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Inhibition of Starch Digestion by Gallic Acid and Alkyl Gallates

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Abstract

As phenolic compounds, alkyl gallates may inhibit the activity of digestive enzymes for starch. Furthermore, their alkyl chains may facilitate starch inclusion complexation and results in an increased resistant starch (RS) content or slowly digestible starch (SDS) content, which may further retard starch digestion. The significance of such inhibition is that the rate of starch hydrolysis into glucose is reduced, thereby preventing hyperglycemia and related metabolic diseases. This study examined the inhibitory effects on *in vitro* enzymatic digestion of starch by gallic acid and five alkyl gallates of varying alkyl chain lengths, i.e., butyl gallate, octyl gallate, dodecyl gallate, hexadecyl gallate, and octadecyl gallate. Raw and cooked potato starch (PS) and high-amylose maize starch (HAMS) were tested. For both types of raw starch, gallic acid and all the alkyl gallates significantly ($p < 0.05$) increased RS content except for octadecyl gallate. In the case of cooked starch, the RS contents were markedly decreased ($p < 0.05$) as compared to those in raw starch, because gelatinization caused an overall greater susceptibility to enzymatic digestion. The reduction in RS content was less in cooked HAMS than that in cooked PS, due to a higher gelatinization temperature range of HAMS. The aforementioned effect of gallates on RS content was also found for cooked PS, while cooked HAMS experienced increased RS only with gallic acid and butyl gallate. Overall, for the digestion of both starches, regardless of raw or cooked, gallic acid and alkyl gallates with shorter chains demonstrated the strongest inhibitory effects. Our findings indicate that shorter alkyl gallates are effective in inhibiting enzymatic digestion of starch and therefore may be potential in controlling postprandial hyperglycemia.

Keywords

Starch; gallic acid; alkyl gallates; *in vitro* digestion; resistant starch; phenolic compound

1. Introduction

With the prevalence of overweight and obesity throughout the world, recent dietary guidelines suggest reducing excessive caloric intake (World Health Organization, 2018). Since starch is the main energy source of the human diet, to retard starch digestion and glucose absorption will provide an effective way for the prevention and treatment of obesity and associated metabolic diseases. While some anti-obesity drugs work to influence digestive enzymes, people are seeking food-based strategies. Fortunately, many food constituents and herbal extracts have shown inhibitory effects on key digestive enzymes for starch (Rasouli, Hosseini-Ghazvini, Adibi, & Khodarahmi, 2017; Sales, Souza, Simeoni, Magalhães, & Silveira, 2012), and thus have the potential to be used in modulating glycemic response.

Among potent enzyme inhibitors, many are phenolic compounds. In fact, a large variety of phenolic compounds, e.g., phenolic acids, flavonoids, and tannins, have been documented to inhibit starch digesting enzymes, such as α-amylase (Rasouli, et al., 2017) and α-glucosidase (Funke & Melzig, 2005). Phenolic compounds are secondary metabolites produced in plants as a response to stressors. Therefore, besides their inhibitory effect on starch digestion, they provide other beneficial bioactivities, such as antioxidant and anti-inflammatory activities. Their bioactivities could be enhanced by altering their hydrophobicity and thus their affinity with the cell membrane. For instance, Sherratt, Villeneuve, Durand, and Mason (2019) found that membrane antioxidant and cholesterol domain inhibition activities of rosmarinic acid esters are dependent, nonlinearly, on alkyl chain length, with octyl rosmarinate showing the greatest antioxidant effect.

Alkyl gallates are a group of phenolipids and are alkyl esters of gallic acid (GA), a phenolic acid widely found in plant foods, such as fruits and tea. Alkyl gallates have been widely studied

for their antioxidant properties. The chemical structure of its gallate moiety has excellent electron-donating capability that allows its derivatives to be potent antioxidants. Moreover, some alkyl gallates have demonstrated indirect, preventive antioxidant properties. For example, dodecyl gallate (DG) inhibits xanthine oxidase (Kubo, Masuoka, Xiao, & Haraguchi, 2002), which normally generates toxic superoxide anions. Additionally, DG was shown to inhibit mitochondrial lipid peroxidation, while the parent compound gallic acid (GA) did not. This result suggests that the hydrophobic alkyl chain is essential for that particular antioxidant activity (Kubo, et al., 2002). Alkyl gallates have also exhibited diverse antimicrobial activities. They can bind to and disrupt the FtsZ-ring in bacteria, which is essential for proper cell division (Król, et al., 2015). Moreover, octyl gallate (OG) has been demonstrated to act as a surfactant and dramatically reduce the fluidity of fungal plasma membranes (Fujita & Kubo, 2002). Several other alkyl gallates have displayed indirect inhibition of ergosterol biosynthesis, which functions to maintain membrane integrity in fungi (Leal, et al., 2009).

