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Inhibition of human α-amylase by dietary polyphenols

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Abbreviations: 3,5-Dinitrosalicylic acid (DNS), (-)-epigallocatechin gallate (EGCG), phosphate buffer saline (PBS), solid phase extraction (SPE), sodium dependent glucose transporter type 1 (SGLT1), glucose transporter type 2 (GLUT2)

Key words: α-amylase, diabetes, amylose, amylopectin, polyphenol

Abstract

Functional foods offer the possibility to modulate the absorption of sugars, leading to benefits for diabetics and those with metabolic syndrome. As part of the characterisation of such foods, inhibition of α-amylase is used to assess components for their potential ability to modify the post-prandial glycaemic response. Many publications on phenolics as potential inhibitors report widely varying assay conditions leading to variable estimates of inhibition. On this basis, we have optimised the in vitro α-amylase inhibition assay and, in particular, we show the importance of removing certain polyphenols after the enzymic reaction when using 3,5-dinitrosalicylic acid since they interfere with this reagent. There was a substantial ~5-fold effect on acarbose IC_{50} values when working just outside optimal conditions. This shows that inappropriate assay conditions, such as excess enzyme, greatly influence IC_{50} values and could explain some discrepancies in the existing literature.

1. Introduction

It is estimated that about 346 million people worldwide suffer from Type 2 diabetes due to pancreatic β-cell dysfunction and/or increased resistance to insulin with impaired glucose tolerance (Danaei et al., 2011). The risk of developing impaired glucose tolerance is increased by regular high postprandial glucose spikes in the blood (Livesey, Taylor, Hulshof, & Howlett, 2008; Manzano & Williamson, 2010). Hydrolysis of starch is one of the main sources of postprandial glucose in the blood, with the enzymes α-amylase and α-glucosidase being involved in starch breakdown. Salivary and pancreatic α-amylases hydrolyse starch to produce maltose and other oligosaccharides by breaking the α-1,4 glycosidic bonds (Hanhineva et al., 2010; Williamson, 2013). Subsequently, the α-glucosidases located in the brush-border surface membrane of intestinal cells hydrolyse the resulting oligosaccharides into glucose, which is then transported into the blood by the transporters sodium dependent glucose transporter type 1 (SGLT1; SLC5A1) and glucose transporter type 2 (GLUT2; SLC2A2) (Scheepers, Joost, & Schurmann, 2004). Drugs such as acarbose (supplementary **Figure 1s**) are used in the management of type 2 diabetes and act by inhibiting α-amylase and α−glucosidases. Other small molecules such as polyphenols (supplementary **Figure 1s**) might have acarbose-like effects (Hanhineva et al., 2010; Williamson, 2013), and so could provide a suitable strategy to manage type 2 diabetes, since acarbose commonly causes side effects including flatulence, diarrhoea and nausea. Functional foods could ultimately be developed containing components able to inhibit α-amylase, an acarbose-like action but without the side effects.

Many reports (**Tables 1-3**) indicate that polyphenols inhibit α-amylase. However, these studies use different methods of detection and assay conditions (reaction time and temperature, pH, enzyme concentration and source, substrate concentration and source) which have a pronounced impact on the reported data. Acker and Auld (2014) recently outlined the importance of experimental conditions when designing enzyme assays in general. The most commonly used method for measuring α-amylase activity involves the DNS reagent for detection of reducing sugars. The presence of a free carbonyl group (C=O) in reducing sugars enables them to participate in an oxidation-reduction reaction with

3,5-dinitrosalicylic acid (DNS). However, due to the reducing potential of the polyphenols, we postulated that they could interfere with the development of the colour and therefore the results of the assay.

In this paper we report optimisation of the critical steps, showing the conditions required to assess α-amylase inhibition, using DNS as the detection method, and compare the measurement of Ki (the dissociation constant of the enzyme-inhibitor complex) and IC_{50} (concentration of inhibitor giving 50% inhibition) values for the potent inhibitor (-)-epigallocatechin gallate (EGCG).

2. Materials and methods

2.1 Reagents and standards

3,5-Dinitrosalicylic acid, potassium sodium tartrate, chromatographically purified human salivary α-amylase type IX-A (1 "Sigma –defined" unit will liberate 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C), and this is the basis of our initial experiments to optimise the assay. The enzyme preparation on this basis contained 276 Sigma-units per mg protein by Bradford assay), maltose, EGCG, quercetin, amylose and amylopectin from potato were all purchased from Sigma-Aldrich. Co., Ltd., Dorset, UK. Phloridzin, quercetin-3-O-glucoside and luteolin were purchased from Extrasynthase, Genay, France. Gallic acid was obtained from Alfa Aesar, Lancashire, UK. Instant green tea was obtained from Nestle Research Center, Lausanne, Switzerland. Oasis MAX cartridge 1 mL (30 mg) and 3 mL (60 mg) were purchased from Waters co-operation Ltd., Milford, MA, U.S.A. The DNS reagent was prepared by adding to 12 mL water, 20 mL of 96 mM DNS in water and 5.3 M sodium potassium tartrate solution (12 g in 8 mL of 2 M sodium hydroxide). All the reagents were of the highest purity and standards were ≥98 %.

2.2 Enzyme concentration and reaction time

Enzyme concentration and reaction time were determined by using different enzyme concentrations (0.5, 1.0, 1.5 and 2.0 U/mL) and assay mixtures were incubated for different times (0, 3, 6, 9, 12 and 15 min). The linearity of plots of absorbance at 540 nm versus time was assessed.

2.3 Determination of K_m and V_{max}

The kinetic parameters K_m and V_{max} were determined by using a chosen enzyme concentration and incubation times giving linear rates of reaction. The substrate concentrations ranged from 0 to 1 mg/mL in the final assay volume. Maltose standard curve was obtained by adding 1 mL of the DNS reagent to a total volume of 500 µL of different maltose concentrations (0-2 mM) and then heated (100 °C for 10 min). The absorbance was recorded at 540 nm in a PHERAstar FS microplate reader

(BMG Labtech, Inc., Cary, NC, USA), and the amount of maltose produced was calculated against the standard curve. The Lineweaver-Burk plot was used to calculate K_m and V_{max}.

2.4 Effect of polyphenols on colour reagent

The effect of polyphenols on the DNS reagent was determined by adding 1 mL DNS to an assay mixture containing 450 µL phosphate buffer saline (PBS, 0.01 M, pH 6.9) and 50 µL of different concentrations of the different polyphenols (0-1 mM). The absorbance was recorded as described previously.

2.5 Retention efficiency of Solid Phase Extraction cartridges by HPLC-PDA

HPLC analysis for efficiency of retention of polyphenols by the Oasis MAX SPE cartridge was carried out with EGCG using a $UFLC_{XR}$ Shimadzu system (Shimadzu, Japan) consisting of binary pump, a photodiode array with multiple wavelength SPD-20A and a LC-20AD Solvent Delivery Module coupled with an online unit degasser DGU-20A3/A5 and a thermostat autosampler/injector unit SIL-20A (C). The column used was a 5 µm Gemini C_{18} (250 x 4.6 mm, i.d.) with a flow rate of 1 mL/min, column temperature set at 35 ^{0}C with an injection volume of 10 µL and detection at 280 nm. A two phase gradient system consisting of water (Millipore grade) with 0.1% trifluoroacetic acid (HPLC grade) as mobile phase A and acetonitrile containing 0.1 % trifluoroacetic acid as mobile phase B. The gradient conditions were as follows: The initial conditions started with 92% A and increasing to 18 % solvent B at 3.50 min, 32% B at 18 min, 60% B at 28 min reaching to 100% B at 32 min for 4 min, returning to the initial conditions for 3.5 min.

2.6 *α*-Amylase inhibition assay

The assay contained 200 µL each of substrate (amylose or amylopectin) and enzyme, 50 µL PBS and 50 µl of inhibitor of different concentrations. For the control assay, the inhibitor was replaced by an equal volume of PBS. Stock amylose and amylopectin solutions (2.5 mg/mL) were prepared in water by heating at 90 °C on a hot plate for 15 min. A second stock solution of amylopectin was prepared at 0.925 mg/mL. Human salivary α-amylase stock solution (1.25 U/mL) was prepared in PBS. The

enzyme stock solution and the assay mixture containing the inhibitor, PBS and substrate were pre-incubated at 37 °C in a water bath for 10 min and the reaction was started by adding the enzyme to the assay solution. The reaction was carried out at 37 °C for 10 min with salivary α-amylase at 0.5 U/mL, substrate at 1 mg/mL and varying concentrations of the inhibitor up to 1 mM (depending on solubility). The reaction was stopped by placing the samples in a water bath (GLS Aqua 12 plus) at 100 °C for 10 min where no further reaction occurred, transferred to ice to cool down to room temperature and centrifuged for 5 min. The sample obtained was used for SPE to remove polyphenols before adding colour reagent solution. To the resulting sample, 1 mL of the DNS reagent was added and heated at 100 °C for 10 min. After cooling to room temperature, 250 µL from each sample was placed in a 96 well plate (Nunc A/S., Roskilde, Denmark) and the absorbance was recorded at 540 nm. Supplementary **Figure 2s** summarises the different stages involved in the α-amylase protocol.

