



Delivering Phenolic Acids in Soy Protein Gels: Noncovalent Interactions Control Gastrointestinal Bioaccessibility

Marina Marinea^{1,2} · Ashling Ellis^{1,2} · Matt Golding^{1,2} · Simon M. Loveday^{1,3}

Received: 24 August 2022 / Accepted: 17 October 2022
© The Author(s) 2022

Abstract

This study aimed to explore how the delivery of added phenolic acids was affected by the soy protein gel matrix using the INFOGEST static *in vitro* digestion protocol. Gels were prepared by two consecutive heating steps and by adding glucono- δ -lactone (GDL) as an acidifier or magnesium sulphate (MgSO_4) as a salt coagulant. The addition of phenolic acids in GDL gels doubled their elastic modulus (G') ($p < 0.05$), without showing the same effect on MgSO_4 gels. Nevertheless, the bioaccessibility of phenolic acids was not significantly different between the gel matrices ($p > 0.05$). The release of all phenolics was almost complete ($> 80\%$) at the oral phase (pH 7) and significantly lower at gastric phase (pH 3), then at intestinal phase, the release was either increased or significantly reduced depending on the phenolic acid structure. The results of this study suggest that the bioaccessibility of the added phenolic acids is controlled by their interactions with the soy protein gels rather than the protein digestion kinetics of the gels.

Keywords Silken tofu · Soy protein gel · Phenolic acids · Functional foods · Phenolics' bioaccessibility

Introduction

An increasing tendency towards healthier food products has been observed in the last few years. Functional foods are food products with demonstrated physiological benefits that promote optimal health. Developing physically stable products with acceptable sensory characteristics and

demonstrated bio-functionality are some of the technological challenges relevant to functional products.

Phenolics are a large class of phytochemicals with various health benefits, such as antioxidant potency, that have been studied for improving the techno- or bio-functional properties of food products. Nevertheless, most of the phenolic compounds are poorly absorbed by the human body [1]. According to some studies, the recovery of phenolics in the urine, which is a rough estimate of the maximum plasma concentration [2], varies depending on their chemical structure [1]. The lowest excretion concentrations were found for anthocyanins (0.005–0.1 %), while the highest were found for caffeic and ferulic acid (5–27 %) [2, 3].

In this study, we selected two types of soy protein isolate (SPI) unpressed gels as a food matrix for studying the release of added phenolic acids. Phenolic acids were selected as model bioactives to probe the effect of chemical structure on gel matrix interactions. This type of soy protein gels represents an existing food format, silken tofu, which has a very soft texture and it is a popular soy product in Asian countries. Due to the industrial significance of soy, the gelation of soy proteins has attracted much attention over the years. Although studies have focused on the complexation of fat-soluble bioactives (e.g. curcumin) into soy protein

✉ Marina Marinea
marinea.marina@gmail.com

Ashling Ellis
a.ellis@massey.ac.nz

Matt Golding
m.golding@massey.ac.nz

Simon M. Loveday
simon_loveday@sifbi.a-star.edu.sg

¹ Riddet Institute, Massey University, Private Bag 11 222, Palmerston North 4474, New Zealand

² School of Food and Advanced Technology, Massey University, Tennent Drive, Massey University, Palmerston North 4474, New Zealand

³ Singapore Institute of Food and Biotechnology Innovation, Agency for Science, Technology and Research, 138673 Singapore, Singapore

nanoparticles [4], there are only a few articles that study the potential use of soy protein gels as a delivery system.

For riboflavin delivery, Maltais et al. produced cold-set SPI hydrogels with either filamentous or particulate microstructure, using different calcium chloride concentrations [5]. The authors studied the effect of riboflavin on the mechanical properties of gels and its release profile during *in vitro* gastrointestinal conditions [5, 6]. Results showed that the addition of riboflavin weakened the mechanical properties of the gels, because of crystal formation. Interestingly, filamentous gels delayed the release of the bioactive during simulated intestinal conditions. In contrast, particulate gels gave a more rapid release, which was attributed to a faster riboflavin diffusion through the particulate gel structure as a result of higher porosity [5].

In our previous study, we tested if model systems of firm tofu, pressed SPI gels, induced by GDL and MgSO_4 could be used as a successful delivery system for phenolic acids [7]. The pressing step during the production of firm tofu-like gels induced significant changes to the composition of the gels, including losses of the added bioactives. Although phenolic acids were only partially retained in the gel after pressing (35–59 %), their final concentration in the gels was increased (depending on the gels' yield and the phenolics structure) [7]. Addition of phenolics in pressed gels did not deteriorate the mechanical properties of the gels. Also, phenolic acids were bioaccessible by approximately 80 % in the intestinal phase, under *in vitro* conditions, with GDL-induced gels delivering significantly higher phenolic acid concentration than the MgSO_4 -induced gels [7].

This work aimed to study the interactions between phenolic bioactives and soy protein gel matrix using two distinct gelation mechanisms (GDL- and MgSO_4 -). The selected gelation inducers are commonly used in tofu making and they produce tofu with different textural characteristics and thus, it will allow to investigate the effect of gel matrix on phenolics' bioaccessibility. Lastly, we selected a homologous series of structurally-similar phenolic acids to investigate the structure-bioaccessibility relationship of phenolic acids embedded in soy protein gels.

Materials and Methods

Soy protein isolate (Wilpro G300) was purchased from Wilmar International (Singapore). The following chemicals were purchased from Sigma-Aldrich Co. (New Zealand): glucono- δ -lactone (GDL), magnesium sulfate ($\text{MgSO}_4 \times 7\text{H}_2\text{O}$), protocatechuic acid (37580, purity ≥ 97 %), o-coumaric acid (o-CMA) (H22809, purity 97 %), p-coumaric acid (p-CMA) (H52406, purity 98 %), m-coumaric acid (m-CMA) (H23007, purity 99 %), vanillic acid (VNA) (94779, purity 97 %), gallic acid (GLA) (G7384, purity 98.5

%), caffeic acid (CFA) (C0625, purity 98 %), ferulic acid (FRA) (128708, purity 99 %), pepsin (P7000), pancreatin 8 x UPS (P7545), and porcine origin bile extract (B8631). Ethanol, methanol, and trifluoroacetic acid were HPLC grade and they were purchased from Merck (New Zealand).

