



Structure-guided unravelling: Phenolic hydroxyls contribute to reduction of acrylamide using multiplex quantitative structure–activity relationship modelling



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ABSTRACT

We reported a structure–activity relationship study on unravelling phenolic hydroxyls instead of alcoholic hydroxyls contribute to the reduction of acrylamide formation by flavonoids. The dose-dependent study shows a close correlation between the number of phenolic hydroxyls of flavonoids and their reduction effects. In view of positions of hydroxyls, the 3',4'(ortho)-dihydroxyls in B cycle, 3-hydroxyl or hydroxyls of 3-gallate in C cycle, and 5,7(meta)-dihydroxyls in A cycle of flavonoid structures play an important role in the reduction of acrylamide. Flavone C-glycosides are more effective at reducing the formation of acrylamide than flavone O-glycosides when sharing the same aglycone. The current multiplex quantitative structure–activity relationship (QSAR) equations effectively predict the inhibitory rates of acrylamide using selected chemometric parameters (R^2 : 0.835–0.938). This pioneer study opens a broad understanding on the chemoprevention of acrylamide contaminants on a structural basis.

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1. Introduction

The occurrence of acrylamide contaminant in heat processing foods was initially found by Swedish agency and scientists in April 2002 (Tareke, Rydberg, Karlsson, Erickson, & Törnqvist, 2002). The chemical and toxicological properties of acrylamide have been demonstrated elsewhere (Lineback, Coughlin, & Stadler, 2012). It has been found to be a carcinogen and genotoxin in rodents with high-dose exposure. Furthermore, it is confirmed to be a neurotoxin in humans, especially peripheral neuropathy. Primarily based on the animal studies, the International Agency for Research on Cancer (IARC) has classified acrylamide as the Group 2A–“probably carcinogenic to humans” substance (IARC, 1994).

In a mechanistic view, acrylamide is generated from the Maillard reaction, which is known as the asparagine route (Fig. S1) (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002). The isotope-labeled studies have demonstrated the backbone of acrylamide originates from the amino acid asparagine (Stadler et al., 2002; Zyzak et al., 2003). In this asparagine route, asparagine can

react with the hydroxycarbonyl moiety of reducing sugars (route A) or reactive dicarbonyls (route B) for the formation of acrylamide (Hamzalıoğlu & Gökmen, 2012; Stadler et al., 2004). An important intermediate, 3-aminopropionamide (3-APA), has been proposed in the reaction pathway, the deamination of which provides a high yield of acrylamide up to 60% (molar ratio) (Granvogl & Schieberle, 2006). Besides, asparagine without the presence of carbohydrates could generate acrylamide in principle via decarboxylation followed by deamination, but the reaction is inefficient with extremely low yields (Granvogl, Jezussek, Koehler, & Schieberle, 2004). Alternatively, acrylamide can be generated from lipid degradation, protein metabolism and/or organic acid decarboxylation, which is known as the acrolein and acrylic acid route (Lineback et al., 2012). However, this route can be regarded as of marginal importance because the reaction between asparagine and carbonyl compounds such as reducing sugars is more active during heat processing of potato- and cereal-based foods. Based on the understanding of formation mechanism via the asparagine route, acrylamide may be reduced via (i) preventing the reaction between asparagine and reducing sugars or reactive carbonyls; (ii) inactivating the Strecker-type degradation of the Schiff base and/or a β -elimination reaction of the decarboxylated Amadori compounds; and (iii) reducing the deamination of 3-APA (Stadler et al., 2004; Zhang, Ren, & Zhang, 2009). Some other studies investigated the

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reduction effect via the scavenging and eliminating of acrylamide. For instance, niacin as an acrylamide scavenger is capable of trapping acrylamide via Michael addition to generate 1-propanamide-3-carboxy pyridinium (Papamokos, Dreyer, Navarini, & Carloni, 2014). Besides, the inhibition of lipid oxidation could be regarded as a reduction way to the formation of acrylamide in the acrolein and acrylic acid route (route C, Fig. S1) (Kocadağlı, Göncüoğlu, Hamzalıoğlu, & Gökmen, 2012).

