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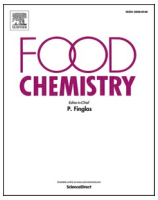

Unraveling the effects of low protein-phenol binding affinity on the structural properties of beta-lactoglobulin

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ABSTRACT

Non-covalent interactions of phenolics with proteins cannot always be readily identified, often leading to contradictory results described in the literature. This results in uncertainties as to what extent phenolics can be added to protein solutions (for example for bioactivity studies) without affecting the protein structure. Here, we clarify which tea phenolics (epigallocatechin gallate (EGCG), epicatechin and gallic acid) interact with the whey protein β-lactoglobulin by combining various state-of-the-art-methods.

STD-NMR revealed that all rings of EGCG can interact with native β-lactoglobulin, indicating multidentate binding, as confirmed by the small angle X-ray scattering experiments. For epicatechin, unspecific interactions were found only at higher protein:epicatechin molar ratios and only with ^{1}H NMR shift perturbation and FTIR. For gallic acid, none of the methods found evidence for an interaction with β-lactoglobulin. Thus, gallic acid and epicatechin can be added to native BLG, for example as antioxidants without causing modification within wide concentration ranges.

1. Introduction

Small hydrophobic compounds (vitamins, fatty acids, as well as phenolics) were found to bind to proteins. The resulting complexes and conjugates formed can be used to fortify beverages (Vieira & Souza, 2022). Further, because of the resulting change of the protein conformation, the processing properties such as solubility, emulsification, gelation, and allergenicity can be changed compared to native proteins (Keppler et al., 2020; Zhang, Cheng, et al., 2021). Therefore, protein–-phenolic complexes and conjugates have received increased attention in the last years. A good understanding of complex formation is crucial to anticipate the resulting effects on protein functionality and bioactivity. However, this also includes information on the absences of non-covalent interactions between phenolics and β-lactoglobulin (BLG): in the case of non-covalently binding ligands, there is an equilibrium between the bound and free states, leading to dynamic and heterogeneous mixtures of associated complexes and free proteins and phenolics. This makes it difficult to extrapolate observed effects in bioactivity tests to structural changes. Thus, compounds that do not bind to proteins or that possess a very low, unspecific binding capacity are often preferred over strongly binding phenolics for reference applications. Furthermore, the absence of interactions makes these phenolics suitable as antioxidants without causing additional non-covalent binding effects such as changing the protein structure and functionality.

Abbreviations: BLG, β-lactoglobulin; CU, Centrifugal ultrafiltration; EC, Epicatechin; EGCG, Epigallocatechin gallate; FTIR, Fourier-transform infrared spectroscopy; FQ, Fluorescence quenching; GA, Gallic acid; SAXS, Small-angle X-ray scattering; STD NMR, Saturation transfer difference nuclear magnetic resonance spectroscopy.

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BLG is a globular whey protein in bovine milk. It is often used as model protein for food products because of its high availability in pure form, its well-known structure and its well-characterized functional properties, such as foaming and emulsification capacity (Keppler et al., 2021; Schestkowa et al., 2019).

The effect of phenolics on the structure of BLG depends on both the phytochemical molecule (He et al., 2020) and its type of binding with the protein (covalent or non-covalent) (Zhang, Cheng, et al., 2021). Especially, the binding of BLG to tea and cocoa phenolics, such as epigallocatechin-gallate (EGCG), epicatechin (EC), and gallic acid (GA) has attracted interest in the literature (Al-Shabib et al., 2020; Cao & Xiong, 2017b; Li et al., 2020; Qie et al., 2020).

Non-covalent interactions of phenolics with proteins (or the absence of significant interactions) are difficult to identify and quantify due to their dynamic nature and inherent weakness. This has led to contradictory results in the literature so far. For example, while the non-covalent interactions of EGCG have been quite reliably established (Keppler et al., 2015; Shpigelman et al., 2012), reports on the respective interactions of EC as well as GA to native BLG are inconsistent. Some authors have observed low-affinity binding of EC by fluorescence quenching and FTIR (Kanakis et al., 2011; Nucara et al., 2013), whereas other authors have found no interaction by the former method (Riihimäki et al., 2008). The same research group, as well as Li et al. (2020) also found no interaction of GA with BLG. In contrast, Chanphai and Tajmir-Riahi (2021) reported that BLG can interact with GA via non-covalent interactions.

We anticipate different interaction affinities of the three phenolics to BLG because multi-allocated galloyl residues at catechines (i.e. EGCG) were suggested to exhibit a higher interaction capacity to proteins than non-allocated galloyl residues (i.e. EC) as well as mono-allocated galloyl residues (i.e. GA) (Cao & Xiong, 2017b). EGCG serves as a positive control for non-covalent interactions as binding has been confirmed by several studies (Keppler et al., 2015; Pu et al., 2021; Qie et al., 2020; Zhang, Li et al., 2021). Based on this, we hypothesize that EGCG has one or multiple binding moieties itself, and several binding sites on the protein, which we aim to determine experimentally among others with STD-NMR, while EC and GA would react very weak or unspecific with native BLG under the tested conditions.

As several studies have reported complex formation between phenols and BLG, the results under the different conditions investigated appear to be partly contradictory and difficult to reproduce in our hands. To clarify the presence or absence of significant non-covalent interactions between BLG and EGCG, EC and GA we elucidated different interaction types and their contributions by various spectroscopic methods and separation methods. Examining both the protein and the phenolic provides a more comprehensive picture. The combination of methods in this study has not been applied for BLG-phenolic analysis yet. Thus, this study also provides a comparative overview of different binding assays and methods using EGCG as a non-covalent binding reference. Analytical methods that allow observing changes for phenolics during the interaction with proteins are ^{1}H NMR shift perturbation and saturation transfer difference (STD) as well as centrifugal ultrafiltration (CU). To elucidate change at the protein, we used fluorescence quenching (FQ) to assess the number of binding sites on the protein level and Fourier-transform infrared spectroscopy (FTIR) for observing changes in the protein structure. In addition, small-angle X-ray scattering (SAXS) investigates protein aggregation behavior and folding (globular or unfolded) during interactions, which helps to identify multidentate binding effects. Different concentration ranges are required for the individual methods, which offers the advantage that concentration effects can be delineated.