SPI Composition

The SPI powder contained 87.76 ± 0.05 % (w/dw) protein, 5.23 ± 0.04 % (w/w) moisture, ≤ 1.0 % (w/w) fat and ≤ 6.0 % (w/w) ash. SPI powder was firstly suspended to water at ambient temperature using an UltraTurrax at 10,000 rpm. Then, the dispersion was magnetically stirred overnight at 4 °C.

Unpressed Soy Protein Gels (Silken Tofu Model System)

A two-step heating method was used for the gelation of the SPI. Initially, 250 g of 4.5 % (w/w) SPI dispersion was heated in a water bath at 80 °C for 15 min (**pre-treatment step**) to unfold the soy protein [8–10] and subsequently cooled at room temperature (approximately 25 °C). Then, a concentrated ethanolic solution of different phenolic acids (300 mmol/l) was added to the SPI dispersion, and the mixture was vortexed for 5 minutes at ambient temperature. Fresh, concentrated GDL (950 mmol/kg) or MgSO_4 (730 mmol/kg) solution was added into the mix and was vortexed for 5–10 seconds (**coagulation step**) and subsequently heated at 80 °C for 30 min in a water bath (**gelation step**). The final concentrations of coagulants were 17.12 ± 0.09 mmol/kg for GDL and 7.09 ± 0.05 mmol/kg for MgSO_4 , while the concentration of phenolic acids was 3.44 ± 0.04 mmol/kg, which corresponds to approximately 53 to 67 mg/ 100 g of tofu depending on the phenolic acid and it is equivalent to 1 to 67–85 on percentage basis ratio between phenolic acid to soy proteins. The selected phenolic acid concentration is within the concentration range found in fruits in vegetables [11]. For the sections “Characterisation of gels” and “Effect of the coagulant on PA bioaccessibility”, the phenolic acids studied were PCA and o-CMA. While in section “PA structure and release profile relationship”, various phenolic acids were tested (VNA, GLA, FRA, CFA, p-CMA and m-CMA). The structures, pKa and logP values of the used phenolic acids can be seen in Supplementary Fig. S1.

Rheological Properties of Gels

Dynamic viscoelastic properties of gels were measured using a controlled-stress dynamic rotational rheometer (Physica MCR 301, Anton Paar, Inc., AU) coupled with a double-gap cylinder geometry (C-DG26.7/T200, Anton Paar) with an

internal diameter 23.822 mm and an external diameter of 27.594 mm.

The pre-treatment step, the cooling, and the addition of the phenolic acids (PCA and o-CMA) and coagulants were conducted outside the rheometer, as described above. After the addition of the fresh coagulants (GDL or MgSO_4), the mixtures were immediately loaded into the preheated rheometer and heated at 80 °C for 30 min. Then, the gels were cooled to 5 °C at a cooling rate of 4 °C/min and held under these conditions for 30 min. The measurements were performed at a constant strain of 0.1, which was within the linear–viscoelastic region, and at a frequency of 1.0 Hz. To prevent solvent evaporation, a thin layer of soy oil was put on top of the samples. The coagulants and phenolic acids were freshly prepared and stored at –20 °C before their use.

Surface Hydrophobicity

The binding of the bromophenol blue salt (BPB) method was used to measure the surface hydrophobicity of the soy protein gels. The method was developed by Chelh et al. [12] and was slightly modified from Lorieau et al. [13]. The protein content of the gels was determined using the Kjeldahl method. Briefly, a mixture of soy protein gel with a volume of phosphate buffer (20 mM) at pH 6 was prepared to a final protein concentration of 2 mg/mL. The mixture was vortexed for approximately 10 min until a dispersion was formed. Then, 1 mL of the mixture or phosphate buffer (control) was transferred to a vial and 80 μL of BPB (1 mg/mL) was added and vortexed for 1 min. Samples and control were agitated for 10 min at room temperature using an orbital shaker, then centrifuged at 2,000 rcf for 15 min. Supernatants were diluted approximately 10 times and the absorbance was measured at 595 nm. The amount that was bound on BPB was calculated using Eq. 1, the higher the bound BPB, the higher the hydrophobicity.

$$\text{Bound BPB}(\mu\text{g}) = 80\mu\text{g} \cdot \frac{A_{595\text{nm}}(\text{Control} - \text{Gel})}{A_{595\text{nm}} \text{Control}} \quad (1)$$

Transmission Electron Microscopy (TEM) on Gels

The gels developed in this study were too weak to be cut into tubes and therefore, they were embedded into a 3 % w/w aqueous solution of agarose before fixation. The rest of the sample preparation was conducted as described in our previous work [7]. Samples were viewed using an FEI Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI Corp., Brno–Černovice, Czech Republic). Image processing and analysis were conducted using ImageJ software (v1.52f, National Institutes of Health, Bethesda, MD, USA).

In vitro Digestion Static Protocol

The *in vitro* digestion experiments were conducted according to the INFOGEST protocol [14] with minor modification. All the gels were soft and thus, they were not mechanically processed (grinding) prior to oral phase.

A mass of 5 g of gelled samples was pipetted to an amber glass bottle and 4 mL of simulated salivary fluid (SSF), 0.025 mL of 0.3 M CaCl_2 and 0.975 mL of distilled water were added. The mixture was then kept at 37 °C under shaking at 40 rpm for 5 min in a shaking water bath. A volume of 0.5 mL of digesta was withdrawn at the end of the oral phase. Next, the remaining of the simulated oral bolus (9.50 g) was mixed with 7.60 mL of simulated gastric fluid (SGF), 4.8 μL of 0.3 M CaCl_2 , the pH was adjusted to 3.0 by adding 0.13 to 0.18 mL of 1 M HCl. The rest of the volume was filled with 0.82 to 0.77 mL of distilled water and 0.95 mL of porcine pepsin stock solution (40,000 U/mL) in order to achieve an activity of around 2,000 U/mL in the final mixture. Then, the mixture was incubated for 2 h in the water bath at 37 °C whilst mixing at 40 rpm. A volume of 0.4 mL was withdrawn at times 30, 60, 120 min of the gastric processing.

Finally, the simulated gastric chyme (17.80 g) was mixed with 9.76 mL of simulated intestinal fluid (SIF), 2.24 mL of bile (approximately 160 mM in SIF) and 36 μL of 0.3 M CaCl_2 , the pH was adjusted to 7.0 by adding 0.15 to 0.21 mL of 1 M NaOH. The rest of the volume was filled with 1.23 to 1.17 mL of distilled water and 4.47 mL of pancreatin stock solution (800 U/mL) in order to achieve a trypsin activity of around 100 U/mL in the final mixture. Both pancreatin and bile salts were vortexed and magnetically stirred for approximately 20 min under cooling conditions, prior to intestinal digestion. The digesta were incubated for 2 h in the water bath at 37 °C whilst mixing at 40 rpm. A volume of 0.4 mL was withdrawn at times 5, 15, 30, 60, 120 min of the intestinal phase.

