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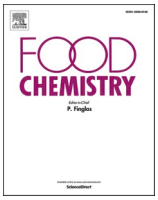

Covalent polyphenol modification of a reactive cysteine in the major apple allergen Mal d 1

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ABSTRACT

Naturally occurring polyphenols can modify the molecular properties of food allergens. For the major apple allergen Mal d 1 it has been postulated that chemical reactions with polyphenols cause permanent changes in the tertiary structure, causing a loss of conformational IgE epitopes and reducing allergenicity. In our study, we investigated the effect that reactions with oxidized polyphenols have on the structure of Mal d 1 by mass spectrometry and NMR spectroscopy. We showed that a surface-exposed cysteine residue in this allergen spontaneously reacts with oxidized polyphenols under formation of a defined covalent adduct. Chemical modification of Mal d 1 did not destabilize or perturb the three-dimensional fold, nor did it interfere with ligand binding to its internal pocket. A structural model of the chemically modified apple allergen is presented, which reveals that the bound polyphenol partially covers a conformational IgE epitope on the protein surface.

1. Introduction

Apples (*Malus* × *domestica* L. Borkh.) are an important domesticated fruit crop of high nutritive and health value. Numerous health benefits have been associated with the consumption of these fruits, which are attributed to their high contents of vitamins, fibers and plant secondary metabolites (Hyson, 2011). In addition, polyphenolic compounds have been attracting particular attention as they reduce risks for cardiovascular diseases and type II diabetes (Boyer & Liu, 2004). Polyphenols are also known for their potential to reduce the allergenicity of various food sources, including apples (Pi, Sun, Cheng, Fu, & Guo, 2022). Not only does the anti-inflammatory activity of polyphenols have beneficial effects on allergic responses, but also can polyphenols interact directly with food allergens and attenuate their allergenicity (Maleki, Crespo, & Cabanillas, 2019).

Apples are a particularly rich source of natural polyphenols, with phenolic acids (mainly chlorogenic acid) and flavan-3-ols (e.g., catechin and epicatechin) accounting for 80 % or more of the total polyphenol content in the flesh (Kschonsek, Wolfram, Stockl, & Bohm, 2018). Allergic reactions to apples very frequently affect individuals who already suffer from birch pollen allergy, caused by the protein Bet v 1 (Geroldinger-Simic et al., 2011). Birch pollen-related apple allergy is triggered by immunologic cross-reactivity of Bet v 1-specific IgE antibodies with a structurally homologous protein in apples, Mal d 1, which is present in the flesh and the skin of the fruits. In some countries more than 70 % of all individuals that are sensitized to birch pollen are affected by this cross-reaction, making Mal d 1 the major allergen in apples. Symptoms of birch pollen-related apple allergy typically occur

Abbreviations: CSP, chemical shift perturbation; ELISA, Enzyme-Linked Immunosorbent Assay; ESI, electrospray ionization; FT-ICR, Fourier-transform ion cyclotron resonance; IgE, Immunoglobulin E; MTSL, (2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methylmethanesulfonothiolate; PPO, polyphenol oxidase; PRE, paramagnetic relaxation enhancement; PR-10, pathogenesis-related protein 10.

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immediately after consumption of the fruit and include itching and swelling of lips, tongue and throat (oral allergic syndrome), as well as food-induced rhinoconjunctivitis and dyspnea.

Mal d 1 belongs to the pathogenesis-related protein 10 (PR-10) family that is upregulated in plants in response to biotic and abiotic stress. We recently determined the three-dimensional structure of Mal d 1 by nuclear magnetic resonance (NMR) spectroscopy (Ahammer, Grutsch, Kamenik, Liedl, & Tollinger, 2017). Mal d 1 folds into a curved β-sheet and three α-helices that surround a large internal binding pocket, which is capable of promiscuously accommodating low–molecular-weight compounds including flavonoids and glutathione (Chebib & Schwab, 2021) The immunologic significance of ligand binding to the internal pocket of PR-10 proteins is currently the subject of intense research (Chruszcz et al., 2021). More than 100 isoforms of this allergen have been identified to date, which are clustered into 12 sub-families (Mal d 1.01-Mal d 1.12) according to their amino acid sequences. The total Mal d 1 content of apples is strongly cultivar dependent and also depends on storage conditions, ripening, and climatic effects (Kiewning & Schmitz-Eiberger, 2014; Vegro et al., 2016). Variability arises from the fact that isoforms are differentially expressed, with those belonging to the Mal d 1.01 and Mal d 1.02 sub-families being most abundant (Chebib et al., 2022; Kaeswurm, Straub, Klußmann, Brockmeyer, & Buchweitz, 2022). Indeed, the isoform composition of Mal d 1 proteins has a decisive effect on the observed tolerability by allergic patients (Romer et al., 2020), which is in accord with the notion that different isoforms of Mal d 1 are distinct regarding their capacity to bind IgE antibodies. Consequently, a simple relationship between the total Mal d 1 content of different apple cultivars and their tolerability by allergic patients is not observed.

Interestingly, however, several studies revealed a correlation between the polyphenol content of apples and their tolerability. Comparing 24 apple cultivars, Vegro et al. showed that old apples with a higher polyphenol content are better tolerated by allergic individuals than more recently developed cultivars (Vegro et al., 2016). This was corroborated by Kschonsek et al., comparing the *in vitro* allergenicity of different apple cultivars with their phenolic profiles (Kschonsek, Wiegand, Hipler, & Bohm, 2019). In this study it was established that a higher content of chlorogenic acid, caffeic acid and epicatechin is associated with lower allergenicity of apples. Groth et al. also showed that apples with a higher content of chlorogenic acid have a lower allergenic potential, while a low content of epicatechin appears have the opposite effect (Groth et al., 2021). Moreover, the enzymatic activity of polyphenol oxidase (PPO) has a significant effect on apple allergenicity (Kiewning, Wollseifen, & Schmitz-Eiberger, 2013). PPO oxidizes polyphenols to form reactive quinones, which subsequently cause enzymatic browning by polymerization, or react with proteins. Garcia et al. showed that addition of PPO to protein extracts from apples significantly reduces the IgE binding capacity of Mal d 1 and proposed that reactive species formed by polyphenol oxidation induce permanent changes in the tertiary structure of this protein, causing a loss of conformational epitopes and a decrease in the IgE binding capacity (Garcia, Wichers, & Wichers, 2006).

