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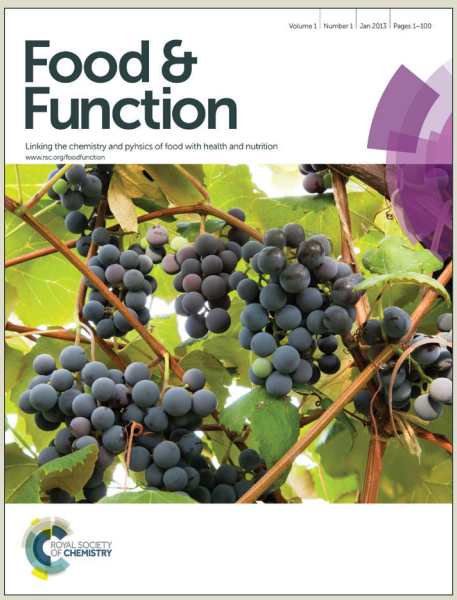

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Protein-phenolic interactions and Inhibition of glycation-combining systematic review and experimental models for enhanced physiological relevance

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Abstract

Background: While antiglycative capacity has been attributed to (poly)phenols, the exact mechanism of action remains unclear. Studies so far are often relying on supra-physiological concentrations and use of non-bioavailable compounds.

Methods: To inform the design of a physiologically relevant *in-vitro* study, we carried out a systematic literature review of dietary interventions reporting plasma concentrations polyphenol metabolites. Bovine Serum Albumin (BSA) was pre-treated prior to in vitro glycation: either no treatment (native), pre-oxidised (incubated with 10nM H_2O_2, for 8 hours) or incubated with a mixture of phenolic acids at physiologically relevant concentrations, for 8 hours). *In-vitro* glycation was carried out in presence of i) glucose only (0, 5 or 10mM), ii) glucose (0, 5 or 10mM) plus H_2O_2 (10nM), or iii) glucose (0, 5 or 10mM) plus phenolic acids (10-160nM). Fructosamine was measured using the nitroblue tetrazolium method.

Results: Following (high) dietary polyphenol intake, 3-hydroxyphenylacetic acid is the most abundant phenolic acid in peripheral blood (up to 338µM) with concentrations for other phenolic acids ranging from 13nM-200µM. Presence of six phenolic acids with BSA during *in-vitro* glycation did not lower fructosamine formation. However, when BSA was pre-incubated with phenolic acids, significantly lower concentration of fructosamine was detected under glycoxidative conditions (glucose 5 or 10mM plus H_2O_2 10nM) ($p<0.001$ vs. native BSA).

Conclusion: Protein pre-treatment, either with oxidants or phenolic acids, is an important regulator of subsequent glycation in a physiologically relevant system. High quality *in-vitro* studies under conditions closer to physiology are feasible and should be employed more frequently.

Introduction

Protein glycation has been implicated in the development of several chronic diseases, particularly diabetic micro and macrovascular complications [1, 2]. The process of glycation, in diabetes, is mainly driven by the elevated blood glucose concentration through non-enzymatic condensation of a sugar molecule on a protein, lipid or DNA molecule [3, 4]. Measurement of glycated haemoglobin is the standard method for monitoring diabetes control [5] and elevated levels are clearly related to tissue damage. Glycated albumin levels in plasma can vary widely, between 1% to up to 16% in normoglyceamic individuals [6, 7] and the reasons for this wide range are not fully understood. In individuals free of diabetes for every 1% increase in HbA1c levels there is an associated 30-50% in the risk of cardiovascular disease [8-10]. Although *in-vitro* studies have been employed to study glycation mechanisms, few have used physiologically relevant glucose concentrations. *In vitro* protein glycation does not easily occur with physiological concentrations of glucose, implying that another factor was necessary. We recently demonstrated that albumin glycation at physiological glucose concentrations (5 and 10mM) was driven by oxidative stress, and that oxidised albumin is more susceptible to glycation than the native form of the protein [11]. We have suggested that the reaction might be considered protein glycoxidation, rather than simply glycation. The study of early glycation, using fructosamine, is of importance for translational value of the model (HbA1c is commonly used in clinical practice). As the first stable glycation product, its production rate is influential on total AGEs production[12].

Antioxidants, and (poly)phenols and their metabolites in particular, have been studied for their *in-vitro* antiglycative properties [13-17]. (Poly)phenols may offer protection by scavenging ROS produced during the glycation reaction, thereby slowing glycation and inhibiting the formation of AGEs [13, 18]. Another possible mechanism involves “physical” protection against glycation. This mechanism suggests that (poly)phenols have the capacity to bind on the protein molecule, most likely with a non-covalent bond, and in this way make glycation targets on the protein molecule (usually amino acids like lysine) inaccessible to take part in the glycation reaction [19, 20].

While these studies may hold value in food science (for example, reduction of AGEs formation during cooking in the presence of polyphenols [21]), their physiological relevance to human health is sometimes questionable. (Poly)phenols are subject to extensive metabolism in the lumen (hydrolysis by the enterocytes’ glucosidase system) and after absorption (glucuronidation/sulfation in the liver) [22, 23]. Most high molecular weight dietary polyphenols have low bioavailability and even though aglycones may reach systemic circulation in small amounts, glycosides do not [23-26]. Those which do not get absorbed instead accumulate in the colon lumen, where they are subject to bacterial degradation, leading to the formation of the phenolic acids.

Phenolic acids have a relatively higher bioavailability [22, 27]. In plasma, an increase in phenolic acid levels is seen 8-10 hours after ingestion, which represents the 'colonic tide' of (poly)phenol metabolites [22, 28, 29]. Studies using foodstuff extracts and mixtures containing aglycones and glycosides thus do not replicate physiology [25, 30] when (poly)phenols in the circulation are mostly phase II metabolites and rarely exceed 1μM concentration (and if so, transiently) [27, 28]. Finally, while single compound studies are informative and allow for mechanisms of action to be dissected, polyphenols and their metabolites are not found or consumed in isolation [23, 31]; they are, also, all consumed within complex food matrices with other nutrients that may modify absorption and metabolism [32, 33].