The withdrawn volumes of digesta were immediately centrifuged for 5 min at 11,000 rcf, then the supernatant was transferred to a pre-weighed tube containing two volumes of ethanol, the tube was weighed again in order to determine the exact amount of sample and finally, it was centrifuged again for 3 min at 11,000 rcf. The dissolved sample was immediately filtered with 0.22 μm syringe filters. All the samples were stored at –20 °C prior to further analysis. Each of these experiments were performed in triplicate.

Bioaccessibility of Phenolic Acids

The quantification of phenolic acids (PA) in digesta was obtained by HPLC analysis. The phenolic acid release was calculated according to Eq. 2, where y was the peak area, a and b were the slope and the intercept of the linear equation

found from the calibration curve of the PA of interest and F was the dilution factor of the samples.

The bioaccessibility of the phenolic acids in digesta was analyzed using an LC–20AD Prominence UFLC (Shimadzu, Japan). UFLC running conditions, eluents and data analysis were the same as previously reported [7]. For the calibration curve, phenolic acids were diluted in an aqueous solution of 63 % (w/w) ethanol. The wavelengths at which the phenolic acids were detected were 280 nm for gallic acid, 295 nm for protocatechuic acid and 325 nm for vanillic acid, caffeic acid, ferulic acid and coumaric acids (p–, m–, and o–), respectively.

$$PA \text{ release (\%)} = \frac{\frac{y-b}{a} \cdot F \cdot \text{Mass of digesta (mg)}}{\text{Total mass of PA added in the gel (mg)}} \times 100 \quad (2)$$

Statistical Analysis

Data were analysed using Minitab 17 statistical software (Minitab Inc., USA). Values were represented as means $\pm$ standard deviations ($n \geq 3$). The pH, hydrophobicity and the rheological properties of the gels as well as the bioaccessibility of PCA and CMA from GDL and $MgSO_4$ gels were analysed with the general linear model (two-factor ANOVA with repeated measures). The factors were the types of gels (control, PCA, CMA) and coagulant mechanism (GDL– and $MgSO_4$ –). Whereas, the release of different added phenolic acids at the end of the gastric ($t=2$ h) and end of intestinal phase ($t=4$ h) were analysed using one-way ANOVA with the release values associated with the two time points (2 h and 4 h) as the main factors. Finally, the significance of the factors' interaction mean values were assessed with the Tukey test and the correlations were considered significant when $p \leq 0.05$.

Results and Discussion

Characterisation of Gels

Gel Composition

The compositions of all the gels were alike, with protein content around 4.3 % and moisture content of approximately 95 %. Although the conditions were identical in the

mixtures, the gels induced by $MgSO_4$ contained a lower concentration of coagulant than the GDL gels (7 and 17 mM, respectively). Higher concentrations of coagulant produced phase separation, which is not desirable for silken-like tofu gels, and lower coagulant concentrations gave weaker gels that collapsed under their own weight. Therefore, the coagulant concentrations were selected to give self-supporting gels without phase separation.

The pH of the gels varied depending on the coagulant and phenolic acid addition (Table 1). As expected, GDL-induced gels were more acidic than the $MgSO_4$ gels. GDL acidifies the mixture by spontaneous hydrolysis to gluconic acid. In contrast, $MgSO_4$ did not affect the pH of the mixture significantly. However, the addition of PCA or CMA reduced the pH to a small extent in both GDL and $MgSO_4$ gels. The surface hydrophobicity of all the gels was similar ($p > 0.05$) (Table 1).

Small-deformation Viscoelastic Properties

The gelation of soy proteins during the second heating step was observed by recording the continuous changes in the small-deformation viscoelastic properties of the SPI mixtures. The changes in G' during the heating-cooling cycles are shown in Fig. 1, with specific points of interest summarised in Table 2.

In general, all gels behaved like elastic solids where $G' > G''$ and $\tan \delta < 1$. The SPI–GDL mixtures formed gels within 10 min from the time that they were loaded to the rheometer. The G' of the GDL gels continued to increase at 80°C which indicates the involvement of hydrophobic interactions, which become stronger at high temperature [15]. Studies have found that hydrophobic interactions are favoured between phenolic ring and apolar side chains of proteins at pH close to the pI, where the buried hydrophobic groups are exposed [15–17]. Upon cooling from 80°C to 5°C, the G' increased at a faster rate until they reached a saturation point at 5°C. The decrease in temperature diminishes the entropy of the system, which leads to short-range, non-covalent, protein–protein and protein–phenolic interaction such as hydrogen bonding, van der Waals and electrostatic interactions [15, 18].

The starting G' values of the control SPI– $MgSO_4$ mixtures were slightly higher than those of GDL controls, while the G' of SPI– $MgSO_4$ gels with PCA or CMA were considerably higher than any other gel. The high initial G' of

Table 1 pH and hydrophobicity of soy protein unpressed gels with added phenolic acids (PCA and CMA)

Properties	GDL-Induced gels			MgSO ₄ -Induced gels		
	GDL control	PCA	CMA	MgSO ₄ control	PCA	CMA
pH of gels	5.7 $\pm$ 0.2 ^a	5.4 $\pm$ 0.2 ^b	5.5 $\pm$ 0.1 ^{ab}	6.7 $\pm$ 0.1 ^c	6.3 $\pm$ 0.1 ^d	6.3 $\pm$ 0.0 ^d
BPB (μ g)	49 $\pm$ 1.2 ^a	50 $\pm$ 0.1 ^a	51 $\pm$ 1.2 ^a	50 $\pm$ 0.4 ^a	50 $\pm$ 1.4 ^a	49 $\pm$ 1.4 ^a

Values that do not share a letter in the same row are significantly different ($p < 0.05$)

Fig. 1 Changes in the rheological properties (A) and final storage modulus (B) of the SPI gels induced by GDL and MgSO_4 (17 and 7 mM). The red indicates the addition of PCA and the yellow the addition of CMA, the grey colour bars and curves are the control gels