The rate of enzyme inhibition was calculated as a percentage of the control (without inhibitor) using the formula:

% = ((Abs Control – Abs sample)/Abs control) x 100

IC_{50} was calculated graphically by dose-dependent inhibition. For Ki values, the Dixon plot method was employed (Dixon, 1953). Ki values were obtained by calculating the intersection point having an associated standard deviation and standard error supplying the uncertainties on the estimate using the following equations:

(Assuming n regressions)

Intersections (x_{ij}, y_{ij}) of each pair-wise combination i,j;

This provides precisely  = N, unique, i.e. non-repetitive pair-wise combinations.

Defining the mean (**x**,**y**) and standard deviations (s_x, s_y) of the unique intersection coordinates x_{ij}, y_{ij}, as

provides the expected intersection point of the regressions and associated standard deviation to supply the uncertainties on the estimate. The lines of each data point were fitted to the intersection point obtained from the equation.

2.7 Statistical analysis

Statistical analysis was performed by one-way analysis of variance using the Number Cruncher Statistical System version 6.0 software (NCSS, LLC). Significant differences were assessed with Tukey-Kramer multiple comparison test ($p \leq 0.05$). The data are expressed as the mean ± SE (n=3).

3. Results

3.1 Effect of enzyme and substrate concentration on inhibition

The IC_{50} of an inhibitor is very dependent on the assay conditions such as enzyme concentration, substrate type, reaction duration, temperature and pH. While temperature and pH have been standardized in most of the published studies on α-amylase inhibition to 37 °C and 6.9, respectively,

there is no consensus regarding the other parameters. In this regard, the effect of acarbose, a well-known α-amylase inhibitor, was tested under two different assay conditions to determine the effect on the inhibition constant. Concentrations of 0.5 and 3.0 U/mL of enzyme were chosen to conduct this experiment where the former represents a suitable concentration of enzyme (linear range, Supplementary **Figure 3As and 3Bs**) and the latter a commonly published but sub-optimal condition (where the substrate is mostly consumed). The experiment was conducted on amylose and amylopectin.

As depicted in **Figure 1**, the two different enzyme concentrations had an effect ($p \leq 0.05$) on the apparent IC_{50} value exhibited by acarbose and was more pronounced when amylopectin was used as substrate. The IC_{50} value of acarbose under non-optimal conditions was 7-8 fold higher than that obtained under optimal conditions for both substrates. Reducing the concentration of amylopectin from 1 mg/mL to 0.37 mg/mL to give the same ratio of km value versus concentration in comparison to amylose, also caused an apparent increase in the inhibitory activity of acarbose, and the same pattern was observed for green tea (lower IC_{50}) (**Table 4**).

3.2 Kinetic studies on amylose and amylopectin

The kinetic parameters of human salivary α-amylase are shown in **Supplementary Figure 4s**. The time dependence assessed for different concentrations of enzyme was linear for up to 15 min for amylose and up to 12 min for amylopectin using 0.5 U of enzyme as depicted in supplementary **figure 3As and 3Bs**. Therefore, 10 min and 0.5 U of enzyme were chosen as the optimum assay conditions to obtain the kinetic parameters, with 1 mg/mL substrate concentration.

Using Lineweaver-Burk plots (Supplemetary **Figure 4s**), the values obtained are: amylose, $K_m = 12.9$ mg/mL and $V_{max} = 1.67$ mmol/min per mg of protein; amylopectin, $K_m = 4.8$ mg/mL and $V_{max} = 0.67$ mmol/min per mg of protein.

3.3 Interference of polyphenols with DNS reagent

The use of the DNS reagent is one of the most widely used methods to quantify the content of reducing sugars and it has been largely applied to measure the inhibition of α-amylase activity by many compounds including polyphenols. Three different classes of polyphenols with different reduction potentials were tested to corroborate this fact (**Figure 2**). Significant differences ($p \leq 0.05$) were found between EGCG, gallic acid and phlorizin. EGCG caused the major interference with the DNS reagent in a dose-dependent manner, followed by gallic acid and phlorizin. The extent of the interference roughly correlates with the number of OH groups in the chemical structure of the polyphenol, which also partially predicts their reduction potential (Rice-Evans, Miller, & Paganga, 1996). While this relationship may not hold for all polyphenols, the removal of polyphenols should be considered in pre-tests involving the DNS reagent (**Figure 2)**. This is something that, to our knowledge, has been ignored in many published studies and may account for the variation in the reported inhibition of α-amylase by EGCG (**Tables 1-3**), since EGCG interacts very strongly with the DNS reagent. Ignoring this contribution would decrease the apparent inhibition, i.e. raise IC_{50} and K_i values.

3.4 *Inhibitory effect of selected polyphenols on salivary α*-amylase activity.

Assays under optimal conditions of enzyme concentration and incubation time were carried out to test the inhibitory activity of selected polyphenols using amylose and amylopectin as substrate (**Figure 3A and 3B**) and compared to that reported in the literature for those compounds (**Table 1**). Polyphenols were removed from the reaction solution using SPE and the efficiency is shown in Supplementary **Figure 5s** for EGCG as an example. The same procedure was carried out with quercetin and luteolin with the same removal efficiency.

All of the tested polyphenols showed dose-dependent inhibition of α-amylase activity on both substrates and, therefore, IC_{50} values could be calculated. The inhibitory activity of quercetin, EGCG and luteolin was higher when amylose was used as substrate. EGCG showed the highest inhibition with maximum inhibition at 20 μM and no significant difference ($p \geq 0.05$) was observed above that concentration. For quercetin and luteolin, the highest inhibition was recorded at the highest

concentration tested (100 µM) owing to limits in solubility, showing significant difference ($p \leq 0.05$) among the tested concentrations. No differences ($p \geq 0.05$) were observed between the three tested polyphenols at a concentration of 100 µM using amylose as substrate.

With amylopectin as substrate, IC_{50} values were higher. The differences in the inhibition behaviour of the polyphenols on α-amylase between amylose and amylopectin could be related to the differences in the affinity (K_m) for each type of substrate, hence the need to calculate the Ki which, for competitive inhibition, represents the dissociation constant of the enzyme–inhibitor complex independently of substrate employed. There was no significant difference ($p \geq 0.05$) between Ki values for amylose (0.28 ± 0.64 µM) and amylopectin (4.50 ± 4.53 µM) (**Figure 4**).

4. Discussion

The results obtained show the importance of determining the kinetic parameters K_m and V_{max} before measuring inhibition constants in any assay. These parameters are then used for assay optimization and are critical to the interpretation of correct and comparable IC_{50} values (Acker & Auld, 2014). Changes in the type of substrate and concentration affect the apparent potency of an inhibitor as shown in **Table 4**. **Tables 1-3** show the α-amylase inhibition data from published studies, and the data even for the same compound can vary widely. Even when acarbose was used as inhibitor, the reported differences in the IC_{50} values ranged from 0.9 to 23100 µM, and when results are compared with same-source enzymes, the range vary from 0.9-6.9 and 1.24-23079 µM for human and porcine, respectively. The measurement of Ki should always be considered for pure compounds but only when they are effective inhibitors, to minimise some of the potential differences between laboratories.

Most of the research regarding the inhibition of α-amylase activity by polyphenols has been carried out using an enzyme from porcine pancreas which possesses 14 % different amino acid composition to that of human origin (Brayer, Luo, & Withers, 1995) and data on the effect of polyphenols on human α-amylase is much more limited when compared to porcine (Lo Piparo et al., 2008). In a previous study, luteolin and quercetin competitively inhibited human salivary α-amylase with IC_{50} of 18.4 and 21.4 µM respectively, similar to those obtained in this study when amylose was used as substrate (Lo Piparo et al., 2008). Using amylopectin, luteolin was shown to be a better inhibitor than quercetin, although the IC_{50} values were higher than those found for amylose. The differences between both substrates are related to the concentration used in the assay and the K_m value of α-amylase. The same effect was also observed for acarbose and the tested polyphenols where the IC_{50} value was reduced as the concentration of amylopectin went far below the K_m value **(Table 4).** The IC_{50} value of a polyphenol is driven by the type and concentration of enzyme and substrate, and by the inhibitory mechanism (competitive, uncompetitive or non-competitive). For example, the IC_{50} value for EGCG in our study was ~5 and ~60 µM for amylose and amylopectin respectively (substrate concentration =1 mg/mL).