Substantial efforts have been undertaken worldwide to formulate recipes that reduce acrylamide in widely-concerned foods, including potato products (potato chips and French fries), cereal-based products (biscuits, bakery foods, breakfast cereals and bread) and coffee. Currently, the acknowledged reduction recipes include the modification of raw materials and optimization of the heat processing parameters, such as change of precursors in raw materials, optimization of heat processing methods and parameters, modification of cultivar techniques, storage temperature of materials, pH modification, fermentation, etc. (Lineback et al., 2012; Zhang et al., 2009). Meanwhile, several studies provided the evidence that the use of the above reduction recipes could simultaneously maintain original physicochemical and sensory properties of foods, such as color, texture and flavor (Mizukami, Yoshida, Isagawa, Yamazaki, & Ono, 2014; Morales, Jimenez, Garcia, Mendoza, & Beristain, 2014; Palazoğlu, Coşkun, Tuta, Mogol, & Gökmen, 2015). Besides, other studies revealed the mechanism of interaction among acrylamide formation, color development and antioxidant capacity during the Maillard reaction (Kotsiou, Tasioula-Margari, Fiore, Gökmen, & Fogliano, 2013; Serpen & Gökmen, 2009).

With the development of chemoprevention as a reduction method, there is much interest in using antioxidants to control acrylamide. As a representative functional group of antioxidants, hydroxyl groups have important antioxidant benefits. Recently, some studies reported that hydroxylic antioxidants, particularly phenolic-rich pure compounds and extracts, could reduce the formation of acrylamide (Bassama, Brat, Bohuon, Boulanger, & Günata, 2010; Kalita, Holm, & Jayanty, 2013; Kotsiou, Tasioula-Margari, Capuano, & Fogliano, 2011). Meanwhile, other studies focused on the effect of hydroxylic antioxidants on the conversion of asparagine into acrylamide during heating on a chemokinetic basis (De Vleeschouwer, Van der Plancken, Van Loey, & Hendrickx, 2009; Hamzalıoğlu, Mogol, Lumaga, Fogliano, & Gökmen, 2013). Unfortunately, it remains unclear how the phenolic and/or alcoholic hydroxyls exert their functions on the reduction of acrylamide in a structure–activity view. Furthermore, corresponding parameters, including the number, positions and spatial steric effects of functional hydroxyls, and the related mode-of-actions remain largely uncertain. The presence of this scientific gap may be ascribed to the complexity of the Maillard reaction, unpredictability of the antioxidant paradox (Halliwell, 2013), lack of appropriate computational approaches, etc. Any breakthrough regarding the chemical structure–activity relationship is desirable to elucidate the chemoprevention of acrylamide formation in the Maillard reaction system.

Flavonoids and their glycosides are a group of polyphenol compounds that contain a benzopyran-based structure with an attached 2- or 3-phenyl group. Flavones, flavonols, isoflavones, flavanols, and their glycosides are four of the most representative groups among flavonoid compounds (Chen, Zheng, Li, & Jiang, 2014). Different numbers and positions of phenolic hydroxyls in flavonoids and alcoholic hydroxyls in their glycosides exert antioxidant capacities to a different extent, which leads to various antioxidant-related physiological functions. Previous study revealed flavonoids effectively reduce the formation of acrylamide in both chemical model systems and real food matrices (Bassama et al., 2010), but mode-of-actions are still unknown. Flavonoids as antioxidants may reduce the formation of acrylamide by pre-

venting lipid oxidation, which limits the accumulation of carbonyls (Kocadağlı et al., 2012). A mechanistic study indicated that naringenin (a citrus flavonoid) probably inhibits the formation of acrylamide by directly reacting with acrylamide precursors, which diverts them from the pathways that lead to acrylamide formation (Cheng et al., 2009). However, this mechanism has not been demonstrated in mode-of-action studies using other flavonoids.