2. Materials and methods

2.1. Chemicals

BLG AB (L0130), (-) epigallocatechin gallate (EGCG, E4268), (-)-epicatechin (EC, E1753), gallic acid (GA, 27645) were of analytical purity > 90 %. These and all other chemicals were purchased from Sigma-Aldrich, Chemie GmbH, Germany.

2.2. Sample preparation

BLG, in 50 mM phosphate buffer pH 7 (0.29 % sodium phosphate monohydrate 0.77 % disodiumphosphate heptahydrate), was either mixed with a phenolic stock solution in ethanol (EGCG, EC or GA) or with pure ethanol and stirred for 1 hr (except the titration experiments). The final ethanol concentration was 1.5 % (v/v), to prevent protein denaturation by the solvent. The molar ratio of BLG:phenolic varies depending on the different methods as listed in Table 1.

2.3. Investigation of the interaction by analysis of phenolic properties

2.3.1. ^{1}H-nuclear magnetic resonance (NMR) analyses

2.3.1.1. Shift perturbation. 1D-^{1}H NMR spectra, with excitation sculpting for water resonance suppression (*zgesgp* pulse program) were recorded on a Bruker 600 MHz NMR controlled by Topspin 3.1 spectrometer operating software (Bruker), using a spectral width of 16 ppm, 1.5 s acquisition time and a repetition delay of 1.5 s. The phenolics were diluted with an aqueous solution containing 10 wt% D_2O and sodium 3-(trimethylsilyl) propionate (TSP) to reach a final concentration of 70 μM. Each phenolic solution was initially measured without added protein. Afterwards, small volumes (2.5 μL) of highly concentrated BLG stock solution (4 mM) were pipetted to 500 μL the phenolic solution (each adding 20 μM protein to the final solution) until a protein concentration of 140 μM was reached. After each titration step the NMR vial was thoroughly shaken and measured at 37 °C.

2.3.1.2. Saturation transfer difference (STD). STD experiments were conducted each with a solution containing 300 μM phenolic and 15 μM BLG. BLG:phenolic molar ratio of 1:20 provided optimum differences in STD-experiments. Spectral widths of 14 ppm were recorded at 25 °C, using an acquisition time of 2 s. The repetition delay of 4 *sec* was split into a relaxation period of 2 *sec* and a saturation delay of 2 *sec*, respectively, followed by saturation delay of 2 *sec*. Irradiation was conducted at 510 Hz/ 0.85 ppm with a series of 50 ms Gaussian pulse to achieve protein saturation. For the reference spectrum without saturation, the irradiation was set to 100 ppm. The data was processed using Topspin 3.1 with zero filling by a factor 2. In the saturated spectra the resonances of the bound phenolics are partially or fully saturated. In the reference spectra neither bound nor free phenolics are saturated. Thus, subtraction of the unsaturated experiment from the saturated one yields a spectrum, in which the remaining line intensity is a measure of protein–phenolic binding properties. Due to the low interactions of EC and GA with BLG, which would not result in sufficient signal resolution, we

Table 1
Overview of the concentrations and molar ratios used in the respective methods.

Method	Phenolic concentration [μM]	BLG concentration [μM]	Molar ratio (phenolic:BLG)
1D-^{1}H NMR	70	20–140	3.5:1–1:2
STD-NMR	300	15	20:1
CU	200; 400; 600	200	1:1; 2:1; 3:1
FQ	0.2–45	15	1:75–3:1
FTIR	2700	270	10:1
SAXS	2700	270	10:1

only investigated the binding sites for EGCG (Supplementary 1).

2.3.2. Centrifugal ultrafiltration (CU)

The complexes between BLG and phenolics were also analyzed by centrifugal ultrafiltration with Vivaspin 2 centrifugal concentrators (Hydrosart® membrane, 10 kDa nMWCO, VS02H02 Sartorius, Stedim Biotech). Before centrifugation, the UF-vials were cleaned with distilled water and passivated by incubating them with the respective BLG-phenolic solution for 5 min to reduce sample losses due to adsorption to the plastic surface and membrane. After that, the passivation solutions were discarded and 2 mL of each of the BLG-phenolic solutions (200 μM protein and 200, 400 or 600 μM phenolic, incubated for 10 min) were filled into the passivated UF-vials and centrifuged at 4000 × *g* in a swinging-bucket rotor at 20 °C for only 1 min. This short centrifugation time should prevent the equilibrium between unbound and bound phenolics from shifting in favor of the unbound ones. The unbound phenolics in the ultrafiltrate were sampled for HPLC analysis.

The HPLC analysis of the unbound phenolics in the filtrate was carried out using an Agilent 1100 system with a diode array detector at a wavelength of 280 nm and a Nucleodur Sphinx RP-C18 column (125 × 4 mm; 5 μM; Macherey-Nagel, Düren, Germany) equipped with a guard column. The mobile phase was isocratic and composed of water, acetonitrile, methanol, ethyl acetate, glacial acetic acid (89:6:1:3:1 v/v/v/v/v) (Saito et al., 2006). The mobile phase flow rate started at 0.7 mL/min was maintained for 15 min and then linearly increased to 1.2 mL/min in 1 min. This flow rate was maintained for 8 min, and then decreased to 0.7 mL/min in 1 min and held for 4 min. All chromatographic analyses were performed at 25 °C, controlled by a column oven.

To prevent an overestimation of the bound phenolic fraction, we calculated the membrane loss at the centrifugal ultrafiltration membrane. Phenolic concentrations without BLG were measured after ultrafiltration and compared to the initial phenolic concentrations in buffer without ultrafiltration. The filtered phenolic solution was used as a 100 % reference. The relative difference between the filtered phenolic solution and the filtered phenolic solution in the presence of BLG semi-quantitatively gave the amount of protein-bound phenolics in “percent bound”.

2.4. Investigation of the interaction by analysis of protein properties

2.4.1. Fluorescence quenching (FQ)

The measurements were conducted as described previously (Keppler et al., 2014; Keppler et al., 2015). In brief, 15 μM native BLG solutions in phosphate buffer pH 7 were used for fluorescence measurements with a Cary Eclipse Fluorescence spectrophotometer (Varian Australia Pty Ltd), utilizing a 10 mm quartz cell with 4 polished sides. A phenolic stock solution was prepared so that each titration step contained 0.2 μM in 2 μL to 15 μM BLG solution. All measurements were performed at 20 °C using a 5 nm excitation and emission bandwidth in the single measurement mode at 294 nm excitation and 340 nm emission. This assured that only tryptophan residues are excited whereas possible tyrosine fluorescence is negligible. For a spectrogram, the emission wavelength was in the range of 300–450 nm. *N*-Acetyl-L-tryptophan amide (NATA) was used to assess the inner filter effect of the phenolics. These effects occur if the phenolics absorb the excitation and/or emission energy used for the intrinsic protein fluorescence analysis (Keppler et al., 2015).