On a molecular level, however, structural data supporting this proposition have not been reported to date and little is known about how allergenic (and non-allergenic) proteins in apples interact with polyphenols. In our present work, we investigate the effect of oxidized and non-oxidized polyphenols on Mal d 1. We show that, *in vitro*, the side-chain thiol of a surface-exposed cysteine residue is prone to spontaneous modification by various polyphenols (chlorogenic acid, epicatechin, caffeic acid) after oxidation by PPO, *via* covalent attachment of the *o*–diphenolic moiety. Combining NMR spectroscopy and mass spectrometry (MS), we provide a structural model of the adduct formed between Mal d 1 and chlorogenic acid. We show that this covalent modification does not destabilize or unfold Mal d 1, nor does it impede its ability to bind non-oxidized polyphenols to its internal cavity. Intriguingly, the covalently bound chlorogenic acid molecule partly covers an immunorelevant IgE epitope on the surface of Mal d 1.

2. Materials and methods

2.1. Allergens

Mal d 1 isoforms were recombinantly produced and purified and their identities were verified by mass spectrometry as described by Ahammer, Grutsch, and Tollinger (2016). Polyphenol modifications were obtained as follows: first, *o*-quinone derived from chlorogenic acid (Merck, Darmstadt, Germany), (-)-epicatechin (Merck, Darmstadt, Germany) or caffeic acid (Carl Roth, Karlsruhe, Germany) was prepared by overnight incubation with mushroom PPO (Merck, Darmstadt, Germany) in 10 mM sodium phosphate buffer (pH 6.9). Subsequently, PPO was removed by centrifugal filtration (5 kDa cutoff, Vivaspin) and Mal d 1 was incubated overnight with 10–250-fold molar excess of *o*–quinone at room temperature in the same buffer. At least 3 replicates were performed for each incubation experiment. Excess polyphenol content was removed by centrifugal filtration (5 kDa cutoff) and samples were desalted using 4.0 × 3.0 mm C18 pre-columns for mass spectrometry.

2.2. Mass spectrometry

Initial electrospray ionization MS (ESI-MS) experiments were performed using 1 μM desalted protein in 1:1 (v/v) methanol/water, 1 % acetic acid (Carl Roth, Karlsruhe, Germany) using a 7 Tesla FT-ICR mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive ionization mode. Detailed analysis of Mal d 1, modified by chlorogenic acid, was performed on a QExactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). MS data were deconvoluted using the UniDec freeware (Marty et al., 2015), and monoisotopic masses were calculated using the Xtract deconvolution function of Free Style V1.3 (Thermo Fisher Scientific, Waltham, MA, USA). Mass Spec Studios' application 'HX PIPE' was employed to identify protein modifications (Rey et al., 2014). 1 × chlorogenic acid ($C_{16}H_{16}O_9$) and 2 × chlorogenic acid ($C_{32}H_{32}O_{18}$) were defined to account for the addition of chlorogenic acid molecules or condensation products. Cysteine and lysine were enabled as possible modification sites. Fragment ions in tandem data were annotated using Mass Spec Studio. Signals related to fragmentation of the attached chlorogenic acid itself were interpreted and annotated manually.

For bottom-up LC-MS/MS, 100 μl of covalently modified Mal d 1 (50 μg) was denatured by a heat shock at 95 °C for one minute and digested overnight by addition of 25:1 (w/w) sequencing grade porcine trypsin in resuspension buffer (Promega, Walldorf, Germany). The digestion was stopped by addition of 1 % formic acid (Merck, Darmstadt, Germany) 1:1 (v/v), and the obtained peptides were separated by liquid chromatography on a C18 column, using a H_2O/acetonitrile (Merck, Darmstadt, Germany) 0.2 % formic acid gradient. The LC column was interfaced with a QExactive mass spectrometer operating in data dependent acquisition mode for peptide fragmentation. MS raw data files were converted using MSconvert (Chambers et al., 2012), and peptide annotation was performed using Mass Spec Studio. The OMSSA + peptide identification algorithm was used for data analysis. Peptide hits were checked manually and deleted in case of insufficient fit. Experimental details are given in the Supporting Information.

2.3. NMR spectroscopy

NMR spectra were recorded using 0.2–0.5 mM ^{15}N or $^{15}N/^{13}C$–labeled Mal d 1 samples in 10 mM sodium phosphate (Carl Roth, Karlsruhe, Germany) buffer (pH 6.9) with 10 % D_2O, at 25 °C using 600 MHz and 700 MHz NMR spectrometers equipped with cryogenic probes (Bruker Biospin, Ettlingen, Germany). Ligand binding experiments were performed as described, using 0.2 mM protein samples

(Grutsch et al., 2014). Residue-specific K_d values were determined from ligand-induced chemical shift perturbations. Mean values and standard deviations are reported. Temperature dependent $^{1}H^{15}N$-HSQC experiments were performed between 10 °C and 65 °C using 1 % (w/v) sodium-3-(trimethylsilyl)propane-1-sulfonate (Merck, Darmstadt, Germany) in the same buffer as external standard. Replicate experiments were recorded at 10 °C after sample heating to verify the integrity of the protein. For paramagnetic relaxation measurements, ^{15}N–labeled Mal d 1.0101 was treated with 3 eq of (2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methylmethanesulfonothiolate (MTSL, Toronto Research Chemicals, Toronto, Canada) in 10 mM sodium phosphate buffer (pH 6.9) at room temperature for one hour. Excess MTSL was removed by centrifugal filtration (3 kDa cutoff, Amicon). A $^{1}H^{15}N$-HSQC spectrum of MTSL–labeled Mal d 1 was recorded with an interscan delay of 5 s, and repeated after reducing the paramagnetic tag with ascorbic acid (3 eq). Peak volumes were employed for data analysis, using ^{15}N–acetylglycine (0.5 eq) as internal reference. Backbone amide resonance assignment of MTSL-labeled Mal d 1 was obtained for the reduced form (92 % completeness) as described (Ahammer et al., 2016).

