Food Chemistry 503 (2026) 147775

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Tannase-catalysed gallic acid release for clean-label inhibition of green tea auto-oxidative browning

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ARTICLE INFO

Keywords:
Catechins
Discolouration
Degalloylation
Tannin acylhydrolase
Colour preservation

ABSTRACT

Ready-to-drink (RTD) green tea is rich in flavan-3-ols, which readily undergo auto-oxidation, causing browning. Conventional browning inhibitors can induce undesirable ingredient interactions, flavour changes, and health concerns. Here, we explored a clean-label strategy to inhibit RTD green tea browning by releasing gallic acid from naturally present galloylated compounds using the enzyme tannase (from *Aspergillus ficuum*). In-depth analysis of green tea by a combination of UHPLC-HRMS, NMR, and enzymatic hydrolysis led to the (tentative) identification of 75 galloylated compounds with four scaffold types. Hydrolysis of these galloylated compounds by tannase was affected by the scaffold type, scaffold stereo configuration, position of gallate attachment, and number of gallate moieties. Tannase treatment resulted in a substantial reduction in auto-oxidative browning of green tea. These results demonstrate that tannase treatment has the potential to decrease reliance on additives for maintaining the colour of RTD green tea and other phenolic-rich beverages.

1. Introduction

Tea is one of the most widely consumed beverages worldwide. Ready-to-drink (RTD) green tea is a convenient and popular option for on-the-go consumption. Furthermore, it appeals to health-conscious consumers as a healthier alternative to carbonated soft drinks and other sugar-rich beverages, due to its low caloric value and natural ingredients. The global RTD tea market (i.e., revenue generated in supermarkets, convenience stores, restaurant and bars) amounted to US$ 110.3 bn in 2024, and is expected to have a compound annual growth rate (CAGR) of 3.7 % from 2024 to 2028 (Statista, 2025).

Green tea is notably rich in flavan-3-ols, a class of phenolic compounds. These compounds are highly susceptible to auto-oxidation, leading to formation of brown or yellow pigments. The resulting discolouration negatively impacts the quality of RTD green tea over its shelf life (Chen et al., 2021). Traditionally, antioxidant additives have been employed to protect flavan-3-ols from rapid oxidative browning. Ascorbic acid (AA) in particular is commonly used (Chen et al., 1998). However, AA can have unintended side-effects due to the sacrificial nature of its antioxidant action leading to transformation into dehydroascorbic acid (DHAA). DHAA can further react with flavan-3-ols, resulting in formation of addition adducts and a wide array of complex brown products (Bradshaw et al., 2001; Hung et al., 2018). Additionally, at higher concentrations of AA addition and during extended storage, AA has been observed to potentially accelerate the degradation of flavan-3-ols (Chen et al., 2021). Therefore, alternatives for AA addition need to be developed. Various anti-browning techniques, like encapsulation, can protect antioxidants, like flavan-3-ols, from oxidative degradation. However, encapsulation introduce additional ingredients, which conflicts with clean-label goals, and encapsulation of tea compounds in aqueous environment presents challenges for long-term stability (Javadi et al., 2024; Karoshi et al., 2025; Xie et al., 2025). Most other approaches, such as using natural plant extracts as stabilizers in tea beverages, also introduce additional ingredients and may be constrained by high costs (Wu et al., 2023). Moreover, there is a lack of conclusive data on the efficacy of such extracts in RTD green tea applications.

Recently, it has been shown that gallic acid (GA), a natural phenolic compound, can function as an inhibitor of auto-oxidative browning in green tea (Tan et al., 2025). In tea, GA is primarily present in bound form. Galloylation refers to the attachment of a galloyl group to various scaffolds, typically through an ester bond (Grundhöfer et al., 2001;

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https://doi.org/10.1016/j.foodchem.2025.147775
Received 27 August 2025; Received in revised form 13 December 2025; Accepted 25 December 2025
Available online 26 December 2025

Haddock et al., 1982; Jiménez-Sánchez et al., 2015; Karas et al., 2017; Kirby et al., 2013). In green tea, these scaffolds include flavan-3-ols (Fig. 1a) (Bhatia & Ullah, 1962), procyanidins (Fig. 1b) (Jiang et al., 2015), organic acids (Fig. 1c) (Roberts & Myers, 1958), and glucose (Fig. 1d) (Nonaka et al., 1984). Well-known examples of galloylated compounds are epigallocatechin gallate (EGCg), epicatechin gallate (ECg) (Hashimoto et al., 1989), theogallin (i.e. 5-*O*-galloyl-quinic acid, according to the IUPAC nomenclature), and galloyl-glucose derivatives that are commonly referred to as hydrolysable tannins (Engelhardt, 2010). Thus, green tea naturally contains a wide variety of galloylated compounds, as well as some free GA (Obuchowicz et al., 2011). The bound gallate (g) moieties present as part of galloylated compounds create a reservoir of potentially releasable GA in green tea, thereby functioning as a natural "GA-bank".

In our previous study, we demonstrated that the presence of GA can reduce the auto-oxidative browning of EC and ECg (Tan et al., 2023). The underlying mechanisms were hypothesised to involve altering the oxidative reaction pathways, reducing the pH of the reaction environment, or a combination thereof. Further investigation revealed that free GA exerts its auto-oxidation inhibitory effect primarily through its role in modulating pH (Tan et al., 2025). In green tea samples, maintaining pH below 4.1 has been shown to effectively inhibit browning (Tan et al., 2025). This led to the following research question: Can we utilise the GA-bank of green tea to develop a clean-label approach to inhibit auto-oxidative browning as an alternative to antioxidant additives? Given the presence of galloylated compounds in green tea, we hypothesise that tannase (EC 3.1.1.20), which catalyses hydrolysis of GA esters (Freudenberg, 1929), can be used to release GA from the GA-bank to inhibit auto-oxidative browning. Tannase treatment, previously recognised for its ability to improve taste (Cao et al., 2019; Zhang et al., 2016), turbidity (Lu et al., 2009), and antioxidant capacity (Battestin et al., 2008) in green tea, is now proposed as a method to reduce auto-oxidative browning of RTD green tea. However, the content of galloylated compounds in the GA-bank is yet unknown and likely dependent

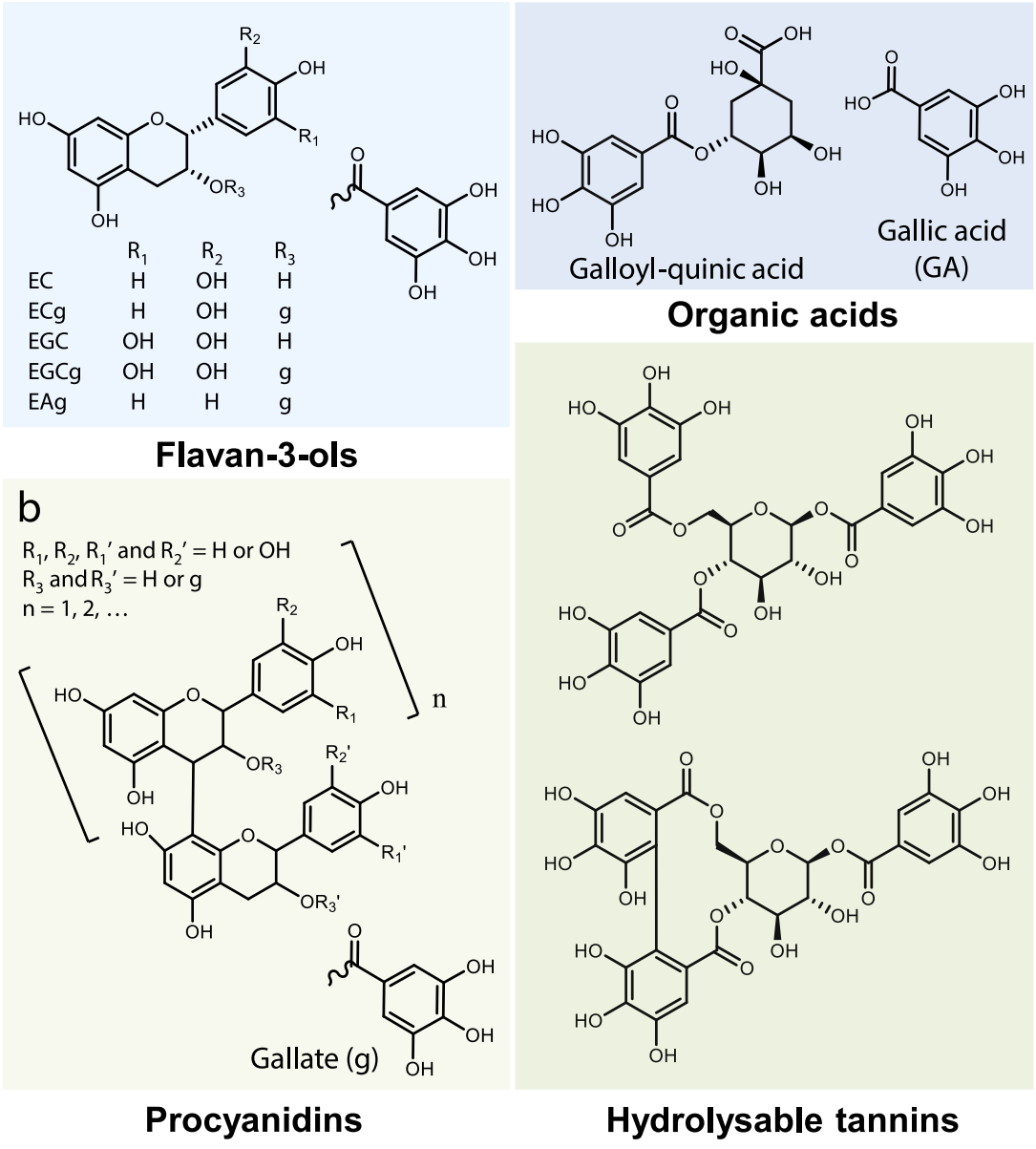


Fig. 1. Representative galloylated compounds with different scaffolds that have been reported in green tea. (a) Flavan-3-ols. (b) Procyanidins. (c) Organic acids. (d) Hydrolysable tannins. The abbreviation GA refers to free gallic acid, whereas g refers to a bound gallate moiety. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on the tea type and origin (Obuchowicz et al., 2011). Additionally, GA can be esterified to various scaffolds (Fig. 1) and the ability of tannase to release GA from these scaffolds has not yet been systematically investigated. Thus, this led to the following research questions: (i) How much free GA can be released from naturally present galloylated compounds by tannase, and (ii) to what extent can browning be inhibited by tannase-catalysed GA release?