The present paper systematically reviews the literature reporting plasma levels of phenolic acids as key polyphenol metabolites, following ingestion of polyphenol rich food products (not under "acute" trial settings). The outcomes of this review are then used to test whether phenolic acids can inhibit the early stages of glycation under physiologically-relevant experimental conditions, using the bench-top design we previously described [11].

Material and methods

Systematic literature review

This review was conducted following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines. A literature research was carried out in PubMed® and ISI Web of Knowledge® for trials reporting plasma phenolic acid levels after a high polyphenol food/diet intake. The search was inclusive of all years up to February 2014. The following search terms were used to identify relevant studies: (phenol*, polyphenol*, phenolic acid*, fruit, vegetable, spice, cocoa, herb, juice, oil, wine, extract, tea or coffee), paired (bolean AND) with (feeding, trial, intervention, consumption OR supplementation). The wild-card term "*" was used to improve the sensitivity of the search by increasing the number of matches. The review was limited to studies utilising chromatographic techniques to identify (poly)phenolic compounds in serum or plasma. Studies on animals were excluded, as well as studies reporting cross-sectional data. Only controlled long-term feeding trials were reviewed. Studies were included in the review if absolute concentrations of phenolic acids were reported.

Experimental procedures

Chemicals

Bovine serum albumin (BSA), sodium azide, nitroblue tetrazolium, d-glucose, PBS, 1-deoxy-1-morpholinofructose (DMF), hydrogen peroxide, caffeic acid, p-coumaric acid, vanillic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and protocatechuic acid were

purchased from Sigma-Aldrich (Dorset, UK). SnakeSkin Dialysis Tubing, 3.5K MWCO was purchased from Thermo Fisher Scientific (Nottinghamshire, UK).

Albumin pre-treatment

BSA (40g/L) was studied in three different forms: native BSA (BSA), pre-oxidised BSA (ox-BSA), and phenolic acid-preincubated BSA (PP-BSA). Ox-BSA was incubated with 10nM hydrogen peroxide (H_2O_2) for 8hrs pre-glucose incubation and PP-BSA was incubated with a phenolic acid mixture for 8hrs pre-glucose incubation. The phenolic acids used were selected based on the results of the literature review aiming to be representative of plasma concentrations of free phenolic acids, with a higher degree of physiological relevance. The acids and concentrations used were: caffeic acid 10nM, p-coumaric acid 8nM, vanillic acid 21nM, protocatechuic acid 40nM, 3-hydroxyphenyl acetic acid 160nM and 3,4-dihydroxyphenyl acetic acid 40nM. Following pre-treatment, ox-BSA and PP-BSA were dialysed against PBS for 24 hrs to remove any free H_2O_2 and/or phenolic acids (Figure 1).

Protein glycation

All incubations took place in PBS (0.137M Sodium chloride, 0.0027M potassium chloride, and 0.010M phosphates) with sodium azide (0.2g/L), in a final volume of 1.5ml, for 14 days. BSA, ox-BSA and PP-BSA (40g/L) were incubated with glucose (0, 5, 10mM) with or without 10nM H_2O_2 or a physiologically relevant phenolic acid mix (as described above). All incubations were replicated 6 times.

Fructosamine measurement

Fructosamine levels were measured at week 2 with the NBT assay, performed in microplates as described previously [34]. Briefly, samples (30µL) were added to of sodium carbonate buffer (100µL, 100mM, and pH 10.8) with Nitroblue Tetrazolium (0.25mM). Microplates were incubated for 15 min at 37^{o}C and measured spectrophometrically against controls at 550nm after 10 and 15 min of incubation. The difference between the two readings was used to calculate concentrations. The fructosamine analog 1-deoxy-1-morpholinofructose (DMF) was used as a standard. All fructosamine measurements were performed in duplicate. The potential interference of H_2O_2 and phenolic acids with NBT colorization was tested, with no evidence of interference detected. Standards and NBT reagent were made fresh every week and stored at -20^{o}C and 4^{o}C respectively. All samples were stored at -20^{o}C prior to assay.

Measurement of AGE fluorescence

AGE fluorescence was measured in diluted samples (1:5) at an excitation wavelength 370nm and an emission wavelength 440nm. AGE fluorescence was measured as arbitrary units and in duplicate using a SpectraMax M2 plate reader.

Protein structure analysis

Protein tryptophan fluorescence intensity was recorded with a Shimadzu RF-5301 PC Spectrofluorophotometer, using 5nm excitation and 10nm emission slit widths. Proteins at a concentration of 0.2g/L in PBS were used after dialysis and prior to glycation. The UV spectra of the protein, measured using a Perkin Elmer Lambda 25 UV/VIS Spectrometer, indicated a maximum absorbance at 277nm for all proteins, which was thereafter used as the excitation wavelength for each protein. Changes in maximum emission were compared between proteins in a semi-quantitative manner and changes in the emission spectra due to pre-treatment were also compared qualitatively. Near UV Circular Dichroism spectra of 1.5g/L protein and far UV Circular Dichroism spectra of 0.2g/L protein were recorded in a 0.5cm and 0.02 cm pathlength quartz cuvette, respectively, using a Jasco J-810 spectropolarimeter [35]. The UniProt database was used to identify structures, sequences and drug binding location on the BSA molecule (http://www.uniprot.org/uniprot/P02769).