Owing to the numerous beneficial bioactivities, further investigation of their effects on starch digestion is valuable. As phenolic compounds, alkyl gallates may inhibit starch digestion. Moreover, the alkyl chain may facilitate the formation of starch inclusion complex, which also decreases starch digestibility. For example, the proportion of resistant starch (RS) was reported to increase when various fatty acids were introduced into the system (Crowe, Seligman, & Copeland, 2000) and starch-lipid inclusion complex could be responsible for the higher RS composition. Since phenolic compounds and fatty acids inhibit starch digestion via different mechanisms, a synergistic effect of alkyl gallates on starch digestion could be exhibited. Additionally, the inhibitory ability may depend on the alkyl chain length. Therefore, the objective of this study was to examine the effects of alkyl gallates with different alkyl chain

length (**Figure 1**), including butyl gallate (BG), OG, DG, hexadecyl gallate (CG), and octadecyl gallate (SG, abbreviated from its common name stearyl gallate to differentiate with OG used for octyl gallate) on starch digestion. Two types of starch, potato starch (PS) and high-amylose maize starch (HAMS) that contain different amylose contents (approximately 25% and 80% (w/w) in PS and HAMS, respectively), were used to assess the possible consequence of differing amylose content in starch. Both raw (uncooked) and gelatinized (cooked) starches were subjected to digestion to study the inhibitory mechanisms of GA and its alkyl gallates on starch digestion.

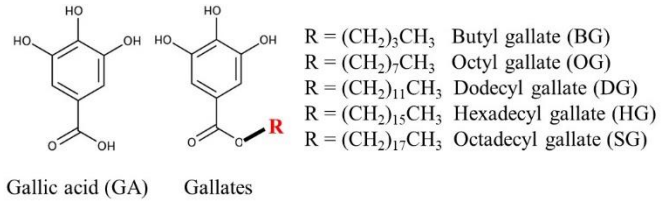

Figure 1. Chemical structures of gallic acid and its alkyl esters.

2. Materials and methods

2.1. Materials:

High amylose maize starch (HAMS; Gelose 80) was kindly provided by Ingredion (Bridgewater, NJ, USA). Potato starch (S2004), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, A7095, 300 U/mL), porcine pancreatin (EC 232-468-9, P7545, 8 x USP), invertase from baker's yeast (EC 3.2.1.26, I4504, 300 U/mg), guar gum (G4129), sodium acetate trihydrate (S7670), gallic acid (G7384), butyl gallate (S764868), octyl gallate (48700), and dodecyl gallate (48660) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). D-glucose assay kit (GOPOD format, K-GLUC) was obtained from Megazyme (Wicklow, Ireland). Hexadecyl

gallate (TCG0013), octadecyl gallate (TCG0019), and ethanol was purchased from VWR International (Radnor, PA, USA).

2.2. Total starch content

The total starch content in PS and HAMS samples was determined using the total starch assay kit purchased from Megazyme (Wicklow, Ireland) following the manufacturer's instruction and AOAC Official Method 996.11 (McCleary, Gibson, & Mugford, 1997).

2.3. In vitro digestion

The *in vitro* starch digestion was conducted according to the method described by Englyst, Kingman, and Cummings (1992) with some modifications. The enzyme solution, containing pancreatin (46.8 mg/mL prior to centrifugation), amyloglucosidase (13 U/mL), and invertase (187.5 U/mL), was prepared immediately before use. Cooked starch was prepared by boiling starch in 100 ^{o}C water for 20 min immediately before *in vitro* digestion and allowed to cool to room temperature (20 °C). Starch (80 mg) alone and starch mixed with 10 mg of GA and alkyl gallates were weighed into 20 mL round-bottom test tubes, followed by addition of 10 mg guar gum and 6 mm glass beads to each tube. A test tube with no starch and inhibitors was used as the blank. Four mL of 0.1 M acetate buffer was added into each tube, and the reaction mixtures were mixed and placed in a water bath at 37 °C for 5 min. Enzyme solution (1 mL) was added into each tube at a certain time interval and this time interval was accurately timed for further sampling. The test tubes were capped and placed into a shaking water bath at 37 $^{\circ}$C and 150 strokes per min. After incubating for 20 and 120 min, aliquots of 200 µL were mixed into 900 µL of 66% (v/v) ethanol and mixed vigorously. The digesta samples were kept at 4 °C overnight. The samples were centrifuged (2700 g, 3 min) and the glucose concentrations in the supernatant