In this work, we aimed to investigate whether tannase-catalysed GA release can be used to inhibit auto-oxidative browning in RTD green tea. Firstly, we performed a comprehensive identification of galloylated compounds in green tea using ultra-high performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS), including diagnostic fragment-dependent compound discovery. Secondly, we determined the ability of tannase (from *Aspergillus ficuum*) to hydrolyse these galloylated compounds. Thirdly, we quantitatively analysed the tannase-catalysed GA release, and its effect on green tea pH and auto-oxidative browning.

2. Materials and methods

2.1. Chemicals

Tannase from *Aspergillus ficuum* (specific activity: 250 U/g protein), gallic acid monohydrate (98 % [*w/w*]), ellagic acid (99 % [*w/w*]), chlorogenic acid (5-caffeoylquinic acid, 95 % [*w/w*]), procyanidin B_2 (90 % [*w/w*]), D-(+)-glucose (99.5 % [*w/w*]), and kaempferol (90 % [*w/w*]), caffeine (99 % [*w/w*]) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-*O*-Galloyl-β-D-glucose (98 % [*w/w*]) was purchased from Cayman Chemical Company (Michigan, USA). Methyl 3-*O*-methylgallate (99 % [*w/w*]) was purchased from MedChemExpress (NJ, USA), 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (99 % [*w/w*]) was purchased from Biosynth (Compton, UK). Quercetin hydrate (99 % [*w/w*]) was purchased from Acros Organics (Geel, Belgium). Taxifolin (99 % [*w/w*]) and dihydromyricetin (97 % [*w/w*]) were purchased from TargetMol Chemicals (MA, USA). Epicatechin (97 % [*w/w*]) and myricetin (97 % [*w/w*]) were obtained from TCI Chemicals (Tokyo, Japan). Epicatechin gallate (97 % [*w/w*]) and epigallocatechin gallate (97 % [*w/w*]) were purchased from Yuanye (Shanghai, China). Epigallocatechin was prepared from EGCg Sunphenon-94 (Taiyo, Schwelm, Germany) by treatment with tannase, as described elsewhere (Bohin et al., 2012). UHPLC-HRMS grade acetonitrile (ACN), methanol (MeOH), and formic acid (FA, 99 % [*v/v*]) were obtained from Biosolve (Valkenswaard, The Netherlands). Water for other purposes than UHPLC was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). In-house standards of 1,4,6-tri-*O*-galloyl-β-D-glucose, 1-*O*-galloyl-4,6-hexahydroxydiphenoyl-β-D-glucose (strictinin) and 5-*O*-galloyl-quinic acid (theogallin) were obtained by extraction from green tea, and subsequent purification by flash chromatography and preparative HPLC. Their identities and purities were confirmed by UHPLC-HRMS and NMR. The details of the purification method and the compound identification are described in Supplementary information IV, *Appendix A*.

2.2. Plant materials

Four green tea samples from different cultivars of *Camellia sinensis* var. *sinensis*, namely Longjing 43 (LJ-43), Fudingdabai (FD), Longjingchangye (LJ-CY), and Echayihao (ECha) were obtained from the Tea Research Institute, Chinese Academy of Agricultural Sciences (Hangzhou, PR China). Ten grams of each green tea sample was milled (Tefal Moulinette Compact MB300138, Rumilly, France) into powder. To obtain the mixed green tea sample, the four individual green tea powders were mixed in equal quantities (5 g each). The individual and mixed green tea powders were extracted using 90 °C water (tea powder:water = 1:25 [*w/v*]) for 30 min, and filtered by grade 595 filter paper (Whatman, Buckinghanshire, UK). The filtrate was then centrifuged at 4695×*g* at 20 °C for 20 min. The supernatant of the mixed tea sample was used for tannase treatment. The supernatants of all extracts were analysed by UHPLC-HRMS after being diluted 10 times with MeOH containing 1 % (*v/v*) FA, and being further diluted 4 times with water containing 1 % (*v/v*) FA.

2.3. Tannase treatment of green tea

The mixed green tea was used as a representative sample that was assumed to cover all the GA-containing compounds present in the individual green teas. UHPLC-HRMS chromatograms of four individual green teas and the mixed green tea are shown in Supplementary information IV, Fig. S1. To 20 mL the mixed green tea, 400 μL of tannase (10 mg/mL, dissolved in water) was added. Subsequently, the tea-tannase mixture was mixed and incubated at 40 °C and 100 μL of the reaction mixture was taken at time points 0, 30, 60, 120, 180, 300, 480, and 1140 min. Samples taken from the reaction mixture were diluted 10 times with methanol containing 1 % (*v/v*) FA to inactivate the enzyme, followed by centrifugation. The supernatant was diluted 4 times with water containing 1 % (*v/v*) FA prior to UHPLC-HRMS analysis. To test the residual tannase activity after 1140 min reaction, a known substrate of tannase, EGCg (0.5 mM) was added to the tea-tannase mixture and the incubation was continued at 40 °C for another 300 min. Samples (100 μL) of this new reaction mixture were taken at time points 0, 120, and 300 min after EGCg addition. The samples were diluted 10 times with methanol containing 1 % (*v/v*) FA, followed by centrifugation. The supernatant was diluted 4 times with water containing 1 % (*v/v*) FA prior to UHPLC-HRMS analysis.

2.4. Analysis of galloylated compounds by ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS)

Samples were separated on a Vanquish UHPLC system (Thermo Scientific, Waltham, MA, USA) equipped with a pump, autosampler, and UV–Vis detector. Accurate mass spectrometric data were acquired using an Orbitrap IQ-X Tribrid mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a heated electrospray ionisation (ESI) probe coupled in-line to the Vanquish UHPLC system. The details of the UHPLC-HRMS analysis are described in Supplementary information IV.

2.5. Identification of galloylated compounds by nuclear magnetic resonance (NMR) spectroscopy

For the structure elucidation of the in-house purified strictinin (1,4,6-tri-*O*-galloyl-β-D-glucose) and theogallin (5-*O*-galloyl-quinic acid), and tannase-treated 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, between 1.2 and 1.5 mg of each tested compounds was dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6) (Sigma-Aldrich). Analysis was performed on a Bruker Avance HD 700 MHz spectrometer (Bruker, Billerica, MA, USA). The details of the NMR analysis are described in Supplementary information IV.

2.6. Anomeric separation of galloyl-glucoses by cyclic ion mobility spectrometry (cIMS)

A Select Series cyclic ion mobility spectrometer (cIMS) (Waters Corporation, Wilmslow, U.K.) was used to assess the presence of different anomeric configurations of glucose in galloyl-glucoses. The samples used for this analysis were glucose standard, 1-*O*-galloyl-β-D-glucose standard, and a sample of tannase-treated 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose. To obtain this latter sample, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose was treated with tannase for 1140-min in an identical way as described for tannase treatment of green tea. All samples were diluted to a concentration of 1 μg/mL. The details of the cIMS analysis are described in Supplementary information IV.

2.7. Quantification of tannase's preference for hydrolysis of different galloylated compounds

The $t_{¼}$ value was introduced to describe the time required for 75 % of the galloylated compounds to be hydrolysed by tannase. Calculation of $t_{¼}$ was performed by prediction using a one-phase decay model based on normalised UHPLC-HRMS peak areas over time in GraphPad Prism 9.3.1 (Boston, MA, USA). For compounds with a straightforward decrease in UHPLC-HRMS peak areas, the initial UHPLC-HRMS peak areas were set as 100 %, with peak areas at subsequent time points expressed relative to the initial peak area. For compounds with a hydrolysis pattern involving an initial increase followed by a decrease or stabilisation after reaching a maximum, this maximum UHPLC-HRMS peak area was set as 100 %, with peak areas at subsequent time points expressed relative to the maximum peak area. The hydrolysis efficiency of each compound was further categorised into one of six levels by corresponding $t_{¼}$: very good (< 30 min), good (30–60 min), medium (60–120 min), poor (120–300 min), very poor (300–1140 min), and 'non-hydrolysable' (> 1140 min).

2.8. Colour analysis of green tea samples

The tannase-treated samples (30, 60, 120, 180, 300, 480, and 1140 min) and blank samples (mixed green tea extracts without tannase treatment) were incubated at 80 °C for 6 h to compare their auto-oxidative browning. A Genesys-150 UV–Vis spectrophotometer (Thermo Scientific, Madison, USA) was used to record a UV–Vis spectrum (400–500 nm, 2 nm intervals) at each timepoint. This wavelength range was specifically chosen for characterizing the yellow-brown colour formed upon flavan-3-ol auto-oxidation (Tan et al., 2020). A high precision quartz cell with a 10 mm light path (Hellma Analytics) was used. The brown colour intensity (BCI) was calculated by integrating the area under the UV–Vis absorbance spectrum in the 400–500 nm range. Baseline correction was applied by subtracting the BCI value of the blank control (non-heated sample without tannase treatment) from the BCI values of all experimental samples. The colour of samples was also visualized by taking an image (Huawei P40, China) of the glass tubes standing next to each other at room temperature under laboratory lighting conditions. The images were evaluated using the $L^*a^*b^*$ colour space (i.e., L^* dark or light, a^* red vs green, b^* yellow vs blue). The values were extracted using image analysis software (Photoshop CC 2021, Adobe). In this procedure, the $L^*a^*b^*$ value was computed based on 5 randomly picked pixels in the image of samples. The difference between the samples and blank sample (without tannase treatment) was calculated as ΔEab according to following equation (Araki et al., 2017).

$$\Delta Eab = \left[\left(L_0^* - L_x^*\right)^2 + \left(a_0^* - a_x^*\right)^2 + \left(b_0^* - b_x^*\right)^2\right]^{1/2}$$

In which L_0^*, a_0^*, b_0^* and L_x^*, a_x^*, b_x^* are the colour space values for the corresponding non-heated blank sample (without tannase treatment) and the tannase-treated or heated blank samples, respectively.