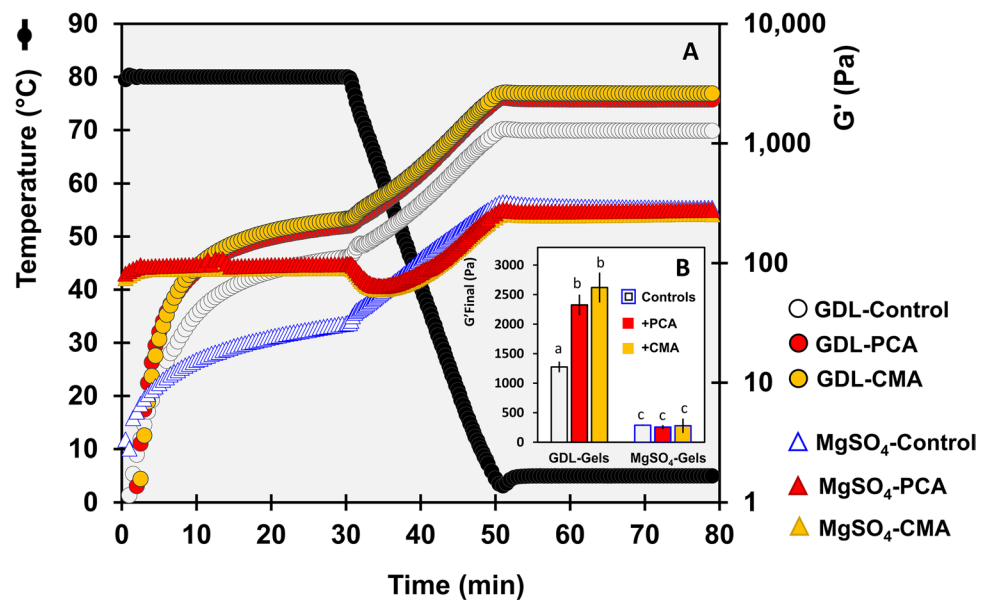


Table 2 Rheological properties of the unpressed gels with added phenolic acids (PCA and CMA)

Properties	GDL-Induced gels			MgSO_4 -Induced gels		
	GDL control	PCA	CMA	MgSO_4 control	PCA	CMA
G'_{80} (Pa)	113 ± 14.2^a	205 ± 19.3^b	236 ± 20.5^b	32 ± 0.08^c	91 ± 17.9^a	98.3 ± 34.7^d
G'_{Fin} (Pa)	1275 ± 91.1^a	2327 ± 173.9^b	2620 ± 253.6^b	289 ± 11.4^c	257 ± 33.1^c	279 ± 122.1^c
G''_{Fin} (Pa)	217 ± 18.4^a	408 ± 27.5^b	479.3 ± 49.5^c	53 ± 3.4^d	57 ± 9.4^d	67 ± 31.4^d
$\text{Tan} \delta_{\text{Fin}}$ (-)	0.17 ± 0.0^a	0.18 ± 0.0^a	0.18 ± 1.0^a	0.18 ± 0.0^a	0.22 ± 0.0^a	0.24 ± 0.0^a

Values that do not share a letter in the same row are significantly different ($p < 0.05$)

SPI- MgSO_4 was consistent with other reports of soy protein gelation induced by divalent salts [19]. GDL is a lactone that gradually hydrolyses to gluconic acid, which reduces the pH of the solution [20]. Thus, the gelling action of GDL occurred more slowly.

All the MgSO_4 -induced gels had a significantly lower G'_{Fin} than the GDL-induced gels ($p < 0.05$) and therefore they were much weaker (Fig. 1, Table 2). The addition of the phenolic acids PCA and CMA affected the rheological properties of the gels to a large extent, especially the gels induced by GDL. The G'_{Fin} of the GDL gels was doubled after the addition of the phenolic acids, with CMA-GDL mixtures giving slightly higher G'_{Fin} ($p > 0.05$).

Previous studies showed that the addition of mono- or polyphenols could enhance the mechanical properties of gelatin gels [21–23] or films [24, 25]. The mechanism that was proposed was either cross-linking through quinone formation under oxidising conditions or via hydrogen bonding, based on the fact that phenols are excellent hydrogen donors [23, 25]. The conditions used during gelation (pH < 7 and lack of oxygen exposure for long time) do not favour the oxidation and as a consequence the polymerisation of the

phenolic acids. Thus, the hydrogen bonding or electrostatic associations between phenolic acids and the amino acid residues of the soy protein might be more likely. The protonated —OH of phenolics ($\text{p}K_{\text{a}2} > 9$) can form hydrogen bonds with the oxygen from the carbonyl group (—C=O) of the peptide bonds [17] and the nitrogen from the amino groups (—NH₂) of lysine and arginine [26]. A recent study found that the higher the number of —OH groups on the phenolic acid ring the greater the binding affinity towards the β -conglycinin [27]. Surprisingly, CMA (with only one —OH group) increased G' more than PCA (two —OH groups) ($p < 0.05$) (Fig. S1). In addition, the deprotonated —COO[−] groups of the phenolic acids can interact electrostatically with the positively charged amino groups of lysine and arginine at pH above the isoelectric point [15, 16].

In the case of MgSO_4 gels with added PCA or CMA, the starting G' was substantially higher than the control, and G' remained constant until the end of the heating step (Fig. 1). This might be partly due to an rapid reduction of the pH by 0.4 units (Table 1). However, during the cooling phase, a slight decrease with a subsequent increase in the G' was observed. At 80 °C the phenolic acids contributed

to gel structure through a reversible temperature-dependent mechanism that was abolished by cooling, e.g. enhancement of hydrophobic interactions. The loss of this contribution in the early stages of cooling (dip in G' between 80 °C and 60 °C) was subsequently counterbalanced by a cooling-induced strengthening, e.g. hydrogen-bonding, with the result that the G'_{Fin} of $MgSO_4$ with PCA/CMA was the same as for control $MgSO_4$ gels.

Upon cooling, the phenolic acids did not strengthen the gel network of $MgSO_4$ gels, as seen with GDL gels. As mentioned before the pH of the $MgSO_4$ gels is between 6.2 to 6.7, where most of the carboxyl groups of the protein are ionised ($-COO^-$) and able to interact with the divalent salt (Mg_{2+}). Similarly, the carboxyl groups of the phenolic acids are ionised too which might also interact with the magnesium ions to some extent and limit protein—phenolic interactions that could potentially enhance the gelling network as in the case of GDL-induced gels.

