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Metal chelation, radical scavenging and inhibition of Aβ₄₂ fibrillation by food constituents in relation to Alzheimer’s disease

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Abstract

Various food constituents have been proposed as disease-modifying agents for Alzheimer’s Disease (AD), due to epidemiological evidence of their beneficial effects, and for their ability to ameliorate factors linked to AD pathogenesis, namely by: chelating iron, copper and zinc; scavenging reactive oxygen species; and suppressing the fibrillation of amyloid-beta peptide (Aβ). In this study, nine different food constituents (L-ascorbic acid, caffeic acid, caffeine, curcumin, (−)-epigallocatechin gallate (EGCG), gallic acid, propyl gallate, resveratrol, and α-tocopherol) were investigated for their effects on the above factors, using metal chelation assays, antioxidant assays, and assays of Aβ₄₂ fibrillation. An assay method was developed using 5-Br-PAPS to examine the complexation of Zn(II) and Cu(II). EGCG, gallic acid, and curcumin were identified as a multifunctional compounds, however their poor brain uptake might limit their therapeutic effects. The antioxidants L-ascorbic acid and α-tocopherol, with better brain uptake, deserve further investigation for specifically addressing oxidative stress within the AD brain.

Keywords
Polyphenols

Vitamins

Metal chelators

Antioxidants

Amyloid-beta peptide

**Chemical compounds**

L-Ascorbic acid (PubChem CID: 54670067); Caffeic acid (PubChem CID: 689043); Caffeine (PubChem CID: 2519); Curcumin (PubChem CID: 969516); (−)-Epigallocatechin gallate (PubChem CID: 65064); Gallamide (PubChem CID: 69256); Gallic acid (PubChem CID: 370); Propyl gallate (PubChem CID: 4947); Resveratrol (PubChem CID: 445154); α-Tocopherol (PubChem CID: 14985)

1. Introduction

Alzheimer’s disease (AD) is the most common form of dementia, and in 2013 there were 44 million people with dementia worldwide, with this number expected to increase to 76 million by 2030, and 135 million by 2050 (Prince, Guerchet, & Prina, 2013). AD involves a gradual worsening in memory and cognitive function, deficits that can be improved modestly with the use of symptomatic drugs, such as acetylcholinesterase inhibitors and N-methyl-D-aspartate receptor antagonists (R. Anand, Gill, & Mahdi, 2014). However, there is currently no disease-modifying treatment that can halt or slow the progression of AD. Various foods and their chemical constituents have been proposed as disease-modifying agents for AD, supported by epidemiological evidence of their beneficial effects. For example, better cognitive function has been observed in older populations that have a high
intake of curries (Ng et al., 2006) and teas (Ng, Feng, Niti, Kua, & Yap, 2008). This phenomenon has been attributed to the medicinal properties of curcumin, which is found in the spice turmeric (Ringman, Frautschy, Cole, Masterman, & Cummings, 2005) that is an ingredient of curries, as well as (−)-epigallocatechin gallate (EGCG) and gallic acid which are found in teas (Bastianetto, Yao, Papadopoulos, & Quirion, 2006). It has been reported that higher consumption of coffee in mid-life is associated with a lower risk of dementia and AD in the later years of life (Eskelinen, Ngandu, Tuomilehto, Soininen, & Kivipelto, 2009). A lower dementia risk was also seen in groups consuming wine, but the consumption of spirits increased the risk, suggesting that wine components, such as polyphenols, are responsible for the risk-modifying effect rather than ethanol (Mehlig et al., 2008). Other food constituents, such as L-ascorbic acid found in citrus fruits, and α-tocopherol found in nuts, have been promoted as antioxidants of benefit in AD (Dysken et al., 2014; Harrison, Bowman, & Polidori, 2014). The beneficial effects of the aforementioned foods might be attributed to the ability of their constituents to ameliorate specific factors linked to AD pathogenesis. According to the amyloid cascade hypothesis, amyloid-beta peptide (Aβ) accumulation is the primary event in AD pathogenesis, and soluble Aβ oligomers are the main neurotoxic species due to cell membrane perturbation, oxidative stress, and other mechanisms (Fandrich, 2012). Aβ contains metal binding sites, and metals, such as copper, zinc and iron, are present at elevated concentrations in Aβ plaques (Greenough, Camakaris, & Bush, 2013). Metals can affect the morphology of Aβ aggregates, accelerate Aβ fibrillation, and increase the cytotoxicity of Aβ (Viles, 2012). Redox active copper and iron in complex with Aβ can generate hydrogen peroxide, which may be converted to hydroxyl radicals via Fenton chemistry, resulting in oxidative cellular damage (Greenough et al., 2013). Consequently, foods constituents that can suppress Aβ fibrillation, chelate metal ions, and scavenge free radicals, may be of benefit in AD.
In this study, nine different food constituents (Fig. 1; L-ascorbic acid, caffeic acid, caffeine, curcumin, (-)-epigallocatechin gallate (EGCG), gallic acid, propyl gallate, resveratrol, and α-tocopherol) were investigated, in vitro, for their effects on the above factors using assays that examine metal chelation, antioxidant properties and inhibition of Aβ_{42} fibrillation. An aim of the study was to determine the relative potencies of the food constituents, and to identify multifunctional agents with good metal chelation, antioxidant, and anti-fibrillation activities. As part of this work, a new assay method was developed to examine the complexation of Zn(II) and Cu(II). The bioavailability and brain uptake of the food constituents was considered via a literature review, and this helped to identify compounds worthy of further investigation in relation to AD.