2.9. Statistical analysis

Statistical analysis was performed by SPSS 27 (IBM SPSS Statistics) or GraphPad Prism 9 (GraphPad Software) using one-way ANOVA with Tukey's post hoc test. Data were visualized as the averages of three replicates with standard deviations as error bars on the line or bar graphs.

3. Results and discussion

3.1. Characterisation of hydrolysable galloylated compounds by a combination of UHPLC-HRMS and enzymatic hydrolysis

The (tentative) identification of galloylated compounds and screening of their hydrolysability by tannase was achieved through a combined approach of UHPLC-HRMS and enzymatic hydrolysis.

First, MS^2 fragmentation data from UHPLC-HRMS analysis were screened for peaks with diagnostic fragments and neutral losses (NLs) associated with bound gallic acid (GA) moieties. Typically, galloylated compounds with one, two, or multiple GA moieties exhibit NLs corresponding to loss of the GA moiety (152.01096 u, $C_7H_4O_4$) or the GA moiety plus water (170.02152 u, $C_7H_6O_5$), in combination with a GA fragment (m/z 169.01425, $C_7H_5O_5$) (Tan et al., 2023). The candidate galloylated compounds were (tentatively) identified based on a comparison of their elemental composition and/or fragmentation as determined by HRMS to literature or The Human Metabolome Database (HMDB, https://www.hmdb.ca/), or by comparison of their retention time and spectral data to that of authentic commercial standards or in-house purified standards (Supplementary information I).

Second, treatment with tannase (from *Aspergillus ficuum*) was conducted to confirm whether these GA moieties are hydrolysable. Using the analytical approach developed here, approximately 70 GA-containing compounds (Supplementary information I) with various scaffolds were identified in a mixed green tea. All galloylated compounds with a flavan-3-ol or dimeric flavan-3-ol scaffold, and most of the galloylated compounds with a hexose or organic acid scaffold were confirmed to be hydrolysable (Fig. 2a), but the preference of tannase for the hydrolysis of these compounds varied (Supplementary information II). Based on this experiment, tannase's preference for the (tentatively) identified hydrolysable galloylated compounds was expressed as $t_{¼}$, which represents the time needed for 75 % of the compound to be hydrolysed. For quantitative comparison of tannase's preference for the hydrolysis of different galloylated compounds, they were categorised into five levels of $t_{¼}$: very good (<30 min), good (30–60 min), medium (60–120 min), poor (120–300 min), and very poor (>300 min) (Supplementary information II).

Only 0.1 % of the initial flavanol and 0.2 % dimeric flavanol peak area remained after 300 min of tannase treatment, with complete hydrolysis of these compounds after 1140 min (Fig. 2 and Supplementary information III). However, 43.2 % of hydrolysable tannins (glucose and ellagoylglucose as scaffolds) remained after 300 min, and 33.3 % was still present after 1140 min. For galloylated organic acids, 59.1 % of the peak area remained after 300 min, and 19.9 % was still present after 1140 min. Given the varying preference of tannase (from *Aspergillus ficuum*) for the different scaffold types present in green tea, a systematic study on the substrate scope of tannase from *Aspergillus ficuum* and its hydrolysis characteristics related to galloylated compounds was initiated.

3.2. Tannase exhibits a preference for the epi-forms over the non-epi-forms of flavan-3-ols

The flavan-3-ols ECg and its epimer catechin gallate (Cg), and EGCg and its epimer gallocatechin gallate (GCg) are the major naturally present mono-galloylated flavan-3-ols in mixed green tea, and are responsible for approximately 63.4 % of the total UHPLC-HRMS peak area of galloylated compounds (Supplementary information III). All four galloylated flavan-3-ols were hydrolysed within 300 min, although the $t_{¼}$ values varied (Fig. 3a). Based on the $t_{¼}$ values, the four major flavan-3-ols are categorised as follows: ECg (very good) vs. Cg (medium), and EGCg (good) vs. GCg (poor) (Supplementary information II). Thus, the epi-forms were hydrolysed faster than their respective non-epi counterparts.

3.3. Tannase can hydrolyse monomeric flavan-3-ol digallates and dimeric flavan-3-ol gallates

Flavan-3-ols with two gallate moieties, i.e. monomeric flavan-3-ol digallates, were also present in minor amounts in green tea (1.5 % of the total UHPLC-HRMS peak area of galloylated compounds in mixed

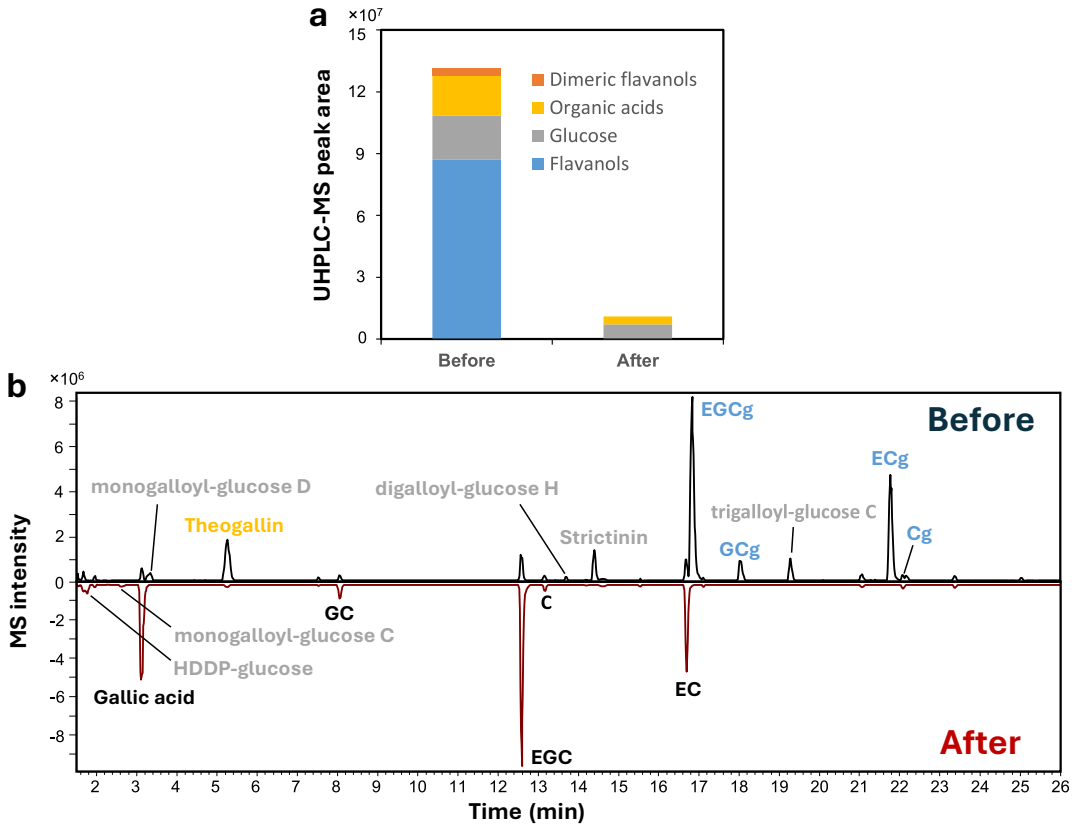


Fig. 2. Effect of tannase treatment on major galloylated compounds present in mixed green tea. (a) Absolute UHPLC-HRMS peak areas of galloylated compounds per scaffold type before and after 1140 min tannase treatment. (b) UHPLC-HRMS base peak chromatograms of mixed green tea before and after 1140 min tannase treatment. (a) was created using the data presented in Supplementary Information III. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

green tea). Monomeric flavan-3-ol digallates were all 'very good' substrates for tannase from *Aspergillus ficuum* with $t_{¼}$ values <30 min (Supplementary information II), generally resulting in close to full hydrolysis within 30 mins of tannase treatment (Fig. 3b). The rapid decrease in flavan-3-ol digallates could be the result of these compounds possessing two GA moieties, with the hydrolysis of either or both of these resulting in loss of the original compound.

Galloylated dimeric flavan-3-ols with one or two gallate moieties were also detected in mixed green tea, accounting for approximately 1.8 % and 1.0 % of the total UHPLC-HRMS peak areas of galloylated compounds, respectively (Supplementary information III and Supplementary information IV, Fig. S2). The detected dimeric flavan-3-ols could be proanthocyanidins or bisflavanols (Engelhardt, 2010). All of these compounds were hydrolysed within 480 min (Fig. 3b). Unfortunately, the exact interflavanic linkage types and stereochemical configurations of these compounds could not be confirmed due to the lack of standards. However, their hydrolysis varied from 'very good' to 'poor' (Supplementary information II), suggesting that these varied structural elements influence enzymatic susceptibility.

3.4. Hydrolysable tannins are not necessarily hydrolysable by tannase

Galloylated compounds with glucose and ellagoylglucose as scaffolds accounted for approximately 16.2 % of the total UHPLC-HRMS peak area of galloylated compounds in mixed green tea (Supplementary information IV, Fig. S2). The most abundant hydrolysable tannin in the mixed green tea sample was identified as 1,4,6-tri-*O*-galloyl-β-D-glucose by comparison with our in-house purified standard (Supplementary information IV, *Appendix A,* Table S1). The compound with most galloyl moieties in mixed green tea was tetragalloyl-glucose, as pentagalloyl-glucose was not detected. Additionally, several digalloyl-glucose and monogalloyl-glucose isomers were also identified (Supplementary information I).

The hydrolysis of tetragalloyl-glucoses (Supplementary information III) and trigalloyl-glucoses (Fig. 4a) resulted in a straightforward decrease (i.e. concentration steadily decreases without any increase throughout the process). Among the three naturally occurring trigalloyl-glucose isomers, 1,4,6-tri-*O*-galloyl-β-D-glucose isomer C was preferred by tannase ('very good' substrate) over isomers A and B, which were classified as 'medium' and 'good', respectively.