DNS is used as a detection reagent for the measurement of reducing sugars. The results show that certain redox-active compounds participate in the reaction involving DNS (**Figure 2)** where EGCG with the highest number of OH groups gave the greatest effect and ferulic acid did not react. This implies that each inhibitor should be tested for any possible interference because any small change in absorbance units entails a major impact on the final inhibition value. This may explain the discrepancy between data, where EGCG was estimated to be less potent: values of 1.5 mM using the DNS reagent as detection method (Koh, Wong, Loo, Kasapis, & Huang, 2010) and 2.3 mM using Nelson-Somogyi were obtained (Miao et al., 2014).

Currently the improvement of glucose homeostasis by reducing intestinal absorption of dietary glucose by alternatives to acarbose through the inhibition of carbohydrate digesting enzymes is of increasing interest. We assessed the existing literature and report here an optimised assay for estimation inhibition of α-amylase by polyphenols. Potential functional foods in the future could use this parameter as an indicator of acarbose-like activity of the constituent polyphenols.

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381 **Figure legends**

382

383 Figure 1. The inhibition of α-amylase by acarbose using amylose and amylopectin at 1 mg/mL. Data
384 points are expressed as mean ± SE (n=3).

385

386 Figure 2. (A) Reaction of selected polyphenols with DNS reagent. Data points are expressed as mean
387 ± SE (n=3). (B) Pearson correlation coefficient of linear regression between the number of OH groups
388 and absorbance at 100, 500 and 1000 μM of selected polyphenols.

389

390 Figure 3. Inhibition of α-amylase by selected polyphenols using amylose (A) and (B) amylopectin as
391 substrate. IC_{50} is indicated by the dotted line. Data points are expressed as mean ± SE (n=3)

392

393 Figure 4. Dixon plot showing the kinetic analysis of EGCG against human salivary α-amylase on (A)
394 amylose and (B) amylopectin. The intercept value represents –Ki. Data points are expressed as mean
395 ± SE (n=3).