were measured using the glucose oxidase-peroxidase (GOPOD) method. Starch contents at 20 and 120 min corresponded to the proportion of rapidly digestible starch (RDS) and slowly digestible starch (SDS), respectively. The amount of RS was determined as the amount remaining after 120 min. Results were adjusted by the blank and the measured total starch content (81.10±5.72% and 100±2.80% in PS and HAMS, respectively), and expressed as a percentage of total starch.

2.4. Statistical Analysis

All experiments were conducted in duplicates. Data were analyzed by linear regression and one-way analysis of variance (one-way ANOVA) followed by Tukey multiple comparison test using the OriginPro software (OriginLab, Northampton, MA, USA). The letters a, b, c, d, and e indicate statistically significant differences, $p < 0.05$ (a > b > c > d > e).

3. Results and Discussion

3.1. Raw starch

The inhibitory effects of GA and alkyl gallates on enzymatic starch digestion were first evaluated using raw samples of two types of starch, PS and HAMS. Results are shown as the digestibility profiles containing relative proportions of RDS, SDS, and RS (**Figure 2**). Starches with no inhibitor (NI) represent a control to demonstrate baseline starch digestion. The RS content of raw HAMS was 62%, which is very close to previous findings in high amylose maize starches, e.g., the Hi-Maize 260 starch (Ingredion, 2019). The RS content in PS was surprisingly high at 89%, higher than that determined by the Englyst method, yet close to the reported value using the Faisant method (Champ, Kozlowski, & Lecannu, 2000). Both HAMS and raw PS have been classified as type 2 RS, or RS2. This group of RS refers to native granular starch that is

inaccessible to digestive enzymes due to densely packed molecular order in the surface region of starch granules (Zhang, Dhital, & Gidley, 2015). The use of RS2 in food is the most convenient among all types of RS, probably due to its availability and minimum processing required. Furthermore, RS2, specifically the high amylose starches, is the only type of RS that has been identified as dietary fiber by the U.S. Food and Drug Administration (2018a). Although the current literature shows inconsistent results, many have demonstrated the beneficial physiological effects of this type of RS in human health, such as reductions in postprandial insulin response and low postprandial glycemic response (Food and Drug Administration, 2018b).

For both raw HAMS and PS, the RS content was significantly increased ($p < 0.05$) in the presence of all alkyl gallates except for SG. This exception may be explained by the fact that longer alkyl chains, particularly in SG, have lower solubility and have been found to self-associate in an aqueous solution (Takai, Hirano, & Shiraki, 2011). In both starches, the RS content appears to decrease with increasing alkyl chain lengths. GA had the most potent inhibitory effect on enzymatic activity and increased the nominal RS contents to 95%, and 98% in HAMS and PS, respectively. The inhibitory effect of GA on starch digestive enzymes, including amylase and maltase, was reported before (Gupta, Gupta, & Mahmood, 2007; Lu, Chen, Zhao, Ge, & Liu, 2016). Linear regression analyses reveal that RDS and SDS contents increased with the alkyl chain length of gallates, whereas RS contents decreased with the alkyl chain length (**Figure 3**). Among the GA and alkyl gallates, water solubility could have played an important role such that gallates with longer alkyl chains were less available in the solution to inhibit the enzymatic activity. Besides, the decrease in inhibitory effect with alkyl chain length could also be due to one limitation of our experimental design that used constant weight ratio