Hence, a new structure–activity method to evaluate the reduction effect of flavonoids on the formation of acrylamide is an unmet challenge and urgently required. Quantitative structure–activity relationship (QSAR) modeling is one of the major computational tools for estimating the functionality of active components on a structural basis, which have widely been employed in high-throughput screening and medicinal chemistry (Cherkasov et al., 2014). However, the use of QSAR approach in the reduction of acrylamide and related food chemistry researches has not been found yet. Here, we report a multiplex dose-dependent QSAR system to determine the role of flavonoids in the reduction of acrylamide formation in the Maillard reaction and provide a pioneer view of the mode-of-action investigations for the chemoprevention of acrylamide contaminants.

2. Materials and methods

2.1. Materials and chemicals

The potato powder (Atlantis variety) was obtained from Sanjiang (Group) Potato Products Co., Ltd. (Lintao, China). Homoorientin (luteolin-6-C-glucoside), orientin (luteolin-8-C-glucoside), vitexin (apigenin-6-C-glucoside), isovitexin (apigenin-8-C-glucoside), luteolin, apigenin, luteolin-7-O-glucoside, luteolin-4'-O-glucoside, apigenin-7-O-glucoside, kaempferol, kaempferol-3-O-glucoside, myricetin, daidzein, daidzin, genistein and genistin were purchased from Extrasynthese (Lyon, France). Quercetin, quercetin-3-O-glucoside, rutin, acrylamide, L-asparagine monohydrate, D-(+)-glucose monohydrate and D-(–)-fructose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tricin (purity $\geq 95\%$) was self-prepared by preparative HPLC according to our previous work (Jiao, Zhang, Liu, Wu, & Zhang, 2007). Epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) were given as presents by the Tea Research Institute of Chinese Academy of Agricultural Sciences (Hangzhou, China). D₃-Acrylamide (isotope purity $\geq 99\%$) was provided by Cambridge Isotope Laboratories (Andover, MA, USA). Methanol and formic acid were of HPLC grade while other chemicals used in this study were of analytical grade.

2.2. Characterization of the potato-based equimolar asparagine–glucose model system

A batch of potato powder (Atlantic variety) was used for the asparagine–glucose model reaction. Since 90% of all sugars in potatoes are glucose, fructose and sucrose, the sugar contents were determined for these three components. The concentration of sugars was analyzed using HPLC, which was performed on a Waters 2695 HPLC chromatograph with a Capcell Pak C₁₈ A.Q column (5 μ m, 150 mm $\times$ 2.0 mm I.D.) and protected by a RP₁₈ guard column (5 μ m, 4.0 mm $\times$ 3.0 mm I.D.), both of which were purchased from Phenomenex (Torrance, CA, USA). The mobile phase was composed of acetonitrile and water (75/25) while the flow rate was 1.0 mL/min. The injection volume was 30 μ L whereas the column temperature was maintained at 25 °C. Asparagine was monitored at 254 nm with the diode array detector, whereas glucose, fructose and sucrose were monitored using a differential refractive index detector. Then, the contents of asparagine and three sugars in the

potato powder were quantified using the external standard method. The potato powder in the present work contains 370 mg/kg asparagine, 153 mg/kg glucose, 136 mg/kg fructose, and 608 mg/kg sucrose. Based on these contents, the potato matrix was spiked with the standard solutions of both asparagine and glucose to prepare an equimolar asparagine–glucose model system.

2.3. Preparation of current Maillard reaction system in potato matrices

The stock solutions of asparagine (0.2 mol/L) and glucose (0.5 mol/L) were prepared in a phosphate buffer (0.1 mol/L, pH 6.80) according to our previous study (Cheng, Chen, Lu, Chen, & Zhang, 2014). The working concentrations of both precursor substances were set to a final equimolar level (0.14 mol/L). A portion of potato powder (10 g) can be configured into a 200 mL of Maillard reaction solution.