396

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Figure 1

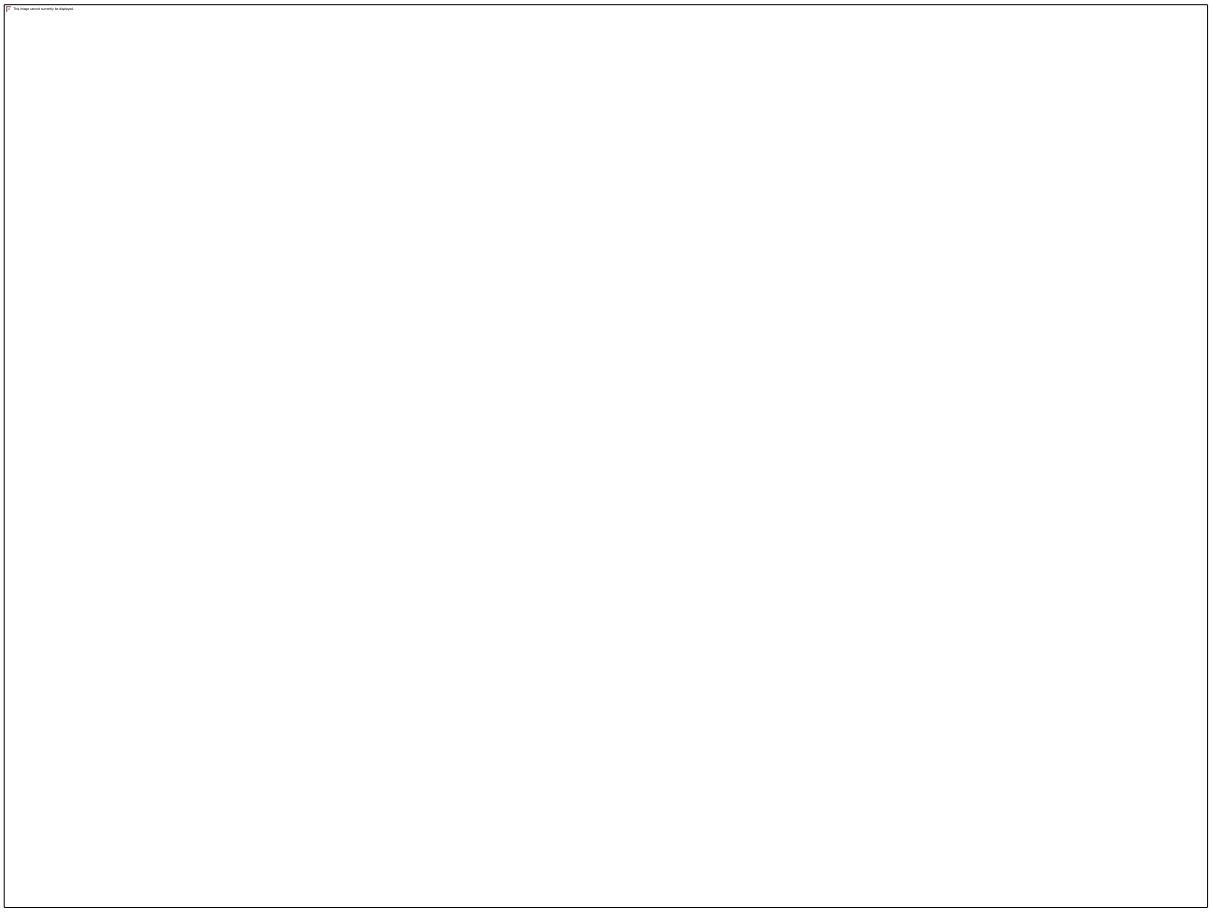

Figure 2

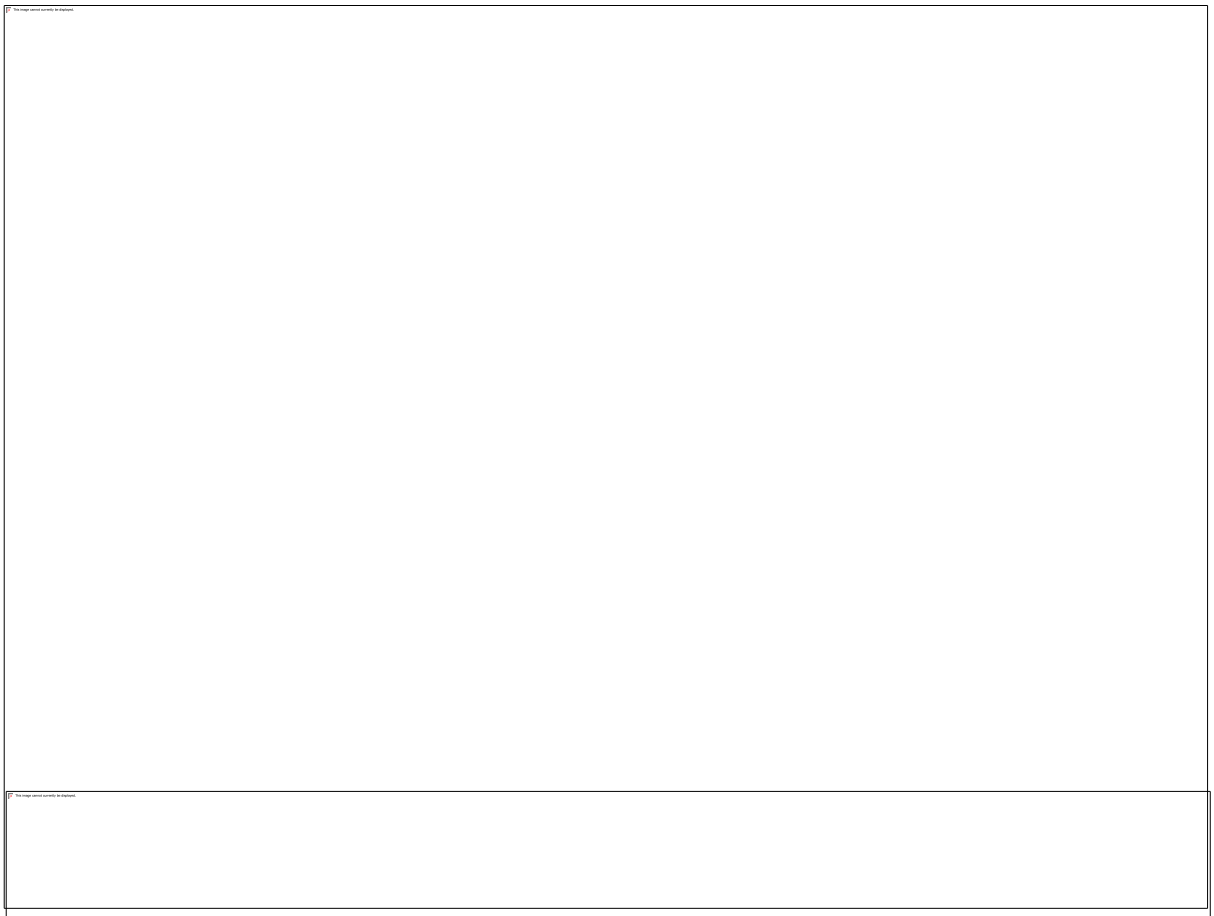

Figure 3

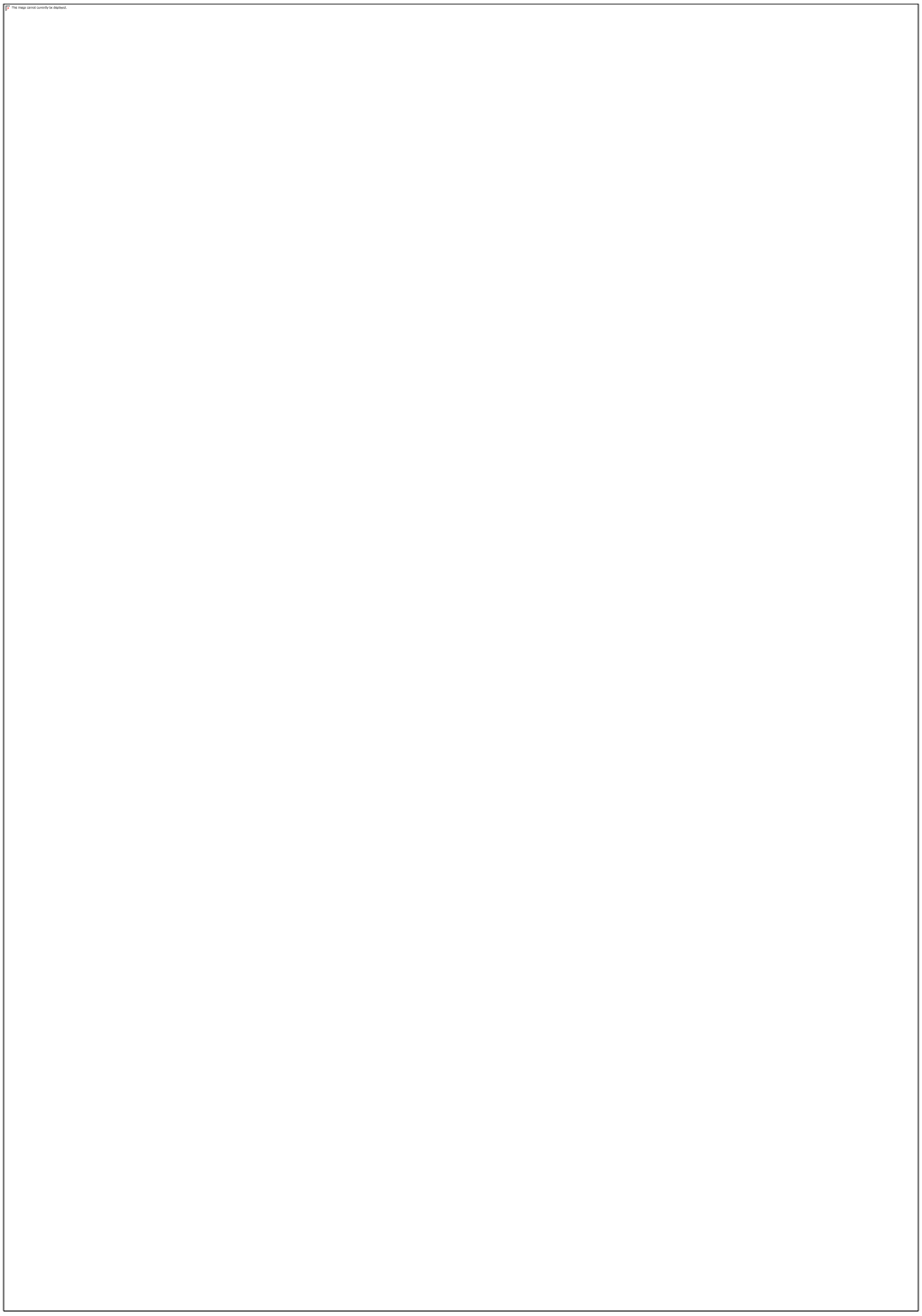

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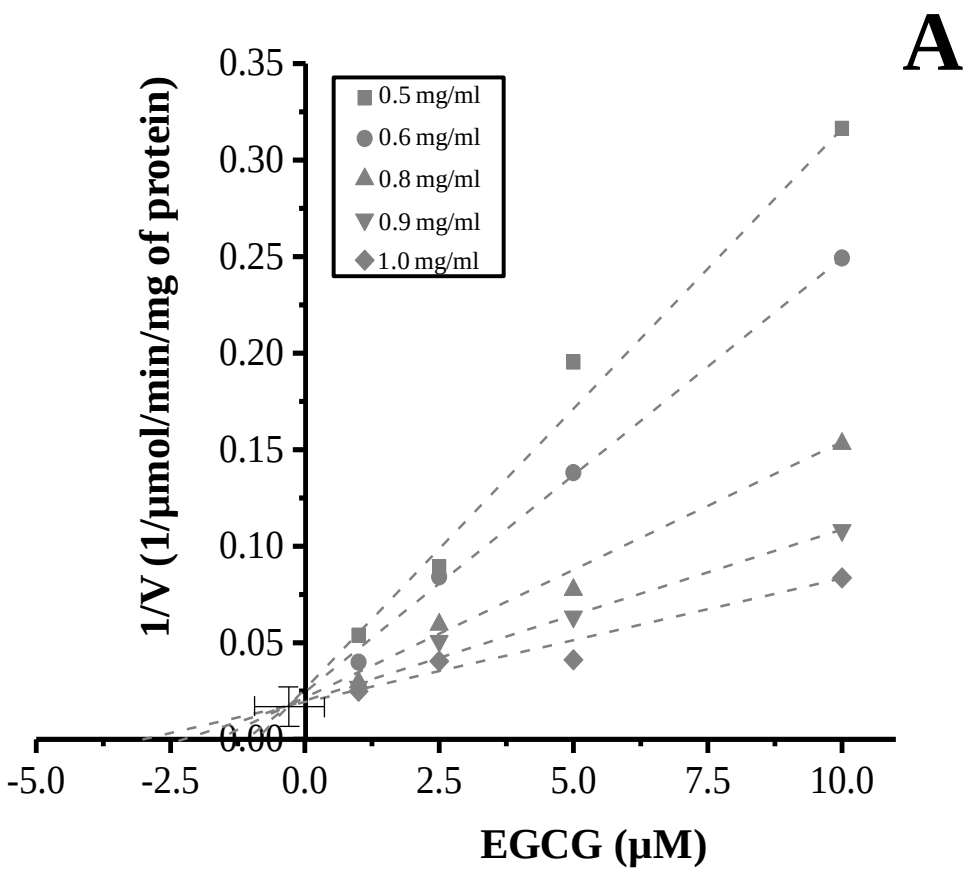
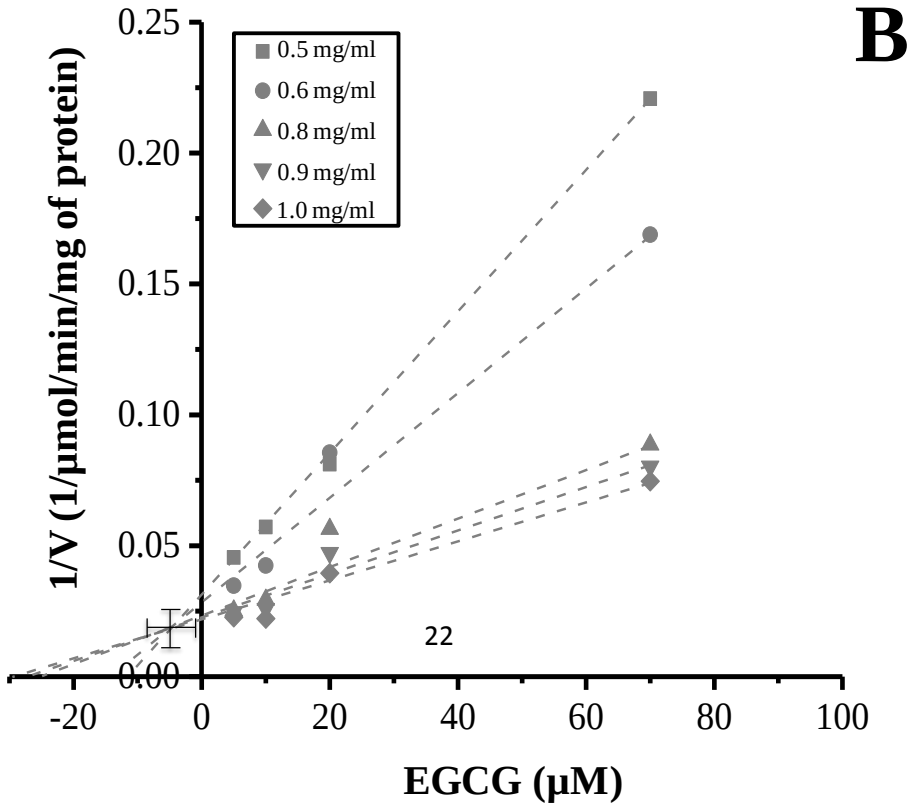
Figure 4