The data obtained with fluorescence quenching was analyzed as described by Cogan and co-workers (1976). A plot of $P^*\alpha$ versus $L^*\alpha/(1-\alpha)$ gives a straight line with $\alpha = \frac{F_{max}-F}{F_{max}-F_0}$ = fraction of free binding sites, P is the BLG concentration, L is the phenolic concentration at a particular fluorescence, F_{max} is the fluorescence intensity upon saturation, F is the fluorescence and F_0 is the initial fluorescence of BLG only.

The reciprocal value of the slope gives the number of binding sites n, whereas $K'd$ is the intercept with the abscissa. All measured values were corrected beforehand for self-fluorescence (phenolic solution titration of the buffer solution without protein) and for inner filter effects (phenolic solution titration of NATA) as described previously (Keppler et al., 2014; Keppler et al., 2015).

2.4.2. Fourier-transform infrared spectroscopy (FTIR)

FTIR measurements were performed with a Tensor 27 spectrometer (Bruker Optics, Ettlingen, Germany) in the mid-infrared, i.e. in the wave number range 4000–900 cm^{-1}. For detection, a LN-MCT Photovoltaic detector (Bruker Optics) cooled with liquid nitrogen was used. The specific software OPUS Version 7.5 (Bruker Optics) was applied for recording the spectra as well as for monitoring and control of the spectrometer and Haake DC 30 (K20) thermostat (Thermo Haake, Karlsruhe, Germany). FTIR was performed using an AquaSpec measuring cell (transmission modus) at 40 °C. Per analysis, 120 scans were made. All samples were corrected by measurement of the respective phenolic in ethanol dissolved in PBS pH 7 as reference. Based on the obtained spectra, Fourier self-deconvolution (FSD) was performed with a resolution enhancement factor of 3.0 cm^{-1} and a bandwidth (full width at half height) of 10 cm^{-1} using OPUS Version 7.5 over the wavenumber range 1770–1450 cm^{-1}. The resulting bands in the amide I range (1700–1590 cm^{-1}) were integrated for the calculation and quantification of the structural components.

2.4.3. Small angle-X-ray scattering (SAXS)

SAXS experiments were performed at the BioSAXS Beamline P12 at PETRA III (EMBL/DESY, Hamburg, Germany). The scattering patterns were measured using Pilatus 2 M pixel detector. The X-ray beam energy was 10 keV, the beam size 0.1 mm (V) × 0.2 mm (H), the sample to detector distance was 3.1 m and the q-range was 0.03 to 4 nm^{-1}. Before and after each SAXS measurement, a signal from 50 mM phosphate buffer pH 7 with 1.5 % EtOH or with 1.5 % EtOH/ phenolic solution was measured and used for background subtraction. All samples were moved slightly during the exposure in order to reduce the risk of radiation damage. The temperature was 20 °C. For each measurement, 20 diffraction patterns were recorded for the same sample volume, using an exposure time of 0.045 s per frame. The background corrected SAXS data were used to calculate one-dimensional scattering curves by angular averaging. The data were corrected for the transmitted beam. Radiation can cause artefacts, which falsify the measurements. To verify that no artefacts had occurred, all scattering curves for a recorded dataset were compared to a reference curve (typically the first exposure). Then, the curves were integrated using an automated acquisition and analysis program (GNOM) (Franke et al., 2012). The range of the reciprocal space vector q was calibrated using diffraction patterns of silver behenate (Blanton et al., 2000).

2.5. Statistics

All measurements were performed at least in independent triplicates. The data were compiled and analyzed with GraphPad PRISM (version 6.07, GraphPad Software, San Diego, USA) unless otherwise stated. The same software was used for the Shapiro-Wilk test ($\alpha = 0.05$) and ANOVA. All ANOVAS were calculated using Tukey multiple comparison test and $\alpha < 0.05$.

3. Results

First, effects of the interactions were examined for the phenolics (^{1}H NMR and CU). The protein-bound fraction of phenolics was quantified by the difference between unbound portion and the total amount. We chose molar ratios of BLG:phenolic with a maximum of 1:3.5 to avoid large amounts of unbound phenolics overlaying the signal of bound phenolics in ^{1}H NMR chemical shift titration and CU. In addition, we determined the involvement of the EGCG rings in the interaction with BLG at an excess of EGCG (20 fold). Secondly, BLG was analysed for its interaction with phenolic by FQ (binding constants) and FTIR

(secondary structure changes). Finally, protein aggregation and protein unfolding were evaluated using SAXS. To get clear signals in protein changes without having a large surplus of unmodified protein, we used a molar ratio of BLG:phenolic of 1:10 for these experiments.

3.1. Investigation of the interaction by analysis of phenolic properties

3.1.1. ^{1}H- nuclear magnetic resonance (NMR) analyses

The interactions of EGCG, EC and GA to BLG were analyzed by ^{1}H NMR to detect interactions in a broad affinity range (nM to mM) (Aguirre et al., 2015). For shift perturbation experiments, the chemical shift of the unbound phenolic is followed while protein is being added. As the putative interactions are overall weak, it can be anticipated that a rapid equilibrium exchange occurs between the free- and protein-bound phenolic, i.e. binding kinetics are fast on the NMR-time scale. Thus, complex formation with a protein results in a single population-averaged resonance signal set of the phenolic compounds, whose chemical shifts move between the two extremes of free and 100% bound compound. Thus, the concentration dependent changes in chemical shift can be fitted to binding curves, provided that significantly high population of bound phenolic can be achieved. If on the other hand the equilibrium binding occurs slowly relative to the NMR-shift time scale, then separate signals are observed for the free and bound phenolic. In this case, the resonance intensities of the respective population or state reflect the respective, equilibrium- or affinity determining concentration.

^{1}H resonances of the phenolics were measured before and after the addition of the protein to the phenolic solutions (Fig. 1a). The phenolics were kept in excess through the addition of only small aliquots of protein (Fig. 1b).