Statistical analysis

All combinations of oxidative damage and glycation drivers were tested as six true replicates, according to the experiment. Assays were conducted in duplicate. Differences in fructosamine production between hydrogen peroxide levels were tested using a one-way ANOVA and Tukey's post-hoc tests, at each glucose level separately. The interaction between glucose levels and hydrogen peroxide levels, as well as the overall dose response effect, were studied using two-way ANOVA. Statistical analysis was performed using SPSS statistical software package version 19.0.0 (IBM, SPSS Software, Armonk, NY, USA).

Results

Using the primary search terms, 436 papers were identified, with 40 excluded as duplicate entries. Titles from the remaining 396 articles were screened and 43 were excluded as not relevant to the topic of the review (mostly studies focusing on phenylalanine and other phenol ring-containing substances). During abstracts screening, 246 reports were excluded from the analysis with the majority being in-vitro or animal studies' reports, alongside with reports from

cross-sectional studies. Review papers, conference proceedings, and reports written in languages other than English were also excluded. A total of 107 full papers were screened, leading to the exclusion of 28 additional reports. These reports either performed a qualitative analysis of metabolites in plasma or did not provide data on the concentrations of the metabolites measured in plasma. The reference list of each publication was screened in order to identify other publications from the same study, in case the data on the metabolites concentrations were published elsewhere. Finally, two reports [36, 37] were excluded as phenolic acids were reported as relative concentrations rather than absolute (Figure 1).

A total of 79 published studies satisfied the inclusion criteria and reported plasma levels of polyphenol and polyphenol metabolites following a dietary intervention. From those, 9 reports focused on longer-term feeding studies while the rest covered acute changes in polyphenol levels following a single consumption of a test food (Figure 1). Acute trials mainly focused on polyphenol bioavailability in the 5-24 hour window following ingestion, while longer-term feeding studies investigated changes in polyphenol metabolites levels after 5 days to 8 weeks supplementations [38, 39] (Table 1).

Most studies (Table 1) focused on a single food product, from a diverse range including tea (black and green) [40], coffee [41], olive oil [42], cocoa [43] and berries, either as a juice [44] or a mixed berry diet [39]. Two studies used extracts instead of a food product [38, 45]. Regarding sample treatment prior to phenolic acids measurements, only two of the studies measured free phenolic acids (i.e. without employing a prior hydrolysis step to derive aglycones from glucuronide and sulfate esters) [42, 46]. One of these two studies did not detect sufficient phenolic acids in plasma for quantification, despite using a HPLC/MS (LCQ Fleet quadripole ion-trap MS) detection system [46], and the other focused on hydroxytyrosol as a marker of compliance (olive oil intake) [42].

The major phenolic acids identified in the studies were: 3-hydroxyphenylacetic acid (120nM-338µM), 3,4-dihydroxyphenylacetic acid (110nM-135µM), homovanillic acid (90nM-199µM), m-coumaric acid (12.8-58.8nM), p-coumaric acid (15-30nM), caffeic acid (13.4nM-62.2µM), protocatechuic acid (99.4nM-10.52µM), ferulic acid (55.1-210nM) and vanillic acid (70nM-2.71µM). The compounds measured and identified all represent concentrations of aglycones after hydrolysis and not concentrations of free compounds.

A combination of 3-hydroxyphenylacetic acid (160nM), 3,4-dihydroxyphenylacetic acid (40nM), vanillic acid (20nM), p-coumaric acid (10nM), caffeic acid (10nM) and protocatechuic acid (40nM) was selected for the *in-vitro* glycation of BSA. These phenolic acids represent only a selection of all the acids identified in plasma after feeding interventions, but they are the ones for which data on absorption and metabolism are more extensive. The glucuronidation of polyphenols and phenolic acids may impact on the antiglycative capacity of the molecules [13]. These concentrations take into

consideration the fact that phenolic acid levels in all studies in the literature review were measured after hydrolysis, therefore a factor was applied to estimate what percentage of the reported values would be true non-conjugated phenolic acids.

This percentage was calculated based on findings from previous studies and more specifically, data from acute feeding interventions identified during the process of the present literature review. From acute feeding interventions measuring both the conjugated and non-conjugated form of phenolic acids, only 20-26% of the total caffeic found in plasma after coffee consumption is in free form [47, 48]. Similarly only 25% of the total vanillic acid, 40-50% of the total p-coumaric acid and 15-25% of the total ferulic acid are found as free phenolic acids[48, 49]. In the contrary 4-hydroxyphenylacetic acid is less extensively metabolised (80% present as free form) [49].

The published reports were of variable methodological quality. Although the detection systems used for phenolic acids measurement were based on mass spectrometry (MS, MS/MS, quadripole MS), some studies used less sensitive detection systems like ultraviolet, fluorimetric and electrochemical detectors[38, 41, 45]. One of the major methodological limitations was that 7 out of 9 studies employed some form of hydrolysis prior to phenolic acids determination. The measurement of aglycones following hydrolysis leads to a significant increase in the concentrations reported. The only study measuring phenolic acid concentrations without hydrolysis did not detect concentrations high enough for quantification even with a sensitive quadripole MS system [46].

The variable duration and dose of the compounds tested also make comparisons between the studies available difficult. The compounds tested were delivered in various matrices, some as supplements [38, 45], others as drinks [41, 43, 50, 51], or whole foods [46] and even as dietary pattern (combination of various food items) [39]. Such matrices will have a significant effect on bioavailabilty and potential long-term changes in absorption through changes in gut microbiota cannot be excluded/ controlled for. The lack of successful control conditions is another point to be mentioned, as some studies either had no control groups or used a cross-over design without allowing for sufficient wash-out periods [38, 41, 43]. When bioavailability of (poly)phenolic compounds is the main focus of the study the lack of a control group may be of lesser importance but not allowing for sufficient wash-out periods can introduce a carry-over effect between the interventions making difficult to compare between doses/groups tested.