2. Materials and methods

2.1. Materials and general methods

The synthetic non-food related molecule gallamide was included in these studies to help to decipher structure-activity relationships, and the known metal chelators EDTA and D-penicillamine were used as positive control comparator compounds in the metal chelation assays. All experiments were conducted at room temperature (23 °C) unless otherwise specified. Commercial chemicals and reagents of at least analytical grade were used. Caffeic acid, curcumin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-(5-bromo-2-pyridylazo)-5-[N-propyl-N-(3-sulfopropyl)amino]phenol disodium salt dihydrate (5-Br-PAPS), 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine monosodium salt hydrate (ferrozine), iron (II) sulfate heptahydrate, zinc sulfate heptahydrate, EGCG, gallic acid, propyl gallate, 3,4,5-trihydroxybenzamide (gallamide), α-tocopherol, resveratrol, D-penicillamine, caffeine, EDTA, thioflavin T (ThT) and sodium chloride were purchased from Sigma-Aldrich. L-Ascorbic acid was obtained from Chem-Supply. Methanol (HPLC grade) was purchased from
Burdick and Jackson. Copper (II) sulfate pentahydrate was obtained from Scharlab. Synthetic Aβ42 with purity > 95% was purchased from Mimotopes and stored at –80 °C. Type 1 ultrapure water was used throughout the study. Buffers were prepared by reference to ChemBuddy Buffer Maker software (version 1.0.1.55), using disodium hydrogen phosphate and sodium dihydrogen phosphate dihydrate, or HEPES and HEPES sodium salt, purchased from Sigma-Aldrich, and buffers were filtered through a 0.45 µm regenerated cellulose membrane before use. The buffer ionic strength (I) was adjusted with NaCl for use at 37 °C. Bath sonication was conducted using a 70 W Soniclean 160HD benchtop ultrasonic cleaner operating at 43 kHz ± 2 kHz sweep bandwidth with 20 Hz pulses. Ultracentrifugation was carried out using a Beckman Coulter Optima L-100 K Preparative Ultracentrifuge with a Type 100 Ti fixed angle rotor and 2 ml quickseal, bell-top polypropylene tubes. UV absorbance was measured using a Cary 50 UV visible spectrophotometer and a 1 cm path length quartz cell, or a Bio-Rad iMark Microplate Absorbance Reader. Fluorescence intensity was measured using a BMG Fluostar Omega plate reader. For the ThT assay, protein LoBind Eppendorf tubes and Corning non-binding surface assay plates (96 well half area black with clear flat bottom polystyrene NBSTM microplate) were used to minimize Aβ42 binding to surfaces. To minimize evaporation, the plates were sealed with Corning non-sterile aluminum sealing tape. Copper grids, 200 mesh square for transmission electron microscopy (TEM), were purchased from ProSciTech. Corning polystyrene assay plates (96 well clear flat bottom polystyrene microplates) were used for metal chelation and antioxidant kinetics studies. Regression and statistical analysis was performed using GraphPad Prism 6 (version 6.04). The CLogP of α-tocopherol was calculated using the CLogP function of ChemBioDraw Ultra 13.0; CLogP licensed from BioByte.

2.2. Metal chelation assays

2.2.1. Chelation of Cu(II) and Zn(II)
Type 1 ultrapure water was filtered through a 0.45 μm regenerated cellulose membrane, degassed by sonication for 1.5 h under vacuum, and then immediately sealed with an airtight lid. This water was used to prepare the solutions for the metal chelation assays. A new assay method was developed to examine the complexation of Zn(II) and Cu(II) using the indicator ligand 5-Br-PAPS which was first used by Makino, Saito, Horiguchi, & Kina (1982) to determine serum zinc concentrations. The absorbance of the metal-5-Br-PAPS complex at 560 nm was used to estimate the concentration of free metal. The assay was conducted using 96 well plates and solutions were prepared using HEPES buffer (15 mM, pH 7.5). A thirteen-point plot was obtained for the absorbance at 560 nm of solutions of Zn(II) ranging from 0 to 12 µM, containing 60 µM of 5-Br-PAPS ($r^2 > 0.99$) (Supplementary Fig. S1). A sixteen-point plot was obtained for the absorbance at 560 nm of solutions of Cu(II) ranging from 0 to 15 µM, containing 60 µM of 5-Br-PAPS ($r^2 > 0.99$) (Supplementary Fig. S2). These plots assisted with the choice of metal concentrations that gave absorbance values of < 1.0 for the chelation experiments, and they demonstrated that a linear response in absorbance could be achieved for the concentrations of free metal present in the assay. Stock solutions of ligands (food constituents) were prepared as described in Supplementary Table S1. To ensure complete dissolution, L-ascorbic acid, caffeic acid, caffeine, curcumin, resveratrol, and α-tocopherol were dissolved in a small volume of DMSO prior to dilution with buffer, with the concentration of DMSO in the final assay solution ≤ 0.67% v/v. Solutions of various concentrations of ligand (prepared from the stock solution by dilution with buffer; 100 µl, concentrations in the final assay solution are described in Supplementary Table S1) were mixed with a solution of metal ion (100 µl, total metal ion concentrations in the final assay solution, 10 µM Cu(II) or 7 µM Zn(II)) and stored at room temperature for 30 min to allow complex formation. A solution of 5-Br-PAPS (100 µl, concentration in the final assay solution, 60 µM) was added and the absorbance at 560 nm was recorded. The delay
time between addition of 5-Br-PAPS and the initial absorbance measurement was generally 30 s. Absorbance readings were taken every 10 s for the first 5 min, then every 1 min until 15 min was reached. In cases where the absorbance increased over time, an exponential one-phase association equation (1),

\[ A_t = A_0 + (A_\infty - A_0)(1 - e^{-kt}) \]

was fitted to the absorbance-time data and the absorbance at time = 0 was obtained and used to estimate the concentration of free metal at time = 0, where: \( A_t \) is the absorbance at time \( t \); \( A_0 \) is the absorbance at time = 0, corresponding to the \( A_{M+L+In} \) term in equation 2; \( A_\infty \) is the absorbance at infinite time; \( k \) is the rate constant, expressed in units of inverse time.

In cases where the absorbance was constant over the time-course of the experiment, the first absorbance reading was used to estimate the concentration of free metal at time = 0 (corresponding to the \( A_{M+L+In} \) term in equation 2) (Supplementary Fig. S4 and Supplementary Table S2). The chelation (%) was estimated using the equation (2),

\[ \text{Chelation (\%)} = \left(1 - \frac{A_{M+L+In} - A_{M+In}}{A_{M+In} - A_M}\right) \times 100 \]

where \( A \) is the absorbance of a solution containing one or more of the following solutes: \( M \), metal; \( L \), ligand (food constituent); and \( In \), indicator ligand (5-Br-PAPS). Three independent experiments were conducted.

2.2.2. Chelation of Fe(II)

Complexation of Fe(II) was examined using the ferrozine assay whereby the absorbance of the Fe(II)-ferrozine complex at 560 nm was used to estimate the concentration of free metal (Carter, 1971). The assay was conducted using the same method that was used to study the chelation of Cu(II) and Zn(II), with the following exceptions. Solutions were
prepared using HEPES buffer (15 mM, pH 6.8). A fourteen-point plot was obtained for the absorbance at 560 nm of solutions of Fe(II) ranging from 0 to 30 µM, containing 1 mM of ferrozine ($r^2 > 0.99$) (Supplementary Fig. S5). Supplementary Table S1 describes the preparation of the solutions of ligands. In the assay, the final total concentration of Fe(II) was 25 µM and the final concentration of ferrozine was 1 mM. Three independent experiments were conducted.