For EGCG and EC, the ^{1}H resonances of ring A (between 6.2 ppm and 6.1 ppm) were chosen for the binding analysis since these signals do not overlap with protein signals (Fig. 1b) (Keppler et al., 2014). However, with increasing addition of BLG, protein resonances increase and overlap with the phenolic signal. Together with linewidth broadening, this makes line shape fitting for affinity calculations more difficult in the presence of proteins (Aguirre et al., 2015), which is why no further calculations were conducted. The addition of 20 μM BLG to 70 μM EGCG (1:3.5) already resulted in chemical shift changes of the ring A resonances and a concurrent line width increase. These effects were more pronounced with further addition of BLG up to a molar ratio of 1:0.5 (protein:phenolic) at a BLG concentration of 140 μM BLG) with a final shift of 0.01 ppm when all BLG was added.

Similarly to EGCG, resonance shifts of EC were observed after addition of BLG (Fig. 1b). However, these shifts were less pronounced. Only after a protein addition of >60 μM (1: 1.2) a minor linewidth broadening and a shift variation of < 0.01 ppm were observed.

No changes in resonance properties were observed in the GA spectrum (Fig. 1b). Despite a slight shift of the baseline probably caused by protein signals, we suggest that no interaction of GA with BLG is present.

Thus, the ^{1}H NMR shift perturbation showed the most interactions for EGCG, followed by EC, but none for GA.

In addition to determining the binding capacity to BLG, we also aimed to differentiate between the extent of the binding of the three rings in the EGCG moiety. STD-NMR is used to analyze the binding ability and binding sites of reactants by comparing different H atomic absorption spectra. In STD experiments, the protein is selectively saturated by irradiating a specific spectral region for the protein, and any

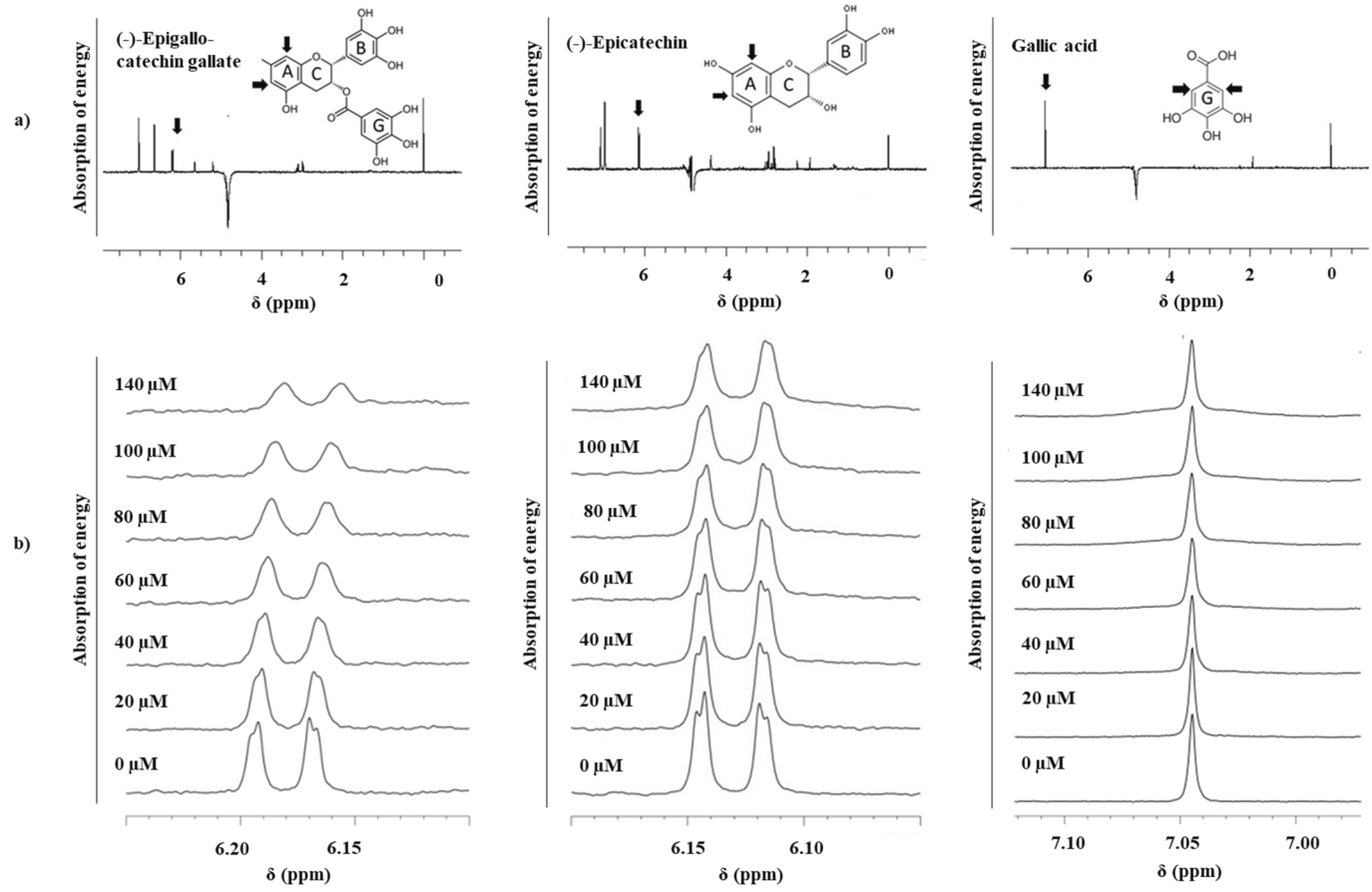

Fig. 1. (a) 1D-^{1}H NMR spectra of 70 μM EGCG, EC and GA in deuterated phosphate buffer pH 7 with TSP as reference. Arrows indicate resonances that were followed in detail in the following titrations. (b) ^{1}H NMR spectrum of relevant phenolic resonances (6 to 7 ppm) of 70 μM EGCG, EC and GA titrated with increasing concentrations of BLG (20 to 140 μM). The protein concentration [μM] is indicated on the left side of each spectrum.

ligand moiety in close contact to a binding site is saturated via spin diffusion as well. When subtracting the saturated and the non-saturated spectra, the intensity of remaining ligand signals depend on the transferred saturation in the bound form and is thus a (semi-)quantitative reflection of the protein–ligand interaction. (Viegas et al., 2011).