Microstructure of Gels

The microstructure of the gels can be found in the TEM micrographs in Fig. 2. The dark areas correspond to the protein network or aggregates and the white areas to the aqueous phase. A convoluted, thin protein network can be observed in both GDL and $MgSO_4$ control gels. In both cases the amount of coagulant added was low (17 mM and 7 mM, respectively). Therefore, the net charge was negative in both cases (Table 1), thus there was electrostatic repulsion to some extent. However, the repulsion was not large enough to form fine-stranded networks. The addition of CMA induced significant changes in the microstructure of both GDL and $MgSO_4$ gels (Fig. 2b–d). The aggregates became thicker and longer. Although this phenomenon was more pronounced in GDL–CMA gels (Fig. 2d), the network was irregular, and the areas fluctuated in density and thus gel's porosity. In contrast, $MgSO_4$ –CMA gels had a more regular network and the micrograph presented in Fig. 2b is a representative one. The increase in thickness and aggregate size after the addition of the CMA could also explain the increase in elastic modulus in GDL gels presented in Fig. 1. However, it is unclear why the microstructural changes did not affect the elasticity of the $MgSO_4$ gels.

Bioaccessibility of Phenolic Acids

Effect of the Coagulant on Phenolic Acid Bioaccessibility

The bioaccessibility profile of the CMA (pK_{a1} 3.85 [28]) and PCA (pK_{a1} 4.26 [29], Fig. S1) from $MgSO_4$ - and GDL-induced gels during *in vitro* digestion can be found in Fig. 3. The coagulants used and thus, the texture of the gels, had a negligible effect on the phenolic acid release.

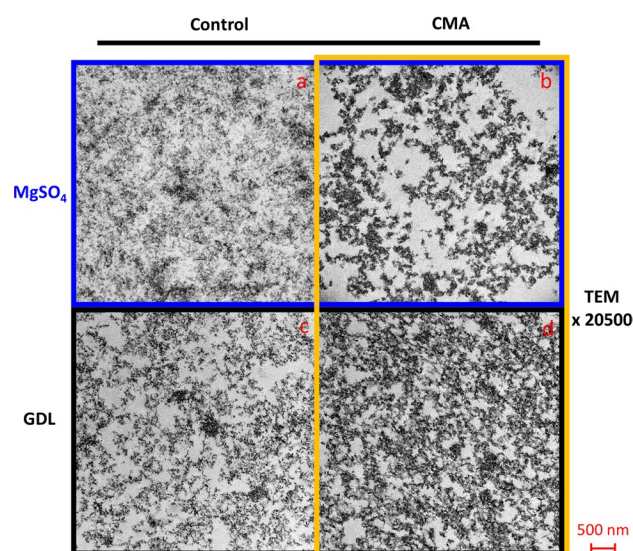


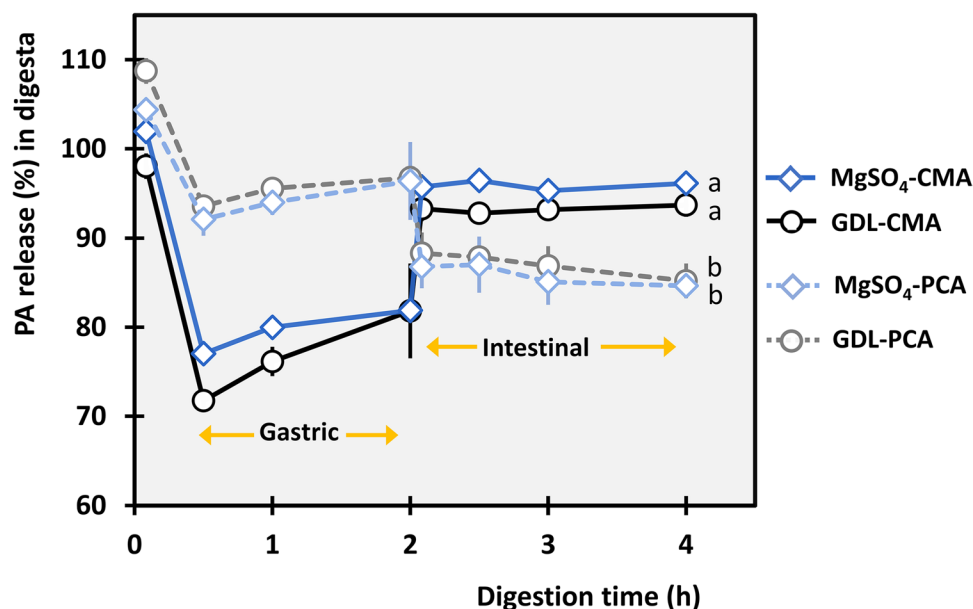
Fig. 2 Microstructural characteristics of unpressed gels induced by GDL and $MgSO_4$. $MgSO_4$ -induced control gel (a) and with the addition of 3.5 mM CMA (b). GDL-induced gels control (c) and with the addition of 3.5 mM CMA (d)

However, CMA and PCA gave different bioaccessibility patterns. A similar trend was found in SPI gels formed by pressing [7], even though the general bioaccessibility profile was distinct [7].

A noticeable phenomenon is that both phenolic acids from both gel matrices were released rapidly and completely ($\geq 95\%$) at the end of the oral incubation ($pH \sim 7$). Therefore, the neutral pH favours the bioaccessibility of the phenolic acids from the gel matrix. This tendency was not pronounced in the oral incubation of the pressed gels, studied in our previous work [7]. The latter can be explained by the higher protein content and thus, the higher buffering capacity [30] of the pressed protein gels. Our experiment presented in Supplementary Fig. S2 clearly showed that the lower the pH of the gel the lower the PCA release in the oral phase. In the gastric and intestinal phase, the pH was adjusted and checked before and during the incubations, and it remained at the target levels in all gel samples ($pH \sim 3$ and ~ 7 , respectively).