2.3. Antioxidant assays

2.3.1. Antioxidant stoichiometry

The following procedure was adapted from the method of Shi & Niki (1998). Solutions of DPPH (112.5 µM), L-ascorbic acid (180 µM), α-tocopherol (180 µM), caffeic acid (180 µM), caffeine (900 µM), curcumin (180 µM), EGCG (45 µM), gallamide (45 µM), propyl gallate (45 µM) and resveratrol (180 µM), were prepared in methanol immediately prior to use. The initial absorbance value ($A_0$) was obtained by taking the mean of the absorbance, at 515 nm, of three 1 ml fractions of DPPH solutions each diluted with 125 µl of methanol (final concentration of DPPH, 100 µM). One hundred and twenty five µl of each solution containing antioxidant (food constituent) was mixed with 1 ml DPPH in a microcentrifuge tube and sealed with laboratory film to minimise evaporation (final concentrations: DPPH (100 µM), L-ascorbic acid (20 µM), α-tocopherol (20 µM), caffeic acid (20 µM), caffeine (100 µM), curcumin (20 µM), EGCG (5 µM), gallamide (5 µM), propyl gallate (5 µM) and resveratrol (20 µM)). After storage for 24 h in the dark, the absorbance of each solution at 515 nm was measured ($A_{24}$). The stoichiometric factor ($n$) was calculated using the equation (3),

$$n = \frac{\Delta A}{e \cdot [AO]} \quad \text{– Equation 3,}$$
where: $\Delta A$ is the change in absorbance at 515 nm ($A_0 - A_{24}$); $\varepsilon$ is the molar extinction coefficient of DPPH; $l$ is the optical length of the cuvette; and $[AO]$ is the concentration of antioxidant at time = 0. The molar extinction coefficient of DPPH at the absorbance wavelength of 515 nm is 10450 L mol$^{-1}$ cm$^{-1}$ based on a calibration curve (Supplementary Fig. S6) for concentrations of DPPH between 0 and 150 µM ($r^2 = 0.995$). Three or more independent experiments were conducted.

2.3.2. Antioxidant reactivity

The following procedure was adapted from the method of Campos, Duran, Lopez-Alarcon, & Lissi (2012). Solutions of DPPH (67.5 µM) were prepared in methanol (for normal kinetic studies), or in methanol containing 11.25, 112.5, 1125 mM acetic acid (for kinetic studies at lower pH to suppress phenoxide formation (Litwinienko & Ingold, 2007)). Solutions of antioxidants (45, 90, 135, 180 and 225 µM (for antioxidants that react rapidly) or 90, 225, 450, 675, 900 µM (for antioxidants that react slowly)) were prepared in methanol. The assay was conducted using 96 well plates. The absorbance at time = 0 ($A_0$) was obtained from the average absorbance of 5 wells each containing 25 µl of methanol and 200 µl of DPPH solution (final concentration of DPPH, 60 µM). The kinetics measurements were conducted in batches, by measuring 5 wells concurrently as follows. DPPH solution (200 µl) was added to 25 µl of each of the 5 concentrations of the same antioxidant using a multichannel pipette, and the absorbance at 515 nm was recorded (final concentrations: DPPH, 60 µM; and the antioxidants, 5, 10, 15, 20 and 25 µM or 10, 25, 50, 75, and 100 µM). Readings were taken every 10 s for the first 200 s, then every 30 s until 500 s was reached. The delay time between the addition of DPPH solution and the first absorbance reading was 12 s, and this was taken into account when plotting absorbance-time curves. An empirical bi-exponential equation (4) was fitted to the data,
\[ A_t = A_\infty + A_f e^{-kt} + A_s e^{-k_s t} \]  \quad \text{-- Equation 4,}

with the y-intercept constrained to equal \( A_0 \), and where: \( A_t \) is the absorbance at time \( t \); \( A_f \) and \( A_s \) are the amplitudes associated with the fast and slow decays; \( k_f \) and \( k_s \) are the first order rate constants of the fast and slow decays; and \( A_\infty \) accounts for unreacted DPPH at infinite time. The bleaching rate is described by the first derivative of Equation 4,

\[- \frac{dA}{dt} = A_f k_f e^{-kt} + A_s k_s e^{-k_st} \]  \quad \text{-- Equation 5.}

Substituting \( t = 0 \) into equation 5 affords an equation for the bleaching rate extrapolated to zero time,

\[- \left( \frac{dA}{dt} \right)_0 = A_f k_f + A_s k_s \]  \quad \text{-- Equation 6.}

A plot of \( (dA/dt)_0 \) against \( A_0[AO] \) gives the overall bimolecular rate constant \( (k_b) \) as the gradient of the linear fit. Plots of \( (dA/dt)_0 \) against \( A_0[AO] \) were linear with \( r^2 \) values > 0.85, as shown in the example with EGCG in Supplementary Fig. S7. Stoichiometric \( k'_b \) values were calculated by dividing the overall bimolecular rate constants \( (k_b) \) by the stoichiometric factors \( (n) \) (Shi & Niki, 1998),

\[ k'_b = k_b/n \]  \quad \text{-- Equation 7.}

Three independent experiments were conducted.