Saturation ratios differed between 13 % and 18 % (Table 2). These differences are comparatively small and indicate that no strong preference for a binding site exists. The resonances of polyphenol ring A protons H6, H8 (at approx. 6.2 ppm) showed the most intense reaction to BLG in relation to the EGCG spectrum (Supplementary 1) with 17.6 % saturation (Table 2). Consequently, EGCG ring A is in closest proximity to the protein, possibly through interactions via hydrogen bonds of the hydroxyl groups. The H"2, H"6 proton resonances (7.01 ppm) of the galloyl residue (ring G) was also very responsive with 15.9 %, followed by the ring B hydrogens (6.6 ppm). Saturation of the EGCG ring C was more difficult to determine, since the H4 resonances were superimposed by residual protein signal. The H3 saturation was only 12.9 %, which was the lowest saturation detected together with ring B resonances (13.0 %). Nevertheless, the H2 saturation at ring C was more intense, with 15.7 %.

To conclude, the relatively small percentage range of saturation observed in the STD-experiment suggests that there is no specific ring for EGCG involved in the interaction at the protein:phenolic molar ratio used for the experiment. All aromatic rings of EGCG were involved in the protein interaction in the present study, with only a slight preference for ring A, over G and B.

3.1.2. Centrifugal ultrafiltration (CU)

Unbound phenolics were separated from the protein by size difference via CU. The method only gives a rough indication of complex formation, because the passivation of the filtration vial with phenolic compounds to account for losses caused by adsorption to the membrane and the surface of the vial, as well as dynamic dissociation of the complex during the short centrifugation time, will affect the outcome. CU is especially useful to get an impression on different affinities in ligand mixtures. The molar proportion of unbound phenolics was assessed via HPLC in the filtrate using three different molar ratios of protein and phenolic in the range used in the NMR experiment (1:1, 1:2 and 1:3) (Fig. 2a–c). At higher ratios the results were no longer significant as difference between the phenolic solution and unbound phenolics became comparatively smaller with respect to the increasing concentration of phenolics in both solutions.

EGCG showed interaction with the filter membrane, thus the area under curve was not only decreased in the filtrate of protein-containing solutions but also in the filtrate containing only EGCG (Fig. 2a). Nevertheless, we found that approximately 33% EGCG was retained by the protein at a protein:phenolic molar ratio of 1:1 (200 μM each). The percentage of bound EGCG decreased with increasing concentration (1:2 = 30 % and 1:3 = 23 % bound EGCG). Thus, the concentration of bound EGCG increased roughly from 65.4 μM to 138 μM for BLG with 1:1 to 1:3 ratio.

Table 2
Percentage saturation of 300 μM EGCG to 15 μM BLG. Nd, not detectable; + low, ++ medium, +++ high binding contribution.

Resonance signal [ppm]	Resonance assignment	Proton location	Percentage saturation [%]	Interaction preference
7.01	Ring G	H2́H6́	15.9	++
6.59	Ring B	H2́H6́	13.0	+
6.19	Ring A	H6 H8	17.6	+++
6.17	Ring A	H6 H8	15.0	++
5.63	Ring C	H3	12.9	+
5.18	Ring C	H2	15.7	++
3.10	Ring C	H4	nd	Nd
2.69	Ring C	H4	nd	Nd

In contrast, no interaction effect was observed for EC and for GA in CU for all ratios (Fig. 2b and c). This is evident by the unchanged concentration after filtration of the phenolic solutions with protein compared to the filtered phenolic solution without protein. There seems to be a minor increase in the area under curve for the filtered BLG-GA complex as compared to the unfiltered phenolics, which can be traced back to the mentioned inaccuracies of the CU method.

3.2. Investigation of the interaction by analysis of protein properties

3.2.1. Binding analysis with fluorescence quenching (FQ)

Binding of ligands changes the local ambient polarity of the tryptophan in the protein, resulting in a decrease in intensity. The relative fluorescence quenching of BLG, as well of the reference amino acid Trp alone (NATA), was measured with increasing concentrations of EGCG, EC or GA until saturation was reached (i.e., a molar ratio for protein: phenolic of 1:3) (Fig. 2A–C).

EGCG exhibited strong filter effects, which is evident by the NATA fluorescence decrease with increasing phenolic addition (Fig. 2A). In the case of a decrease of the fluorescence of NATA, the quenching is not due to an interaction, but to the fact that the phenolic quenches or absorbs at the same emission and/or excitation wavelength of NATA, the so-called filter effect (Keppler et al., 2014; Keppler et al., 2015; Kimball et al., 2020). After correction of the filter effect, only the quenching curve of EGCG remains to show significant quenching effects. When considering the addition of 20 μM EGCG (ratio 1:1.3) as the maximum possible quenching (so-called plateau when all protein binding sites are covered) (Fig. 2A), n = 1.2 interaction sites and a dissociation constant K'd of 0.3 μM can be calculated based on the Cogan plot (Supplementary 2). One interaction site of EGCG with BLG AB at pH 7 in phosphate buffer is consistent with literature (Kanakis et al., 2011; Keppler et al., 2014; Keppler et al., 2015; Zorilla et al., 2011).

A minor interaction effect was visible for EC after correction for self-fluorescence, but the effect was too small to allow accurate calculations.

Similarly to EGCG, GA exhibited strong filter effects. No plausible binding constant could be calculated after correction for the inner filter effect because GA showed also self-fluorescence.

3.2.2. Secondary structure with Fourier-Transform infrared spectroscopy (FTIR)

FTIR analyses were conducted to understand the effect of phenolics on the protein conformation. For a clear signal on the protein, a molar BLG:phenolic ratio of 1:10 was chosen.

For the native protein 41 % intramolecular (1675 and 1630 cm^{-1}) β-sheet content, 20.6 % α-helix (1657–1659 cm^{-1}) and 24 % turns and unordered conformations (1689/1690/1669, and 1647 cm^{-1}) were detected (Table 3). The addition of EGCG and EC altered the protein conformation significantly. EGCG increased the turn portion (1669 and 1689 cm^{-1}) from 13% to 29%. Furthermore, additional intermolecular β-sheets at 1618 cm^{-1} were evident after EGCG addition. EC increased the α-helical conformation to 32 % (1658 cm^{-1}). Both structural changes resulted at the expense of disordered structures (1647 cm^{-1}), whereas the β-sheet concentrations (1676 cm^{-1} and 1630 cm^{-1}) were not influenced.