The release levels of both phenolics reduced rapidly at the beginning of the gastric phase ($p < 0.05$) where the pH of the media was below the pK_a of phenolics' carboxyl groups, indicating that phenolic acids were in a protonated state. In the intestinal phase, the bioaccessibility levels of CMA increased significantly ($p < 0.05$) reaching approximately 95 %. In contrast, PCA levels further dropped by 10 % ($p < 0.05$) and reached 85 %. From Fig. 3 and Fig. S2 it is clear that the release of both phenolic acids is purely dependant on the pH conditions during *in vitro* digestion and not on gels' coagulation mechanism

Fig. 3 Bioaccessibility (%) of PCA and CMA (dashed and full lines, respectively) from unpressed gels during *in vitro* digestion. The black and grey lines correspond to GDL-induced gels and the blue to MgSO_4 -induced gels



and physical characteristics. In general, changes in the pH affect the number of the protein sites that are able to interact either with proteins or phenolics [16]. At pH 7 there is electrostatic repulsion because the carboxyl groups of both phenolic acids and glutamic acid and aspartic acid are deprotonated ($-\text{COO}^-$), which could explain the high release percentages of CMA in oral and intestinal phase. The reduction of PCA release in the intestinal phase might indicate degradation which is in agreement with other studies [31, 32]. The release pattern of PCA and CMA from unpressed SPI gels was distinct than the pressed gels, where both bioactives levels had an upward trend at the intestinal phase [7]. In addition, the end quantities delivered from the pressed SP gels were overall higher than unpressed gels and dependant on the gelation mechanism used [7]. Also, current results did not show a gradual release pattern as in the case of the riboflavin from cold-SP- CaCl_2 gels [5]. The differences of our results with the later study can be mainly attributed on the different *in vitro* digestion protocol used and on the lipophilic nature of riboflavin.

Phenolic Acid Structure and Release Profile Relationship

Digestion experiments of GDL-induced gels enhanced with phenolics were expanded using a range of homologous phenolic acids, the chemical structure of which can be found in Supplementary Fig. S1. The peak for all phenolic acids' release was at the end of the oral incubation ($\text{pH} \sim 7$) and varied depending on the structure, with caffeic acid having the lowest release value ($87 \pm 2.4\%$) (Fig. 4). Digestive enzymes were absent in the oral phase and thus, the release of the phenolic acids was mainly affected by the pH conditions rather than the protein hydrolysis of the gels.

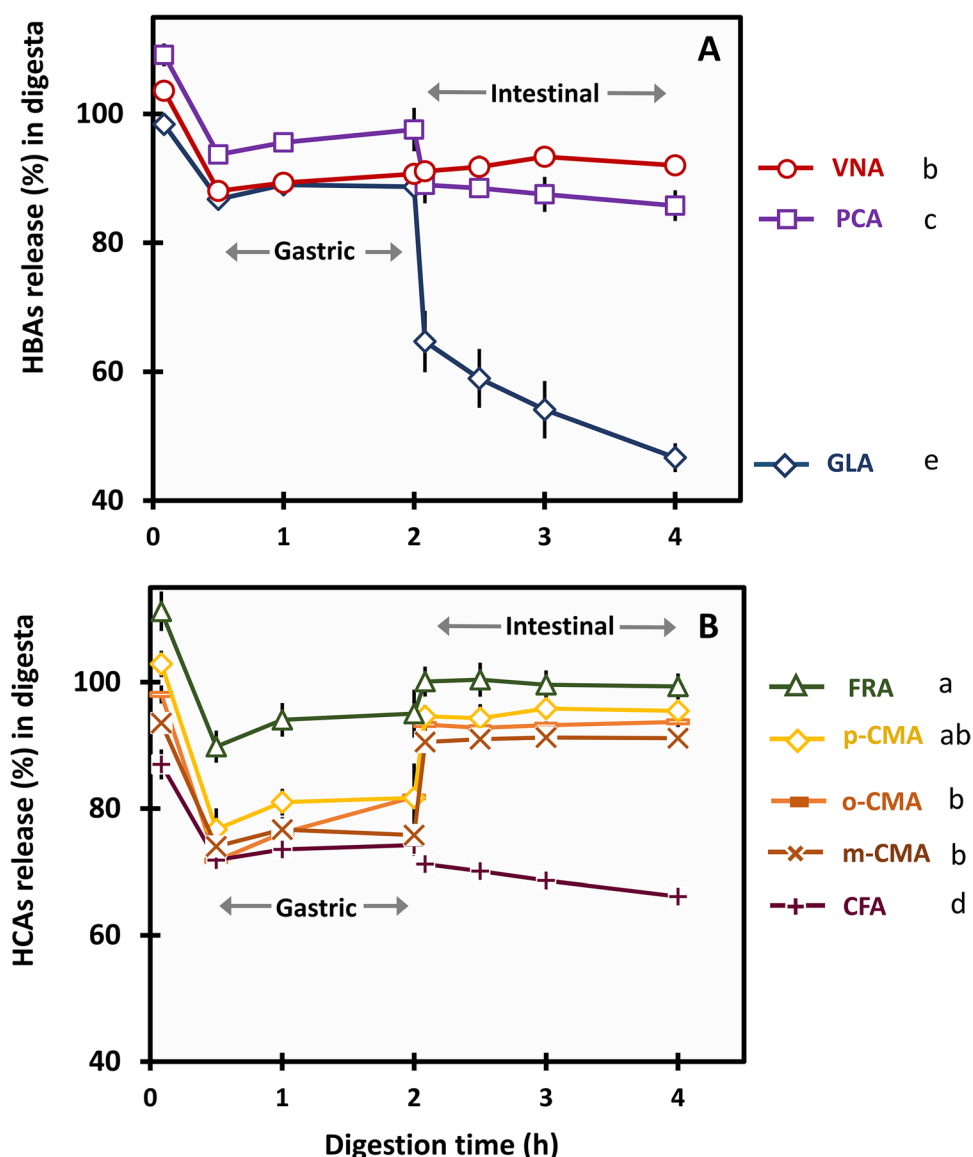
Neutral pH favours the release of all phenolic acids, which might be due to electrostatic repulsion. Unfortunately, most of the available studies present only the end release values from the gastric and/or intestinal phases thus, we cannot compare our oral phase results.

After the oral phase, the concentration detected in the liquid fraction of the digesta was reduced at the early stages of gastric incubation ($\text{pH} \sim 3$). At acidic pH, phenolics are less soluble due to protonation of $-\text{OH}$ and $-\text{COOH}$ groups [33]. Research has shown that at acidic pH, porcine pepsin can interact with tannins which results in precipitation [34]. Similarly, a study found that pepsin was able to interact with cinnamon polyphenols, under gastric conditions, resulting in the precipitation of complexes [35]. Other studies, however, have demonstrated an increase or no substantial effect on polyphenol release during gastric incubation [33, 36].