### 2.4. ThT fluorescence assay of Aβ42 fibrillation

The method used was based on our published protocol (Rajaram et al., 2014), with the main difference being that Aβ42 was used instead of Aβ40. Briefly, low molecular weight (LMW) Aβ42 rich in monomers and small oligomers was prepared by dissolution of Aβ42 (2.5 mg) in dilute aqueous NaOH solution (1.0 ml of water containing 7.5 µl of 1 M aqueous NaOH)
followed by dilution with phosphate buffer (1.0 ml, 40 mM, pH 7.4), sonication for 60 s, and ultracentrifugation (513000 × g at r_{max} for 90 min at 4°C). The upper 500 µl of supernatant was mixed with 100 µl of phosphate buffer (20 mM, pH 7.4) to afford a stock solution of LMW Aβ_{42}. One part of test compound and 1 part of ThT solution (60 µM), both in phosphate buffer (20 mM, pH 7.4, I 0.225 M), were added to an Eppendorf tube, followed by the addition of 1 part of LMW Aβ_{42} stock solution (81 µM in phosphate buffer (20 mM, pH 7.4); the concentration of Aβ_{42} was determined by UV absorbance). The solution was gently mixed with a pipette and an aliquot (70 µl) was transferred to the well of a half-area 96-well plate. The final concentrations of components were: test compounds, 10 or 100 µM; ThT solution, 20 µM; and Aβ_{42}, 27 µM; in phosphate buffer (20 mM, pH 7.4, I 0.17 M). DMSO (final concentration 0.1% v/v) was present in resveratrol and α-tocopherol samples as a cosolvent to ensure complete dissolution. The plate was sealed with aluminium sealing tape and incubated at 37°C under quiescent conditions for 21 h with fluorescence readings (λ_{ex} 440 nm, λ_{em} 480 nm) taken every 5 min. Fluorescence intensity readings were corrected for the fluorescence of ThT alone (20 µM). Supplementary equation S1 was fitted to the data to obtain the half-life and amplitude values, which are reported as the mean + SEM of three independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey’s multiple comparisons test.

2.5. Transmission electron microscopy

Formvar-coated copper grids were activated by irradiation at 254 nm using a UV lamp. Samples (7 µl) were taken from the wells of assay plates used in the ThT assay, immediately after the completion of the fluorescence studies, and placed in contact with the grid for 2 min. The excess solution was removed using filter paper. Aqueous ammonium molybdate solution (10 µl, 3% w/v) was added and the sample stained for 1 min; excess
solution was removed using filter paper. The grid was allowed to air dry and TEM images were captured using a JEOL 1011 microscope at 100 kV with magnification at ×100k.

3. Results and discussion

3.1. Metal chelation

The ability of the food constituents to chelate Fe(II) was examined using the ferrozine assay, and a new assay method was developed using 5-Br-PAPS to study the complexation of Zn(II) and Cu(II). Solutions of ligand (L; food constituent) and metal (M; Fe(II), Cu(II) or Zn(II)) were stored for 30 min to allow complex (ML) formation: M + L ⇌ ML. A solution of indicator ligand (In; either ferrozine for Fe(II) or 5-Br-PAPS for Cu(II) and Zn(II)) was then added to detect free (uncomplexed) metal: M + In ⇌ MIn. Both ferrozine and 5-Br-PAPS react rapidly with free metal (Supplementary Fig. S8) to form water soluble, purple to orange-red coloured, metal-indicator ligand complexes (MIn). The absorbance at 560 nm was used to estimate the concentration of free metal. In some cases the absorbance increased over time after the addition of indicator ligand, demonstrating that ML was unstable in the presence of ferrozine or 5-Br-PAPS, with metal of the ML complex being sequestered by the indicator ligand until equilibrium was reached typically within 5 min (Supplementary Fig. S3 and Supplementary Table S2). In these cases, an exponential one-phase association equation was fitted to the absorbance-time data and the absorbance at 0 s was obtained and used to estimate the concentration of free metal at 0 s. In other cases, the absorbance was constant over the time-course of the experiment, signifying that ML was stable in the presence of ferrozine or 5-Br-PAPS, and the first absorbance reading was used to estimate the concentration of free metal at 0 s (Supplementary Fig. S4 and Supplementary Table S2). The percentage chelation was estimated, and Fig. 2 shows the logarithm of the concentration of ligand required for 50% chelation of 25 µM Fe(II), 10 µM Cu(II), and 7 µM Zn(II)
(Supplementary Table S3 contains the concentration of ligand in µM for 50% chelation). The positive control comparator compound EDTA was a strong chelating agent, and the other positive control comparator compound, D-penicillamine, strongly chelated Cu(II) but it was only a moderate and weak chelator of Zn(II) and Fe(II) respectively, which is consistent with its clinical application as a Cu(II) chelating agent in the treatment of Wilson’s disease. L-Ascorbic acid, α-tocopherol, caffeine, caffeic acid, and resveratrol were observed to be weak chelating agents, with concentrations over 600 µM required for 50% metal chelation. Phenolic compounds that contained the pyrogallol moiety (gallic acid, propyl gallate, gallamide, and EGCG) were all strong chelators of Fe(II). EGCG was also a strong chelator of Cu(II) and Zn(II), however gallamide, propyl gallate, and gallic acid were moderate-to-weak chelators of Cu(II) and only weak Zn(II) chelating agents. It is known that phenols that are capable of acting as bidentate ligands, such as those bearing oxygen donor atoms in ortho or peri positions, are good chelators of many metals, especially of iron (Hider, Liu, & Khodr, 2001). In this study, EGCG was more potent than gallamide, propyl gallate, and gallic acid, which is partly explained by EGCG having more than one metal binding site, whereas gallamide, propyl gallate and gallic acid contain a single metal binding site. Deprotonation of the phenolic group is required for metal chelation, and Perron, Hodges, Jenkins, & Brumaghim (2008) showed that phenolic pKₐ values correlate with inhibition by polyphenols of Fe(II)-induced DNA damage. A similar trend was observed in this study, in terms of direct correlation between phenolic pKₐ values (Table 1) and metal chelation: the pyrogallol compound with the lowest phenolic pKₐ value, EGCG, was the most potent chelator; the pyrogallol compound with the highest phenolic pKₐ value, gallic acid, was the least potent chelator; and propyl gallate and gallamide with intermediate phenolic pKₐ values had intermediate potency. Curcumin, which coordinates with metals through its keto-enolic moiety (Zhao et al., 2010), was observed to be a moderate chelator of all of the three metals.
(Bernabe-Pineda, Ramirez-Silva, Romero-Romo, Gonzalez-Vergara, & Rojas-Hernandez, 2004; Zhao et al., 2010) with concentrations of 35 to 41 µM affording 50% metal chelation. Significantly, curcumin formed stable complexes with all three metals, whereas the strong-to-moderate chelation by EGCG, gallamide, propyl gallate, and gallic acid, formed complexes that were unstable in the presence of ferrozine or 5-Br-PAPS (Supplementary Table S2).

