine et l'épigallocatéchine gallate (EGCG). A cette fin, un protocole basé sur la séparation par ultrafiltration de la fraction du composé phénolique n'ayant pas interagit avec la protéine, suivie par une quantification par UV de cette même molécule dans le filtrat, a été utilisé. Parmi les protéines testées, la β-caséine et les hydrolysats de gélatine, en particulier de celle de poisson, sont apparus comme les vecteurs les plus prometteurs de par leurs affinités pour l'EGCG de l'ordre de 10^4 M^{-1}. En tenant compte de l'absence de turbidité en solution, la β-caséine prévaut comme vecteur prometteur. Une structure ouverte et flexible des protéines, comme dans le cas des protéines non structurées, a montré son importance. Des flavonoïdes monomériques représentant plusieurs classes (flavan-3-ols, flavanones, flavones et flavonols) n'ont montré qu'une faible capacité d'interaction avec les protéines globulaires testées et également avec la β-caséine. En considérant la capacité maximale d'interaction de la β-caséine pour l'EGCG (c'est-à-dire sa saturation exprimée en mole de composés phénoliques/mole de protéines), il apparaît que seules des quantités de protéines de l'ordre du gramme seraient nécessaires pour transporter

la quantité d'EGCG associée à une dose quotidienne induisant des effets positifs sur la santé.

Dans le **Chapitre 3**, le potentiel des caséines du lait en tant que vecteurs pour les monomères de flavan-3-ols et les dimères de types A et B (procyanidines) a été étudié par la technique d'extinction de la fluorescence. Les affinités mesurées pour ces ligands ont ensuite été reliées à leurs structures tridimensionnelles respectives. Il a été observé que l'estérification d'un flavan-3-ol par l'acide gallique, comme pour l'EGCG, augmentait son affinité par un facteur 5 à 10 comparé à l'addition d'une unité de catéchine ou d'épicatéchine, comme dans les dimères de type B. Cette différence est possiblement due au plus grand degré de liberté de rotation des cycles aromatiques de l'EGCG, comparé aux autres molécules testées. La procyanidine A1 a montré une affinité plus grande pour la α-caséine comparé aux dimères de type B, malgré sa liaison interflavanique additionnelle pouvant potentiellement réduire son degré de liberté de rotation. Cependant, cette liaison additionnelle expose les cycles B des deux unités monomériques, ce qui peut potentiellement favoriser l'interaction avec la protéine. Étonnamment, aucune interaction n'a pu être mesurée entre la procyanidine A1 et la β-caséine ; et ce, malgré le fait que la β-caséine ait montré l'affinité la plus élevée pour les autres flavan-3-ols, comparé à la α-caséine. De plus, la β-caséine contient plus de prolines et de doublets proline-proline, décrits comme étant importants pour ces interactions, que la α-caséine. Ces résultats suggèrent que l'interaction entre les composé phénoliques et les caséines n'est pas seulement gouvernée par la quantité de proline et le nombre de doublets proline-proline.

Le **Chapitre 4** décrit une méthode d'évaluation de l'affinité pour la β-caséine de composés phénoliques donnés présents dans une mixture. Cette méthode est basée sur de l'ultrafiltration (UF) couplée à une analyse du filtrat par UHPLC-MS-MS et a été développée en utilisant une fraction de procyanidines trimères isolé de pépins de raisins comme modèle. Les composés phénoliques d'intérêt dans le filtrat ont été analysés par Selected Reaction Monitoring (SRM) dans un spectromètre de masse avec un analyseur de type trappe linéaire, et quantifiés relativement à leur quantité initiale dans l'extrait utilisé. Cette méthode a permis le suivi rapide et sensible de quatre composés phénoliques de façon simultanée. La comparaison des paramètres déterminés pour les interactions de plusieurs procyanidines d'intérêt de la mixture a montré que la galloylation augmentait l'affinité des procyanidines pour la β-caséine. Le degré de polymérisation et la galloylation des procyanidines ont montré une action synergétique promouvant les interactions protéines-composés phénoliques. De plus, les oligomères ont été suggérés comme initiateurs de l'interaction des monomères avec la β-caséine dans une même mixture. Des données préliminaires avec un extrait de thé noir a permis d'illustrer la possibilité d'étendre cette méthode UF-MS à d'autres extraits de plantes. La galloylation de la théasinensine, un produit de l'oxidation des flavan-3-ols présent dans le thé noir, semble favoriser son interaction avec la β-caséine. La méthode UF-MS présentée apparaît comme un outil

d'intérêt pour l'évaluation simultanée de l'interaction avec la β-caséine de composés phénoliques individuels présents dans un mélange complexe.