2.4. Mimicking the Maillard reaction via microwave digestion

The generation and reduction of acrylamide using flavonoids were performed in microwave reaction systems using the Ethos D microwave digestion labstation (Milestone Inc., Shelton, CT, USA) according to our previous study (Cheng et al., 2014). The potato model system consisted of a 20% potato matrix, where the asparagine and glucose concentrations were selectively adapted to a final equimolar level of 0.14 mol/L for both components. Meanwhile, each flavonoid was added into the model system, where the concentrations of all 24 flavonoids were adapted to a final sequence of 0 (control), 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} mol/L. The remaining 80% consisted of a 0.1 mol/L phosphate buffer with a pH value of 6.8. Each concentration was performed in triplicates ($n = 3$).

These reaction solutions (10 mL) were added into hermetically closed microwave digestion vessels and microwave-heated for 5 min at 180 °C under a working power of 500 W after a prepared temperature programming of the microwave digestion labstation as follows: room temperature $\rightarrow$ 120 °C (200 W, 5 min); 120 °C $\rightarrow$ 180 °C (500 W, 5 min). At the end of heating, the microwave digestion vessels filled with the final reaction products were retrieved from the labstation and immediately cooled in prepared ice water to stop any further reaction. The entire cooling procedure was performed in a special room with stable air temperature (20 °C) adjusted by air-conditioning.

2.5. Pre-treatment of Maillard reaction products and UHPLC–MS/MS analysis of acrylamide

All final reaction products were centrifuged at 15,000 rpm for 15 min with a Microfuge 18 centrifuge (Beckman Coulter Inc., Indianapolis, IN, USA). The aliquot of the supernatant (0.2 mL) was sampled and diluted to 10 mL using phosphate buffer. Then, the sample solution was pre-treated by adding D_3 -labeled acrylamide, performing liquid–liquid extraction with ethyl acetate and cleaning up with Oasis HLB SPE cartridges (Waters Corp., Milford, MA, USA). Finally, the acrylamide content in pre-treated samples in all investigated groups was quantified using UHPLC–MS/MS based on our previous work (Zhang, Jiao, Cai, Zhang, & Ren, 2007). The reduction of acrylamide using different addition levels of different flavonoids was finally estimated via the inhibitory rate, which was calculated as the percentage ratio of decreasing level of acrylamide via the effect of flavonoid to the acrylamide level in the control group.

2.6. Multiplex QSAR modeling and statistical analysis

Genetic algorithms and multiple linear regression (MLR) analyses were used to select the descriptors and generate the correlation

models that relate the structural features of flavonoids to the reduction effects. At first, the conformational search script in the HyperChem 7.52 software package was used to minimize the energy of initial structures of all selected flavonoids and explore their conformational space using the AM1 semiempirical method (Silva-Junior & Thiel, 2010). Therefore, the optimal structures that were derived from the conformational searches were ordered according to the energy. Then, 1664 molecular descriptors were calculated using the Dragon 5.4 software for a set of 24 optimal flavonoid structures with the reduction effect of acrylamide. Based on the descriptor computation, the genetic algorithm was applied to select the best combinations of calculated descriptors and the most relevant combination to obtain the multiplex QSAR models with the highest predictive power of the reduction effects. The data for the inhibitory rates of acrylamide using each flavonoid in each addition level were expressed as the mean $\pm$ SD ($n = 3$). The genetic algorithm (GA) technique began with a population of 50 random models, 150 iterations to evolution, crossover rate of 50%, and mutation rate of 1%. In total, 33 descriptors were optimized using the GA Toolbox in the Matlab 7.1 software. Subsequently, the stepwise regression program of MLR in the SAS 8.2 software was used to derive the final multiplex QSAR models. Using the stepwise regression method, the 4 most relevant descriptors among the 33 descriptors were selected to build 8 multiplex QSAR models for each addition level of flavonoids. The final set of multiplex QSAR models was validated by applying the leave-one-out technique with its predictive ability, which was estimated and confirmed using the cross validation coefficient (Q^2).