In contrast, the addition of GA to BLG had only a minor effect on the protein conformation. Primarily a shift in the maximum wavelength for β-sheets from 1675 cm^{-1} to 1680 cm^{-1} was evident. The FTIR measurements revealed all three phenolics had an effect on the secondary structure of BLG, but to different extents.

3.2.3. Protein aggregation by Small-angle X-ray scattering (SAXS)

SAXS not only yields very sensitive results on protein aggregation before and after addition of substances, it also provides information on the protein folding state (i.e. compact or unfolded). For these experiments we chose again, a molar BLG:phenolic ratio of 1:10 to induce a clear interaction effect. The measurements reveal a molecular weight of

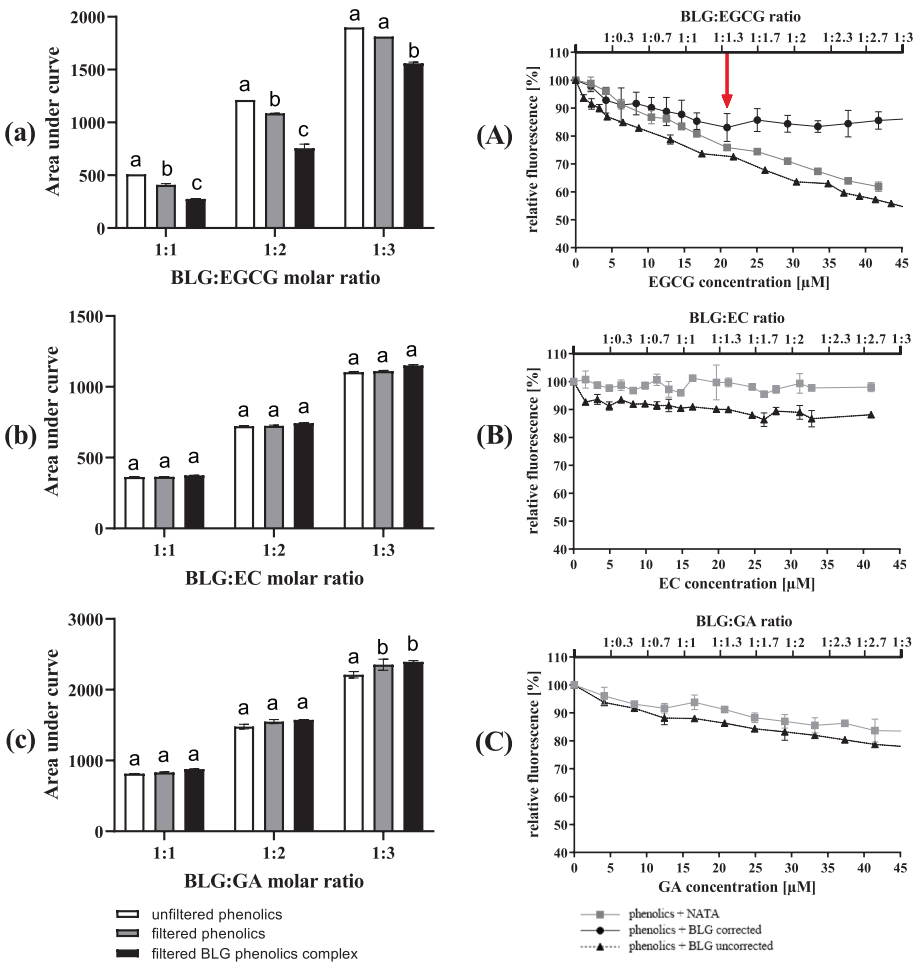

Fig. 2. Ultrafiltration experiments (a-c), showing content (AUC) of free phenolics measured by HPLC in filtered phenolic solutions without BLG (filtered phenolic) and with BLG (filtered BLG phenolic complex) and in the phenolic control solution without previous filtration (unfiltered phenolic). BLG was used at concentration of 200 μM and the phenolic concentrations were 200 μM (1:1), 400 μM (1:2) and 600 μM (1:3). Means (n = 3) with different letters (a-c) within a protein:phenolic molar ratio differ significantly ($p < 0.05$). Relative fluorescence quenching curves (A-C) of 15 μM BLG with increasing concentrations of (a) EGCG, (b) EC and (c) GA. All curves were corrected for self-fluorescence. The inner filter effect was determined analyzing the fluorescence of *N*-acetyl-tryptophan amide (NATA) as a function of phenolic addition. The arrow in 3a indicates the onset of the quenching plateau after correction. All measurements were done in individual triplicates.

~ 35 kDa (Table 4), which corresponds to twice the molecular weight of the monomeric protein (i.e., 18.4 kDa). These results confirm that BLG in phosphate buffer at pH 7 existed in a dimeric form with no further aggregation. This is evident in the low intensity at the low scattering angle (<0.05 nm^{-1}) region (Supplementary 3). The apparent radius of gyration (Rg) was 2.2 nm and the shape of the Kratky plot revealed a compact folding with an estimated maximum intramolecular distance (R) of 6.5 nm. All these findings were reported previously (Chen et al., 2020; Keppler et al., 2015; Panick et al., 1999).

The addition of EGCG to BLG resulted in significant protein aggregation. A high molecular weight of 290 kDa, the Rg of 5.96 nm and the Dmax (the maximal distance within the aggregate) of 26.3 nm were obtained. Additionally, the scattering intensity of the BLG-EGCG complexes increased ten-fold compared to the native BLG. The high Dmax of the p(r)-function of the BLG-EGCG complex suggests somewhat elongated aggregates. The presence of these aggregates confirms the interaction between EGCG and BLG. Protein aggregation occurring after addition of EGCG was also reported previously (Keppler et al., 2015; Shpigelman et al., 2012). The aggregation was found to increase with an increase of the BLG:phenolic ratio in favour of EGCG, until insoluble cross-linked protein complexes were formed (Charlton et al., 2002).