instead of constant molar ratio, so that less ligand molecules were available to bind enzymes as molecular mass increased with alkyl chain length. Yet, solubility should have been the main limiting factor. The decrease in solubility with increasing alkyl chain length was noticed during the experiment. It is worthwhile to note that the GA and alkyl gallates influenced the starch digestibility mainly by inhibiting the enzymatic activities and delaying the digestion, at least in the case of raw starch digestion (their influence on starch structure and actual RS/SDS content will be discussed in the next subsection). The inhibitory ability of GA on α-amylase was suggested to occur via binding to the active site and subsequent induction of conformational change of α-amylase(Lu, et al., 2016). Furthermore, although the inhibitory mechanism of alkyl gallates have not yet been studied, other gallate derivatives were reported to inhibit α-amylase and α-glucosidase via binding mechanisms (Li, et al., 2018; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). So, the nominal increase in RS content actually resulted from retarded enzymatic activity. Accompanying the increase in RS content, the RDS and SDS contents gradually decreased with the addition of gallates with shorter alkyl chains. These lost RDS and SDS were found as RS, without showing a preferential increase in either RDS or SDS. It reaffirms that the GA and alkyl gallates mainly affected the enzymatic activity rather than forming new structures with starch, in the case of raw starch digestion.

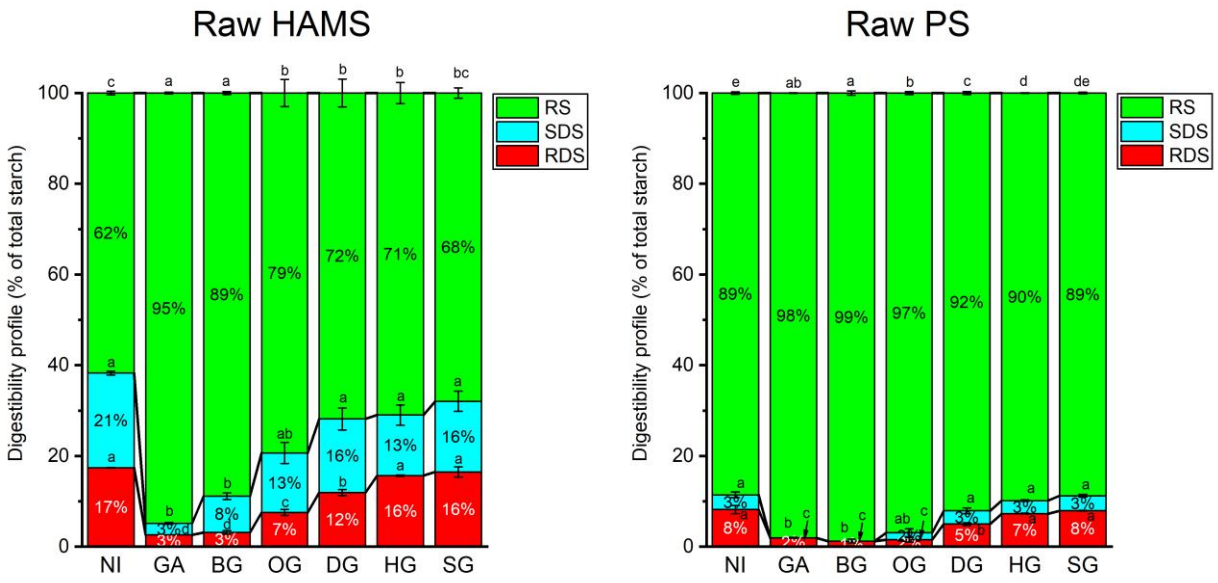

Figure 2. Starch digestibility profiles, presented as rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) contents, of raw high amylose maize starch (HAMS) and raw potato starch (PS) without inhibitors (NI) and with the presence of gallic acid (GA), butyl gallate (BG), octyl gallate (OG), dodecyl gallate (DG), hexadecyl gallate (HG), and octadecyl gallate (SG). Values below 1% were not shown. Error bars show standard deviation; n=2. Significant differences among treatments within each starch digestion fraction are denoted by different letters (a > b > c > d > e, $p < 0.05$).