3.2. Antioxidant properties

Antioxidants reduce the deep violet coloured DPPH free radical to yellow DPPH-H, so the antioxidant properties of the food constituents were studied by monitoring their ability to bleach the absorbance of a methanolic DPPH solution at 515 nm. Two parameters that are important to consider when studying antioxidants are: a) the antioxidant stoichiometry – the number of radicals scavenged by one molecule of antioxidant; and b) the antioxidant reactivity – the rate of the reaction between the antioxidant and radicals. Mixing the food constituents with an excess of DPPH, and measuring the amount of DPPH remaining after 24 h, enabled estimation of the antioxidant stoichiometry (Table 1, Stoichiometric factor, n). All of the polyphenols were antioxidants, with stoichiometric factors ranging from 14.8 for EGCG to 2.8 for resveratrol. Polyphenols that contained the pyrogallol moiety (EGCG, gallamide, propyl gallate, and gallic acid) had the highest stoichiometric factors, with values around twofold higher than the number of OH groups present in these molecules. Similarly, caffeic acid had a stoichiometric factor of 3.3, which is higher than would be anticipated from the two OH groups contained in its pyrocatechol moiety. The ability of these polyphenols to reduce more DPPH radicals than their number of available OH groups is consistent with the literature (Supplementary Table S4), and it is proposed to be due to regeneration of the OH groups from their oxidation products via various mechanisms, including nucleophilic addition of methanol (solvent) with an ortho-quinone (Saito, Gao, & Kawabata, 2006). α-Tocopherol is known to donate two H atoms and form α-tocopheryl quinone via a quinone methide.
intermediate (Tappel, 1972), which explains its ability to scavenge two DPPH radicals despite possessing only one OH group. The other food constituents had stoichiometric factors aligned with their number of phenolic and enolic OH groups.

The antioxidant reactivity of the food constituents was estimated via kinetic analysis of the rate of DPPH bleaching immediately after mixing the food constituents with DPPH (Fig. 3). In accordance with the study of Campos et al. (2012), we observed that the reaction was best modelled by an empirical bi-exponential decay equation incorporating both fast and slow decay processes (Fig. 3 and equation 4, $R^2 \geq 0.9552$). By studying the reaction over a range of food constituents’ concentrations, and analysing the data as explained in the experimental section, an estimate of the overall bimolecular rate constant was obtained (Table 1, Overall $k_b$). As illustrated in Fig. 3 and quantified by the overall $k_b$ values (Table 1), there were distinct differences in reactivity between compounds. For example, resveratrol reacted very slowly with DPPH and steady state was not achieved in 500 s. In contrast, L-ascorbic acid reacted very quickly and achieved steady state in less than 15 s. The reaction was so rapid that we were unable to estimate a bimolecular rate constant for L-ascorbic acid using the techniques available to us in this study. Based on overall $k_b$ values, the order of antioxidant reactivity was: L-ascorbic acid > EGCG > propyl gallate > gallamide > gallic acid > curcumin > caffeic acid > α-tocopherol > resveratrol. It is seen from this sequence that, with the exception of L-ascorbic acid, compounds with higher $n$ values tend to have higher overall $k_b$ values. This is not surprising because both the number of active H atoms in each antioxidant molecule, as well as their reactivity, will influence the overall reaction rate. Stoichiometric $k'_b$ values were calculated by dividing the overall bimolecular rate constants ($k_b$) by the stoichiometric factors ($n$) (Table 1) (Shi & Niki, 1998). These $k'_b$ values can be considered to be the average reactivity of the active H atoms in the antioxidant molecule, and they facilitate comparison of the reactivity of a series of molecules with different
stoichiometries. The stoichiometric $k'_b$ values of the phenolic molecules were similar in magnitude, while L-ascorbic acid had a substantially larger $k'_b$ value.

The reaction between phenolic antioxidant and radical is known to proceed by at least three mechanisms: hydrogen atom transfer (HAT); proton-coupled electron transfer (PCET), which both involve the transfer of proton and electron in one kinetic step; and sequential proton-loss electron transfer (SLEET), in which the phenol dissociates into a phenoxide anion, followed by electron transfer from the phenoxide anion to the radical that is subsequently protonated (Supplementary Fig. S9) (Di Meo et al., 2013; Litwinienko & Ingold, 2007). In addition to being influenced by the properties of the reactants, the relative contribution of each mechanism to the overall process is highly dependent on the ability of the solvent to promote ionisation of the phenol and to accept hydrogen bonds (HBs) (Litwinienko & Ingold, 2007). The rate constant for electron transfer ($k_{ET}$) (the rate limiting step of SLEET (Di Meo et al., 2013)) is normally much greater than the rate constant for HAT ($k_{HAT}$), hence SLEET can be the prevailing mechanism even at very low phenoxide concentrations (Litwinienko & Ingold, 2007). In solvents with high dielectric constants and good anion solvation abilities (e.g. water, methanol), phenoxide formation is encouraged and usually SLEET dominates, whereas in solvents with low dielectric constants and poor ability to solvate anions (e.g. alkanes, ethyl acetate), phenoxide formation is deterred and typically HAT or PCET is the main mechanism (Litwinienko & Ingold, 2007). In HB-acceptor solvents, H-bond formation between the solvent and the H atom of the antioxidant OH group suppresses HAT/PCET, with only the non-H-bonded fraction of OH groups reactive (Litwinienko & Ingold, 2007). Consequently, HAT/PCET is relatively slower in HB-acceptor solvents (e.g. ethyl acetate, methanol) than in non-HB-acceptor solvents (alkanes) (Litwinienko & Ingold, 2007). In our antioxidant study, methanol was used as the solvent hence SLEET is likely to be the predominant mechanism, although the non-SLEET
mechanisms may still contribute. The free radical (DPPH) was identical in all experiments, thus its influence on the reaction is constant, and differences in reactivity should therefore be attributable to differences in antioxidant structure. The order of antioxidant reactivity was broadly aligned with the $pK_a$ values for the most acidic OH group in each of the molecules, that is, the greater the acidity of the OH group, the larger the rate constant (Table 1). This can be interpreted in terms of the SPLET mechanism, since the more acidic the OH group, the greater the concentration of phenoxide that is available for the rate-limiting electron transfer step of SPLET, and normally $k_{ET} >> k_{HAT}$ (Litwinienko & Ingold, 2007). One exception to this trend was $\alpha$-tocopherol, which was greater in relative reactivity, based on $k_b$ and $k'_b$, than its relatively high $pK_a$ of 11.7 would forecast. A high reactivity despite a low degree of ionisation suggests that a non-SPLET mechanism, such as HAT, may be a major contributor to the observed activity of $\alpha$-tocopherol. To further investigate this aspect, the reactions between DPPH and some of the food constituents ($\alpha$-tocopherol, propyl gallate, and EGCG) were studied in methanol containing acetic acid, which was added to lower the pH and suppress phenoxide formation, thus impeding SPLET (Litwinienko & Ingold, 2007). The $k_b$ values were lower in the presence of acetic acid (Table 1), but the reactivity of $\alpha$-tocopherol was less affected than was the reactivity of propyl gallate and EGCG (31%, 98% and 95% reduction in $k_b$ respectively, between 0 and 1000 mM of acetic acid). This confirms that in methanol, SPLET is a minor contributor to the $k_b$ of $\alpha$-tocopherol, but it is a major contributor to the $k_b$ values of propyl gallate and EGCG, in alignment with the ionisability signified by the $pK_a$ values. In addition, the $k_b$ of $\alpha$-tocopherol was greater than the $k_b$ values of propyl gallate and EGCG when 100 mM and 1000 mM of acetic acid were present. This suggests that the $k_{HAT}$ of $\alpha$-tocopherol is much greater than the $k_{HAT}$ of propyl gallate and EGCG, and supports the notion that $\alpha$-tocopherol is more reactive in methanol than is predicted by its $pK_a$ value because it is a potent HAT mechanism-based antioxidant. Another
exception to the link between antioxidant reactivity and $pK_a$ values was EGCG, which appeared lower in relative reactivity, based on $k'_b$, than its relatively low $pK_a$ would predict. This discrepancy may be because the $pK_a$ value in Table 1 is for the most acidic OH group and therefore it is predictive of the reactivity of the most reactive H atom, whereas the stoichiometric $k'_b$ value represents the average reactivity of all of the active H atoms, which for EGCG with a large $n$ of 14.8 may be much less than the reactivity of the most reactive H atom. The EGCG $k_b$ value did, however, place EGCG second only to L-ascorbic acid in reactivity which is aligned with it having the second lowest $pK_a$ value.