Finally, as (poly)phenolic compounds are nearly ubiquitous, controlling for the effect of the background diet is important when studying bioavailability. In the studies reviewed, background diet control was variable ranging from the subjects being requested to abstain from polyphenol sources throughout the experimental period [46, 51] to being asked to consume their habitual diet [42]. One study requested from the participants to avoid food products similar to those provided

during the study period [39] and another gave no advice but requested from the participants to keep records of the flavonoid rich foods consumed during the study [45]. In order to control for the effect of background diet, Grimm et al requested from their volunteers to follow a flavonoid free diet 24 hours prior to blood sampling [38]. Unfortunately three of the studies did not provide with any information on whether and how they attempted to control for the effect of the background diet [41, 43, 50]. In this instance lack of adequate control of the background diet was not considered as a bias given the purpose of the review.

In-vitro assessment of antiglycative capacity of phenolic acids

The antiglycative capacity of phenolic acids was investigated in a two dimensional design. Phenolic acids were tested for their capacity to reduce fructosamine production when i) added in the reaction solution alongside glucose and H_2O_2 and ii) when pre-incubated with albumin (BSA) prior to the glycation incubation.

Effect of addition of hydrogen peroxide and phenolic acids in the reaction solution

Addition of 10nM H_2O_2 in the reaction solution throughout the incubation has a significant but opposing impact on fructosamine production of both native and phenolic pre-incubated albumin. Incubation of native albumin in the presence of H_2O_2 led to significantly higher levels of fructosamine at 10mM glucose compared to the glucose-only control (11% increase), whereas a reduction in fructosamine production was seen in the phenolic pre-incubated albumin (Table 2). A two-way ANOVA analysis showed that H_2O_2 affects fructosamine production of native and phenolic pre-incubated albumin independently of glucose ($p<0.001$ for both albumin forms; H_2O_2 plus glucose vs. glucose only control).

On the contrary addition of phenolic acids in the reaction solution throughout the incubation period had no significant effect on fructosamine production compared to glucose alone in any of the three albumin forms used (Table 2).

Effect of protein pre-treatment

Pre-oxidised and phenolic-preincubated albumin was more prone to glycation than the native molecule in the presence of 10mM glucose ($p=0.001$ and $p=0.02$, respectively) (Table 2). This effect was independent of glucose concentration, for both the pre-oxidised and the phenolic-preincubated albumin ($p=0.001$, two-way ANOVA). In the presence of H_2O_2, native and pre-oxidised albumin showed similar glycation levels ($p=0.52$; two-way ANOVA).

Preincubation with phenolic acids, on the other hand, significantly reduced glycation in the presence of H_2O_2 ($p=0.001$ vs native; two-way ANOVA). This effect was seen at both 5 and 10mM glucose ($p=0.01$ and $p<0.001$, respectively, vs. native albumin) (Figure 3) and the glycation

reduction was greater with increasing glucose levels ($p<0.001$ for the interaction pre-treatment*glucose levels).

Effect on AGEs production

After two weeks incubation, AGEs levels were not higher in any experimental condition compared to the 0mM glucose control, but for oxidised BSA exposed to 10mM glucose with 10nM H_2O_2 ($p = 0.048$, 205±15.8 vs.284±76.1 AU ; oxBSA 0mM glucose plus 10nM H_2O_2 vs. oxBSA 10mM glucose plus 10nM H_2O_2).

Effect of protein pre-treatment on protein structure and characteristics

The circular dichroism (CD) analysis showed no effect of the pre-treatment on the secondary protein structure (Figures 5 & 6). However, exposure to 10nM H_2O_2 for 8hrs lead to a 25% decrease in tryptophan fluorescence. Pre-incubation with phenolic acids for 8hrs resulted to a 38% reduction, indicative of protein-phenolic acid binding (Figure 4).

Discussion

Our mechanistic study results indicate that pre-treatment of albumin with phenolic acids inhibits fructosamine production, especially in the presence of oxidative stress or oxidative damage. This antiglycative activity was apparent when comparing the glycation achieved using phenolic-enriched albumin to those with the native and pre-oxidised BSA. A two-way ANOVA analysis showed that, in the presence of H_2O_2, albumin pre-incubated with phenolic acids had a significantly lower fructosamine content compared to the native BSA and the pre-oxidised BSA molecule. Phenolic acid preincubation only offered protection against fructosamine production in the presence of H_2O_2 10nM: it provided no protection against glycation by glucose alone. In the presence of H_2O_2, the antiglycative activity of phenolic acid pre-incubation was greater with higher glucose levels (10% vs. 22.5% decrease at 5 and 10mM glucose respectively). No effect was seen for AGEs production, with the exception of pre-oxidised BSA exposed to a combination of 10nM H_2O_2 and 10mM glucose. This maybe due to the duration of the experiment, too short to lead to AGEs formation in the given glucose concentrations. In contrast with most of the literature to date, which suggests that polyphenols and phenolic acids added to the incubation solution provide potent antiglycative activity[14, 18, 24, 52, 53], our results show that physical protection from glycation through protein-phenolic acid interaction is the most likely antiglycative mechanism especially in oxidative environments. In the previous investigations, concentrations used were

non-physiological, with the lowest glucose concentration being 30mM (>5-6 fold higher than normoglycaemia), generating higher glycation than our use of physiologically relevant 5 and 10 mM concentrations, representative of normoglycaemic and diabetic conditions respectively [11]. Our results suggest that pre-incubation of albumin with phenolic acids is the most likely mechanism to offer protection against glycation in a physiologically relevant system. Pre-incubation of BSA with either 10nM H_2O_2 or phenolic acids did not affect the secondary structure of the molecule but both led to the reduction of tryptophan fluorescence. Tryptophan is an established oxidation target in the BSA molecule [54], and oxidation would modify its fluorescence. The BSA molecule has only two tryptophans: one inside a α-helix in domain I and another one inside the hydrophobic binding pocket of domain II [55, 56]. A proposed mechanism of action from our result involves steric hindrance, with phenolic acids binding in a BSA locum which includes a tryptophan and consequently preventing the amino acids in this locum are to participate in oxidation and subsequent glycation reactions. (since BSA oxidation increases its susceptibility to glycation [11].