Dans le **Chapitre 5**, les paramètres de l'interaction de plusieurs protéines laitières pures, ainsi que de leurs équivalents comestibles, avec l'EGCG ont été reliés à leur effet sur la réduction de l'activation d'un récepteur de l'amer par EGCG *in vitro* et leur potentiel pour masquer l'amertume *in vivo*. La β-caséine a montré l'affinité la plus grande pour l'EGCG ($K = 45.0\ (\pm 7.2) \times 10^3\ M^{-1}$) et l'effet le plus fort sur le récepteur de l'amer hTAS2R39, avec une réduction de son activation par l'EGCG de 93%. Les caséinates de sodium ont montré un potentiel similaire à la β-caséine. La β-lactoglobuline n'a eu que très peu d'effet sur l'activation du récepteur de l'amer étudié, comme cela pouvait être prévu de par sa faible affinité pour l'EGCG. Le potentiel des caséinates de sodium pour masquer l'amertume a été confirmé *in vivo* avec un panel sensoriel entraîné. La β-lactoglobuline a également réduit significativement la perception de l'amertume de l'EGCG, mais de façon moins important que les caséinates de sodium. Cette observation n'a pas pu être reliée directement et uniquement à l'interaction de l'EGCG avec ces protéines. Il est conclus que la méthode d'évaluation par activation d'un récepteur de l'amer est un outil valable pour étudier *in vitro* l'efficacité des protéines alimentaires comme agents masquant l'amertume des composés phénoliques.

Le **Chapitre 6** combine les information décrites dans les chapitres précédents et adresse les interactions entre les protéines et les composés phénoliques aux vues des connaissances acquises avec les protéines alimentaires. De plus, les avantages et les inconvénients de plusieurs méthodes communément utilisées pour l'étude des interactions protéines-composés phénoliques sont présentés. Dans ce cadre, le besoin de développer des méthodes pour évaluer l'interaction de mixtures de composés phénoliques est mis en avant. Par ailleurs, pour étendre la gamme de protéines pouvant être utilisées comme vecteurs au-delà des protéines non structurées, des stratégies alternatives pour améliorer le potentiel des protéines globulaires alimentaires comme vecteurs des composés phénoliques sont présentées. L'ouverture des protéines globulaires par protéolyse n'est pas une alternative valable pour l'interaction avec des composés phénoliques. Au contraire, la dénaturation thermique suivie d'une restructuration en présence de composés phénoliques présente certaines opportunités. De bons vecteurs pour les flavonoïdes monomériques n'ont pas été identifiés, mise à part l'albumine de sérum bovin qui a un coût trop élevé pour des applications alimentaires. L'interaction par l'intermédiaire d'ions métalliques, comme dans les complexes phosvitine-composés phénoliques, semble être une alternative pour le transport des flavonoïdes monomériques. Cependant, cette interaction forme des complexes colorés qui peuvent être une contrainte pour des applications alimentaires. Enfin, des perspectives sont données sur l'application des protéines alimentaires en tant que vecteurs des composés phénoliques.

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Seven years ago, I set foot in Wageningen for the very first time as an Erasmus MSc student to study Nutrition and Health with the plan to stay for 6 months. Well, plans are made to be changed and now I am writing the last pages of my PhD thesis in Food Chemistry, after more than 6 years living in Wageningen. These past years and the work for this thesis would not have been the same without all the people I have been surrounded with; and for that, I am very thankful to all of them.