3.3. Inhibition of $\alpha$B42 fibrillation

The effect of the food constituents on Aβ42 fibrillation was studied using the ThT fluorescence assay and TEM. ThT fluorescence can be used to detect the formation of Aβ fibrils because the fluorescence emission of ThT is enhanced upon binding to amyloid fibrils. Fig. 4A shows the kinetic traces for Aβ42 fibril formation in the presence and absence of the food constituents, monitored by in situ ThT fluorescence. Typically a sigmoidal kinetic accrual curve was observed, in keeping with an initial lag phase of fibril formation mostly via slow primary nucleation and elongation, followed by exponential growth due to an autocatalytic process involving fast fibril-catalysed secondary nucleation and elongation which becomes the main source of new oligomers, and finally an equilibrium plateau phase (Arosio, Cukalevski, Frohm, Knowles, & Linse, 2014). An empirical sigmoidal model was fitted to each of the fluorescence intensity versus time data sets and the amplitude of the sigmoidal curve (Fig. 4B) and half-life of the process (Supplementary Fig. S10) was obtained. α-Tocopherol, L-ascorbic acid, and caffeine had little effect on the fibrillation of Aβ42. This is consistent with previous observations that α-tocopherol and L-ascorbic acid are weak inhibitors Aβ40 fibril formation (Tomiyama et al., 1996). All of the polyphenols
substantially decreased the amplitude, indicating that they inhibit Aβ42 fibril formation. The most active compound was EGCG, which at 100 µM prevented the formation of a sigmoidal kinetic accrual curve, instead producing a response curve that had a slight downward trend, with the final FI less than the initial FI (Bastianetto et al., 2006; Ehrnhoefer et al., 2008). This not only indicates complete inhibition of Aβ42 fibril formation, but also suggests that the quantity of fibrils was lower at the end of the experiment than at time zero, possibly due to the known ability of EGCG to remodel preformed fibrils (Bieschke et al., 2010) that may have been present at the start of the experiment. When EGCG was tested at 10 µM, a sigmoidal curve was observed similar to those obtained for the other polyphenols at 100 µM. The order of activity for inhibition of fibril formation by 100 µM of polyphenol, and % of the Aβ42 control amplitude was: EGCG, < 0% (Bastianetto et al., 2006; Ehrnhoefer et al., 2008); gallic acid, 24% (Di Giovanni et al., 2010); resveratrol, 27%; gallamide, 35%; propyl gallate, 39%; and caffeic acid, 44% (Di Giovanni et al., 2010); with 10 µM of EGCG, 54% (Bastianetto et al., 2006; Ehrnhoefer et al., 2008).

TEM was used to study the morphology of the aggregated Aβ42 after 21 h incubation at the end of the ThT assay. Aβ42 control exhibited long fibrils of varying lengths and widths of approximately 7-10 nm (Fig. 5A and Supplementary Fig. S11). In the presence of 100 µM of EGCG (Bastianetto et al., 2006), gallic acid (Di Giovanni et al., 2010), propyl gallate, resveratrol and caffeic acid (Di Giovanni et al., 2010), there were fewer fibrils compared with the Aβ42 control sample, consistent with the results of the ThT assay. It was also observed that there were a large number of amorphous and spherical aggregates, ranging from 20 to 200 nm in size. Although the occasional amorphous and spherical aggregate was found in the Aβ42 control, they were substantially more prevalent in the treated samples, which also contained markedly fewer fibrils, and this suggests that these food constituents promote aggregation along an “off-pathway” rather than the “on-pathway” of fibril formation.
Ehrnhoefer et al., 2008). The aggregates formed in the presence of the EGCG are known to be less toxic to PC12 cells than are protofibrils and fibrils (Ehrnhoefer et al., 2008). Curcumin was not examined in the ThT assay or by TEM, due to its poor aqueous solubility. A high concentration of DMSO cosolvent (33% v/v) was required to solubilise curcumin, and this concentration of DMSO adversely affected fibril formation in the control Aβ42 sample. Nevertheless, curcumin has been shown in other studies to be an inhibitor of Aβ fibril formation (Ono, Hasegawa, Naiki, & Yamada, 2004).

The results of this study indicate that polyphenolic food constituents are good inhibitors of Aβ fibril formation, and EGCG is a relatively more potent inhibitor than other polyphenols. The ability of polyphenols to inhibit Aβ fibril formation has been attributed to interactions of polyphenols and their quinone auto-oxidation products with hydrophobic binding sites in amyloid fibrils, and the formation of quinoprotein adducts via Michael addition of quinones with the amino groups of Aβ lysine residues (Palhano, Lee, Grimster, & Kelly, 2013; Sato et al., 2013).