2.4. Molecular dynamics simulations

A structural model of Mal d 1 with chlorogenic acid attached to Cys107 was obtained by molecular dynamics in explicit solvent (Ahammer et al., 2017). Parameters for the Cys107-modification were based on a generalized amber force field (GAFF), and partial charges were fitted using the restrained electrostatic potential (RESP) procedure (Wang, Wolf, Caldwell, Kollman, & Case, 2004). The three-dimensional solution structure of Mal d 1.0101 (pdb: 5MMU) was used as starting structure. Simulations were carried out with a harmonic restraining potential on all residues but the Cys107-modification. We performed 13 replica trajectories of 100 ns length, totaling to 1.3 μs of simulation time. Representative structures were extracted using density-based DBSCAN clustering.

3. Results

3.1. Covalent modification of Mal d 1 by polyphenols

To probe the reactivity of the major apple allergen toward polyphenols, we incubated the isoform Mal d 1.0101 with *o*-quinones that

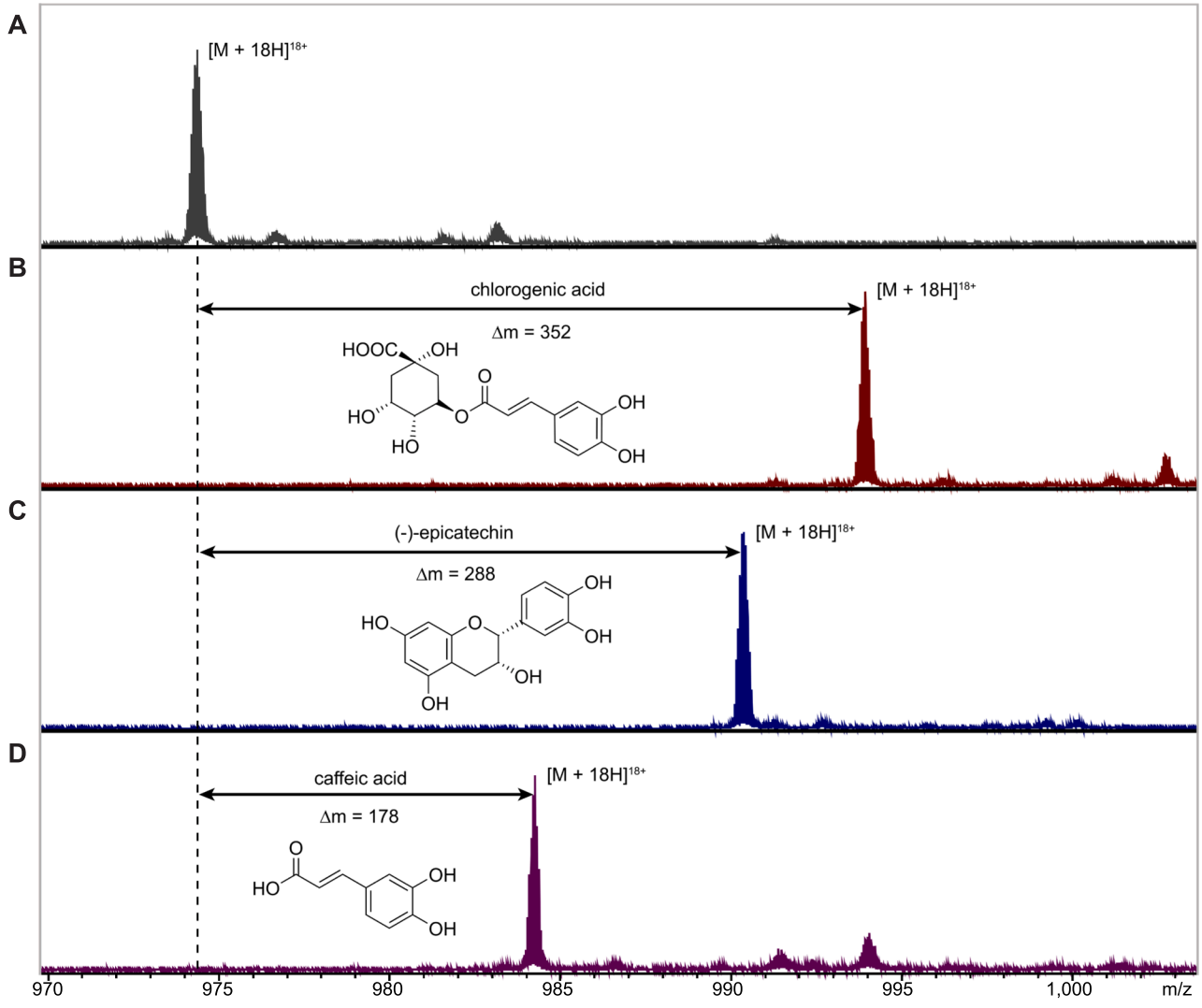

Fig. 1. Modification of the major apple allergen by oxidized polyphenols. Section of electrospray ionization mass spectra of 18-fold positively charged Mal d 1.0101, recorded on an FT–ICR mass spectrometer (A), compared to spectra after incubation with enzymatically generated *o*-quinones derived from chlorogenic acid (B), epicatechin (C) or caffeic acid (D). Mass shifts, Δm, indicate the formation of adducts with these polyphenols. The nominal molecular masses of chlorogenic acid, epicatechin and caffeic acid are $M_r = 354$, $M_r = 290$ and $M_r = 180$.

were obtained by *in vitro* oxidation of chlorogenic acid, (-)-epicatechin or caffeic acid with polyphenol oxidase. In all cases, mass spectrometric analysis of the reaction products revealed that the allergen had formed defined adducts (Fig. 1). The observed mass shifts, Δm, indicate that these adducts are predominantly formed with a single polyphenol molecule. We next characterized the reaction product with the most abundant polyphenol in apples, chlorogenic acid, in detail. Intact mass analysis shows that adduct formation is accompanied by the loss of two hydrogen atoms, in accordance with the formation of a single covalent bond (Fig. 2A). Covalent adduct formation of proteins with oxidized polyphenols has been studied in detail, suggesting that the side-chains of surface-exposed lysine and cysteine residues act as primary reaction sites (Rawel & Rohn, 2010). Mal d 1.0101 contains 16 lysine residues, the majority of which have their side-chains located on the protein surface, and a single, surface-exposed cysteine residue that might react with oxidized polyphenols.