The hydrolysis of digalloyl-glucoses exhibited a more diverse trend compared to trigalloyl-glucoses (Fig. 4b). Naturally abundant digalloyl-glucose isomer (H) exhibited a straightforward decrease over time. However, its isomers B, C, D, E, and F, presumably resulting from the hydrolysis of tetra- and trigalloyl-glucoses, initially accumulated during the early stage (0 to 60 min), followed by full hydrolysis within 1140 min.

The hydrolysis pattern of monogalloyl-glucoses appears to be the most complex among the galloylated compounds with a glucose scaffold (Fig. 4c). The naturally abundant monogalloyl-glucose isomer D solely decreased over time. The galloyl-glucose isomers A, B, E, and F accumulated during the early stage (0–120 min) and then slowly decreased after reaching their maximum. Interestingly, monogalloyl-glucose isomer C was not naturally present in the mixed green tea, accumulated, and its content then remained stable. To the best of the authors' knowledge, accumulation of one specific monogalloyl-glucose isomer, in this case isomer C, upon hydrolysis of multi-galloyl-glucoses by tannase was not previously reported. This observation clearly indicates that the hydrolysis of multi-galloyl-glucoses does not necessarily result in a quantitative yield of glucose and free GA. This contradicts the

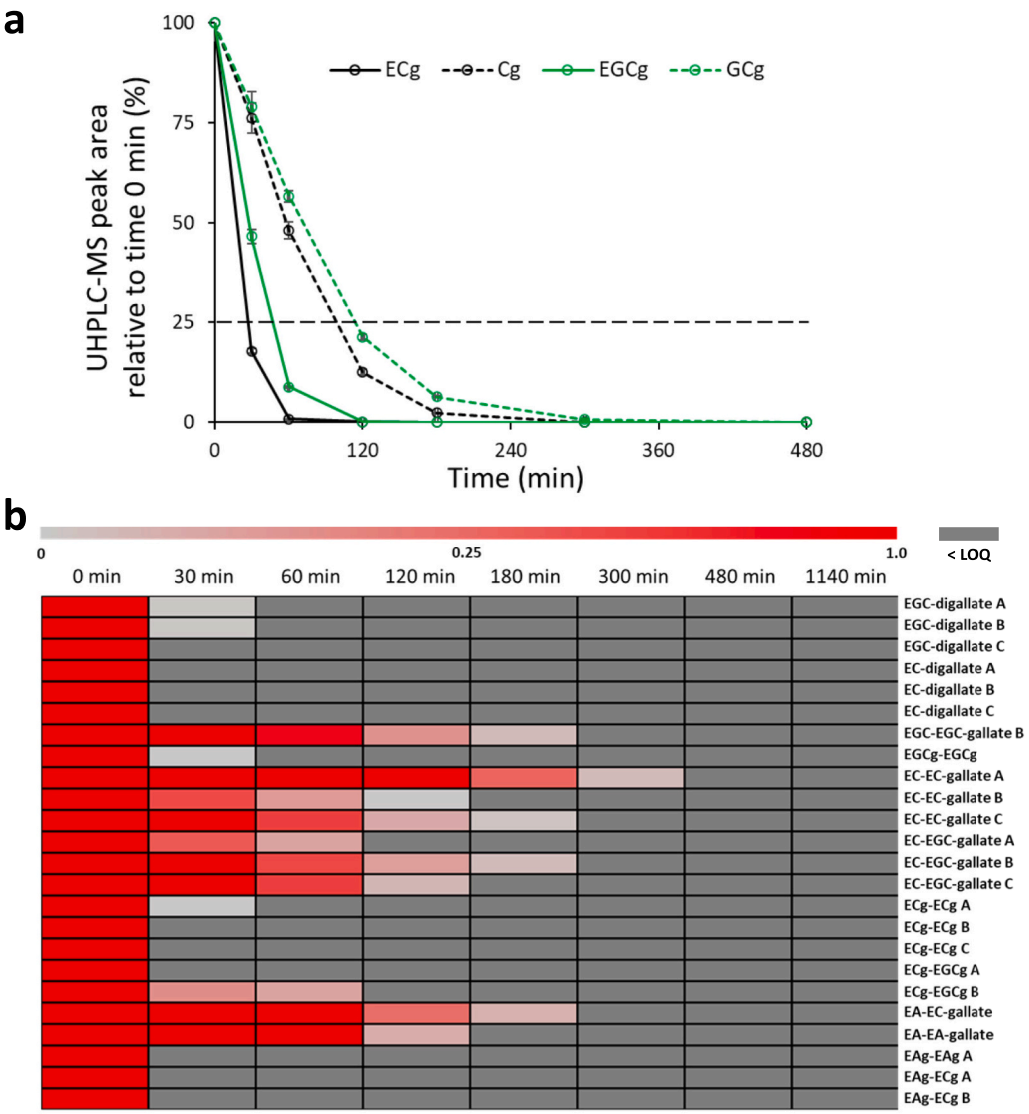


Fig. 3. Straightforward hydrolysis of galloylated flavan-3-ol monomers and dimers. (a) Epimerisation affects the hydrolysis efficiency of EGCg vs. GCg, and ECg vs. Cg. The dashed line in panel (a) represents a visual reference for 75 % reduction in peak area. (b) Heat map illustrating the hydrolysis of monomeric flavan-3-ol digallates and dimeric flavan-3-ol gallates. The colour gradient from red to light grey represents the relative UHPLC-HRMS peak area, whereas dark grey indicates that the concentration was below the limit of quantification (LOQ). Figure data are averages ± standard deviation ($n = 3$) based on the information from Supplementary information III. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conventional suggestion that treatment of galloyl-glucoses with tannase always results in complete hydrolysis and full GA release (Chávez-González et al., 2018; Lekshmi et al., 2021). Our results also indicate that tannase from *Aspergillus ficuum* is unable to hydrolyse the galloyl ester at one specific position on the glucose scaffold. To gain more insight into the positional preference of tannase, we aimed to identify the position of the non-hydrolysable galloyl-group. By comparison with a commercial standard, we could only identify isomer D as 1-*O*-galloyl-β-D-glucose. As this standard did not match with monogalloyl-glucose isomer C, and no other monogalloyl-glucose standards were available, we performed additional experiments to elucidate the position of the gallate moiety on the glucose scaffold for this non-hydrolysable monogalloyl-glucose isomer.

3.5. Elucidating the position of non-hydrolysable gallate substitution on the glucose scaffold

To identify the non-hydrolysable monogalloyl-glucose isomer C, we first performed tannase treatment of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, 1,4,6-tri-*O*-galloyl-β-D-glucose, and 1-*O*-galloyl-β-D-glucose standards (Fig. 5a– c). The 1140-min tannase treatment of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose and 1,4,6-tri-*O*-galloyl-β-D-glucose primarily resulted in formation of several monogalloyl-glucose isomers, with isomer C as the predominant product in both cases (Fig. 5a and b). The 1-*O*-galloyl-β-D-glucose standard was found to be easily hydrolysed (Fig. 5c). Based on these results, we concluded that the glucose scaffold of isomer C should be galloylated on either *C*4 or *C*6.

Next, the tannase-treated 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, which is relatively rich in monogalloyl-glucose isomer C (Fig. 5a), was analysed by NMR (Fig. 5d). The ^{1}H NMR spectrum showed one prominent peak at δ_H 6.92 ppm (s) with an integral 6.7 times larger than the second largest peak at δ_H 6.96 ppm (s), indicating that this peak originated from a compound that is highly abundant and lacks other protons. Thus, this peak was attributed to the two aromatic protons of the free gallic acid that was released during tannase treatment (Supplementary information IV, Fig. S14). Several other aromatic singlets were observed between δ_H 6.85 and 7.05 ppm, likely originating from intermediate galloyl-glucose hydrolysis products. The most abundant of these peaks,

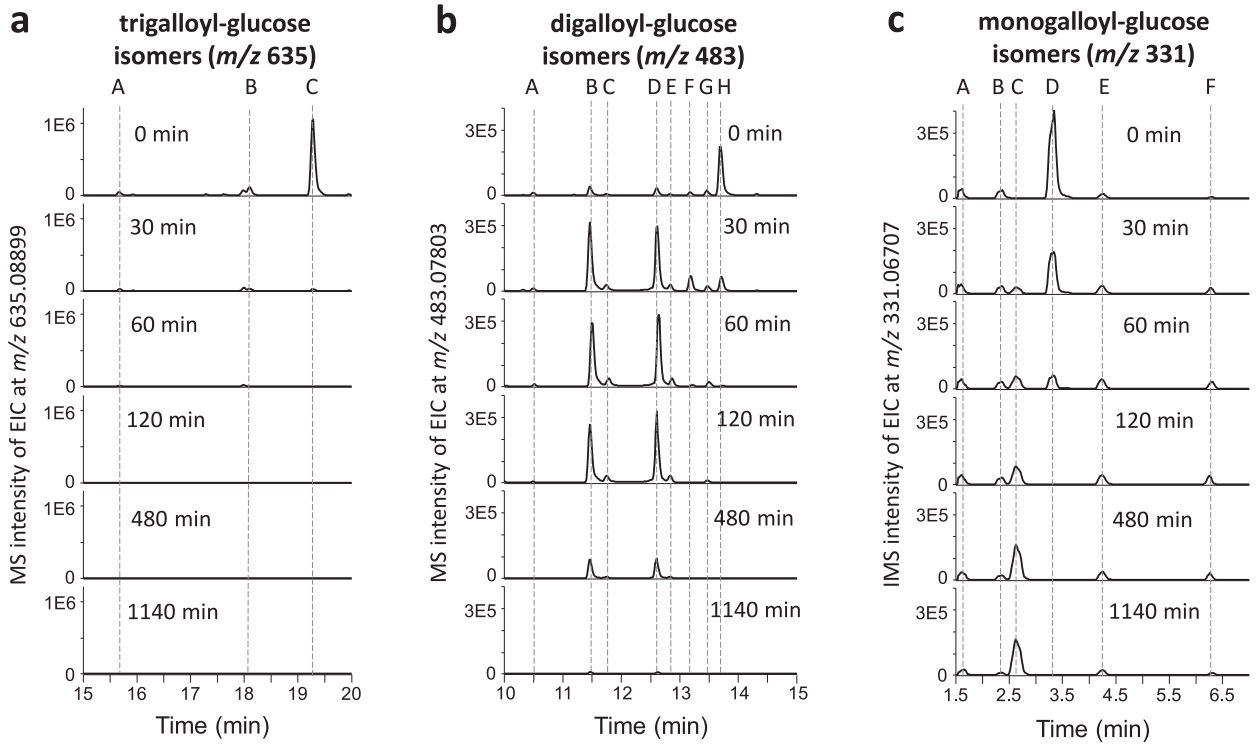


Fig. 4. Varied hydrolysis patterns of hydrolysable tannins from green tea: Tannase preference is affected by position of gallate substitution on the glucose scaffold. Dashed lines with labels A-H in the UHPLC-HRMS extracted ion chromatograms (EIC) of *m/z* 635, *m/z* 483, and *m/z* 331 indicate the corresponding tri-, di-, and monogalloyl-glucose isomers, respectively. a Straightforward decrease of all trigalloyl-glucose isomers. b Naturally abundant digalloyl-glucose isomer H exhibited a straightforward decrease, whereas several other isomers that are generated from trigalloyl-glucoses initially accumulated followed by full hydrolysis within 1140 min. c Naturally abundant monogalloyl-glucose isomer D exhibited a straightforward decrease, whereas monogalloyl-glucose isomer C that is generated from digalloyl-glucoses accumulated. This suggests that the tannase-catalysed hydrolysis of galloyl-glucoses does not always lead to full conversion to glucose and free GA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