3. Results and discussion

3.1. Overall dose–response effect

To investigate and compare the effect of 24 selected flavonoids (see the names and chemical structures in Fig. S2) on the reduction of acrylamide, we investigated the dose–response effect in a potato-based model system with the addition of asparagine, glucose and a selected flavonoid using microwave heating. The results of the reduction study show a bell-shaped dose-dependent relationship in all 24 asparagine–glucose–flavonoid model systems and demonstrate the reduction effect of acrylamide by flavonoids in the range of 10^{-11} – 10^{-4} mol/L (Figs. S3–S6). Furthermore, we finalized the maximal reduction effect on acrylamide formation when the addition level of all selected flavonoids was 10^{-9} mol/L.

We employed an equimolar asparagine–glucose potato model system for mimicking the Maillard reaction and generating acrylamide via microwave heating conditions. First, potatoes are in the food category with probably the highest concentrations of acrylamide generated after thermal processing so far because potato tubers contain substantial amounts of acrylamide precursors, including free asparagine, glucose, and fructose (Mottram et al., 2002; Stadler et al., 2002), and in which most studies have been performed to control acrylamide (Taeymans et al., 2004; Zhang et al., 2009). Second, an equimolar amount of asparagine and glucose generates more significant concentrations of acrylamide than other molar ratios of two substrates (Stadler et al., 2002; Zhang, Fang, & Zhang, 2008). When developing current potato-based equimolar asparagine–glucose system, we initially considered original contents of both substrates in current potatoes and then compensated their concentrations to an equimolar level using their stock solutions. Third, microwave heating has been recognized as an effective way to generating acrylamide in thermally processing food compared with other conventional heating methods (Yuan et al., 2007). In the present work, considerable levels of acrylamide were produced in Maillard reaction systems via

microwave heating, the characteristics of which refer to high heating temperature and short heating time and promote the formation kinetics of acrylamide. Taken together, current equimolar asparagine–glucose Maillard reaction system based on a potato matrix is close to actual food system for generating acrylamide. Besides, current system simulates a binary asparagine–glucose reaction in an equimolar level, which fulfils the requirements for structure–activity relationship investigation.

Current study revealed flavonoids exert their reduction effects on the formation of acrylamide in a nonlinear and bell-shaped dose–effect way. Such bell-shaped dose–effect relationship may be ascribed to the following two factors. First, the increase of acrylamide content significantly correlates with an enhancement of the antioxidant activity of food matrices or Maillard reaction model systems (Haase, Grothe, Matthaeus, Vosmann, & Lindhauer, 2012; Serpen & Gökmen, 2009). Second, the acrylamide content may reduce because of the reaction that is explained by the quinine–amine interaction between the phenolic-rich antioxidants and the direct precursor of acrylamide, e.g., the oxidation of flavonoids to quinones, which subsequently react with a key intermediate, such as 3-aminopropionamide (3-APA) (Granvogl & Schieberle, 2006). The inhibitory rate of acrylamide via the addition of flavonoids may be judged by considering both factors and alternatively estimating which factor plays a predominant role in current Maillard reaction system.