3.4. Identification of multifunctional compounds, and consideration of bioavailability and brain uptake

The results above indicate that the food constituents EGCG and gallic acid are multifunctional compounds with strong and moderate activity, respectively, in the metal chelation assays, the antioxidant assays, and assays of Aβ42 fibrillation. The structurally related polyphenols, gallamide and propyl gallate, were also found to be multifunctional, however, these are synthetic compounds that do not naturally occur in foods, although propyl gallate is commonly used as a food additive. The naturally occurring food constituent, curcumin, exhibited moderate metal chelation and antioxidant activity, and it is a known inhibitor of Aβ fibril formation (Ono et al., 2004). For EGCG, gallic acid, and curcumin to be
beneficial in AD via the mechanisms discussed in this paper, must be absorbed via the oral route, cross the blood-brain barrier, and attain concentrations within the brain that are sufficient to exert a therapeutic effect. Manach, Williamson, Morand, Scalbert, & Remesy (2005) compiled pharmacokinetic data from multiple bioavailability studies of polyphenols. They found a mean $C_{\text{max}}$ for EGCG of $0.12 \pm 0.03 \mu\text{mol/l}$ after a 50 mg (0.110 mmol) oral dose, suggesting poor systemic availability, although EGCG is absorbed predominately in free form (77-90%) whereas other catechins are highly conjugated. In comparison, gallic acid was relatively well absorbed after a 50 mg (0.294 mmol) oral dose, with a mean $C_{\text{max}}$ of 4.00 ± 0.57 µmol/l, although 60% of this was the 4-0-methylgallic acid metabolite. Higher doses of these polyphenols may result in even greater plasma concentrations, for example a 1600 mg (3.49 mmol) oral dose of EGCG afforded a $C_{\text{max}}$ of 7.4 µmol/l, and this might be achieved by taking supplements of pure compounds or plant extracts, or by consuming foods that are naturally rich sources of the relevant polyphenols (Manach et al., 2005). The bioavailability of curcumin is very poor, with oral doses of 4000 to 12000 mg (10.9 to 32.6 mmol) resulting in curcumin levels ranging from 0.011 to 0.63 µmol/l (P. Anand, Kunnumakkara, Newman, & Aggarwal, 2007). Reliable data on the brain uptake of polyphenols is limited, but evidence from rodent studies suggests that polyphenols usually accumulate at levels below 1 nmol/g tissue, with a 500 mg/kg (1.09 mmol/kg) oral dose of EGCG leading to 12.3 µmol/l in the plasma and 0.5 nmol/g in the brain tissue of rats (Schaffer & Halliwell, 2012). Thus, although some polyphenols can enter the brain, the literature suggests that polyphenols may not attain concentrations within the brain that are sufficient to exert a therapeutic effect via the mechanisms studied in this paper. Further research is necessary to identify those dietary polyphenols and their metabolites that have good bioavailability and brain uptake, and studies should focus especially on understanding the activities of those compounds. In addition, polyphenols with strong activity but with poor bioavailability and brain uptake might form a
basis for the design of compounds with improved ADMET profiles. Aside from the classical direct radical scavenging antioxidant properties of polyphenols, these molecules are known to directly interact with cell signalling pathways (Spencer, 2008), and further research into this activity is also warranted. This study highlighted the superior antioxidant reactivity of L-ascorbic acid compared with polyphenols under conditions that favour SPLET. In addition, α-tocopherol is known to be a potent antioxidant under conditions that favour HAT/PCET, which are likely to prevail within the lipid membrane environment that is preferred by the highly lipophilic α-tocopherol (CLogP 12.0) [α-tocopherol $k_b$ 8500 M$^{-1}$ s$^{-1}$ in $n$-heptane (Musialik & Litwinienko, 2005)]. L-Ascorbic acid (Schaffer & Halliwell, 2012) and α-tocopherol (Vatassery, Brin, Fahn, Kayden, & Traber, 1988) have very good and moderate brain uptake respectively, and although these compounds are not multifunctional, they deserve further investigation for specifically addressing oxidative stress within the AD brain.

### 4. Conclusion

A new assay method was developed, demonstrating that the indicator ligand 5-Br-PAPS can be used to examine the complexation of Zn(II) and Cu(II). EGCG, gallic acid, and curcumin are multifunctional food constituents with strong to moderate abilities to chelate Cu(II), Zn(II), and Fe(II), scavenge free radicals, and inhibit $A\beta_{42}$ fibrillation. SPLET is the prevailing mechanism of radical scavenging by the polyphenols, with antioxidant reactivity broadly aligned with the $pK_a$ values for the most acidic OH group in each of the molecules. L-Ascorbic acid and α-tocopherol are potent antioxidants but they are poor metal chelators and they do not inhibit $A\beta_{42}$ fibrillation. The literature indicates that L-ascorbic acid and α-tocopherol have very good and moderate brain uptake respectively, hence they deserve further investigation for specifically addressing oxidative stress within the AD brain. In contrast, the literature suggests that the brain uptake of EGCG, gallic acid, and curcumin is relatively poor, and so they may not exert a therapeutic effect via the mechanisms studied in
this paper. Further studies are needed to identify dietary polyphenols and their metabolites that have good bioavailability and brain uptake, and these compounds should be the focus of biomedical investigations.

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References


Sato, M., Murakami, K., Uno, M., Nakagawa, Y., Katayama, S., Akagi, K.-i., et al. (2013). Site-specific inhibitory mechanism for amyloid β42 aggregation by catechol-type flavonoids targeting the Lys residues. *Journal of Biological Chemistry, 288*(32), 23212-23224.


Fig. 1. Chemical structures of the food constituents examined in this study, and their main dietary sources. The synthetic non-food-related molecule gallamide (R$_1$ = NH$_2$) was included in the study to assist in understanding structure-activity relationships.

Fig. 2. The logarithm of the concentration of compound for 50% chelation of 7 µM Zn(II), 10 µM Cu(II) and 25 µM Fe(II), deduced using ferrozine and 5-Br-PAPS assays. The solvent was HEPES buffer [15 mM, pH 7.5, for Zn(II) and Cu(II); or 15 mM, pH 6.8, for Fe(II)] and the concentrations of indicator ligands were ferrozine, 1 mM, and 5-Br-PAPS, 60 µM. L-Ascorbic acid, caffeic acid, caffeine, curcumin, resveratrol, and α-tocopherol samples contained DMSO ≤ 0.67% v/v. Values are the mean + SEM of three independent experiments.