To identify the site of covalent adduct formation in Mal d 1 we employed bottom-up LC–MS/MS analysis. MS^2 of an 11-residue peptide, comprising residues Leu104-Lys114, yielded a full y–ion series (fragments y1-y10) with y8*-y10* bearing the chlorogenic acid modification, as indicated by prominent mass shifts (Fig. 2B). Moreover, the two fragments y8* and y9* show characteristic neutral loss fragments (–192 Da and –386 Da) that can be attributed to the fragmentation of the bound polyphenol. The latter fragment equates to the mass of a chlorogenic acid-sulfur adduct, which unambiguously shows that chlorogenic acid must be linked to the sole cysteine residue in the peptide, Cys107. Neither for fragments y1–y7, nor for any other tryptic peptide, adducts were detected. We thus conclude that the primary modification site in Mal d 1.0101 is Cys107, with the polyphenol covalently linked to the sulfur atom in the side–chain.

To complement these data, we examined the reactivity of cysteine towards polyphenols using caffeic acid as substrate for PPO and glutathione as low–molecular-weight model of cysteine (Fig. S1). NMR and MS experimental data confirm the formation of single bond between the sulfur atom of the single thiol group in glutathione and the carbon atom C2′ of the *o*-diphenolic moiety resulting from nucleophilic (Michael-type) addition, in accordance with the chemical structure shown in Fig. 2 (Le Bourvellec & Renard, 2012). The same reaction pattern has previously been reported for cysteine in a model study (Schilling, Sigolotto, Carle, & Schieber, 2008).

We further investigated the reactivity of the apple allergen toward *o*-quinones by performing experiments with the two isoforms Mal d 1.0105 and Mal d 1.0201. For Mal d 1.0105, which shares 98.7 % sequence identity with Mal d 1.0101 and contains two surface-exposed cysteines (Cys3 and Cys107), the MS data show the covalent attachment of two chlorogenic acid molecules (Fig. S2). On the other hand, the isoform Mal d 1.0201 (90.5 % sequence identity), which has no cysteine residues, does not react with chlorogenic acid under the same conditions. Clearly, as with Mal d 1.0101, both isoforms are not susceptible to chemical modification at other sites. Taken together, the accumulated experimental data suggest that surface exposed cysteine residues act as the primary reaction sites for polyphenolic derived *o*–quinones in Mal d 1, while reaction with lysine residues is not observed in our study.

3.2. Effect of covalent binding of polyphenol on the structure of Mal d 1

Using NMR spectroscopy, we investigated the impact of Cys107 modification by chlorogenic acid on the three-dimensional structure of Mal d 1. Fig. 3A shows that the backbone amide $^1H^{15}N$-HSQC spectrum of Mal d 1 after modification is very similar to that of the unmodified allergen. Backbone amide $^1H^{15}N$-HSQC spectra represent fingerprints of proteins, with resonance positions being sensitive reporters of the local geometry. These data thus demonstrate that the backbone of Mal d 1 is not significantly perturbed by covalent modification of Cys107 by chlorogenic acid. The largest chemical shift perturbation (CSP) values are found for amino acids in the direct neighborhood of the modification site, i.e. the loop that harbors Cys107 and the two surrounding β-strands, β6 and β7, covering residues Val105-Lys114 (Fig. 3B and Fig. S3). CSP values are also found for residues Thr10, Ser11 and Glu12 at the tip of β–strand β1, and for amino acids in the central part of helix α3. These residues form a coherent patch on the protein's surface, suggesting that the covalently bound polyphenol is predominantly located within this area. In fact, this agrees well with the orientation of the reactive side chain of Cys107, which points toward the β-sheet. The observed chemical shift differences between modified and unmodified Mal d 1 may be due to the spatial proximity of the bound polyphenol, or result from minor adjustments of the local geometry in response to the covalent modification at Cys107. Major structural changes or unfolding of the protein backbone would significantly displace backbone amide resonances in $^1H^{15}N$-HSQC spectra, typically by values exceeding 0.5 ppm and 2 ppm in the ^1H and ^{15}N dimensions, respectively (Tollinger et al., 2006) This is clearly not the case when chlorogenic acid attaches to Cys107.