A
0.5 mg/ml
0.6 mg/ml
0.8 mg/ml
0.9 mg/ml
1.0 mg/ml
1/V (1/µmol/min/mg of protein)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-5.0
-2.5
0.0
2.5
5.0
7.5
10.0
EGCG (µM)


B
0.5 mg/ml
0.6 mg/ml
0.8 mg/ml
0.9 mg/ml
1.0 mg/ml
1/V (1/µmol/min/mg of protein)
0.25
0.20
0.15
0.10
0.05
0.00
-20
0
20
40
60
80
100
EGCG (µM)

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476 **Table 1.** Assay parameters used for measuring the inhibition of human salivary α-amylase by polyphenols.

Method of detection	Inhibitor	Substrate		Enzyme (mg/mL)	Buffer	Incubation time (min)	Temperature (°C)	Kinetic parameters	IC_{50} (µg/ml)	Acarbose (µM)	Reference
		Source	Concentration (mg/mL)								
DNS reagent	Almond nut seeds skin polyphenols	*	5	*	Sodium phosphate (100 mM, 17 mM NaCl, pH 6.9)	30	37	*	2.74	*	(Tsujita, Shintani, & Sato, 2013)
DNS reagent	Chestnut extract (Tannins and procyanidins)	*	5	*	Sodium phosphate NaCI, (17mM pH 6.8)	30	37	*	3.17	*	(Tsujita et al., 2011)
EnzChek Ultra Amylase Assay Kit	Plant extracts Grape seed Green tea White tea Teavigo Catechins	DQ starch from corn	0.005	0.0000025	NH_2PO4, 50 mM NaCl, 0.5 mM $CaCl_2$, and 0.1% bovine serum (50 mM pH 6.0)	30	25	*	Grape seed 8.7 Green tea 34.9 Catechin 160 EGC 27 EGCG 24 GCG 17	6.9	(Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012)
DNS reagent	Phlorotannins	Corn starch	0.0476	0.0083	Sodium phosphate (20 mM, NaCl 6.7 mM, pH 6.9)	10	20	*	2.8	*	(Roy et al., 2011)
DNS reagent	Black tea Green tea Oolog tea Catechins Theaflavins	Rice starch	0.16	0.0044	Sodium phosphate (50 mM, 6.85 mM NaCl, pH 6.9)	12	37	*	Black tea 420 TDG 2.2 EGCG 642	5.7	(Koh et al., 2010)

Nelson-Somogyi	Flavonoids	Potato starch	*	*	50 mM NH_2PO_4, 50 mM NaCl, 0.5 mM $CaCl_2$, and 0.1% bovine serum albumin, pH 6.0.	10	25	*	Scutellarein 2.75 Quercetagetin 3.24 Luteolin 5.26 Fisetin 5.61 Quercetin 6.46 Myricetin 9.61 Eupafolin 15.18	0.9	(Lo Piparo et al., 2008)
DNS reagent	Chestnut extract	*	5	pancreatic*	Sodium phosphate (100 mM, 17 mM NaCl, pH 6.8)	30	37	*	9.4	*	(Tsujita, Takaku, & Suzuki, 2008)
DNS reagent	Chesnut extract	*	5	*	Sodium phosphate (100 mM, 17 mM NaCl, pH 6.8)	30	37	*	7.5	*	(Tsujita et al., 2008)
DNS reagent	Polyphenol-rich pine bark extract	*	*	*	Phosphate *	5	37	*	1.7	3.9 µM	(Y. M. Kim, Jeong, Wang, Lee, & Rhee, 2005)
Nelson-Somogyi	Catechins	Maize starch	0.2	4.95	Phosphate* (0.2 M, pH 5.2)	15	37	*	GCG 503.8 ECG 618.8 EGCG 1053 EGC 11689 Epicatechin 11745 Catechin 13310	*	(Miao et al., 2014)
DNS reagent	Almond nut seed skin polyphenols	*	5	*	Sodium phosphate (100mM, 17 mM NaCl, pH 6.8)	30	37	*	2.74		(Tsujita et al., 2013)

*Not stated or clearly defined

DQ starch is a starch derived-substrate labelled with a fluorescent group (BODIPY ® FL dye)

TDG: Theaflavin digallate

480

481

482 **Table 2.** Assay parameters used for measuring the inhibition of porcine α-amylase by polyphenols.

Method of detection	Inhibitor	Substrate		Enzyme (mg/mL)	Buffer	Incubation time (min)	Temperature (°C)	Kinetic parameters	IC_{50} (µg/mL)	Acarbose (µM)	Reference
		Source	Concentration (mg/mL)								
DNS reagent	Phaleria macrocarpa fruit extracts	*	*	0.16	Sodium phosphate (0.02 M, pH 6.9)	10	25	*	n-butanol fraction 58.5 Methanol extract 43.90	49.6	(Ali et al., 2013)
DNS reagent	Almond nut seeds skin polyphenols	*	5	*	Sodium phosphate (100mM, 17 mM NaCl, pH 6.8)	30	37	*	2.2	*	(Tsujita et al., 2013)
Chromogenic red starch method	EGCG	Red Starch	7	*	Sodium phosphate (20 mM, 6.7 mM NaCl, pH 6.9)	10	37	*	9.2 (IC_{34})	*	(Forester, Gu, & Lambert, 2012)
DNS reagent	Polyphenol rich extracts of C.olitorius leaf.	*	0.01	0.25	Sodium phosphate (0.02M, 0.006 M NaCl, pH 6.9)	10	25	*	26.8	*	(Oboh et al., 2012)
DNS reagent	Cyanidin-3-rutinosode	*	*	*	Phosphate buffer saline (0.1M, pH 6.9)	10	*	*	15.4	18.1	(S. Akkarachiyasit, Yibchok-Anun, Wacharasindhu, & Adisakwattana, 2011)

*	Rowanberry extract Raspberry extract Red raspberry extract Yellow raspberry extract	Potato starch	0.003	0.025	Synthetic saliva buffer	*	*	*	**	1.24	(Grussu, Stewart, & McDougall, 2011)
DNS reagent	Polyphenols from chestnut	*	5	*	Sodium phosphate (100 mM, NaCI 17mM, pH 6.8)	30	37	*	5.71 *		(Tsujita et al., 2011)
DNS reagent	Cyanidin Cyanidin-3-glucoside	*	1	*	Sodium phosphate (pH 6.9)	10	*	*	Cyanidin 109 Cyanidin-3-glucoside 145	120	(Sarinya Akkarachiyasit, Charoenlertkul, Yibchok-anun, & Adisakwattana, 2010)
DNS reagent	Dinkum raspberry extract	*	*	0.167	Sodium phosphate, (0.02M, 6 mM NaCl, pH 6.9)	10	25	*	16.8	*	(Zhang et al., 2010)
DNS reagent	Andrographis paniculata extract Andrographolide	*	*	0.17	Phosphate (20 mM, pH 6.9)	10	25	*	Andrographis paniculata extract 50900 Andrographolide 11300	23079	(Subramanian, Asmawi, & Sadikun, 2008)
Starch iodine test	Cyanidin-3-sambubioside	*	0.0036	*	Sodium phosphate (pH 7)	*	37	*	592	*	(Iwai, Kim, Onodera, & Matsue, 2006)
Liberation of p-nitrophenol	Quercetin Luteolin Myricetin EGCG Apigenin	Synthetic substrate non reducing end blocked p-nitrophenyl maltoheptaoside (BPNPG7)	*	0.03	HEPES buffer (pH 6.9)	10	37	*	Quercetin 151 Luteolin 103 Myricetin 98 EGCG >229 Apigenin >135		(Tadera, Minami, Takamatsu, & Matsuoka, 2006)
DNS reagent	Polyphenol-rich Pine bark extract	*	*	*	Sodium phosphate (pH 6.9)	5	37	*	1.69	2.71	(Y. M. Kim et al., 2005)
Reducing Termini Using PAHBAH	Berry extracts	Potato starch	0.003	0.025	Synthetic saliva	*	*	*	***		(McDougall et al., 2005)