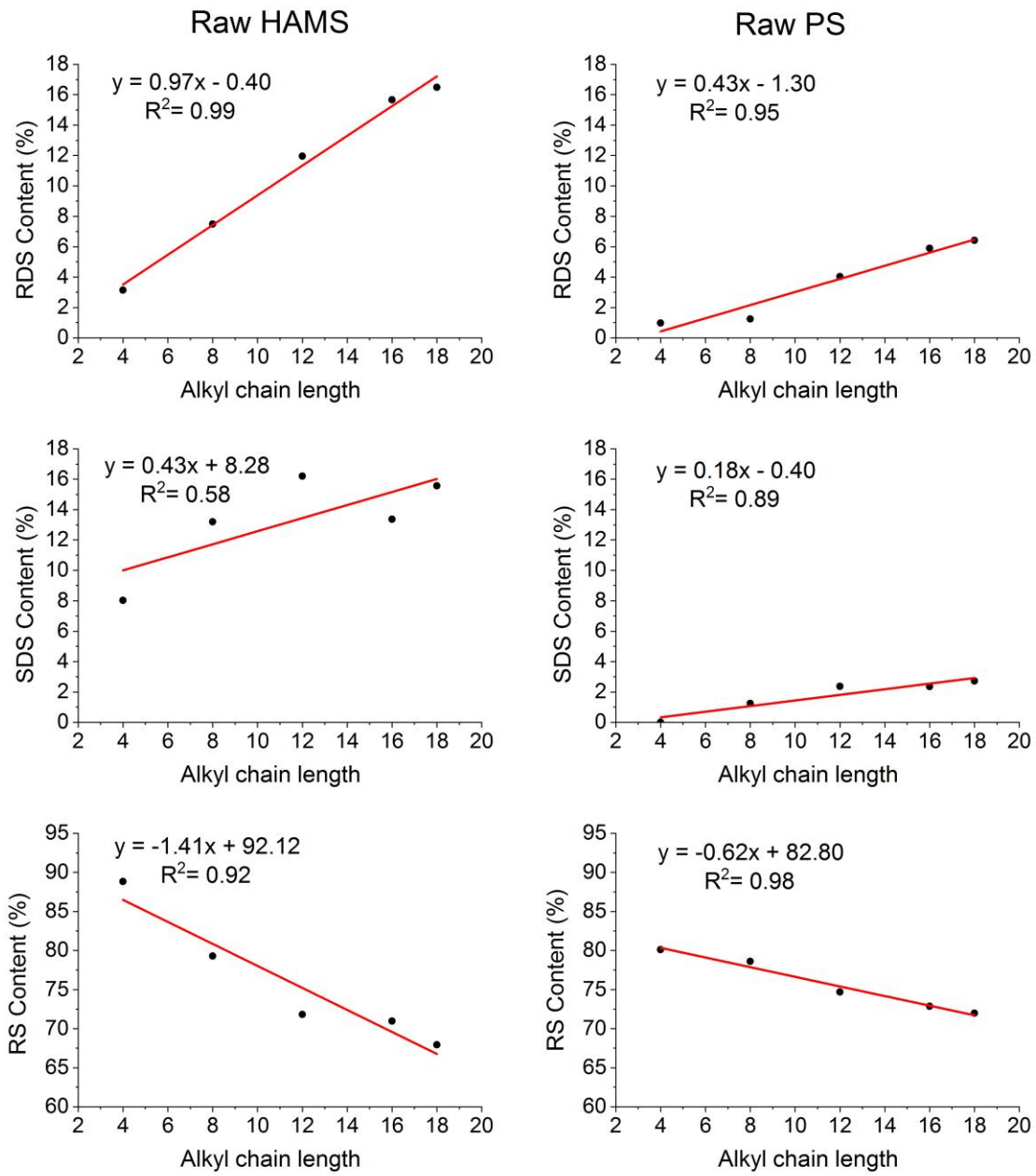

Figure 3. Relationships of alkyl chain length and rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) contents in raw high amylose maize starch (HAMS) and raw potato starch (PS).

3.2. Effect of cooking (gelatinized starch)

Starch digestibility is heavily influenced by the processing of starch, e.g., starch gelatinization. Starch can be gelatinized by heating in the presence of water, i.e., cooking. Starch gelatinization results in the disruption and swelling of starch granules, the leaching of dissolved