In PR-10 proteins, loops connecting secondary structure elements are

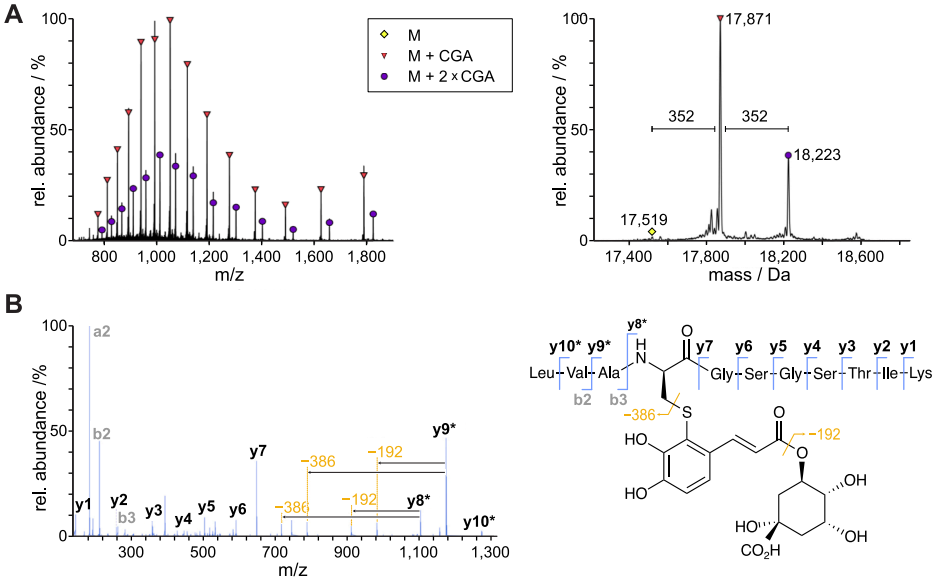

Fig. 2. Mass spectrometric analysis of the adduct formed between Mal d 1 and chlorogenic acid. Positive-mode (left) and deconvoluted zero-charge (right) ESI-MS spectra of Mal d 1.0101 (A). Prominent mass shifts of 352 Da correspond to a covalent modification by chlorogenic acid. A fraction of Mal d 1 forms an adduct with dimeric chlorogenic acid. LC–MS/MS data of a tryptic digest (B). MS^2 spectrum of the doubly charged precursor peptide Leu104–Lys114 (m/z = 694.3165) harboring the chemical modification (left), along with the peptide sequence, the postulated chemical structure and the fragmentation pattern of the adduct (right). Chlorogenic acid is bound to Cys107, *via* thioether linkage to its *o*-diphenolic moiety.

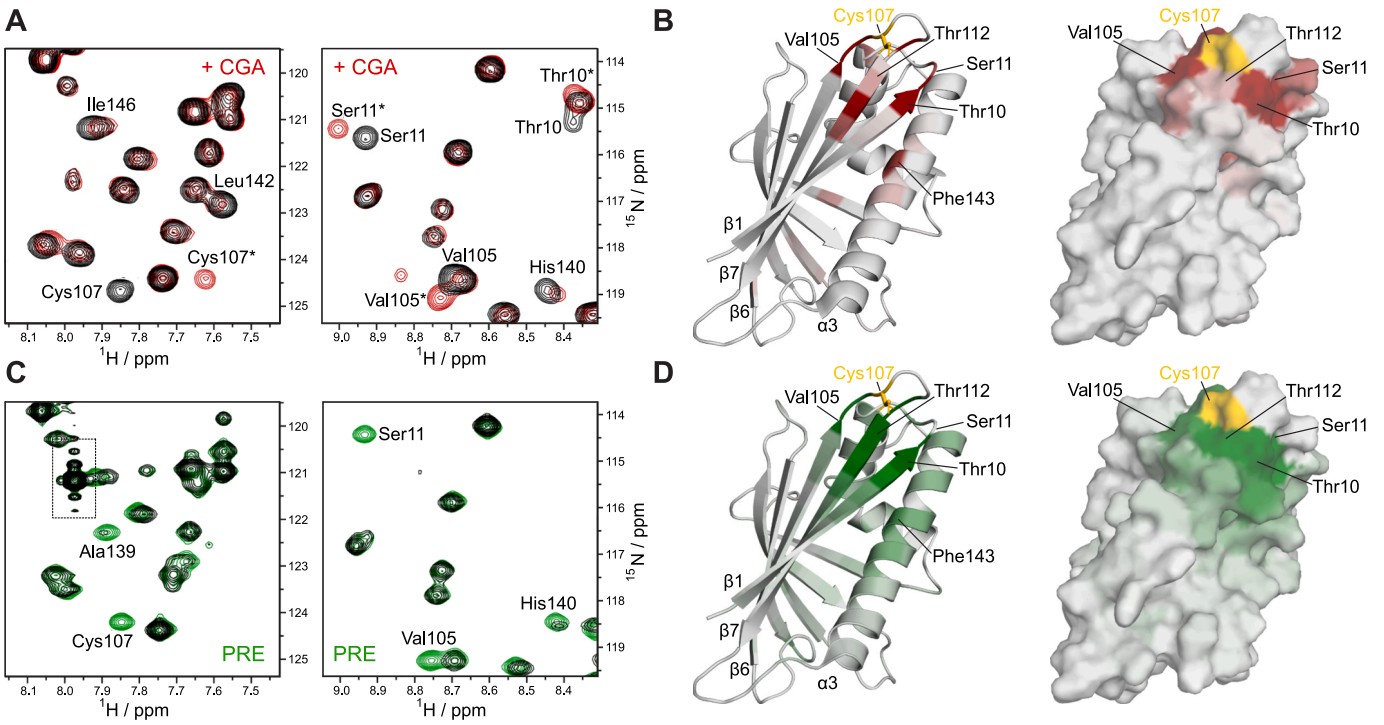

Fig. 3. NMR spectroscopy of covalently modified Mal d 1. Sections from backbone amide ^{1}H^{15}N-HSQC spectra of Mal d 1.0101, recorded at 600 MHz, before (black) and after (red) reaction with oxidized chlorogenic acid (A). Backbone and surface representations of Mal d 1, highlighting residues with chemical shift perturbations in red (B). Darker color indicates larger CSP values. Spectra of Mal d 1.0101 with a paramagnetic spin label attached to the side-chain of Cys107 (C). Reduction of the spin label leads to enhanced intensities of nearby backbone amides (from black to green). Residues affected by paramagnetic relaxation (green); the dashed box indicates the refence compound ^{15}N–acetylglycine (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