Detecting the release of chromophore from synthetic substrate	Flavonoids	p-nitrophenyl-α-D-maltopentoglycoside	1.05	*	Phosphate buffer (100 mM, 0.2 % w/v bovine serum albumin, 1.80 mM $CaCl_2$, pH 7)	5	*	*	Luteolin (50-500) Luteolin-7-O-glucoside 4540 (IC_{100}) Kaempferol-3-O-glucoside 4540 (IC_{100}).	7.74-77.44	(J. S. Kim, Kwon, & Son, 2000)

*Not stated or clearly defined

** Inhibition values reported as µg of gallic acid equivalent/mL

*** Inhibition values reported as µg of phenols/assay

Where IC_{50} not given, data is presented as IC_n, where n= % of inhibition reported

Table 3. Assay parameters used for measuring the inhibition of microorganism α-amylase by polyphenols.

Method of detection	Inhibitor	Substrate		Enzyme (mg/mL)	Buffer	Incubation time (min)	Temperature (°C)	Kinetic parameters	IC_{50} (µg/mL)	Acarbose (µM)	Reference
		Source	Concentration (mg/mL)								
Microorganism											
DNS reagent	Phenolics from the pericarp of red pepper	Potato starch	0.005	*	Phosphate buffer (20 mM, 6.7 mM NaCl, pH 6.9)	3	25	*	Pericarp A 3000 (IC_{36}) Pericarp B 5000 (IC_{36})	*	(Chen & Kang, 2014)
DNS reagent	Geraldone, Isookanin and Luteolin	*	0.001	*	Sodium phosphate buffer (20 mM, pH 6.7)	5	37	*	Geraldone 10000 (IC_{94}) Isolokanin 10000 (IC_{84}) Luteolin 10000 (IC_{90})	15480	(Ahmed, Kumar, Sharma, & Verma, 2014)
DNS reagent	Almond nut seeds skin polyphenols	*	5	*	Sodium phosphate (100 mM, 17 mM NaCl, pH 6.8)	30	37	*	200−50 kDa (49.5)	*	(Tsujita et al., 2013)
starch-iodine method	Tannins from Cocoa, Pomegranates, Cranberries and Grapes	Potato starch	*	*	*	2	55	*	Cranberry 5000 (IC_{55}) Pomegranate 5000 (IC< 50) Grapes 5000 (IC<50) Cocoa 5000 (IC <50)	*	(Barrett et al., 2013)
DNS reagent	Polyphenols from chesnut	*	5	*	Sodium phosphate (100 mM, 17 mM NaCl, pH 6.8)	30	37	*	300−100 kDa (23.95)	*	(Tsujita et al., 2011)
Starch Iodine method	Polyphenols from different Bangladesh fruits	*	*	0.019	Phosphate buffer (0.02 M, 0.006 M NaCl, pH 7.0).	10	37	*	D. indica (410) Polyphenols from other fruits had < 50%	*	(Hossain et al., 2008)
DNS reagent	Polymers and Oligomers from Proanthocyanidins of Persimmon peel.	*	*	*	Phosphate buffer (20 mM, pH 6.7)	3	20	*	Polymers 100 (IC_{53}) Oligomers 100 (IC<50)	*	(Lee, Cho, Tanaka, & Yokozawa, 2007)

*Not stated or clearly defined

Where IC_{50} not given, data is presented as IC_n, where n= % of inhibition reported

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494

495 **Table. 4** Experimental IC_{50} values of acarbose, selected polyphenols and green tea extract.

Inhibitor	Substrate		
	Amylose (1 mg/mL)	**Amylopectin (1 mg/mL)**	**Amylopectin (0.37 mg/mL)**
Acarbose	3.5±0.3	10±1	7.6±0.8
EGCG	5.3±0.6	60±2	24±4
Quercetin	19.8±0.3	83±7	22±1
Luteolin	26.3±0.6	75±1	42±9
Green tea	8.9±0.1	60±2	25±1

496 The IC_{50} value of the tested pure compounds is expressed in µM. For green tea, IC_{50} values are
497 expressed in µg/mL.

498 Green tea comprises EGCG, epigallocatechin, epicatechin gallate and epicatechin (200, 124, 34 and
499 23 µg/mg) as analysed by HPLC (Manzano & Williamson, 2010).

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502

503 **Supplementary figure legends**

504

505 Figure 1s. Chemical structures of acarbose and polyphenols studied in this article.

506

507 Figure 2s. Schematic design of the α-amylase inhibition assay.

508

509 Figure 3s. Time-dependence of salivary α-amylase hydrolysis of (A) amylose and (B) amylopectin.
510 The hydrolysis of both substrates was measured by the amount of maltose produced at 4 different
511 amounts of enzyme. Data points are expressed as mean ± SE (n=3).