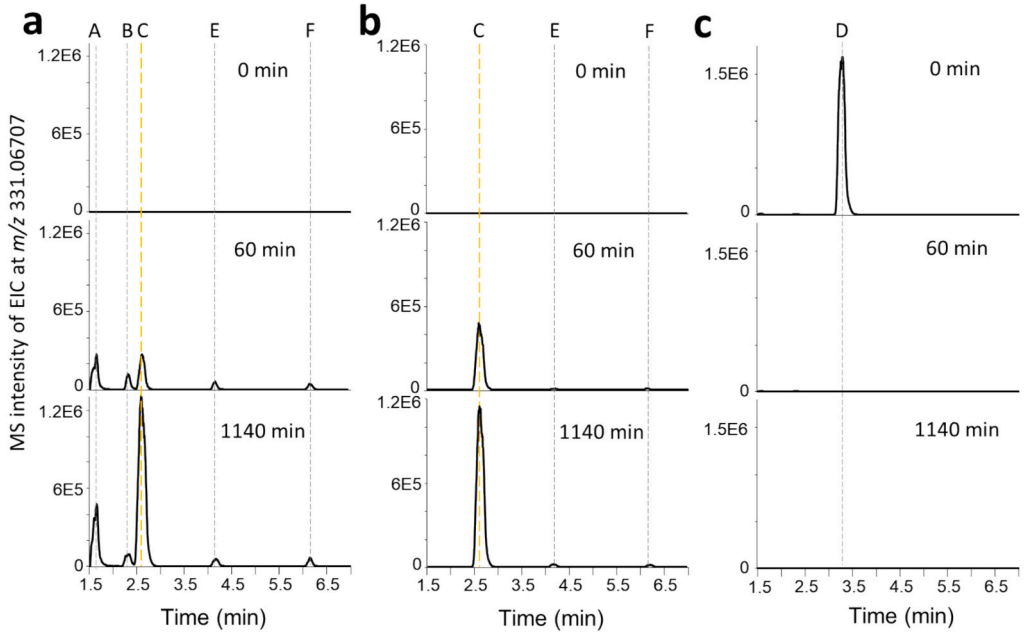
at δ_H 6.96 ppm (s, 2H), was attributed to the major monogalloyl-glucose product observed in Fig. 5a, i.e. monogalloyl-glucose isomer C (HRMS: $C_{13}H_{15}O_{10}$ found for $[M-H]^-$ *m/z* 331.06689 with error − 0.5 ppm). The integral of this peak matched with six other signals [δ_H 4.91 ppm (t, J = 6.5 Hz, 1H); δ_H 4.27 ppm (d, J = 7.7 Hz, 1H); δ_H 3.66 ppm (d, J = 11.9 Hz, 1H); δ_H 3.11 ppm (t, J = 8.4 Hz, 1H); δ_H 3.04 ppm (t, J = 9.4 Hz, 1H); δ_H 2.89 ppm (t, J = 8.7 Hz, 1H)], with 2D NMR confirming that a peak at δ_H 3.42 ppm (below water signal) also belonged to this structure. HSQC correlations from δ_H 3.42 ppm and δ_H 3.66 ppm to δ_C 61.31 ppm indicated that these signals corresponded to the protons of *C*6, the only carbon in the glucose scaffold that possesses two protons. Compared to 1,4,6-tri-*O*-galloyl-β-D-glucose (Supplementary information IV, *Appendix A*, Table S1 and Fig. S3), the protons of *C*6 shifted upfield, indicating that the monogalloyl-glucose isomer C lost its GA moiety at *C*6. The most downfield shift, besides those of the aromatic proton signals in the range of δ_H 6.85–7.05 ppm, was at δ_H 4.91 ppm (t, J = 6.5 Hz, 1H), which suggested that this proton is attached to the carbon whose hydroxyl-group is involved in the ester bond. For comparison, in 1,4,6-tri-*O*-galloyl-β-D-glucose, the carbons whose hydroxyl-groups are involved in the ester bonds had the following corresponding proton signals: H*1* [δ_H 5.72 ppm (d, J = 8.1 Hz, 1H)] and H*4* [δ_H 5.02 ppm (d, J = 9.7 Hz, 1H)] (Table S1), suggesting that the proton at δ_H 4.91 ppm (t, J = 6.5 Hz, 1H) is H*4*. The attachment of the remaining GA moiety at *C*4 was further supported by an HSQC correlation between δ_H 4.91 ppm and *C*4 (δ_C 92.24 ppm) and a HMBC correlation from δ_H 4.91 ppm to δ_C 165.18 ppm (*C*1′ of the gallate moiety). Moreover, we already established that isomer C is not 1-*O*-galloyl-β-D-glucose (Fig. 5a, b, and c). The β-configuration of glucose was confirmed by the coupling constant observed for the anomeric proton *H*1 [δ_H 4.27 ppm (d, J = 7.7 Hz, 1H)] and NOESY correlations from *H*1 to *H*3 [δ_H 3.11 ppm (t, J = 8.4 Hz, 1H)] and *H*5 [δ_H 3.04 ppm (t, J = 9.4 Hz, 1H). Thus, the monogalloyl-glucose isomer that is seemingly non-hydrolysable by tannase (isomer C) is 4-*O*-galloyl-β-D-glucose (Fig. 5a and b). Other signals were annotated based on ^{1}H NMR, ^{13}C NMR, HSQC, NOESY, HMBC, and COSY (Fig. 5e–g).

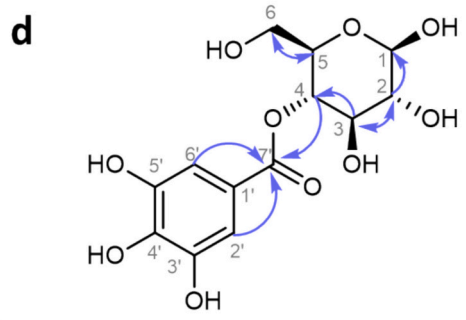
These results demonstrate, for the first time, the distinct differences in hydrolysability by tannase among different positional isomers of galloyl-glucoses. Most notably, the accumulation of 4-*O*-galloyl-β-D-glucose versus the rapid hydrolysis of other monogalloyl-glucoses, such as 1-*O*-galloyl-β-D-glucose, demonstrates that tannase from *Aspergillus ficuum* has a clear preference for hydrolysis of specific galloyl-glucoses based on the position of gallate attachment to the glucose scaffold. Based on our results, the expected order of GA release by tannase, from the easiest to the hardest position, is as follows: *C*1 > *C*6 > *C*4. The preference for release of GA from *C*2 and *C*3 should be between that for *C*1 and *C*4, although the exact order of all five positions cannot be conclusively determined due to the lack of authentic standards for 2-, 3-, and 6-*O*-galloyl-β-D-glucose for comparison. Thus, tannase's substrate preference is more intricate than simply hydrolysing any galloyl-glucose substrate into free GA and glucose, as is traditionally suggested.

3.6. Galloyl-glucose anomers were formed upon tannase-catalysed hydrolysis

There are 5 possible positional isomers of monogalloyl-glucose, yet at least six monogalloyl-glucose isomer peaks (A–F) were detected after 1140-min tannase treatment of mixed green tea (Fig. 4c). Thus, the number of monogalloyl-glucose isomer peaks detected cannot be adequately explained by positional isomerism alone.

It is well-established that glucopyranose in solution can undergo mutarotation to interconvert between its α and β anomer if the *C*1





4-O-galloyl-β-D-glucose

Position	δ_C	δ_H (mult.; *J* [Hz]; int.)
1	96.96	4.27 (d, *J*=7.7, 1H)
2	75.02	2.89 (t, *J*=8.7, 1H)
3	76.88	3.11 (t, *J*=8.4, 1H)
4	92.24	4.91 (t, *J*=6.5, 1H)
5	70.38	3.04 (t, *J*=9.4, 1H)
6	61.27	3.42 (below water peak), 3.66 (d, *J*=11.9, 1H)
1'	165.18	-
2'	119.55	-
2' + 6'	108.8	6.96 (s, 2H)
3' + 5'	145.56	-
4'	138.51	-

Note: mult = multiplicity, int. = number of protons based on peak integral, s = singlet, d = doublet, and t = triplet.