In contrast, the addition of EC or GA to BLG did not significantly affect the shape of the SAXS curves. This was also evident for the molecular weight of the protein, which only marginally increased from 35 kDa for the native BLG to 38 kDa and 37 kDa for the EC and GA addition, respectively. The Rg was not significantly affected after phenolic addition and ranged from 2.2 nm for native BLG to 2.2 nm and 2.3 nm for EC or GA addition. The intramolecular distance (Dmax) was 6.2 nm and 7.9 nm, respectively for EC and GA. These results illustrate that the addition of both phenolic compounds had no significant influence on the BLG dimer form.

4. Discussion

We clarified which of the selected phenolics react with native BLG (non-covalently) and which do not in a specified concentration range, by employing and comparing the results of various analytical methods. The overall results confirmed our hypothesis that while EGCG and EC have different affinities for BLG in the order EGCG ≫>≫ EC, GA does not interact with the protein under the selected conditions. EC can be considered as unspecific binding, and not detectable when below a ≤ 1:3 ratio with the protein. However, both GA and EC cause some structural changes in the protein (FTIR), which are discussed in the following sections.

4.1. Epigallocatechin gallate

We could confirm in our study with all methods that EGCG binds non-covalently with BLG in phosphate buffer at pH 7 (Keppler et al., 2015; Pu et al., 2021; Qie et al., 2020; Zhang, Li et al., 2021). In addition, our experiments further specify the mode of binding of EGCG to BLG in solution: STD-NMR gives experimental evidence that all rings of EGCG are involved in protein interactions at a molar ratio of 1:20 for BLG:EGCG (Table 2). The reason likely is that EGCG has two interactions sites at least, to induce protein aggregation as shown by SAXS already at a 1:10 ratio (Table 4). In addition to the SAXS measurements, protein aggregation at the 1:10 ratio is also visible in the shifted FTIR band from intramolecular to intermolecular β-sheets at 1618 cm^{-1} (Table 3). The

Table 3
Portion of secondary structure components of pure BLG and after addition of EGCG, EC and GA. Values are listed as mean of 3 ± standard deviation.

Max. [cm^{-1}]	Secondary structure component	BLG native, not loaded/ Ref. a [%]	BLG native, loaded with EGCG/ Ref. b [%]	BLG native, loaded with EC/ Ref. c [%]	BLG native, loaded with GA/ Ref. d [%]
1690	turns	13.1 ± 0.1		12.6 ± 0.3	13.1 ± 0.2
1689	turns		17.2 ± 1.2		
1680	β-sheet				11.5 ± 0.5
1676	β-sheet			8.7 ± 0.2	
1675	β-sheet	10.5 ± 0.1			
1669	turns		11.3 ± 1.0		
1659	α-helix	20.6 ± 0.1			
1658	α-helix			32.4 ± 0.3	23.8 ± 1.5
1657	α-helix		22.8 ± 1.0		
1647	disordered	11.0 ± 0.3			11.5 ± 0.4
1635	Intra β-sheet		32.8 ± 0.1		
1630	Intra β-sheet	30.3 ± 0.7		32.3 ± 0.0	30.3 ± 0.1
1618	Inter β-sheet		8.6 ± 0.1		
1612		8.0 ± 0.2			
1611				8.1 ± 0.1	9.8 ± 0.6
1602			7.3 ± 0.6		
1597		6.6 ± 0.1		5.9 ± 0.0	
1596					

Table 4
Molecular weight, apparent radius of gyration, maximum intramolecular distance and scattering intensity and RG/RH ratio of 0.27 mM BLG with or without addition of 2.7 mM phenolics (EGCG, EC and GA). Values are listed as mean of 3 ± standard deviation.

Phenolics	Molecular weight [kDa]	Apparent radius of gyration (appRg) [nm]	Apparent number of monomers	Max. intramolecular distance (Dmax) [nm]
–	35	2.20 ± 0.10	1–2	6.5
EGCG	290	5.96 ± 0.04	12–16	26.3
EC	38	2.22 ± 0.28	1–2	6.2
GA	37	2.25 ± 0.02	1–2	7.9

β-sheet reduction for EGCG can be caused by a hydrogen bond of EGCG with the protein backbone, thus disrupting the β-sheet folding. However, Dai and co-workers (2019) reported for rice-glutelin that hydrophobic interactions are the main driving forces for the interaction with flavonoids. The aggregation caused by EGCG is dependent on the phenolic structure and probably mediated by multidentate binding of the different galloyl groups of EGCG to several BLGs via hydrogen bonds (Dönmez et al., 2020). Such aggregation reactions correlate in general positively with the molecular weight of the phenolic (Charlton et al., 2002). Usually, studies of the binding moiety of phenolics to proteins are conducted via simulations and rarely by experimental approaches such as in the presented study. Therefore, our experimental results can only be compared with simulations: For BLG monomers, molecular docking simulations of EGCG by different research groups showed different results both in terms of the number of bonds and the rings involved (Huang et al., 2021; Pu et al., 2021; Wu et al., 2013; Zhang, Li et al., 2021). An explanation for the discrepancies between these studies could be the usage of different simulation softwares (GROMOACS, ICM, AutoDock Vina, Discovery studio) that take different parameters into account. However, recently, Zhang and co-workers (2021) considered molecular docking with BLG dimers instead of monomers (Supplementary 4), which is the preferable quaternary structure of BLG at pH 7 (Gołębiowski et al., 2020). The calculations with AutoDock Vina revealed a preferred interaction to the interface of two BLG subunits with the EGCG rings A, B and G, however not with ring C (Zhang, Li et al., 2021). Our experimental findings by STD-NMR are actually in line with Zhang and co-workers (2021). This is particularly noteworthy, because many molecular docking studies are evaluating the interaction of a ligand without taking environmental factors (e.g., buffer) or several ligand molecules and protein molecules at the same time into account.

In summary, EGCG interacts non-covalently with BLG in the phosphate buffer pH 7 environment, leading to cross-linking effects that prevent the interaction from being confined to individual phenolic rings.

4.2. Epicatechin

In contrast to EGCG, for EC we confirm only weak interactions with BLG in phosphate buffer pH 7. Only a minor ^{1}H NMR shift perturbation was observed starting at a molar ratio of protein to EC of approx. 1:1.2 (Fig. 1) and was not pronounced enough for further analysis with STD-NMR. At high excess of EC (1:10), conformational changes (FTIR) were also only slightly visible (Table 3). Also, the results for FQ in our study likewise indicated that EC did not interact with BLG, because the interaction we observed was too weak to allow a calculation of binding sites or the binding affinity with this method. In addition, we could not detect significant interactions between BLG and EC (ratios of 1:1, 1:2 and 1:3) using CU (Fig. 2B), but this method is also less sensitive for detecting weak non-covalent interactions as compared to ^{1}H NMR or fluorescence quenching.