Later, at the intestinal phase, the levels of bioaccessible phenolic acids either significantly increased ($p < 0.05$), in the case of FRA and o-, p-, m-CMA or significantly decreased ($p < 0.05$) such as PCA, GLA, and CFA. An exception was the VNA, the levels of which remained constant during this transition (Fig. 4). The reduction during the intestinal phase has been observed in several studies, [31, 33, 36–38] and has been described as degradation due to alkaline conditions of the intestinal phase. However, if the degradation that was implied in the other studies was oxidation, a more gradual reduction of the phenolics levels would be expected. Gallic acid gave a very interesting release pattern (Fig. 4), with an initial rapid drop and an additional gradual reduction throughout the intestinal incubation, which suggests oxidation.

Taking into consideration the phenolic acid structures from Fig. S1 and the Fig. 4 we can observe some important

Fig. 4 Bioaccessibility (%) of different phenolic acids during *in vitro* digestion. Hydroxybenzoic acids (HBAs) (A): vanillic acid (VNA), protocatechuic acid (PCA), gallic acid (GLA) and hydroxycinnamic acids (HCAs) (B): ferulic acid (FRA), p-coumaric acid (p-CMA), o-coumaric acid (o-CMA), m-coumaric acid (m-CMA), caffeic acid (CFA)



structural features that affect the bioaccessibility of phenolics at the intestinal phase. Firstly, a comparison of VNA and FRA showed that the phenolic acid with the longer carbon backbone (FRA) had a higher bioaccessibility ($p < 0.05$). Whereas a contradictory trend was found between PCA and CFA, thus it is unclear whether the presence of the propionic group can affect the bioaccessibility of phenolics. Secondly, it was found that the more numerous the hydroxyl groups ($-\text{OH}$) attached to the benzene ring, the less bioaccessible the bioactive was at the intestinal phase (i.e. $\text{GLA} < \text{CFA} < \text{PCA}$) ($p < 0.05$). Also, methylation of the $-\text{OH}$ group attached to the C_3 of the benzene ring resulted in a significant increase of the intestinal phase bioaccessibility (i.e. VNA vs PCA and FRA vs CFA) ($p < 0.05$). Furthermore, a comparison of the bioaccessibility concentrations at the intestinal phase between CMA isomers (o-, p-, m-) did not reveal any

significant differences ($p > 0.05$). Thus, the position of the $-\text{OH}$ group did not make any difference.

Nevertheless, the number of $-\text{OH}$ groups attached to the benzene ring appears to have a higher impact on the bioaccessibility of the phenolic acids. Some of our findings are correlated well with the findings of Friedman et al. [39] who studied the stability of different phenolics between pH 3 to 11. The authors found that the UV-Vis spectrum of CFA and its derivatives dramatically changed with increasing pH and these changes were irreversible. In another study, oxidative products of CFA were detected at acidic conditions (pH 3–5), although the rate of oxidative reaction increased drastically over pH 5 [40]. At alkaline pH, a non-enzymatic oxidative reaction of CFA can be progressed rapidly (within minutes) [40–42]. Thus, the low bioaccessibility rates of CFA during our incubations might suggest oxidation (Fig. 4).

According to Cilliers et al. [41], the oxidation products of CFA do not absorb at 325 nm (max for cinnamate derivatives) but at 200 nm [41], which might explain why the new peaks did not appear during our HPLC analysis. Similarly, GLA, which has 3 —OH attached to the phenolic ring, was also unstable at high pH. In contrast, FRA with a single —OH free was stable at high pH and for more than 24 hours [39]. The authors suggested that the molecules with two or more —OH attached to the phenolic ring are more susceptible to chemical transformations at high pH. These transformations can be due to the formation of unstable quinone intermediates which may eventually oxidize in the presence of oxygen to degradation products [39]. Another important finding of the same study was the irreversibility of the induced changes in some phenolics [39]. Although the phenomenon was tested from alkaline conditions (pH 8–11) to neutral (pH 7) (conditions not present in our study), it shows that incubations at different pH can cause permanent changes to the phenolics. This could explain why the concentration of the phenolic acids was not recovered in the intestinal phase. Therefore, the history of samples (i.e. pH used in oral and gastric incubations) might affect the bioaccessibility of the phenolic acids in the intestinal phase.

In order to test whether oxidation was possible under the current *in vitro* digestion conditions, gastric and intestinal incubation mixtures were flushed with nitrogen after the addition of the digestive enzymes, as it was suggested by Bermudez et al. [36]. According to the authors, this resulted in reduced levels of oxygen during digestion and therefore gave a more realistic representation of gastric and intestinal conditions [36, 43]. Interestingly, with N₂ flushing GLA bioaccessibility increased by approximately 30 % at the end of the intestinal phase, whereas PCA was not affected (Supplementary Fig. S3). Hence, the gradual decline of GLA during intestinal incubation was indeed an effect of oxidation, which was most probably a result of the three —OH groups attached to the aromatic ring. But it is still unclear the cause of the rapid reduction in the bioaccessibility of PCA, CFA and GLA during the transition from gastric to intestine conditions. Nevertheless, apart from the GLA and CFA, the rest of the phenolic acids were bioaccessible by more than 80 %, which was a very high percentage and indicated that these compounds could be used as antioxidants in soy protein food formulations.

Conclusion

Two types of soft SPI gels were used to study how phenolic acids interact with the soy protein gel matrix. It was found that added phenolic acids can alter the microstructure of the SPI gels and significantly increase the elastic behaviour of

the acidified but not the salt-induced gels. Interestingly, the release profile of the phenolics was not affected by the gelation mechanism, but it was rather dependant on the pH conditions during *in vitro* digestion and the phenolic acid structure. Further experiments with a homologous series of phenolic acids showed that protocatechuic acid, caffeic acid and gallic acid were less bioaccessible than the other phenolics, which was attributed to the higher number of hydroxyl groups. Overall, the bioaccessibility rates for most of the phenolic acids were above 80 %, which indicates that unpressed soy protein gels could be a good delivery matrix for phenolics.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11483-022-09763-6>.

Acknowledgements The authors would like to thank Manawatu Microscopy and Imaging Centre (MMIC) at Massey University for conducting the preparation for the TEM imaging.

Author Contributions M.M.: conceptualization, formal analysis, investigation, methodology, validation, visualization, writing-original draft, writing-review and editing. A.E.: conceptualization, methodology, supervision, writing-review and editing. M.G.: conceptualization, methodology, supervision, writing-review and editing. S.M.L.: conceptualization, funding acquisition, methodology, supervision, writing-review and editing. All authors read and approved the final manuscript.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions This research was supported by the Riddet Institute, a New Zealand Centre of Research Excellence, funded by the Tertiary Education Commission.