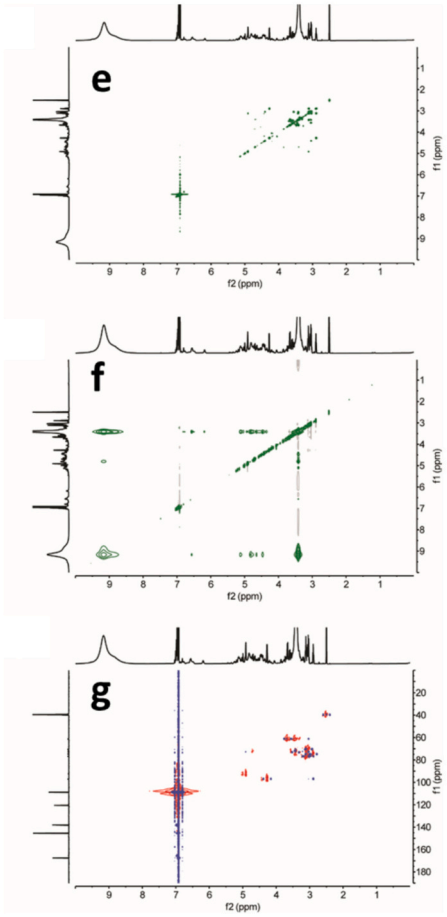


Fig. 5. Tannase-catalysed hydrolysis of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose and 1,4,6-tri-*O*-galloyl-β-D-glucose both resulted in accumulation of monogalloyl-glucose isomer C and NMR analysis confirmed that 4-*O*-galloyl-β-D-glucose is the non-hydrolysable monogalloyl-glucose isomer (i.e. isomer C). EIC of *m/z* 331 corresponds to monogalloyl glucoses. Dashed lines with labels A-F indicate the monogalloyl-glucose isomers, with the yellow dashed line corresponding to isomer C. a Monogalloyl-glucoses detected from hydrolysis of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose. b Monogalloyl-glucoses detected from hydrolysis of 1,4,6-tri-*O*-galloyl-β-D-glucose. c Rapid hydrolysis of 1-*O*-galloyl-β-D-glucose standard. d ^{1}H (700 MHz) and ^{13}C (176 MHz) NMR spectral data for 4-*O*-galloyl-β-D-glucose in DMSO-d_6. Key HMBC correlations (blue arrows) are shown in the structure. Assignments were based on ^{1}H and ^{13}C NMR signals and 2D spectra from e COSY, f NOESY, and g HMBC (blue) and HSQC (red). Full size 2D NMR spectra are shown in Supplementary information IV, *Appendix B*, Fig. S7 and S8. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydroxyl-group is unsubstituted (Angyal, 1984; Kaufmann et al., 2018). Due to the rapid hydrolysis of 1-*O*-β-D-galloyl-glucose (Fig. 5c), we hypothesised that the majority of galloyl-glucose hydrolysis products are unsubstituted at *C*1 and can thus undergo mutarotation from the naturally present β anomer to the corresponding α anomer, thereby explaining the additional peaks observed in our tannase-treated samples.

To test this hypothesis, cyclic ion mobility spectrometry (cIMS) was employed, the results of which are presented in Supplementary information IV, *Appendix C*, Fig. S9. The cIMS results confirmed our hypothesis that galloyl-glucoses with an unsubstituted *C*1 position can freely mutarotate, resulting in an anomeric equilibrium, much like that of free glucose. Based on this finding, we theorize that up to 9 monogalloyl-glucose isomers, including positional isomers (gallate at *C*1, *C*2, *C*3, *C*4, or *C*6) and anomers (when gallate is attached at *C*2, *C*3, *C*4, or *C*6) could be formed from the hydrolysis of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose. In the current study, we detected six of these nine theoretical isomers of monogalloyl-glucose in mixed green tea. Besides this, we detected a complex product profile containing both positional isomers and anomers of di-, tri- and tetragalloyl-glucoses due to anomeric inversion of their glucose scaffold after tannase-catalysed release of the gallate moiety at *C*1. Based on our results, the number of galloyl-glucose isomers observed in tea and other plants (Gómez-Caravaca et al., 2016; Liu et al., 2019) can now be explained by the presence of both positional isomers and anomers.

3.7. The hydrolysis pattern of galloyl-HHDP-glucoses is similar to that of galloyl-glucoses

Galloylated compounds with ellagoylglucose, i.e. hexahydroxydiphenoyl-glucose (HHDP-glucose), as a scaffold accounted for approximately 6.0 % of the total UHPLC-HRMS peak area of galloylated compounds in mixed green tea. The most abundant naturally occurring galloyl-HHDP-glucose in the mixed green tea was identified as 1-*O*-galloyl-4,6-HHDP-β-D-glucose (galloyl-HHDP-glucose isomer E), commonly referred to as strictinin, by comparison with our in-house purified standard (Supplementary information IV, *Appendix A*, Fig. S4 and Table S2). The HHDP moiety consists of a *C*-*C*-linked gallate dimer attached to the glucose scaffold by two ester bonds (Fig. 1d, strictinin). Besides 1-*O*-galloyl-4,6-HHDP-β-D-glucose, four other naturally occurring galloyl-HHDP-glucose isomers (A, B, C, and D) and a digalloyl-HHDP-glucose were also detected (Supplementary information I). The hydrolysis of these mono- and digalloyl-HHDP-glucoses resulted in a straightforward decrease, demonstrating that the HHDP moiety does not

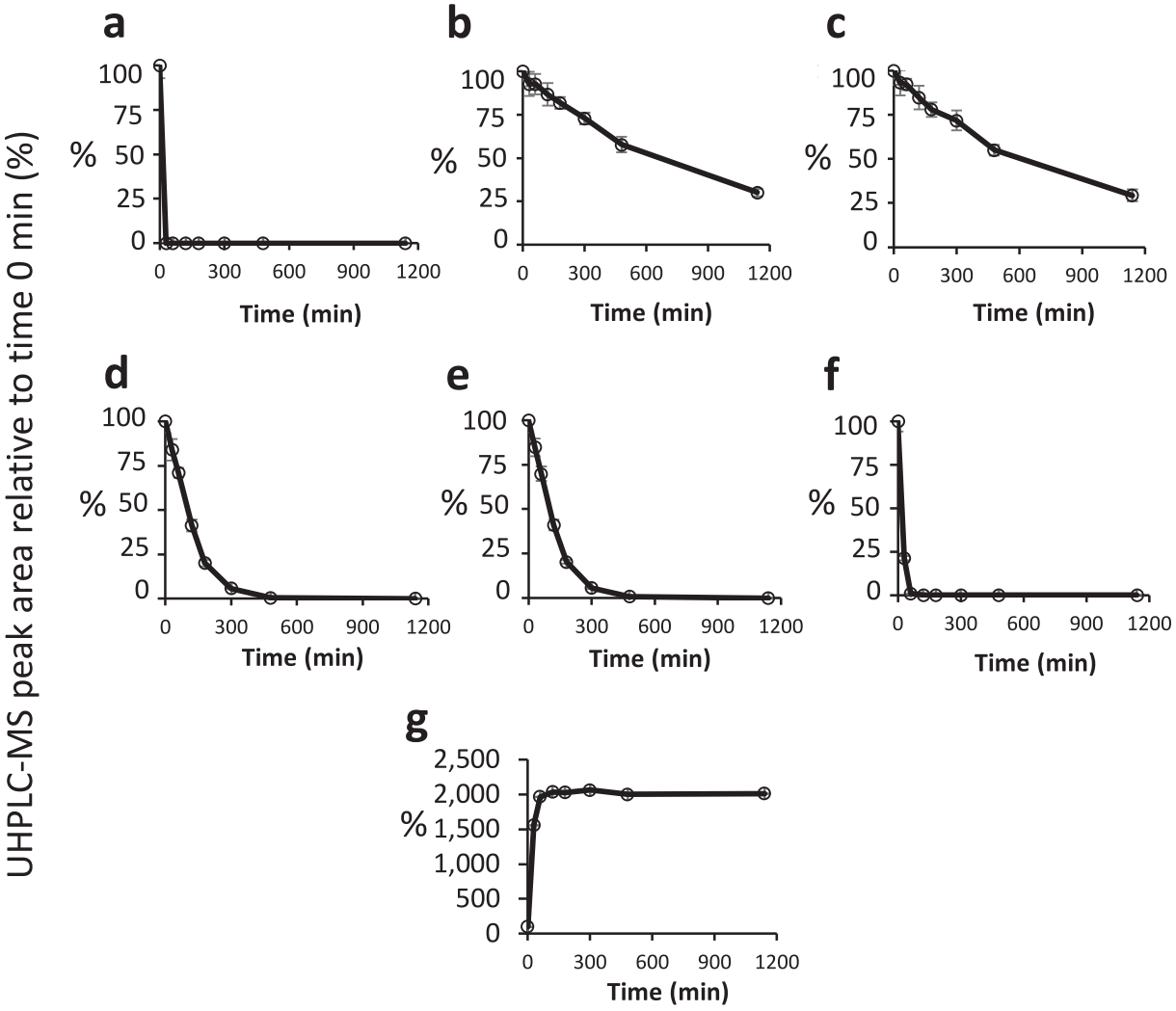


Fig. 6. Tannase-catalysed hydrolysis of galloyl-HHDP-glucoses from mixed green tea over time. (a) Corresponds to relative UHPLC-HRMS peaks areas of digalloyl-HHDP-glucose; (b)–(f) correspond to galloyl-HHDP-glucose isomers A, B, C, D, and E, respectively; and (g) shows accumulation of HHDP-glucose. Data are shown as averages ± standard deviation ($n = 3$) based on the information from Supplementary information III. Error bars indicate the standard deviation of triplicates of tannase treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hinder tannase-catalysed hydrolysis (Fig. 6). Galloyl-HHDP-glucose isomer E (strictinin) is a 'very good' substrate ($t_{3/4}$ = 26 min) and it was completely hydrolysed by tannase within 60 min, which is significantly faster than hydrolysis of its less abundant isomers A and B ('very poor'), and C and D ('poor'). These results indicate that hydrolysis of gallate esters attached to a HHDP-glucose scaffold is position-selective, similar to what was observed for the hydrolysable tannins with a glucose scaffold. This result matches well with our previous result that the GA moiety at *C*1 of galloyl-glucoses is easily hydrolysable by tannase.

To clarify whether the decrease in HHDP-glucoses solely resulted from the release of the gallate moiety or also from release of the HHDP moiety, the tannase-catalysed hydrolysis behaviour of naturally occurring HHDP-glucose was investigated. The concentration of HHDP-glucose was found to continuously increase during the first 60 min of tannase treatment and then remained stable (Fig. 6g). This indicated that the HHDP moiety in HHDP-glucoses could not be released from the glucose scaffold.