This shows that EC interacts only weak and unspecific with BLG at the given conditions. Interestingly, although EC has the aromatic rings A, C and B, these seem not to be sufficient to induce significant interactions or cross-linking with BLG. EC does not possess a G ring compared to EGCG and has in total 4 hydroxyl OH-groups less (3 OH-groups at the ring G and as well as one OH-group less at ring B). The lower number of OH-groups seem to be insufficient to stabilize the interaction between EC and BLG and stresses the importance of the galloyl-residue of ring G to induce cross-links. For EC, probably hydrophobic interactions or aromatic stacking are predominant, because the β-sheets were not disturbed and no aggregation was evident in SAXS (Table 4). Al-Shabib and co-workers (2020) and Kanakis and co-workers (2011) revealed in their docking study that EC binds in the vicinity of Phe-105 which shows the possibility of π-π stacking. In addition, the calculated thermodynamic parameters support the assumption of hydrophobic interactions between EC or catechin and BLG (Al-Shabib et al., 2020). Nucara and co-workers (2013) also found only minor changes in the BLG conformation at lower molar ratios (≤2) which increased by further EC addition (≥5). However, their FTIR measurements showed a conversion of antiparallel to exposed β-sheets at ratios ≤ 2 which decreases at higher ratios at the expense of disordered structures. In contrast to the unspecific EC interaction to BLG in the present study (Fig. 2B), 0.9 binding sites and a weak affinity of 3.2×10^3 M^{-1} were reported using FQ analysis with double logarithmic plot for complexes without residual fluorescence in a similar pH 7 buffer (Kanakis et al., 2011). However, other research groups reported for fluorescence quenching that EC did not interact with native BLG (Riihimäki et al., 2008). It is possible that the controversy in literature about phenolic binding is caused by variances in the nativity of the protein source, because denatured BLG has more unspecific binding sites (Keppler et al. 2014). Another possibility is based on the unspecific and low affinity binding of EC, which was barely evident even in ^{1}H NMR, making detection with less precise methods very difficult.

To conclude, EC can be added to a native BLG solution at pH 7 without showing significant effects on the protein when the phenolics are kept at low concentrations.

4.3. Gallic acid

With the exception of FTIR (Table 3), all our methods showed unequivocally that GA did not interact with BLG (Figs. 1, 2). The addition of GA also had no effect on protein cross-linking as shown by SAXS (Table 4). One reason could be, that GA is in its dissociated form at pH 7 (Badhani & Kakkar, 2017), and there might be repulsion effects with some negatively charged amino acids in BLG at pH 7 because the protein is above its isoelectric point at this pH value.

Since the addition of GA to BLG had a minor effect on the protein conformation (alpha-helical structure in FTIR), we cannot completely rule out that interactions by ionic bond (for example with remaining positively charged amino acid resides) or hydrophobic forces are still possible under the protein:GA ratio tested. However, it should be noted that GA (as well as the other phenolic compounds) has an absorption spectrum in FTIR that overlaps with the wavelength of the proteins (1600–1700 cm^{-1}). Therefore, slight deviations in the subtraction of the GA signal from the protein GA sample could also be the cause of the inconsistent results in FTIR.

Chanphai and co-workers (2021) confirmed with thermodynamic experiments that GA would generally be able to interact via ionic contact with BLG. Whereas another research group reported that the interaction of GA with BLG is predominantly driven by hydrophobic forces (Li et al., 2020). Molecular docking experiments identified hydrophobic interaction forces between GA and the amino acids Val-41, Ile-71 and Ile-56 whereas hydrogen bonds were not present with BLG (Li et al., 2020).

Similarly to EC, controversy in the literature regarding the GA interaction with BLG can be based on the nativity of BLG (Cao & Xiong, 2017a) as well as variances in the solvent environment (e.g. buffer). In our experimental setting, the results show that GA in its non-oxidized form can be added to BLG solutions without provoking (significant) protein modifications.

5. Conclusion

From the results obtained in this study it can be concluded that interactions between phenolics and BLG depend on the type of phenolic and the protein:phenolic molar ratio.

In this study, the phenolics ECGC, EC and GA showed varying interactions with BLG. EGCG gave clear evidence of non-covalent interactions but no specific interaction site with BLG as well as several equally preferred binding moieties, which could be due to multidentate binding. This assumption is supported by a strong aggregation behaviour in the SAXS measurement. EC only led to a weak interaction at higher protein concentrations and we could not determine the binding affinity. It appears that due to the absence of ring G, EC did neither lead to protein aggregation nor bind to a specific site on the protein. No interactions were found for GA with BLG with any of the used methods, with the exception for FTIR. It is a common phenomenon for phenolic compounds that poly-allocated gallic acids have a significantly higher binding affinity and cross-linking capacity to proteins, while the GA moiety alone or in EC are hardly reactive. The higher binding affinity of phenolics with multi-allocated galloyl residues (>3 GA) in general can be explained by the size of the molecule, offering more moieties to interact with multiple proteins.

Overall, the results give experimental evidence, that EC and GA can be added to native BLG solutions within certain concentration limits without provoking protein modifications, which is an important prerequisite when adding them as antioxidants to foods. These findings also pave the way for bioactivity studies, by excluding the presence of protein–phenolic interactions. However, when denaturing the protein or changing the reacting conditions (changes in temperature or pH value), the dynamic complexation will be different than under the presented conditions and a new evaluation is necessary.

A combination of methods that detect protein properties and phytochemical properties enables to analyse protein-phytochemical interactions for both moieties and the results complement each other. Thereby more insights into the interaction can be gained.

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CRediT authorship contribution statement

Kerstin Schild: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Frank D. Sönnichsen:** Methodology, Investigation, Writing – review & editing, Visualization. **Dierk Martin:** Methodology, Investigation, Writing – review & editing. **Vasil M. Garamus:** Methodology, Investigation, Writing – review & editing. **Atze Jan Van der Goot:** Writing – review & editing, Supervision. **Karin Schwarz:** Methodology, Resources, Writing – review & editing, Visualization, Supervision, Project administration. **Julia K. Keppler:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136496.

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