The literature review highlighted that 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, m-coumaric acid, p-coumaric acid, caffeic acid, protocatechuic acid, ferulic acid and vanillic acid are most commonly detected in plasma after long-term feeding interventions. Concentrations of phenolic acids ranged between 13.4nM-338.0µM. The majority of the studies measured phenolic acid levels as aglycones after hydrolysis of conjugated phenolic acids. This could have led towards a systematic overestimation of phenolic acid levels. Phenolic acids are subject to extensive rapid metabolism [22, 27] and since the chemical properties of their conjugates are often different, even opposite, from those of the aglycones [13] it is important for *in-vitro* studies to take their relative abundance into consideration. More *in-vivo* studies, reporting phenolic acid levels as aglycones and conjugates separately, are needed.

Nonetheless, the results of the literature review showcase that the use of food extracts or food-derived polyphenol molecules in mechanistic studies focusing on human metabolism is likely to be of limited physiological relevance. The parent compounds commonly used for *in-vitro* studies are rarely found in circulation, while metabolites like phenolic acids are found in potentially important concentrations in the nM to µM range. These metabolites are also not seen in isolation, but in fairly consistent combinations of phenylacetic acids, caffeic acid, vanillic acid and protocatechuic acid.

Despite increasing evidence that a very limited fraction of dietary polyphenols are absorbed, with low levels of metabolites circulating for limited amount of time, *in-vitro* models to-date utilise designs that make them irrelevant to physiology outside the gut lumen [30, 57-59]. Most of these reports used supra-physiological glucose and albumin concentrations [30, 57, 59] as well as methanolic

extracts of food products [60-62]. While of importance for the food industry [63], they hold limited translational value for human health and may confuse our understanding of the role of glycation in health and disease. Here, a combination of caffeic acid, p-coumaric acid, vanillic acid, protocatechuic acid, 3-hydroxyphenyl acetic acid, and 3,4-dihydroxyphenyl acetic acid was chosen to replicate physiological conditions, according to the systematic review.

This study has several limitations. The *in-vitro* design is in itself limiting and translation to physiology must still be cautious. Concentrations were chosen to mimic physiology for glucose, H_2O_2, and phenolic acids but this made it impossible to draw conclusions on the effect of individual phenolic acid on glycation. Not all our results fit a simple mechanistic explanation. It is intriguing that our results suggest that phenolic-preincubated albumin was more prone to glycation than native albumin, in the presence of 10mM glucose. We can offer no plausible mechanism to explain this, which may have been a random or chance effect. Nonetheless, this study has provided evidence to extend the published literature, showing that research into the antiglycative capacity of polyphenols is still possible at physiologically relevant concentrations of phenolic acids, and at physiological glucose concentrations, much lower than have been shown to generate glycation previously.

Conclusion

Phenolic acids have the capacity to modulate early stages of protein glycation under normoglycaemic, physiologically relevant conditions. Incubation with phenolic acids prior to glycation significantly inhibits the process in the presence of oxidative stress. Designing *in-vitro* studies with a high degree of physiological relevance is very important in order to reach biologically sound conclusions.

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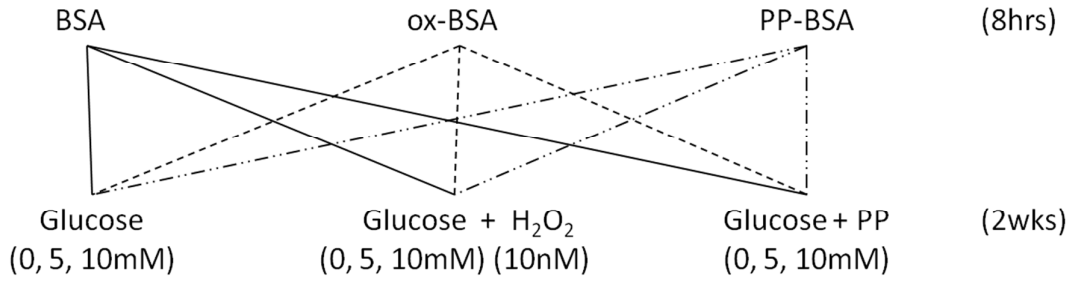
482 **Figure 1** Experimental design for the study of the antiglycative potential of physiologically
483 relevant phenolic acids.



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485 BSA: bovine serum albumin, ox-BSA: bovine serum albumin incubated with 10 nM H_2O_2, PP-BSA: bovine serum albumin
486 incubated with phenolic acids mixture; PP: caffeic acid 10nM, p-coumaric acid 8nM, vanillic acid 21nM, protocatechuic
487 acid 40nM, 3-hydroxyphenyl acetic acid 160nM and 3,4-dihydroxyphenyl acetic acid 40nM