3.8. Tannase is also able to hydrolyse gallate esters from organic acid scaffolds

Organic acid scaffolds approximately contribute 14.7 % to the total UHPLC-HRMS peak area of galloylated compounds (Supplementary information IV, Fig. S2). The quinic acid scaffold is the major organic acid scaffold observed for galloylated compounds in the mixed green tea samples. The most abundant isomer was identified as 5-*O*-galloyl-quinic acid (Fig. 1c, theogallin) by comparison with our in-house purified standard (Supplementary information IV, *Appendix A*, Fig. S5, S6 and Table S3). Additionally, two galloyl-quinic acid isomers (3-*O*-galloyl-quinic acid and 4-*O*-galloyl-quinic acid) and two digalloyl-quinic acid isomers (A and B) were tentatively identified based on diagnostic fragment ratios reported in literature (Supplementary information I) (Clifford et al., 2007; Narváez-Cuenca et al., 2012). The hydrolysis of galloyl-quinic acids showed a slow but constant decrease over time, with their $t_{¼}$ values classifying them as 'very poor' substrates (Supplementary information IV, *Appendix D*, Fig. S10a and Supplementary information II). Among them, 5-*O*-galloyl-quinic acid (theogallin) was preferred by tannase over 3-*O*-galloyl-quinic acid and 4-*O*-galloyl-quinic acid. Thus, position-dependent preference for tannase-catalysed hydrolysis also occurs with the quinic acid scaffold.

Two gallic acid-gallate isomers (A and B) were tentatively identified (Supplementary information I), indicating that GA itself can serve as a scaffold (Supplementary information IV, *Appendix D*, Fig. S11a). The gallic acid-gallates (isomers A and B) were rapidly hydrolysed, classifying them as 'very good' substrates (Supplementary information IV, *Appendix D*, Fig. S10b and Supplementary information II). Ellagic acid also consists of two galloyl moieties, yet these are linked by a C—C bond in addition to two intramolecular ester bonds (Supplementary information IV, *Appendix D*, Fig. S11b). Its stable UHPLC-HRMS peak area over 1140-min of tannase treatment (Fig. S10b and Supplementary information II) suggests that ellagic acid is likely not a substrate for tannase.

To determine whether the ester bonds between organic acids other than gallic acid and a scaffold can also be hydrolysed by tannase, additional compounds containing such ester bonds (e.g. caffeoylquinic acids and coumaroylquinic acid), were monitored during the tannase treatment (Supplementary information IV, *Appendix E*, Fig. S12). Based on these results, we concluded that caffeic acid and coumaric acid esters cannot be hydrolysed from the quinic acid scaffold by tannase from *Aspergillus ficuum*.

3.9. Tannase is capable of hydrolysing methylgallate esters

We also tentatively identified three naturally occurring methyl-galloylated flavan-3-ols and one methylgalloyl-quinic acid (Supplementary information I). Methylgalloylated phenolics only contribute approximately 0.1 % of the total UHPLC-HRMS peak area of galloylated compounds in this mixed green tea, which is relatively low compared to some reports of high methylgalloylated tea varieties (Jin et al., 2023). It has not been previously investigated whether methylgallate is releasable by tannase. In this study, we show, for the first time, that methylgallate esters can be hydrolysed by tannase (Supplementary information IV, *Appendix F*, Fig. S13). Tannase's preference for methylgalloylated flavan-3-ols ranges from 'medium' to 'very good' (Supplementary information II) likely mainly influenced by their flavan-3-ol scaffold, which is in line with what we observed for 'normal' galloylated flavan-3-ols. Methyl-galloyl-quinic acid was hydrolysed slowly, classifying it as a 'very poor' substrate ($t_{¾}$ > 300 min), following a similar pattern to that of 'normal' galloyl-quinic acids. Thus, our results demonstrate that methylation of the galloyl moiety does not have a large impact on the hydrolysability of the ester bond to various scaffolds by tannase from *Aspergillus ficuum*.

3.10. Browning is significantly reduced but not completely inhibited by tannase-catalysed gallic acid release

Given that GA can be released from most scaffolds by tannase, we were particularly interested in determining the extent of pH decrease that can be achieved through tannase-catalysed GA release. Tannase treatment over 1140 min led to a significant increase in GA concentration from an initial 0.8 mM–7.3 mM, with a concurrent pH decrease from 5.8 to 4.6 (Fig. 7a). The majority of total GA release (53.9 %) occurred very rapidly, within the first 30 min, reducing the pH to 4.9. The total GA release reached more than 90 % within 5 h (300 min) of tannase treatment, already reaching the final pH of 4.6. Longer tannase treatment did not result in a significant further increase in GA concentration or decrease in pH (Fig. 7a). An additional experiment was performed to verify that tannase was active for the entire duration of the treatment. EGCg (a known tannase substrate) was added to the tannase-treated mixed green tea sample after 1140 min of reaction. EGCg was completely hydrolysed within 120 min of its addition (Supplementary information IV, Fig. S15), demonstrating that there was considerable residual tannase activity after the 1140-min reaction, thereby excluding that loss of tannase activity was the reason for reaching a plateau in GA concentration and pH after 300 min.

Overall, we estimate that a 1140-min tannase treatment resulted in release of 91.7 % of the total gallate content from the GA-bank of the mixed green tea, based on the observed decrease in total UHPLC-HRMS peak area of galloylated compounds in this sample. Even if it would be possible to hydrolyse the remaining galloylated compounds (i.e. non-hydrolysable compounds in the GA-bank, such as 4-*O*-galloyl-D-glucose and HHDP-glucose) would not release sufficient free GA to result in a significant further decrease in pH, as the 1140-min tannase treatment already lowered the pH to 4.6, which is close to the pK_a of the carboxylic acid group of GA (pK_a = 4.4) (Fernandes & Salgado, 2016). This is close to the desired pH below 4.0 that is required for complete prevention of auto-oxidative browning of flavan-3-ols in model systems and green tea (Tan et al., 2025).

The tannase-treated samples and blank samples (without tannase treatment) were incubated at 80 °C for 6 h to evaluate their auto-oxidation. The images presented in Fig. 7b–e clearly demonstrate that 1140-min tannase treatment had an inhibitory effect on heating-induced auto-oxidative browning. The pH values at each tannase treatment time were positively correlated with both the brown colour intensity (BCI) (R^2 = 0.93) and Δ*Eab* (R^2 = 0.88) values. This is in line with the observation from our previous work that free GA primarily inhibits auto-oxidation via its pH lowering effect (Tan et al., 2025). Despite this, heating after tannase treatment still resulted in increases in BCI and Δ*Eab* values for all samples, indicating that browning could not be fully inhibited (Fig. 7f and g). Following extended tannase treatment, the brown colour increased from 300 to 480 min. This may be due to the prolonged incubation at 40 °C after reaching the minimum pH, which promoted auto-oxidative browning, while there was no longer a

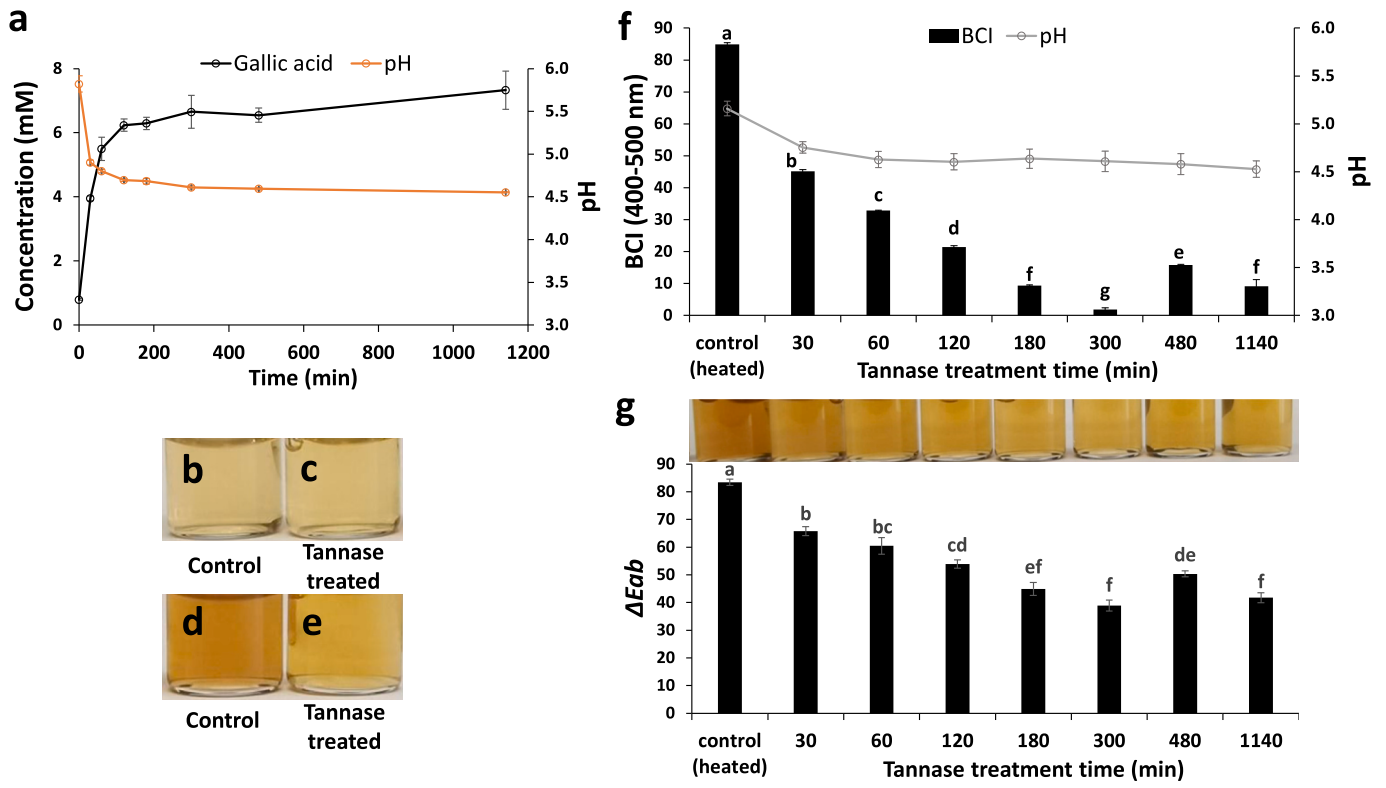


Fig. 7. Effect of tannase treatment over 1140 min on colour, pH, and GA concentration of mixed green tea infusion. (a) Concentration of GA (black line, primary y-axis) and pH (orange line, secondary y-axis) during tannase treatment for 1140 min. Representative images of mixed green tea (b) control (without tannase treatment) and c with tannase treatment for 1140 min. (d and e) respectively, are samples (b) and (c) after heat treatment at 80 °C for 6 h to simulate accelerated auto-oxidation during shelf-life. f Baseline-corrected brown colour intensity (BCI) values of samples heated after tannase treatment (black bars, primary y-axis) and pH of each sample (grey line, secondary y-axis). (g) Baseline-corrected *ΔEab* values and representative images of samples heated after tannase treatment. Data are shown as averages ± standard deviation ($n = 3$). Different letters in (f) and (g) indicate a significant difference in the values compared to the other samples (Tukey's test, $p < 0.05$). Error bars indicate the standard deviation of triplicates of tannase treatment or triplicates of heat treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