amylose molecules into the surrounding water, and the loss of molecular order in amylopectin. A potential consequence of gelatinization is that a larger fraction of starch becomes more susceptible to enzymatic digestion. Since starch is usually cooked in food preparation, the effects of GA and alkyl gallates on the digestibility of gelatinized starch was investigated (**Figure 4**). Compared with raw starches, the cooked starches contain dramatically less RS contents ($p < 0.05$) under all tested experimental conditions. This result is consistent with previous findings that gelatinization causes marked decreases in the RS contents of canna, rice, and potato starches (Inan Eroglu, 2017; Juansang, Puttanlek, Rungsardthong, Puncha-arnon, & Uttapap, 2012). The RS contents in gelatinized HAMS and PS were reduced to 40% and 15%, respectively. PS experienced more reduction in RS content than HAMS, which could be attributed to their different gelatinization behaviors. Gelatinization occurs over a range of temperature and varies among starches. The gelatinization temperature range of PS was reported to be around 59 – 68 °C, while HAMS only swells to a limited extent upon boiling (Ding, et al., 2019; Greenwood, 1976). HAMS requires a temperature as high as 125 °C to effectively gelatinize (Chen, et al., 2017; Greenwood, 1976). The amylopectin branches are longer in HAMS and thus enable stronger helical association, probably also involving amylose molecules (Montgomery, Sexson, & Senti, 1961). Therefore, HAMS may be more able to resist gelatinization and subsequent enzymatic digestion. Meanwhile, the RDS contents drastically increased in HAMS and PS from 17% to 60%, and from 8% to 85%, respectively, whereas the SDS content diminished or vanished after cooking. PS was gelatinized to a higher extent, making it more digestible. The gelatinization of both starches resulted in a highly digestible portion and resistant portion, probably corresponding to gelatinized and ungelatinized proportions. The lack of an intermediate

component (SDS) implies the absence of newly formed inclusion complex immediately following cooking of starch.

In regard to the impact of inhibitors, only GA and BG significantly enhanced RS content in cooked HAMS, while all of the alkyl gallates except for SG increased RS content in cooked PS. The same trends were found that increasing alkyl chain length generally decreased the RS content and increased the RDS content in both starches (**Figure 5**). Similar to the discussion of raw starch, this indicates that longer alkyl chains were less effective in inhibiting starch digestion, due to lower solubility. GA had the strongest effect in enhancing the RS content. The RS content in cooked HAMS was brought back to 64%, the same level as in the raw HAMS without adding GA or alkyl gallates. Yet, the RDS content was still much higher than any of the raw starch samples. After gelatinization, the starch molecules in PS were highly available and therefore even the most potent inhibitor used could not compensate for the loss in RS.

BG, OG, DG, and HG caused a significant increase in SDS contents in cooked HAMS. This was not seen in the raw starches and gelatinized PS. When starch is gelatinized, it leaches amylose and disrupts molecular packing of amylopectin side chains and, therefore, facilitates the formation of inclusion complex when appropriate guest molecules are present. This form of starch can retard digestion, though not to the extent of total resistance, yet may contribute to both SDS and RS portions (Tan & Kong, 2019). The alkyl gallates with intermediate chain length provided the ideal conditions for inclusion complexation, i.e., sufficient solubility in water to interact with amylose and alkyl chain length for helical wrapping. This result is consistent with previous findings by Kaur and Singh (2000) that amylose-lipid complex formation occurred more effectively with myristic acid (C14:0) than stearic acid (C18:0) during cooking. In situ inclusion complexation is not as effective as methods to intentionally prepare starch inclusion

complexes, but was reported to be responsible for changes in physicochemical properties of starch as well as its digestibility. For instance, Crowe, et al. (2000) found that various lipids could significantly retard starch digestion and this effect using lauric acid was as effective as retrogradation. The rapid formation of amylose-lipid inclusion complexes after cooking amylose with lipids was proposed to cause the enzymatic retardation. This study differs with that of Crowe, et al. (2000) in that alkyl gallates were not cooked together with starch and starch was not fully dissociated by alkali solutions. Both treatments in the study of Crowe, et al. (2000) would facilitate amylose inclusion complexation, and hence, this study did not produce as much inclusion complex, reflected by small SDS portions. In contrast to HAMS, the SDS contents in gelatinized PS were not affected by the presence of GA and any type of alkyl gallates. The low amylose content in PS could explain the difference, because guest inclusion complexation prefers long linear chains of amylose.