488 **Figure 2** Flow diagram of the study selection process

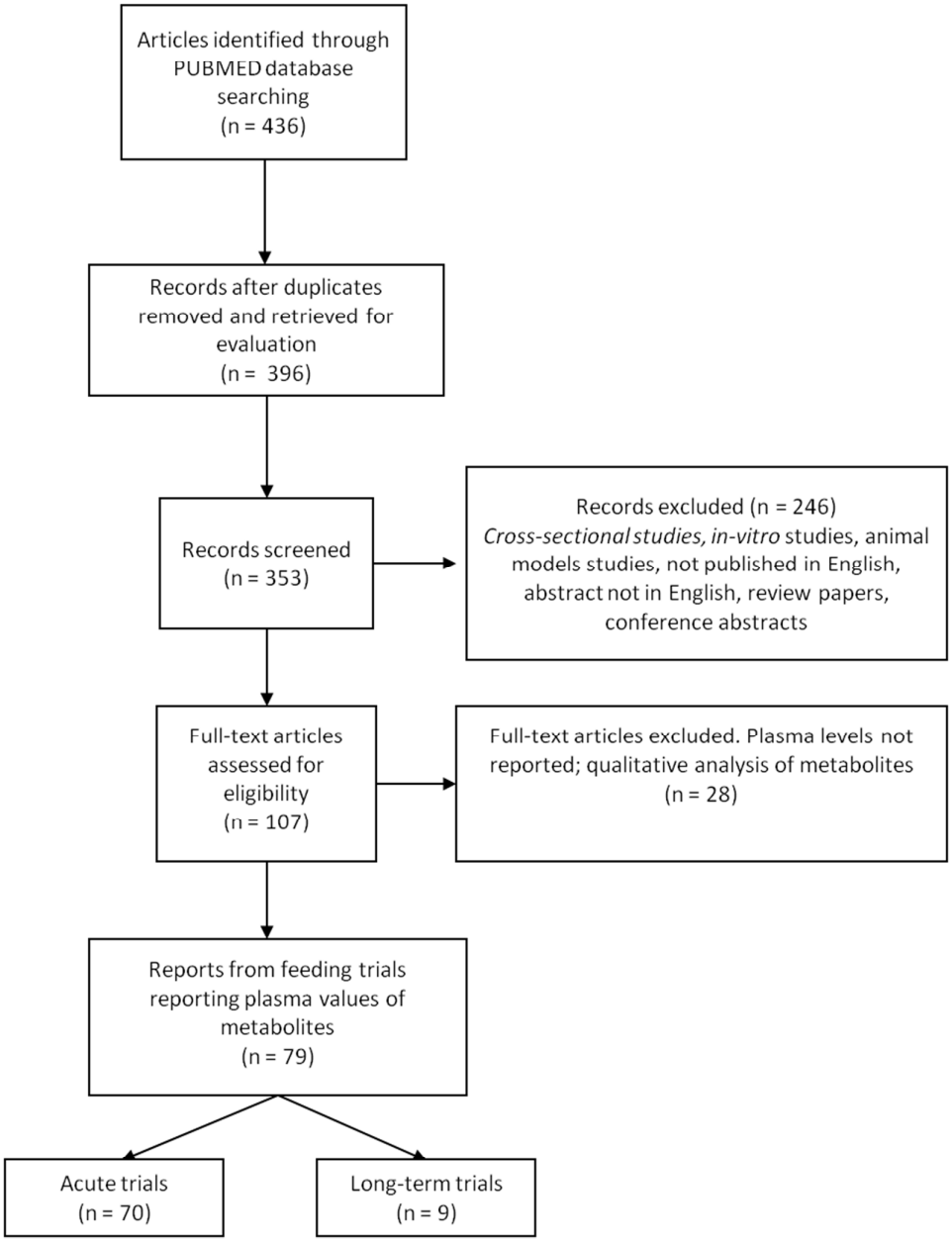

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Table 5.1 Evidence table of long-term supplementation trials measuring phenolic acids in plasma.

Authors	Food	Study design	Duration	Metabolites	Hydrolysis	Comments
Heinrich et al. (2013)	Blue honeysuckle berry (165g/d)	Uncontrolled (n=10)	1 wk	Below limit	No	Low polyphenol diet throughout
Henning et al. (2013)	Black tea (6 cups/d)	Non-blinded RCT, parallel (n=46)	3-6 wks	**3-hydroxyphenylacetic acid:** 261µM **4- hydroxyphenylacetic acid:** 668µM **3,4-dihydroxyphenylacetic acid:** 117µM **Hippuric acid:** 2305µM **Homovanillic acid:** 176µM	Yes	No dietary info
Henning et al. (2013)	Green tea (6 cups/d)	Non-blinded RCT, parallel (n=47)	3-6 wks	**3- hydroxyphenylacetic acid:** 338µM **4- hydroxyphenylacetic acid:** 798µM **3,4-di hydroxyphenylacetic acid:** 135µM **Hippuric acid:** 1950µM **Homovanillic acid:** 199µM	Yes	No dietary info
Oliveras-Lopez et al. (2012)	Extra virgin olive oil as a fat replacement plus 50mL raw	Non-blinded cross over (n=20)	4 wks	**Hydroxytyrosol:** 487nM	No	Habitual diet as control
Karlsen et al. (2010)	Bilberry juice (1L diluted in water)	Non-blinded parallel RCT (n=63)	4 wks	**Quercetin:** 43.6nM **m-coumaric:** 12.8nM **p-coumaric:** 27.4nM **protocatechuic:** 99.4nM	Yes	3wks low antioxidant/berry diet