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Maxime

About the author

CURRICULUM VITAE

Maxime C. Bohin was born on November 26th, 1984 in Blois, France. After graduating from high school (Lycée F.Ph. Dessaignes, Blois (France)) in 2002, he enrolled in an intensive foundation degree (biology, physics, chemistry and mathematics) preparing for the admission to the selective French engineer schools (Grandes Ecoles). In 2004, he was admitted at the National engineer School of Agronomy and Food Industry (ENSAIA) in Nancy (France), and studied Food Sciences and Processing. As part of his curriculum, he left to The Netherlands in 2006 to participate in the Erasmus exchange program of the European Union at Wageningen University. In 2007, after having completed all the required courses for the MSc Food Technology, he returned to France to complete his study in Nancy and specialized in Food Formulation. In 2008, he returned to Wageningen University for his MSc thesis entitled "Monitoring changes in isoflavonoid composition in fungus-challenged soybeans" at the Laboratory of Food Chemistry under the supervision of Rudy Simons and Dr. Ir. Jean-Paul Vincken. The same year, he graduated from the National engineer School of Agronomy and Food Industry (ENSAIA) in France. In 2009, he completed an internship at Unilever R&D in Vlaardingen (NL) and obtained his MSc degree in Food Technology. The same year, he was offered the opportunity to work as a PhD student at the Laboratory of Food Chemistry under the supervision of Dr. Ir. Jean-Paul Vincken and Prof. Dr. Ir. Harry Gruppen. The results of his PhD research are presented in this thesis. Currently, Maxime continues working at Laboratory of Food Chemistry as researcher.

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LIST OF PUBLICATIONS

Simons, R.; Vincken, J-P.; **Bohin, M.C.**; Kuijpers, T.F.M.; Verbruggen, M.A.; Gruppen, H. Identification of prenylated pterocarpans and other isoflavonoids in *Rhizopus* spp. elicited soya bean seedlings by electrospray ionisation mass spectrometry. Rapid Commun. Mass Spectrom., **2011**, 25, 55-65

Bohin, M.C.; Vincken, J-P.; van der Hijden, H.T.W.M.; Gruppen, H. Food proteins as carriers for flavonoids. J. Agric. Food Chem., **2012**, 60, 4136-4143

Bohin, M.C.; Roland, W.S.U.; Gruppen, H.; Gouka, R.; van der Hijden, H.T.W.M., Dekker, P.; Smit, G.; Vincken, J-P. Evaluation of the bitter-masking potential of food proteins for EGCG by a cell-based assay and binding studies. – *Submitted for publication to J. Agric. Food. Chem.*

Bohin, M.C.; Vincken, J-P.; Westphal. A.H.; Tripp, A.M.; Dekker, P.; van der Hijden, H.T.W.M.; Gruppen, H. Interactions of dimeric flavan-3-ols, with varying flexibility, and different caseins is determined by more than proline content and number of proline repeats. – *Submitted for publication to Food Chem.*

Bohin, M.C.; Verloop, A.J.W.; Gruppen, H.; van Erven, G.; van der Hijden, H.T.W.M., Dekker, P.; Vincken, J-P. Quantifying the preferential binding of individual phenolics in complex mixtures to β-casein using UHPLC-MS-MS – *to be submitted*

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Courses

- Isothermal Titration Calorimetry training course (GE Healthcare), Amersham (UK), 2009
- Advanced food analysis† (VLAG), Wageningen (The Netherlands), 2010
- Food enzymology (VLAG), Wageningen (The Netherlands), 2011

Conferences and meetings

- 3rd international symposium on delivery of functionality in complex food systems, Wageningen (The Netherlands), 2009
- International conference on polyphenols and health, Harrogate (UK), 2009
- 4th international symposium on delivery of functionality in complex food systems†, Guelph (Canada), 2011
- 2nd conference on food oral processing†, Beaunes (France), 2012
- 26th international conference on polyphenols†, Florence (Italy), 2012

General courses

- VLAG PhD introduction week, 2010
- PhD competence assessment, WGS, 2010
- Philosophy and ethics of food sciences and technology, WGS, 2010
- Techniques for writing and presenting scientific papers, WGS, 2011
- Career perspectives, WGS, 2012

Optionals

- Preparation PhD research proposal
- Food Chemistry study trip to Ghent, Belgium, WU (FCH), 2009
- PhD trip FCH to Switzerland/Italy‡, WU (FCH), 2010
- PhD trip FCH to Singapore/Malaysia‡, WU (FCH), 2012
- BSc/MSc thesis students presentations and colloquia, WU (FCH), 2009/2013
- PhD presentations, WU (FCH), 2009/2013

† Poster presentation; ‡ Poster and oral presentations

Abbreviations used:

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Science

WGS: Wageningen Graduate Schools

WU: Wageningen University

FCH: Laboratory of Food Chemistry

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