Declarations

Conflicts of interest The authors have declared no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

1. T. Bohn, *Nutr. Rev.* **72**, 429 (2014)
2. C. Manach, A. Scalbert, C. Morand, C. Rémésy, L. Jiménez, *Liliana. Am. J. Clin. Nutr.* **79**, 727 (2004)
3. A. Scalbert, C. Morand, C. Manach, C. Rémésy, *Biomed. Pharmacother.* **56**, 276 (2002)
4. C.H. Tang, *Crit. Rev. Food Sci. Nutr.* **57**, 2636 (2017)
5. A. Maltais, G.E. Remondetto, M. Subirade, *Food Hydrocoll.* **23**, 1647 (2009)
6. A. Maltais, G.E. Remondetto, M. Subirade, *Food Hydrocoll.* **24**, 518 (2010)

7. M. Marinea, A. Ellis, M. Golding, S.M. Loveday, *Foods* **10**, 154 (2021)
8. X. Peng, C. Ren, S. Guo, *Trends Food Sci. Technol.* **54**, 138 (2016)
9. C.M. Bryant, D.J. McClements, *Trends Food Sci. Technol.* **9**, 143 (1998)
10. J. De Wit, *J. Dairy Sci.* **73**, 3602 (1990)
11. V. Neveu, J. Perez-Jiménez, F. Vos, V. Crespy, L. du Chaffaut, L. Mennen, C. Knox, R. Eisner, J. Cruz, D. Wishart, A. Scalbert. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods, Database (2010)
12. I. Chelh, P. Gatellier, V. Santé-Lhoutellier, *Meat Sci.* **74**, 681 (2006)
13. L. Lorieau, A. Halabi, A. Ligneul, E. Hazart, D. Dupont, J. Floury, *Food Hydrocoll.* **82**, 399 (2018)
14. M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Bortrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, A. Brodkorb, *Food Funct.* **5**, 1113 (2014)
15. H. Rawel, S. Rohn, *Phytochem. Rev.* **9**, 93 (2009)
16. S. Schefer, M. Oest, S. Rohn, *Foods* **10**, 2798 (2021)
17. M. Saeed, M. Cheryan, *J. Agric. Food Chem.* **37**, 1270 (1989)
18. I.S. Chronakis, *Food Res. Int.* **29**, 123 (1996)
19. K. Kohyama, Y. Sano, E. Doi, *J. Agric. Food Chem.* **43**, 1808 (1995)
20. Y. Pocker, E. Green, *J. Am. Chem. Soc.* **95**, 113 (1973)
21. X. Zhang, M.D. Do, P. Casey, A. Sulistio, G.G. Qiao, L. Lundin, P. Lillford, S. Kosaraju, *J. Agric. Food Chem.* **58**, 6809 (2010)
22. G. Strauss, S.M. Gibson, *Food Hydrocoll.* **18**, 81 (2004)
23. J. Wu, S.C. Chiu, E.M. Pearce, T. Kwei, *J. Polym. Sci., Part A-1: Polym. Chem* **39**, 224 (2001)
24. T. Le, H. Maki, E. Okazaki, K. Osako, K. Takahashi, *J. Food Sci.* **83**, 1888 (2018)
25. N. Cao, Y. Fu, J. He, *Food Hydrocoll.* **21**, 575 (2007)
26. H. Ojha, K. Mishra, M.I. Hassan, N.K. Chaudhury, *Thermochim. Acta* **548**, 56 (2012)
27. J. Gan, H. Chen, J. Liu, Y. Wang, S. Nirasawa, Y. Cheng, *Int. J. Mol. Sci.* **17**, 1671 (2016)
28. D.S. Wishart, Y.D. Feunang, A.C. Guo, E.J. Lo, A. Marcu, J.R. Grant, T. Sajed, D. Johnson, C. Li, Z. Sayeeda, N. Assempour, I. Iynkkaran, Y. Liu, A. Maciejewski, N. Gale, A. Wilson, L. Chin, R. Cummings, D. Le, A. Pon, C. Knox, M. Wilson, *Nucleic Acids Res.* **46**, D1074 (2017)
29. PubChem. National center for biotechnology information. Pubchem compound summary for cid 72, 3,4-dihydroxybenzoic acid. <https://pubchem.ncbi.nlm.nih.gov/compound/34-Dihydroxybenzoic-acid>. Accessed 19 June (2022)
30. Y.A. Mennah-Govela, R.P. Singh, G.M. Bornhorst, *Food Funct.* **10**, 6074 (2019)
31. D. Tagliazucchi, E. Verzelloni, D. Bertolini, A. Conte, *Food Chem.* **120**, 599 (2010)
32. J.I. Mosele, A. Maciá, M.P. Romero, L. Motilva, L. Rubió, *J. Funct. Foods* **14**, 529 (2015)
33. A. Helal, D. Tagliazucchi, E. Verzelloni, A. Conte, *LWT - Food Sci. Technol.* **60**, 957 (2015)
34. A.E. Hagerman, L.G. Butler, *J. Agric. Food Chem.* **26**, 809 (1978)
35. A. Helal, D. Tagliazucchi, *LWT - Food Sci. Technol.* **89**, 164 (2018)
36. M.J. Bermúdez-Soto, F.A. Tomás-Barberán, M.T. García-Conesa, *Food Chem.* **102**, 865 (2007)
37. F. Vallejo, A. Gil-Izquierdo, A. Pérez-Vicente, C. García-Viguera, *J. Agric. Food Chem.* **52**, 135 (2004)
38. L. Gayoso, A.S. Claerbout, M.I. Calvo, R.Y. Caverro, I. Astiasarán, D. Ansorena, *J. Funct. Foods* **26**, 428 (2016)
39. M. Friedman, H.S. Jürgens, *J. Agric. Food Chem.* **48**, 2101 (2000)
40. H. Fulcrand, A. Cheminat, R. Brouillard, V. Cheynier, *Phytochem.* **35**, 499 (1994)
41. J.J. Cilliers, V.L. Singleton, *J. Agric. Food Chem.* **37**, 890 (1989)
42. P. Hapiot, A. Neudeck, J. Pinson, H. Fulcrand, P. Neta, C. Rolando, *J. Electroanal. Chem.* **405**, 169 (1996)
43. G. He, R.A. Shankar, M. Chzhan, A. Samouilov, P. Kuppusamy, J.L. Zweier, *Proc. Natl. Acad. Sci.* **96**, 4586 (1999)

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.