3.2. Reduction of acrylamide using isoflavones

Considering the nonlinear dose–response relationship and reduction effect of isoflavones (addition level: 10^{-9} mol/L), genistein exerted the maximal reduction efficiency (45.6% inhibitory rate) on the acrylamide formation, whereas two isoflavone glycosides (daidzin and genistin) exhibited relatively weak inhibitory rates (20.4% and 19.6%, respectively) (Table 1). Considering the effect of isoflavones with all addition levels, the reduction efficiency of genistein enhanced significantly due to the introduction of 7-phenolic hydroxyl compared with the effect of genistin bearing an alcoholic hydroxyl-rich 7-O-glucoside (Fig. S3). Similar results were

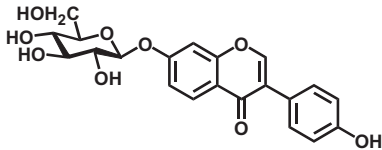
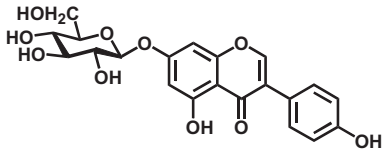
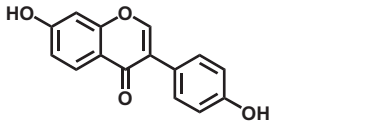
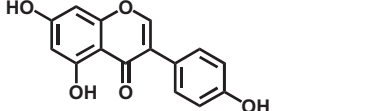
observed when the effects of daidzein and daidzin were compared. The reduction mechanism of isoflavones may be related to its conjugation to free amino groups of asparagine and trapping of dicarbonyl intermediates (Silvan, Srey, Ames, & Del Castillo, 2014).

3.3. Reduction of acrylamide by flavones

Following the effect of flavones with the optimal addition level, the inhibitory rates of flavones ranged from 25.3% to 63.5%, where orientin and apigenin-7-O-glucoside exerted the maximal and minimal reduction efficiencies, respectively (Table 2). Considering the effect of flavones with all addition levels, the reduction effects of flavone 7-O-glucoside or 4'-O-glucoside significantly decreased due to the O-glucoside substitution of 7- or 4'-phenolic hydroxyl compared with the effect of their flavone aglycones (Fig. S4). The inhibitory effect of flavone C-glucosides is obviously better compared with the effect of flavone O-glucosides, which indicates the important role of phenolic hydroxyls instead of alcoholic hydroxyls in the chemical structure of flavonoids to reduce the formation of acrylamide. Besides, these findings allowed us to investigate the reduction effect of flavone glycosides on a structural basis. Flavone C-glycosides are more active in reducing the formation of acrylamide than flavone O-glycosides though they hold the same chemical structure of aglycone (e.g. homoorientin > luteolin-7-O-glucoside). This result may be ascribed to different numbers of phenolic hydroxyls in the structures of flavone glycosides. The insertion of C-glucoside keeps the original number of phenolic hydroxyls and increases the number of alcoholic hydroxyls; however, the replacement of O-glucoside removes one phenolic hydroxyl. In addition, previous study observed a positive correlation between the carbonyl value of selected compounds and acrylamide formation in a model system (Ou et al., 2010). Thus, the role of bioactive carbonyl compounds, such as flavones, with antioxidant properties on the conversion of asparagine into acrylamide has recently attracted wide attention. Flavones with a carbonyl group may compete with the carbonyl group of reducing sugars in Maillard reactions, which affect the formation and kinetics of acrylamide (Hamzalıoğlu & Gökmen, 2012).

Table 1

Relationship between the inhibitory rates of acrylamide by isoflavones with optimized addition levels and phenolic/alcoholic hydroxyls of isoflavone structures in asparagine–glucose Maillard reaction systems ($n = 3$).

Name of isoflavone	Structure of isoflavone	No. of phenolic hydroxyls	No. of alcoholic hydroxyls	Inhibitory rate (%) ^a
Daidzin		1	4	20.4 ± 1.4 ^A
Genistin		2	4	19.6 ± 1.4 ^A
Daidzein		2	0	31.8 ± 3.2 ^B
Genistein		3	0	45.6 ± 2.1 ^C

^a Letters A–C show the statistical results of the Duncan multiple comparison test. Different letters shown in different bars present a significant difference among each other ($p < 0.05$).

Table 2
Relationship between the inhibitory rates of acrylamide by flavones with optimized addition levels and phenolic/alcoholic hydroxyls of flavone structures in asparagine–glucose Maillard reaction systems ($n = 3$).