structurally flexible and heterogeneous, including the 5-residue loop that contains Cys107 (Führer et al., 2022). To probe the structural flexibility of covalently modified Cys107, we attached a paramagnetic spin label to its side-chain by cysteine-specific chemical tagging. Paramagnetic spin labels cause faster relaxation and line broadening of nearby atoms in NMR experiments, known as paramagnetic relaxation enhancement (PRE). The resulting intensity reduction of amino acids in proximity to the paramagnetic label contains spatial information and is exquisitely sensitive to low populated structures in conformationally heterogeneous systems. Fig. 3C shows that several amino acids in Mal d 1 are strongly affected by paramagnetic relaxation, leading to near complete loss of signal intensities in NMR spectra. This includes the segment between residues Val105 and Lys114, as well as residues Phe9 and Ser11 at the tip of strand β1, and to a lesser extent the center of helix α3 (Fig. S4). Additional PRE effects are detected for distances up to ≈ 25 Å away from Cys107, as expected for a 17 kDa protein. The protein surface that is affected by paramagnetic relaxation is restricted to the central β-sheet (strands β1, β6 and β7) and helix α3 (Fig. 3D), and coincides with those parts of Mal d 1 for which covalent binding of chlorogenic acid affects the backbone amide chemical shifts. On the other hand, only minor PRE effects are observed, e.g., for the glycine-rich loop region between strands β2 and β3 (Gly46–Gly51), which is conserved in many PR-10 allergens and ≈ 20 Å away from Cys107 (Fig. S5). The chemical modification at Cys107 obviously does not

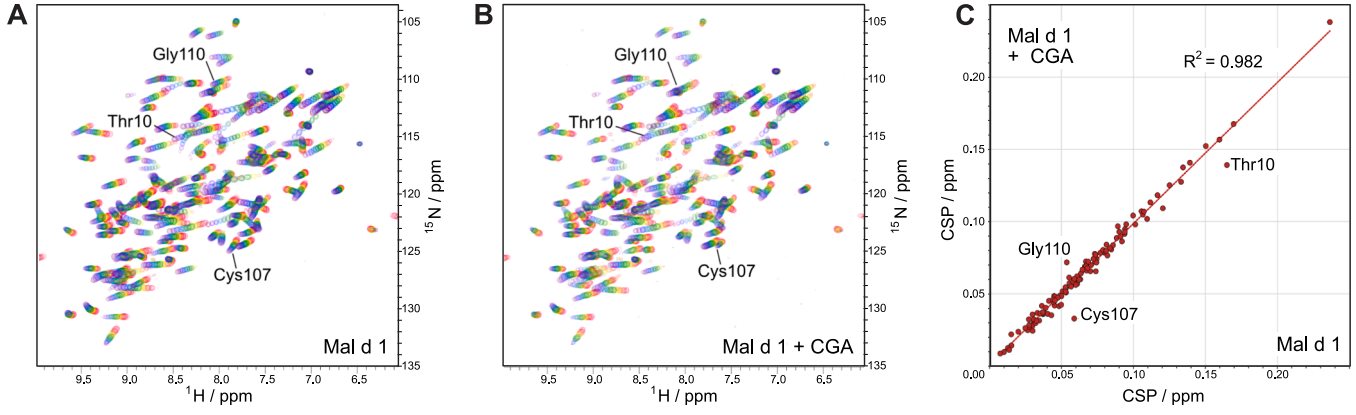

Fig. 4. Temperature-dependent stability of Mal d 1.0101. Superposition of backbone amide ^{1}H^{15}N-HSQC spectra, recorded at 700 MHz, for a temperature range from 10 °C (violet) to 65 °C (red), before (A) and after (B) covalent modification by chlorogenic acid. Comparison of the temperature dependence (CSP values between 15 °C and 35 °C) of backbone amide ^{1}H^{15}N resonances with and without chemical modification by chlorogenic acid (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

contact this part of the protein surface.

We next probed the backbone hydrogen bonding network in Mal d 1 by use of temperature dependent NMR spectroscopy. The temperature dependence of backbone amide ^{1}H chemical shifts contains valuable information about the presence and strength of hydrogen bonds on a per-residue level (Okazaki et al., 2018). As shown in Fig. 4, the temperature dependence of most Mal d 1 resonances is nearly identical with and without chemical modification, suggesting that the hydrogen bonding network remains largely unchanged when chlorogenic acid attaches to the side chain of Cys107. This holds true for all amino acid residues in secondary structure elements in Mal d 1, and for most loops. Only for Thr10, Cys107 and Gly110 small differences in the temperature response of ^{1}H resonances are observed. Their small magnitude is indicative for only minor and local adjustments of hydrogen bonds in the direct neighborhood of the modification. Global destabilization of the three-dimensional scaffold of Mal d 1 can be clearly excluded.

Model studies showed that covalent derivatization of lysozyme by chlorogenic acid is accompanied by a significant decrease of solubility (Rawel, Kroll, & Rohn, 2001). Contrasting these observations, we did not observe differences regarding the solubility of Mal d 1 before and after modification by CGA. Both forms of the protein remain in solution and show no signs for precipitation in samples containing up to 5 mg/mL at pH 6.9. Taken together, covalent modification by chlorogenic acid appears to have little effect on the tertiary structure of Mal d 1 and its biophysical properties.

3.3. Non-covalent binding of polyphenol to Mal d 1

Mal d 1 binds amphiphilic low-molecular-weight compounds, including flavonoids, to its internal pocket in a non-covalent manner (Chebib & Schwab, 2021). We investigated binding of epicatechin to the apple allergen by NMR. Addition of this polyphenol results in gradual chemical shift changes of amino acids in the β-sheet (strands β4, β5 and β6), in helices α1 and α2, and in connecting loops surrounding the internal cavity of the protein (Fig. 5). The location of these amino acid residues suggests that epicatechin binds to the internal pocket in Mal d 1, as observed for other PR-10 proteins (Fig. S6). Of note, Cys107, which is distal from the pocket, is only marginally affected when epicatechin binds to the internal cavity. Fig. 5 shows that Mal d 1 still binds epicatechin when Cys107 is covalently modified by chlorogenic acid, with an affinity that coincides with that of the unmodified protein (K_d values are 5.0 ± 2.8 mM without and 4.3 ± 2.0 mM with modification by CGA). Evidently, ligand binding to the internal cavity is not compromised by covalent modification on the protein's surface.