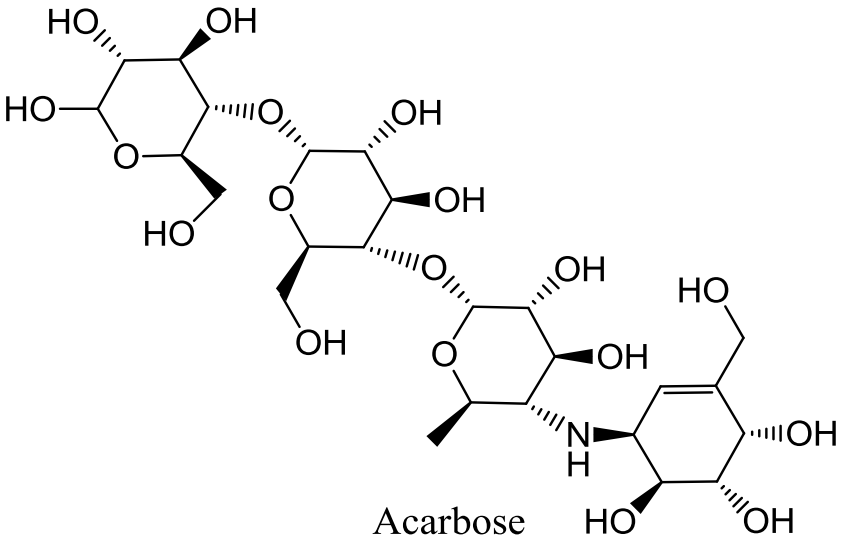
512

513 Figure 4s. Lineweaver-Burk plot for action of salivary α-amylase on amylose and amylopectin.

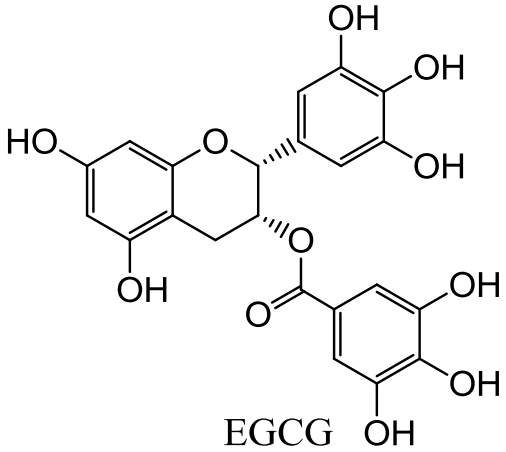
514

515 Figure 5s. HPLC chromatogram of EGCG (A) before SPE and (B) after SPE. The removal of EGCG
516 was > 99 %.

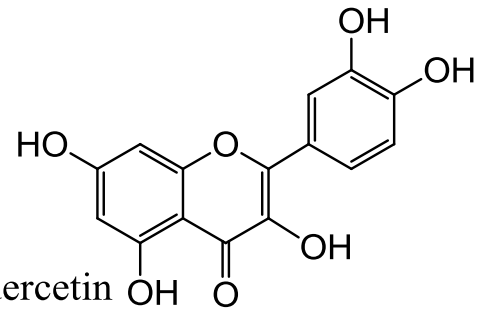
517 Figure 1s



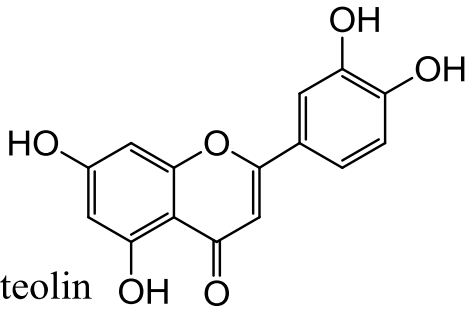
Acarbose



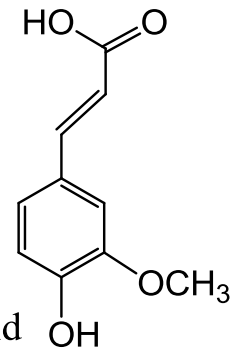
EGCG



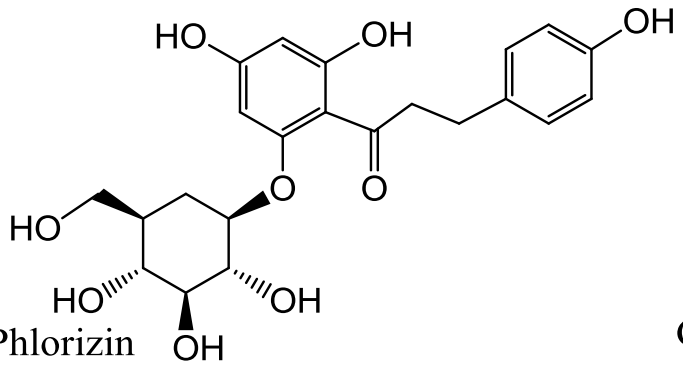
Quercetin



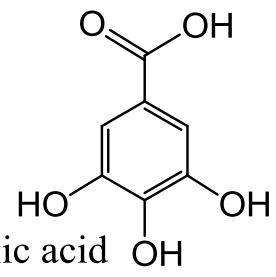