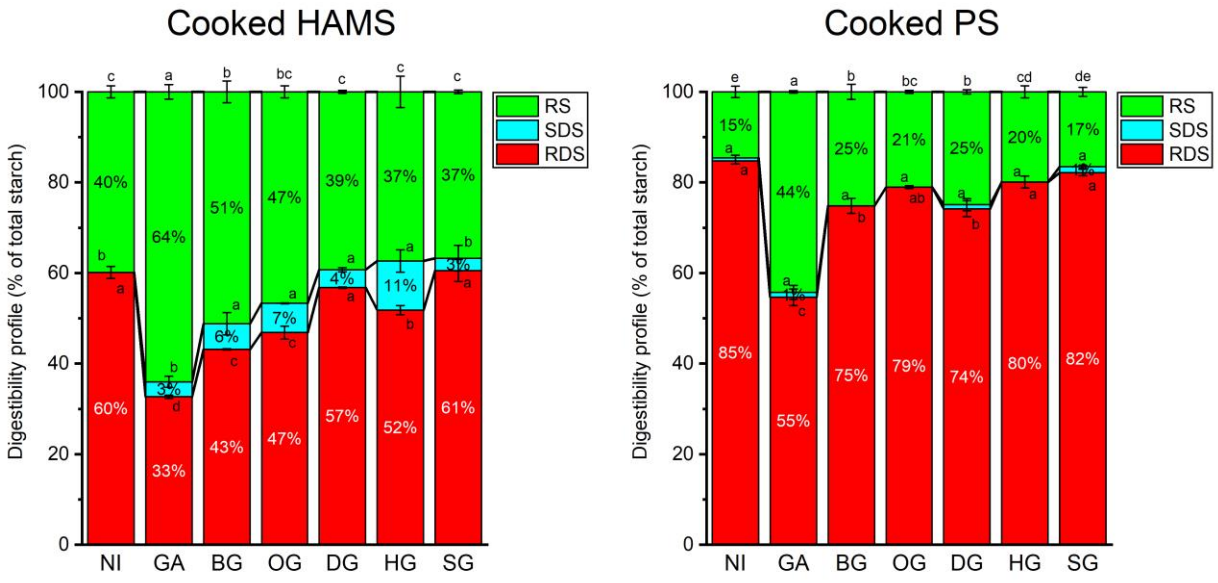

Figure 4. Starch digestibility profiles, presented as rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) contents, of cooked high amylose maize starch (HAMS) and cooked potato starch (PS) without inhibitors (NI) and with the presence of gallic acid (GA), butyl gallate (BG), octyl gallate (OG), dodecyl gallate (DG), hexadecyl gallate (HG),

and octadecyl gallate (SG). Values below 1% were not shown. Error bars show standard deviation; n=2. Significant differences among treatments within each starch digestion fraction are denoted by different letters (a > b > c > d > e, $p < 0.05$).