Fig. 3. Representative absorbance-time plots of the bleaching of DPPH in methanol by selected food constituents. The concentration of antioxidants was 25 µM and the concentration of DPPH was 60 µM. A is the absorbance of the sample and A$_0$ is the absorbance of 60 µM DPPH at 515 nm. When equation 4 was fitted to the data, $R^2$ was ≥ 0.9552.

Fig. 4. Aβ$_{42}$ fibrillation monitored by in situ ThT fluorescence. LMW Aβ$_{42}$ (27 µM) was incubated with food constituents (100 µM, with the exception of EGCG which was assayed at both 10 and 100 µM) in phosphate buffer (20 mM, pH 7.4, I 0.17 M, containing 20 µM ThT) at 37° C under quiescent conditions. Resveratrol and α-tocopherol samples contained DMSO (0.1% v/v). A) Representative kinetic plots corrected for the fluorescence of ThT alone (20
μM). Fluorescence readings ($\lambda_{ex}$ 440 nm, $\lambda_{em}$ 480 nm) were recorded every 5 min. B) Extent of fibrillation depicted as percentage of the Aβ42 control amplitude. Values are the mean ± SEM of three independent experiments with *p < 0.0001 c.f. Aβ control and ^p < 0.0001 c.f. DMSO 0.1%. Aβ control and DMSO 0.1% were not significantly different from each other (p > 0.05).

**Fig. 5.** Morphology of Aβ42 aggregates collected upon completion of the ThT assay. LMW Aβ42 (27 μM) was incubated with food constituents (100 μM) in phosphate buffer (20 mM, pH 7.4, I 0.17 M, containing 20 μM ThT) at 37° C under quiescent conditions for 21 h. TEM images of: A) Aβ42 control; B) Aβ42 + EGCG; C) Aβ42 + gallic acid; D) Aβ42 + propyl gallate; E) Aβ42 + resveratrol + 0.1% v/v DMSO; F) Aβ42 + caffeic acid. Image of Aβ42 + 0.1% v/v DMSO (Supplementary Fig. S11) was similar to (A). The scale bar represents 500 nm.
Table 1
Antioxidant stoichiometry and reactivity of the food constituents with the DPPH radical.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Food constituent</th>
<th>Antioxidant stoichiometry</th>
<th>Antioxidant reactivity</th>
<th>Lit. pK\textsubscript{a} \textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stoichiometric factor (n) \textsuperscript{b}</td>
<td>Bimolecular rate constant \textsuperscript{c}</td>
<td>Overall</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$k_b$ (M\textsuperscript{-1} s\textsuperscript{-1}) \textsuperscript{e}</td>
</tr>
<tr>
<td>EGCG</td>
<td>14.8 ± 0.3</td>
<td>3569 ± 249</td>
<td>242 ± 22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>346 ± 138 \textsuperscript{g}</td>
<td>152 ± 22 \textsuperscript{h}</td>
</tr>
<tr>
<td>Gallamide</td>
<td>7.03 ± 0.20</td>
<td>2182 ± 264</td>
<td>310 ± 46</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>6.55 ± 0.49</td>
<td>3175 ± 214</td>
<td>485 ± 69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55.5 ± 14.6 \textsuperscript{g}</td>
<td>43.1 ± 9.6 \textsuperscript{h}</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>6.30 ± 0.18</td>
<td>1335 ± 316</td>
<td>212 ± 56</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>3.34 ± 0.13</td>
<td>474 ± 61</td>
<td>142 ± 24</td>
</tr>
<tr>
<td>Curcumin</td>
<td>3.18 ± 0.09</td>
<td>948 ± 103</td>
<td>298 ± 41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(enol)</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>2.79 ± 0.14</td>
<td>91.4 ± 3.8</td>
<td>32.8 ± 3.1</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>1.99 ± 0.13</td>
<td>386 ± 35</td>
<td>194 ± 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>342 ± 14 \textsuperscript{g}</td>
<td>271 ± 32 \textsuperscript{h}</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>2.02 ± 0.12</td>
<td>12287 ± 1306&lt;sup&gt;j&lt;/sup&gt;</td>
<td>6080 ± 1001&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
<tr>
<td>-----------------</td>
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</tr>
<tr>
<td>Caffeine</td>
<td>0.147 ± 0.029</td>
<td>Inert&lt;sup&gt;l&lt;/sup&gt;</td>
<td>-&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All data were collected from ≥ 3 experiments, using methanol solvent.

<sup>b</sup> Number of DPPH molecules scavenged by one molecule of food constituent in 24 h. Values are the mean ± SEM.

<sup>c</sup> <i>r</i>² values of plots of (dA<sub>t</sub>/dt)<sub>0</sub> vs. A₀[AO] were between 0.85 and 0.99.

<sup>d</sup> <i>pK</i><sub>a</sub> of the most acidic hydroxyl group in each food constituent. Values are the mean of the literature values listed in Supplementary Table S5.

<sup>e</sup> Values are the mean ± SEM.

<sup>f</sup> <i>k</i>' <sub>b</sub> = <i>k</i><sub>b</sub>/<i>n</i>. Values are the mean ± SEM.

<sup>g</sup>-<sup>i</sup> Rate in presence of acetic acid: <sup>g</sup> 10 mM, <sup>h</sup> 100 mM, and <sup>i</sup> 1000 mM.

<sup>j</sup> Literature value [mean ± SD (n = 6)] for overall <i>k</i><sub>b</sub> was provided because the reaction was too rapid to be estimated by the techniques used in this study (Noipa, Siriajaranai, Tuntulani, & Ngeontae, 2011).

<sup>k</sup> The stoichiometric <i>k</i>' <sub>b</sub> value was estimated by dividing the literature overall <i>k</i><sub>b</sub> value (Noipa et al., 2011) by the value of <i>n</i> obtained in this study.

<sup>l</sup> Antioxidant reactivity was not studied because caffeine is inert to reaction with DPPH.
Highlights

- Studied 9 compounds as chelators, antioxidants, and Aβ fibrillation inhibitors
- Developed an assay using 5-Br-PAPS to examine the complexation of Zn(II) and Cu(II)
- SPLET is the prevailing mechanism of radical scavenging by the polyphenols
- Gallic acid, EGCG, and curcumin are multifunctional agents
- L-Ascorbic acid and α-tocopherol are potent antioxidants but not multifunctional