Luteolin



Ferulic acid



Phlorizin



Gallic acid

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519 Figure 2s

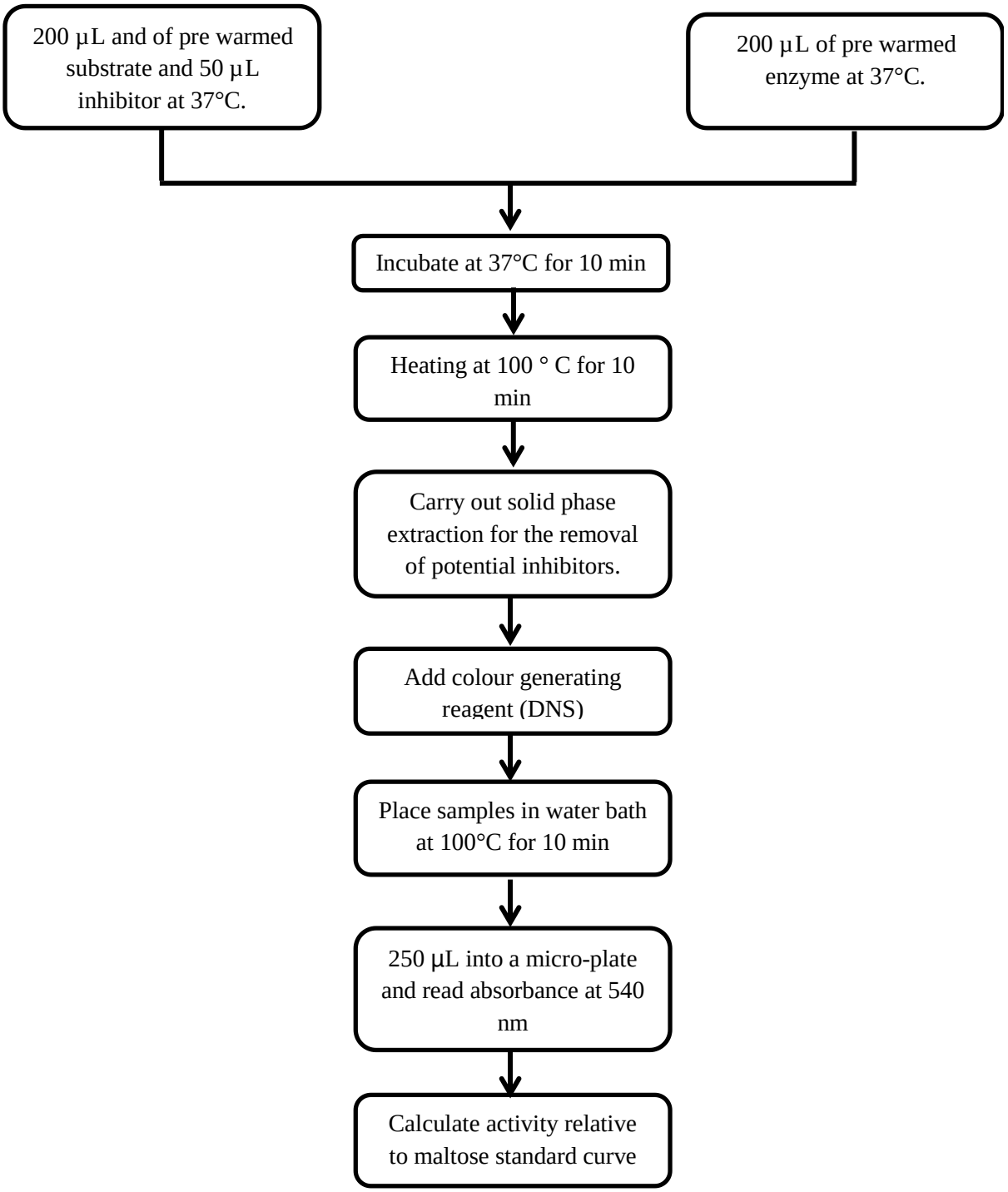

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Figure 3s

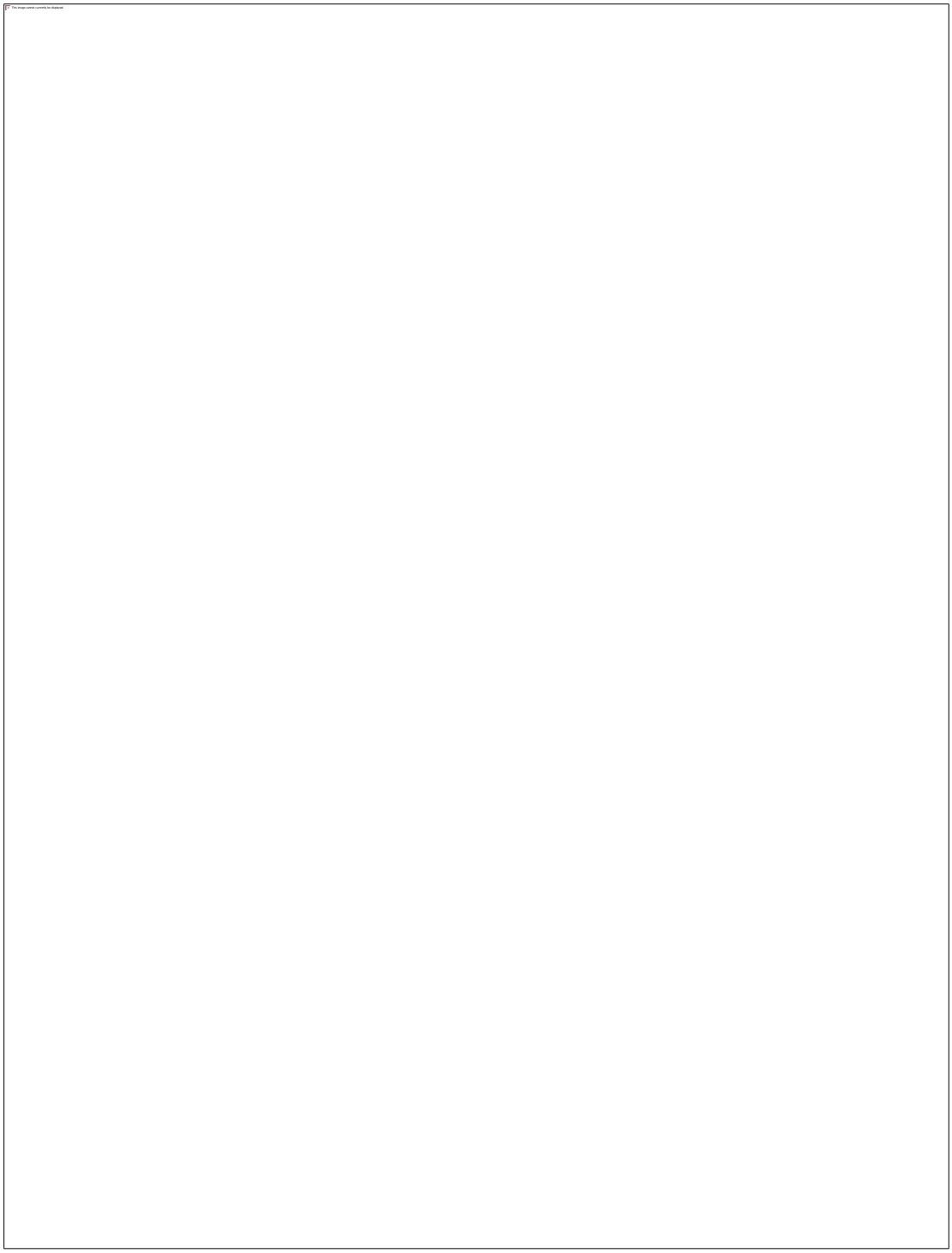

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Figure 4s

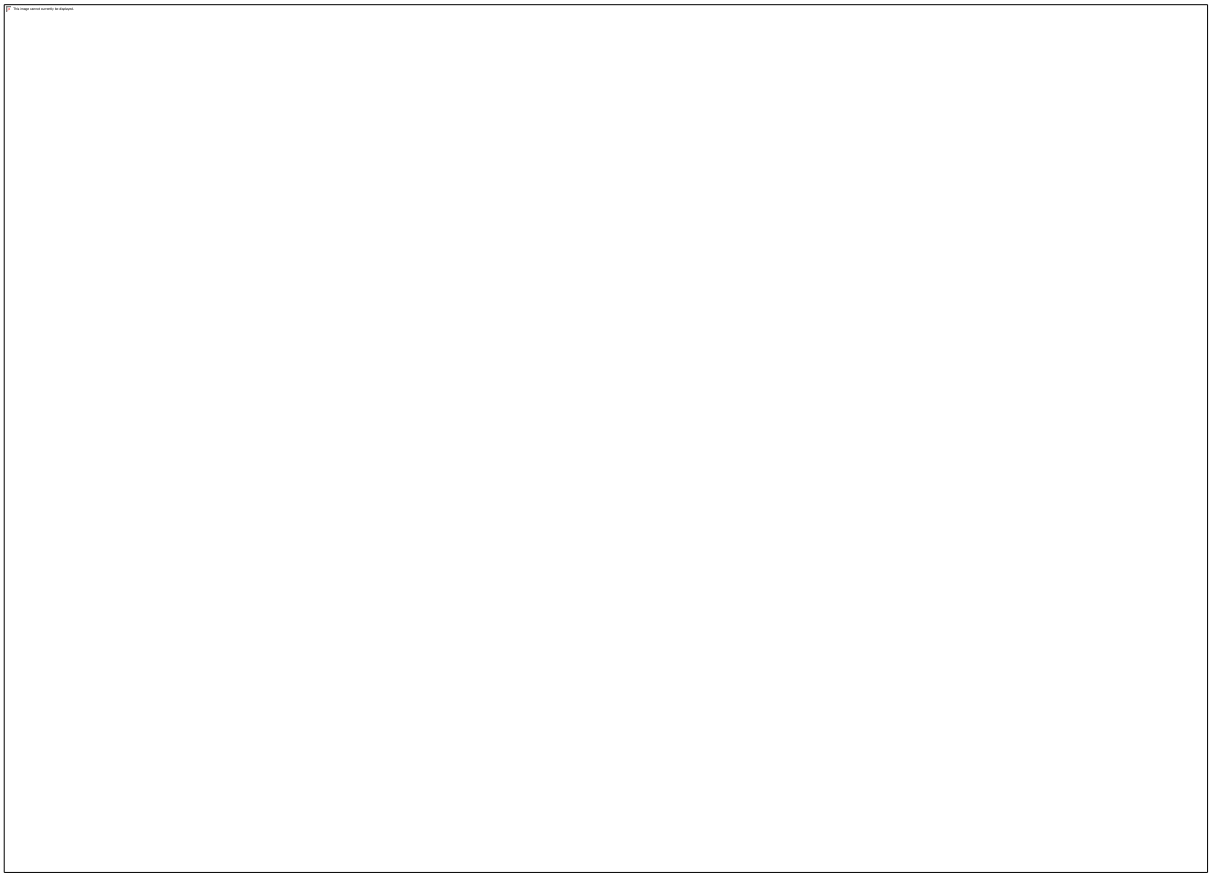

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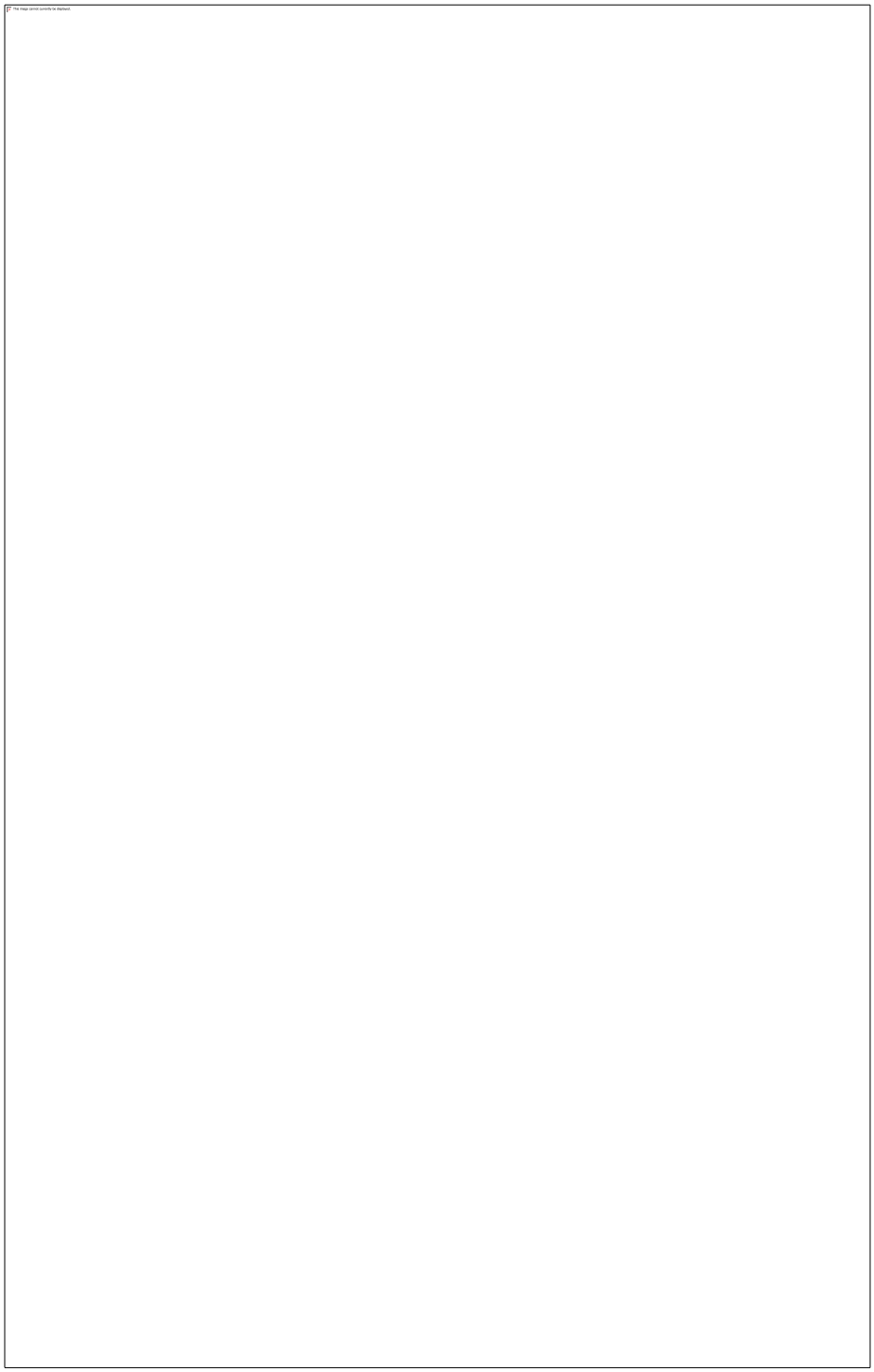
Figure 5s



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