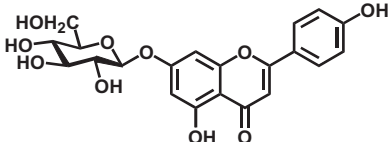
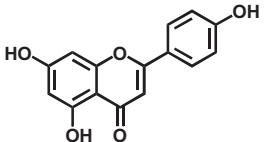
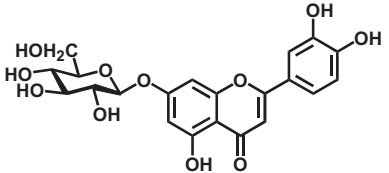
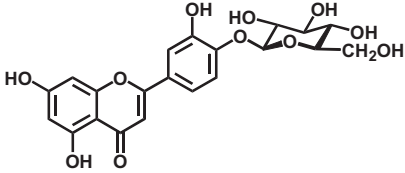
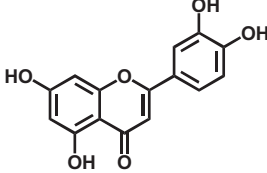
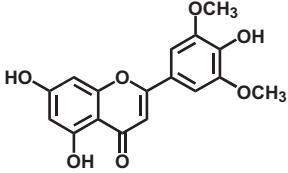
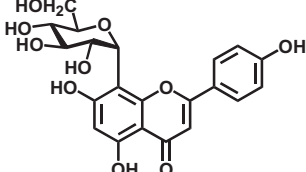
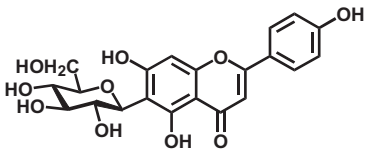
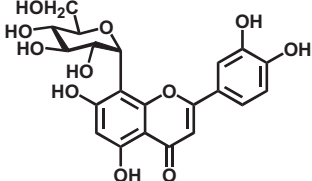
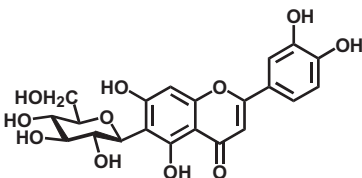
Name of flavone ^a	Structure of flavone	No. of phenolic hydroxyls	No. of alcoholic hydroxyls	Inhibitory rate (%) ^b
Apig-7-OG		2	4	25.3 ± 5.9 ^A
Apigenin		3	0	49.0 ± 1.6 ^B
Lute-7-OG		3	4	37.3 ± 2.0 ^C
Lute-4'-OG		3	4	35.7 ± 6.3 ^C
Luteolin		4	0	52.1 ± 1.5 ^B
Tricin		3	0	38.9 ± 1.8 ^C
Vitexin		3	4	53.0 ± 2.8 ^B
Isovitexin		3	4	54.6 ± 5.5 ^B
Orientin		4	4	63.5 ± 1.5 ^D

Table 2 (continued)

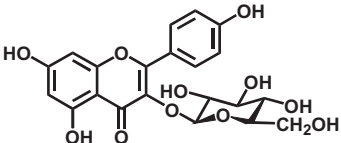
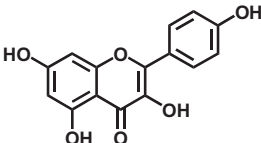
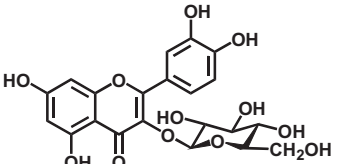
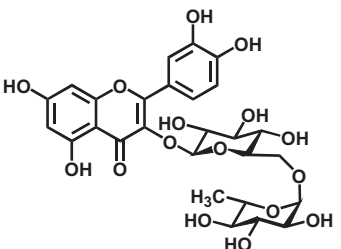
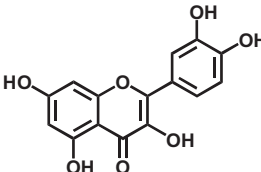
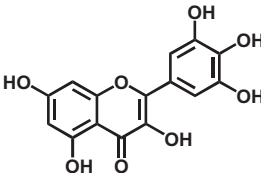
Name of flavone ^a	Structure of flavone	No. of phenolic hydroxyls	No. of alcoholic hydroxyls	Inhibitory rate (%) ^b
Homoorientin		4	4	61.8 ± 4.4 ^D