3.4. A structural model of polyphenol modified Mal d 1

To visualize the structure of covalently modified Mal d 1 we used molecular dynamics (MD) simulations in explicit water (Fig. 6). It is evident that the bound polyphenol (chlorogenic acid) occupies at least two major orientations in the MD ensemble, packing on the allergen's surface near strands β1, β6 and β7, in agreement with the experimental NMR data. Intriguingly, bound chlorogenic acid partially covers the surface-exposed residues Thr10, Ser111 and Thr112, which are part of a conformational IgE epitope in Mal d 1 (Ahammer et al., 2017). The *o*–diphenolic moiety of chlorogenic acid is only 3–4 Å away from the side-chains of Thr10 and Thr112, and less than 7 Å away from Ser111, whose side-chain faces the protein core. Several other amino acid residues in Mal d 1 have been proposed to be relevant for IgE binding, including Ile30, Ile113, Thr57 and a number of residues in or nearby the conserved glycine-rich loop, for which mutation to the corresponding amino acid in Bet v 1 increases the binding affinity of birch-pollen specific IgE (Holm et al., 2011). Ile113 and Ile30 do not reach the protein surface, however, and except for Glu76 all other residues at least 20 Å away from Cys107 and well outside the range that is accessible by the bound polyphenol.

4. Discussion

Polyphenolic compounds are compartmentally separated from proteins in living cells. In apples, polyphenols are almost exclusively (97 %) located in the vacuoles (Beshir et al., 2019), while PR-10 allergens and polyphenol oxidase are present in the cytoplasm and in chloroplasts, respectively (Agarwal & Agarwal, 2014). However, there are several situations in which these molecules meet and react, following destruction of the vacuole by cutting, processing and digestion of the fruit. In the presence of oxygen, enzymatic oxidation of polyphenols by PPO and non-enzymatic autoxidation is induced and reactive *o*-quinones are formed. Our experimental data show that, *in vitro*, *o*–quinones that derive from phenolic acids and flavan-3-ols readily react with the thiol group of Cys107 in Mal d 1, leading to covalent attachment of the polyphenol. The same reaction occurs in apple juice when using isolated cysteine as substrate, suggesting that such modifications may also form in the natural food matrix (Schilling et al., 2008). In accordance with our study, reaction with lysine was not observed in apple juice.

In the three-dimensional structure of Mal d 1, Cys107 is located in the vicinity of a conformational IgE epitope formed by amino acids Thr10, Ser111 and Thr112 (Ahammer et al., 2017). These residues are involved in IgE interactions both in Mal d 1 and in the structurally homologous birch pollen allergen Bet v 1, suggesting that they are part of a common cross-reacting epitope. In fact, mutation of these residues in Mal d 1 results in significantly reduced IgE binding *in vitro* (Ma et al., 2006) and skin prick tests in apple-allergic patients show measurably lower ability to induce skin reactions *in vivo* (Bolhaar et al., 2005). Covalently bound polyphenol at Cys107 partially masks the Thr10-Ser111-Thr112 epitope of Mal d 1 by covering the allergen. This reduces the surface that is available for binding, and potentially hinders access of antibody. It is conceivable that the modification of the apple allergen described here occurs when apple fruits are cut or processed and polyphenols, oxidized by PPO or non-enzymatically, get into contact with Mal d 1. Oxidation of polyphenols in apples occurs within minutes, as manifest from the progression of enzymatic browning upon cutting or

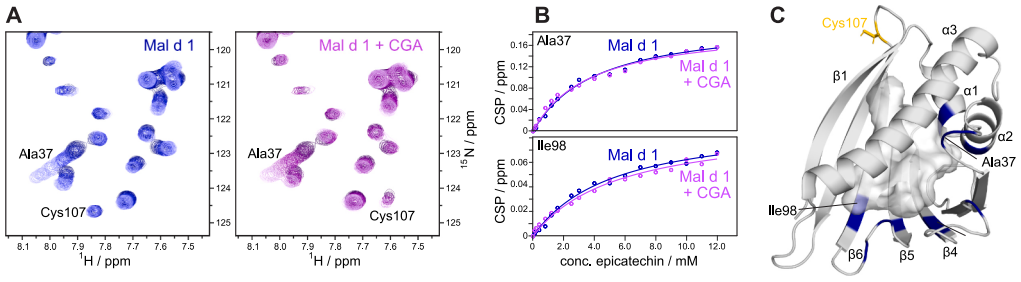

Fig. 5. Binding of the polyphenol epicatechin to the internal cavity of Mal d 1. Superposition of backbone amide ^{1}H^{15}N-HSQC spectra, recorded at 700 MHz, with increasing concentrations (up to 60-fold excess) of epicatechin without (blue) and with (purple) covalent modification at Cys107 (A). Exemplary binding curves (ligand-induced chemical shift perturbations *vs* ligand concentration) for Mal d 1 residues in the internal binding pocket (B). Residues that display significant chemical shift perturbations upon epicatechin binding (blue) surround the internal pocket and are distal from Cys107 (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