corresponding pH decrease. The slight decrease in browning observed in the green tea infusion during even longer incubation (from 480 min to 1140 min) may be caused by further oxidation of phenolics resulting in formation of oligo- and polymeric products with reduced solubility. Considering that the minimum pH value and maximum browning inhibition was reached at 300 min, longer treatment times are not necessary. Moreover, such long tannase treatment times are impractical in industry and, thus, not recommended for future experiments. Overall, we conclude that tannase treatment was able to limit discolouration, reaching a maximum browning inhibitory effect at around 300 min.

3.11. *New insights into the substrate scope and preference of tannase from Aspergillus ficuum*

Our goal was to explore a clean-label approach to extend the shelf-life of RTD green tea by tannase-catalysed release of gallic acid (GA) to inhibit auto-oxidative browning. To effectively implement this strategy, a comprehensive overview of the galloylated compounds present in green tea, as well as detailed understanding of the ability of tannase to hydrolyse the galloylated compounds with various scaffolds is crucial.

Our findings provide a more thorough characterisation of the substrate scope of tannase from *Aspergillus ficuum* than what was previously described. For example, we observed that this enzyme exhibited a distinct preference for the epi- over the non-epi-form of flavan-3-ols, and that it was unable to hydrolyse several hydrolysable tannins. Our detailed insights into the substrate scope and preference of tannase from *Aspergillus ficuum* are summarised in Fig. 8. The enzyme was able to release both gallate and methylgallate moieties that were attached with a single ester bond to various scaffolds, whereas hexahydroxydiphenoyl (HHDP or "ellagoyl") moieties attached to glucose via two ester bonds were not hydrolysable. Release of methylgallate moieties by tannase has not been previously reported, thereby, our findings extend the knowledge of this enzyme's substrate scope. Substrate hydrolysability and preference of tannase was also strongly dependent on the scaffold and the position of gallate attachment (Fig. 8). The observed effect of the position of gallate attachment on the glucose and quinic acid scaffolds illustrates that tannase-catalysed hydrolysis is much more complicated than simply releasing free GA from any scaffold, as suggested before (Aguilar et al., 2007; Chávez-González et al., 2012; Dhiman et al., 2018; Dhiman & Mukherjee, 2022).

3.12. *Considerations for the application of tannase treatment for auto-oxidative browning inhibition*

For consumers, the main advantage of using tannase treatment to control the auto-oxidative browning of beverages or other food products is the potential extension of products' shelf life, via stabilisation of desirable colour and appearance, without the need for additives. The ability to use enzymes like tannase to improve stability aligns with market demands for more natural and high-quality beverages. For industrial implementation, several challenges must be addressed or demand further investigation. These include achieving cost-effective enzyme use, potentially through immobilization technology to enable reuse, while ensuring enzyme stability and activity during processing. The impact of tannase treatment on the final beverage's flavour profile

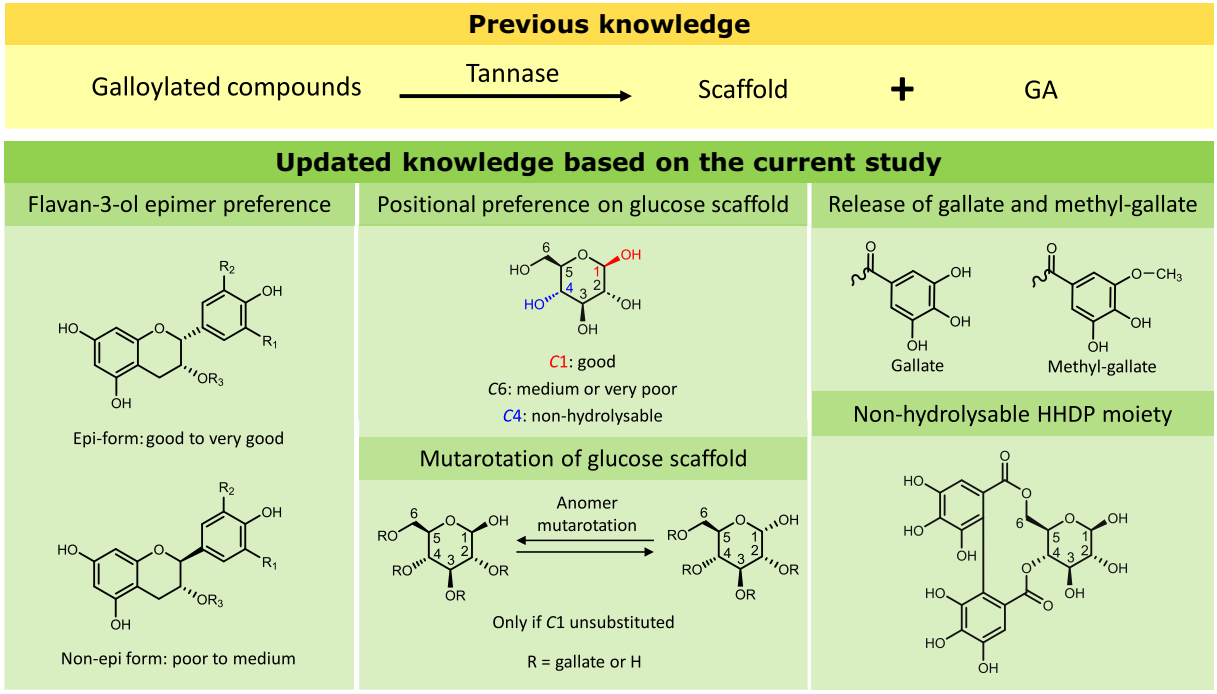


Fig. 8. An updated understanding of the ability and preference of tannase from *Aspergillus ficuum* to hydrolyse galloylated compounds with various scaffolds. The hydrolysability was categorised based on $t_{½}$: very good (<30 min), good (30–60 min), medium (60–120 min), poor (120–300 min), very poor (300–1140 min), and 'non-hydrolysable' (>1140 min).

must also be thoroughly evaluated to ensure consumer acceptance. Furthermore, navigating regulatory hurdles will be essential. In this research, we used tannase from *Aspergillus ficuum*, which is commercially available for research purposes (from Sigma-Aldrich). However, *A. ficuum* does not currently have generally recognised as safe (GRAS) status. For industrial application, it is recommended to use tannase from an organism with GRAS status, such as tannase from *Aspergillus niger*. *A. niger* is approved for use in food in the United States (under GRAS status), China (GB 2760), and the European Union (authorized under EC 1332/2008).

4. Conclusion

One of the challenges in RTD beverage processing and storage is to preserve the desirable light colour of the product, while minimizing the use of additives. Therefore, we aimed to investigate whether tannase-catalysed GA release can be used to inhibit auto-oxidative browning in RTD green tea. Based on in-depth analysis of green tea by a combination of UHPLC-HRMS, NMR, and enzymatic hydrolysis, 75 galloylated compounds with four scaffold types were (tentatively) identified. These analyses provided a much more comprehensive overview of galloylated compounds in green tea than has been reported to date. Hydrolysis of these galloylated compounds by tannase was affected by the scaffold type, scaffold stereo configuration, position of gallate attachment, and number of gallate moieties. Tannase was able to catalyse the release of more than 90 % of naturally present bound gallic acid, thereby resulting in a decrease in the pH of green tea to approximately pH 4.6, which is close to the desired pH for effective inhibition of auto-oxidative browning.

Based on the results of our study, tannase-catalysed gallic acid release is a viable approach to limit discolouration of RTD green tea, thereby by offering a clean-label alternative to extend its shelf-life. While tannase treatment alone was able to significantly reduce auto-oxidative browning, achieving complete inhibition of browning will require combining this approach with other browning inhibition strategies. An integrated strategy including tannase treatment could be more broadly applied to effectively inhibit the auto-oxidation of flavan-3-ols in beverages and other food products, thereby reducing reliance on additives.

CRediT authorship contribution statement

Junfeng Tan: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mark G. Sanders:** Methodology, Investigation, Data curation. **Janniek H. Ritsema:** Visualization, Methodology, Investigation. **Bram van de Put:** Methodology, Investigation. **Jean-Paul Vincken:** Writing – review & editing, Supervision, Funding acquisition. **Zhi Lin:** Supervision, Funding acquisition, Conceptualization. **Wouter J.C. de Bruijn:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Junfeng Tan reports financial support was provided by The China Scholarship Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Part of the presented results were obtained using a Thermo Scientific Vanquish coupled to Orbitrap IQ-X Tribrid LC-MS system which are owned by WUR-Shared Research Facilities. Investment by WUR-Shared Research Facility was made possible by the 'Regio Deal Foodvalley'.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.147775.

Data availability

All processed data needed to evaluate the conclusions presented in this work are presented in the manuscript and in Supplementary information I-IV. Raw data are available on request.

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