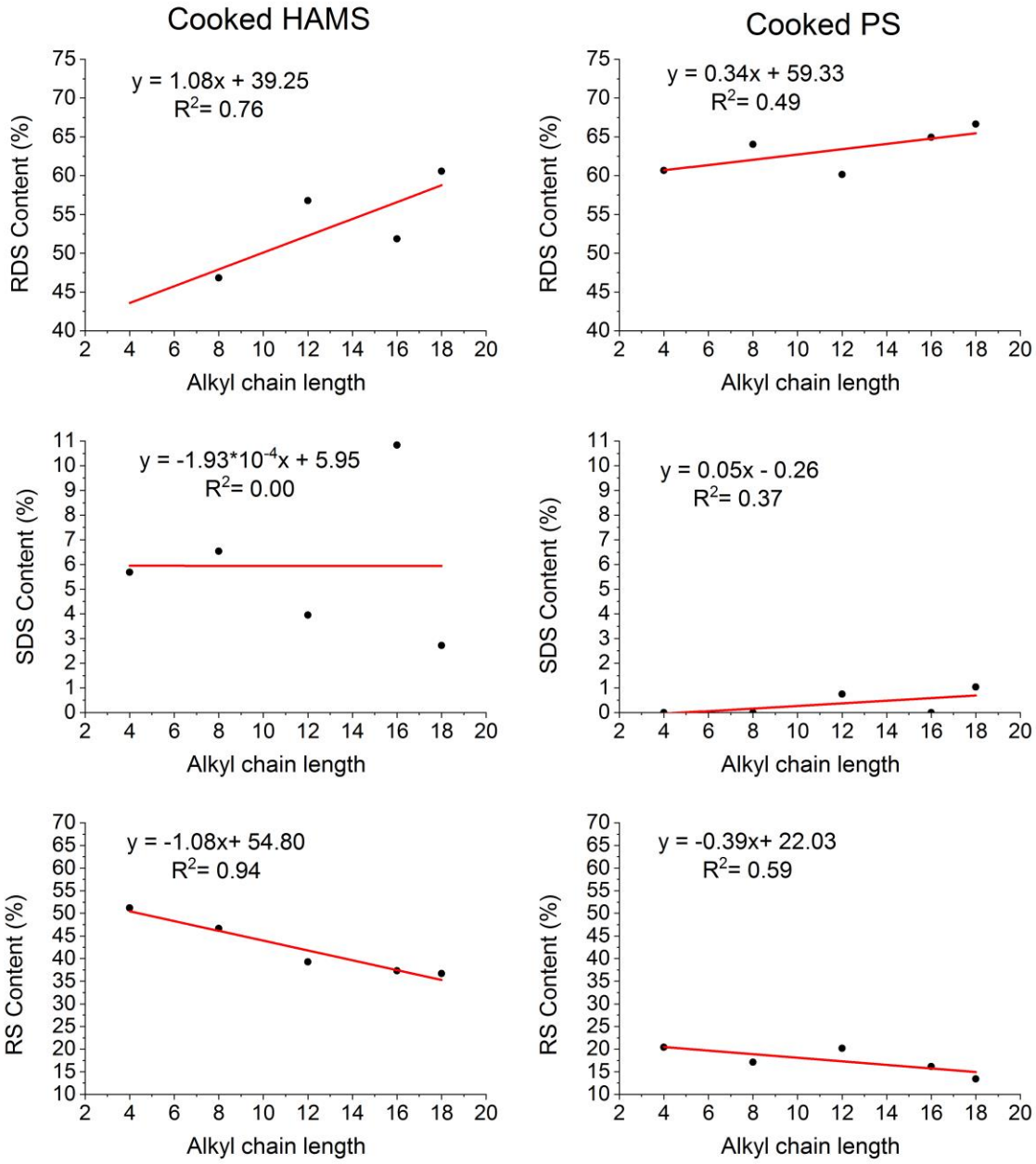

Figure 5. Relationships of alkyl chain length and rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) contents in cooked high amylose maize starch (HAMS) and cooked potato starch (PS).

The findings in this study have practical implications for the use of RS2. Various types of RS2, such as raw potato starch and raw high amylose maize/corn starch, are excellent sources of RS and providing promising roles in managing glucose homeostasis, improving gut health, and

283 increasing satiety (Guo, Tan, & Kong, 2019). However, in reality, these starches are cooked
284 before consumption. Their enzymatic resistance can be mitigated by cooking or gelatinization,
285 and thus losing their function as a RS. Indeed, this study showed that cooked PS lost most of its
286 RS content. To compensate for this loss in RS content, phenolic compounds may be added into
287 the formulation or recipe. GA is one of the simplest phenolic acids and alkyl gallates may affect
288 the starch structure and digestibility more extensively if cooked together with starch.
289 Furthermore, there are numerous other natural phenolic compounds showing profound inhibitory
290 effects on starch digestive enzymes (Sales, et al., 2012; Xiao, Ni, Kai, & Chen, 2013). The
291 inhibitory effect further depends on the type of phenolic compounds, their molecular weight,
292 degree of polymerization, and concentration. For example, Barros, Awika, and Rooney (2012)
293 found that polymerized tannin extract increased the net RS in normal starch by about 2 times
294 more than the monomeric polyphenol extract and this effect was enhanced by the degree of
295 polymerization of tannins (Mkandawire, et al., 2013). Hence, the incorporation of plant food rich
296 in phenolic compounds could be a food-based strategy to reduce caloric intake from starchy
297 food.

298 **4. Conclusion**

299 This study examined the ability of gallic acid and various alkyl gallates to inhibit the
300 enzymatic digestion of HAMS and PS by increasing their RS contents. The RS of both starches,
301 when using raw samples, was significantly increased by GA and every tested alkyl gallate except
302 for SG. Cooking, or gelatinization, significantly reduced the RS contents in both starches, and
303 the reduction was more profound in PS. This may be a consequence of their different
304 gelatinization behavior, where PS has a much lower gelatinization temperature range than
305 HAMS. In both starches, whether raw or cooked, GA and the shorter alkyl gallates exhibited the

strongest inhibitory effects. The inefficiency of longer alkyl chain gallates in inhibiting starch digestion may be attributed to their low solubility and susceptibility to self-association in solution. Although SG lacked inhibitory ability under all tested conditions, the other alkyl gallates showed potential as a means of increasing the RS content of starch. Alongside their already known antioxidant and antimicrobial properties, these compounds may therefore be promising agents in modulating glycemic response.

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