Kempf et al. (2010)	Coffee 4 or 8 cups/d (150mL)	Single blind cross over (n=47)	30 d	**4cups** **Caffeic acid:** 38.3µM **Dihydrocaffeic acid:** 47.9nM **m-Coumaric acid:** 26.4nM **Dihydro-3-coumaric:** 716nM **Ferulic:** 55.1nM **Isoferulic:** 23.5nM **Dihydroferulic:** 93.9nM **Dihydroisoferulic:** 56.5nM **Dimethoxycinnamic:** 77 nM **3-(3,4-Dimethoxyphenyl)-propionic:** 203nM **8cups** **Caffeic acid:** 62.2µM **Dihydrocaffeic acid:** 75.2nM **m-Coumaric acid:** 58.8nM **Dihydro-3-coumaric:** 1583nM **Ferulic:** 67.1nM **Isoferulic:** 49.8nM **Dihydroferulic:** 194.7nM **Dihydroisoferulic:** 90.6nM **Dimethoxycinnamic:** 177.7nM **3-(3,4-Dimethoxyphenyl)-propionic:** 398nM	Yes	No wash out, All subjects consumed 0 cups (1st month), 4 cups (2nd month), 8 cups (3rd month)
Koli et al. (2010)	100g bilberries and nectar containing 50g lingonberries/ 100g black-currant-strawberry puree (80% black currant)	Non blinded RCT, parallel (n=72)	8 wks	**Quercetin:** 40nM **Caffeic acid:** 100nM **Protocatechuic acid:** 120nM **p-coumaric:** 15nM **Vanillic:** 70nM **3-(3-hydroxyphenyl)-propionic:** 800nM **3-Hydroxyphenylacetic:** 275nM **Homovanillic:** 90nM **3,4-dihydroxyphenylacetic acid:** 140nM	Yes	The two supplements were consumed on an alternate day basis

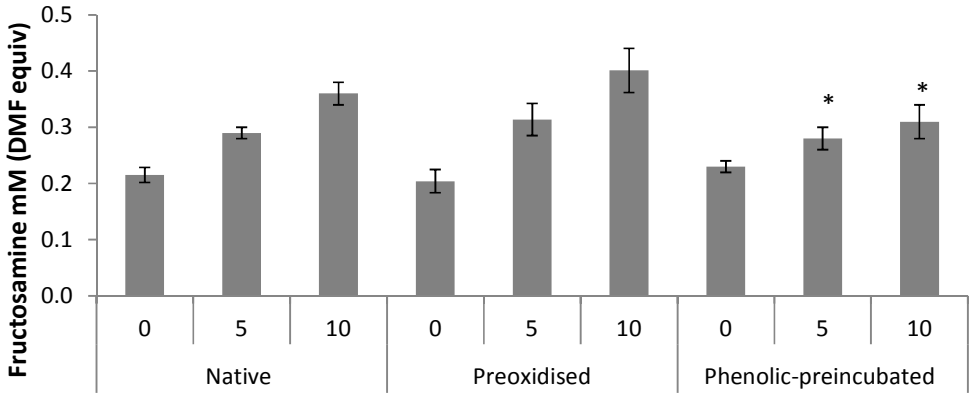
Urpi-Sarda et al. (2009)	Cocoa powder 40g/d with 500mL skimmed milk	Non-blinded RCT Cross-over (n=42)	4 wks	**3,4-Dihydroxyphenylpropionic acid:** 0.2µM **3-Hydroxyphenylpropionic acid:** 0.23 µM **3,4-Dihydroxyphenylacetic acid:** 0.11µM **3-hydroxyphenylacetic acid:** 0.12 µM **Phenylacetic acid:** 20.32 µM **p-coumaric acid:** 0.03 µM **caffeic acid:** 0.08µM **Ferulic acid:** 0.21 µM **Protocatechuic acid:** 10.52µM **Vanillic acid:** 2.71µM **4-hydroxybenzoic acid:** 9.73µM **4-Hydroxyhippuric acid:** 0.11µM **3-Hydroxyhippuric acid:** 0.48µM	Yes	No wash-out
Grimm et al. (2006)	Pine bark extract (200mg/d)	Non-controlled (n=5)	5 d	**Catechin:** 170nM **Caffeic acid:** 13.4nM **Ferulic acid:** 103nM	Yes	Measured 4h after the last dose
Boyle et al. (2000)	Rutin supplement (500mg/d)	Double blind RCT parallel (n=16)	6 wks	**Quercetin:** 166nM **Kaempferol:** 5.24nM **Isorhamnetin:** 9.49nM	Yes	Record flavonoid rich food

Table 2 Fructosamine concentration (mM DMF equivalent) after two week incubation with hydrogen peroxide or phenolic acids.

Glucose level (mM)	Pre-treatment		
	Native Mean (SD)	Pre-oxidised Mean (SD)	Phenolic-rich Mean (SD)
0	0.22 (0.01)	0.23 (0.02)	0.23 (0.02)
5	0.29 (0.01)	0.32 (0.01)	0.32 (0.02)
10	0.36 (0.02)	0.41 (0.03)*	0.40 (0.03)*
0+ H_2O_2 (10nM)	0.19 (0.04)	0.20 (0.02)	0.23 (0.01)
5+ H_2O_2 (10nM)	0.32 (0.03)	0.31 (0.03)	0.28 (0.02)*
10+ H_2O_2 (10nM)	0.40 (0.03)	0.40 (0.04)	0.31 (0.03)*
0+Phenolic acids	0.22 (0.01)	0.22 (0.02)	0.22 (0.01)
5+Phenolic acids	0.31 (0.02)	0.30 (0.02)	0.29 (0.02)
10+Phenolic acids	0.38 (0.04)	0.38 (0.03)	0.36 (0.03)

*p<0.05 vs native

Figure 3 Fructosamine concentration (mM DMF equivalent) after two weeks incubation in the presence of glucose and H_2O_2 (10nM) for native, pre-oxidised and phenolic-preincubated BSA (PP-BSA).



Two-way ANOVA analysis showed a significant effect of phenolic-preincubation inhibiting glycation. *p<0.05

Figure 4 Emission spectra of 0.2g/L BSA, ox-BSA and PP-BSA at λ=277nm showing the quenching effect of protein pre-treatment. Spectra were recorded at pH 7.4.