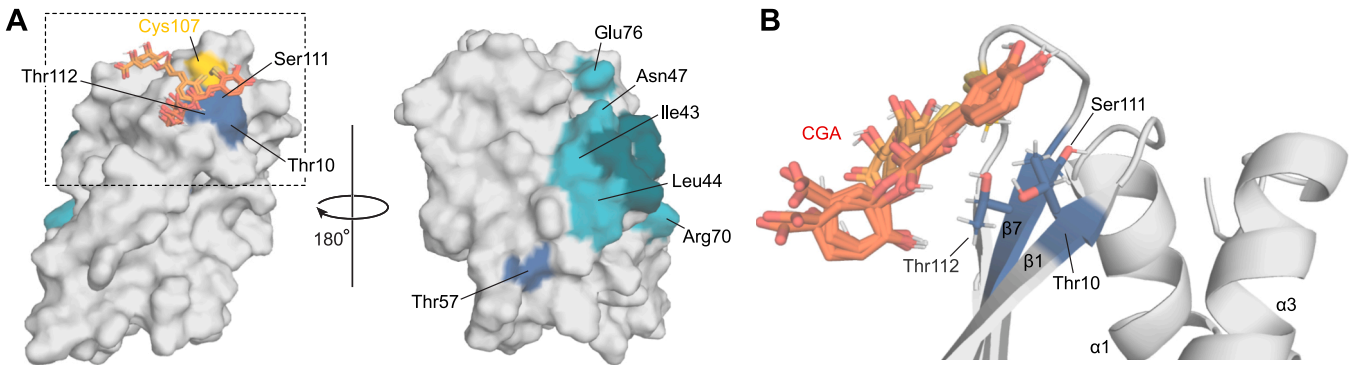

Fig. 6. Structural model of modified Mal d 1, showing six representative orientations of chlorogenic acid (shown in shades of orange) linked to the side-chain of Cys107 (A) and a close-up view highlighting the spatial proximity to the Thr10-Ser111-Thr112 epitope (B). Residues that belong to known IgE binding epitopes are shown in blue and are labeled. Colored in light teal are Mal d 1 residues for which mutation to the corresponding amino acid in Bet v 1 facilitates IgE binding. The conserved glycine-rich loop is shown in dark teal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

processing. A recent study showed that, simultaneously with polyphenol oxidation, apple allergenicity decreases, as judged from time-dependent basophil activation and antigen stimulation tests (Kschonsek et al., 2019).

Contrasting earlier propositions, the reaction of oxidized polyphenols with Mal d 1 does not necessarily induce permanent changes in the tertiary structure of this protein (Garcia et al., 2006). Our spectroscopic data imply that the three-dimensional scaffold of Mal d 1 is not perturbed by covalent polyphenol modification at Cys107. Moreover, a loss of secondary structure, partial unfolding, destabilization and lower solubility, as reported for other proteins (Ali, Homann, Khalil, Kruse, & Rawel, 2013; Rawel, Rohn, Kruse, & Kroll, 2002), are not observed for the major apple allergen. Non-covalent ligand binding to the internal cavity is not affected by covalent modification of Cys107 at the protein surface, and simultaneous covalent and non-covalent binding of polyphenols to Mal d 1 are possible. Naturally, from our data we can not exclude the possibility that additional reactions with polyphenolic compounds may occur in the natural food matrix, including the formation of more complex and cross-linked protein polymers (Rawel & Rohn, 2010).

The total Mal d 1 content of apples is strongly cultivar-dependent, with considerable variation between harvest years, and typically ranges from 1 to 20 μg Mal d 1 per gram of fresh apple. However, a simple relation between the total Mal d 1 concentration, which is routinely determined by ELISA, and allergic potential is not observed (Asero, Marzban, Martinelli, Zaccarini, & Machado, 2006; Romer et al., 2020). This discrepancy can in part be attributed to the fact that for ELISA testing monoclonal or polyclonal antibodies are used. As observed for other PR-10 allergens, different isoforms can possess distinct antibody binding properties even if sequence identities are high (Führer et al., 2021), which introduces uncertainties when determining the total Mal d 1 content by ELISA. Using LC–MS/MS analysis, it was shown that different apple cultivars can indeed display large variations regarding their isoform composition (Romer et al., 2020). Comprehensive proteomic studies recently revealed that isoforms from the Mal d 1.01 and Mal d 1.02 sub-families are generally most abundant, while others are almost not expressed in the fruit (Chebib et al., 2022; Kaeswurm et al., 2022), and several hypoallergenic Mal d 1 isoforms were proposed for which the abundance in the fruit correlates negatively with the observed allergic symptoms (Chebib et al., 2022). These observations demonstrate that not only the total Mal d 1 content but also the isoform composition determine the tolerability of a particular apple cultivar.

Isoform-specific interactions of allergens with the surrounding food matrix, as observed in our study, may represent yet another factor that contributes to the lack of correlation between the Mal d 1 content and tolerability. Cysteine at position 107 is not conserved in all Mal d 1 isoforms. While it is present within the Mal d 1.01 cluster (Mal d 1.0101-Mal d 1.0109), position 107 is occupied by serine in, e.g., the Mal d 1.02 and Mal d 1.03 clusters. In addition, some Mal d 1 isoforms contain two surface exposed cysteine residues, both of which are prone to chemical modification by polyphenols, while others are lacking cysteines. Differential reactivity of Mal d 1 isoforms toward naturally occurring polyphenols is probable. In addition, it was shown that the isoform Mal d 1.0108 has a tendency to dimerize, presumably *via* formation of a disulfide bridge due to cysteine oxidation (Kaeswurm, Nestl, Richter, Emperle, & Buchweitz, 2021). Like Mal d 1.0101, this particular isoform contains a single, surface-exposed cysteine residue at position 107. Oligomerization of Mal d 1 has been proposed to attenuate IgE binding (Pagliarani et al., 2012), as it reduces the allergen surface that is available for interacting with the antibody. It is possible that cysteine-mediated dimerization is impeded by polyphenol binding.

Quite generally, differential reactivity of isoforms toward components of the food matrix may well be a common feature of PR-10 proteins. Our results illustrate the effects that reactions of oxidized polyphenols have on the three-dimensional protein fold and provide a structural rationale for how covalent modifications of the protein surface might attenuate allergenic responses.

CRediT authorship contribution statement

Jana Unterhauser: Investigation, Visualization, Writing – original draft. **Linda Ahammer:** Investigation, Visualization. **Tobias Rainer:** Investigation, Visualization, Writing – original draft. **Reiner Eidelpes:** Investigation. **Sebastian Führer:** Investigation. **Bettina Nothegger:** Investigation. **Claudia E. Covaciu:** Investigation. **Valentina Cova:** Investigation. **Anna S. Kamenik:** Investigation, Visualization. **Klaus R. Liedl:** Resources, Funding acquisition. **Thomas Müller:** Investigation. **Kathrin Breuker:** Investigation. **Klaus Eisendle:** Conceptualization, Funding acquisition. **Norbert Reider:** Conceptualization, Funding acquisition. **Thomas Letschka:** Conceptualization, Supervision, Funding acquisition. **Martin Tollinger:** Conceptualization, Supervision, Funding acquisition, Writing – original draft.

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.2022.135374.

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