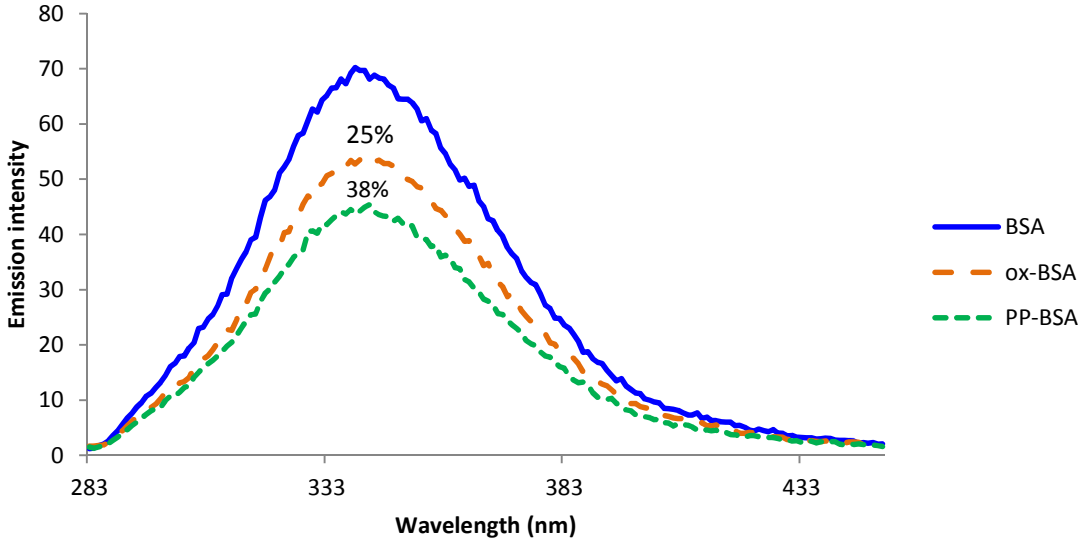


Figure 5 Near UV Circular Dichroism spectra of 1.5mg/ml of native BSA (blue), oxidised BSA (green) and phenolic-treated BSA (red). *Spectra were recorded in a 0.5cm pathlength quartz cuvette using a Jasco J-810 spectropolarimeter.*

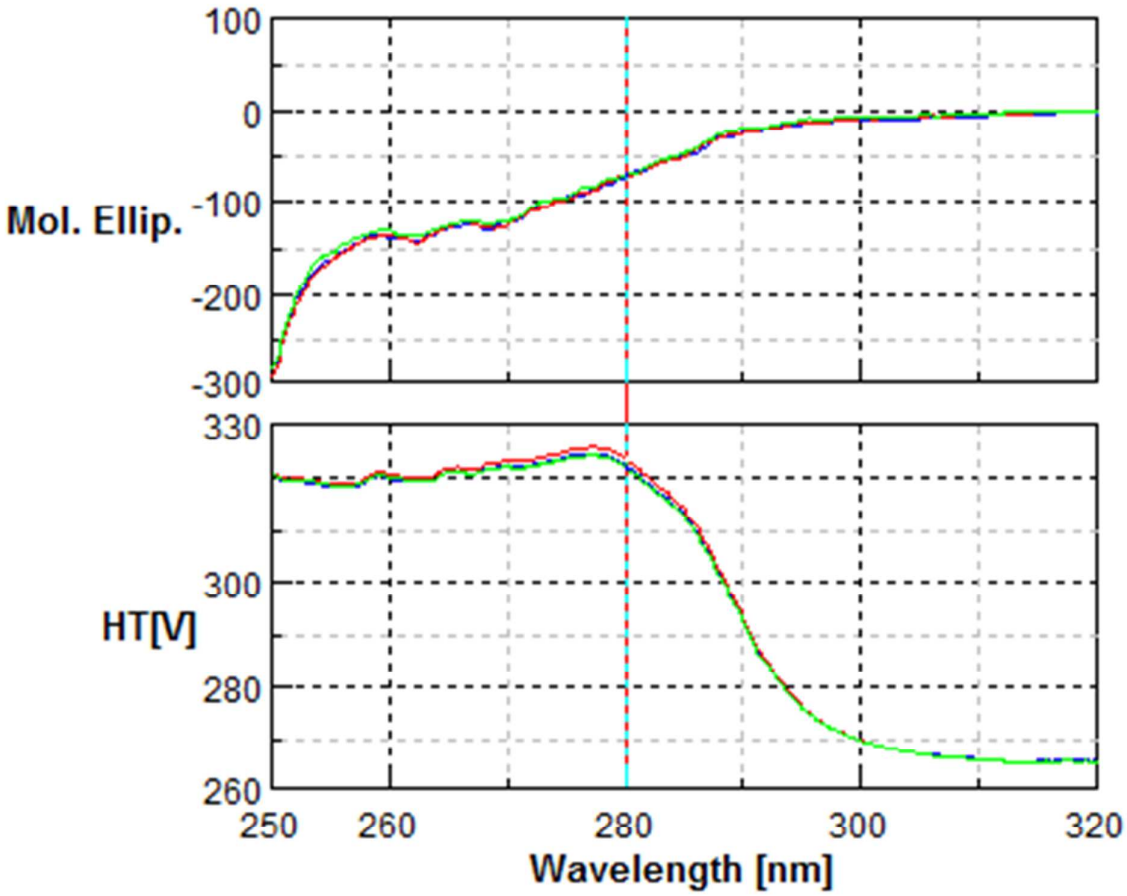


Figure 6 Far UV Circular Dichroism spectra of 0.2mg/ml of native BSA (blue), oxidised BSA (green) and phenolic-treated BSA (red). *Spectra were recorded in a 0.02cm pathlength quartz cuvette using a Jasco J-810 spectropolarimeter*