^a Abbreviations: apig-7-OG, apigenin-7-O-glucoside; lute-7-OG, luteolin-7-O-glucoside; lute-4'-OG, luteolin-4'-O-glucoside.

^b Letters A–D show the statistical results of the Duncan multiple comparison test. Different letters shown in different bars present a significant difference among each other ($p < 0.05$).

Table 3

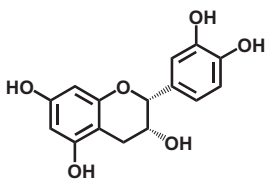
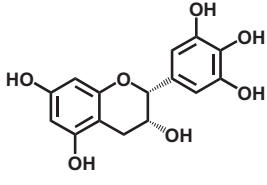
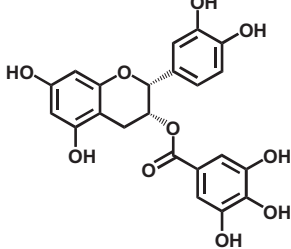
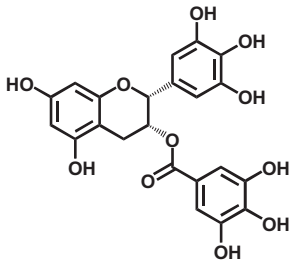
Relationship between the inhibitory rates of acrylamide by flavonols with optimized addition levels and phenolic/alcoholic hydroxyls of flavonol structures in asparagine–glucose Maillard reaction systems ($n = 3$).

Name of flavonol ^a	Structure of flavonol	No. of phenolic hydroxyls	No. of alcoholic hydroxyls	Inhibitory rate (%) ^b
Kaemp-3-OG		3	4	48.9 ± 2.9 ^A
Kaempferol		3	1	51.9 ± 3.9 ^A
Quer-3-OG		4	4	54.8 ± 1.2 ^{AB}
Lutin		4	6	60.8 ± 4.3 ^{AB}
Quercetin		4	1	52.1 ± 1.5 ^B
Myricetin		5	1	69.3 ± 3.6 ^C

^a Abbreviations: kaemp-3-OG, kaempferol-3-O-glucoside; quer-3-OG, quercetin-3-O-glucoside.

^b Letters A–C show the statistical results of the Duncan multiple comparison test. Different letters shown in different bars present a significant difference among each other ($p < 0.05$).

Table 4
Relationship between the inhibitory rates of acrylamide by flavanols and their derivatives with optimized addition levels and phenolic hydroxyls of flavanols and their derivatives in asparagine–glucose Maillard reaction systems ($n = 3$).

Name of flavanol and derivative ^a	Structure of flavanol and derivative	No. of phenolic hydroxyls	No. of alcoholic hydroxyls	Inhibitory rate (%) ^b
EC		4	1	50.1 ± 4.1 ^A
EGC		5	1	58.3 ± 3.4 ^B
ECG		7	0	71.6 ± 1.4 ^C
EGCG		8	0	91.9 ± 1.1 ^D

^a Abbreviations: EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate.

^b Letters A–D show the statistical results of the Duncan multiple comparison test. Different letters shown in different bars present a significant difference among each other ($p < 0.05$).

3.4. Reduction of acrylamide using flavonols

Following the effect of flavonols with the addition level of 10^{-9} mol/L, the reduction effect of flavonols ranged from 48.9% to 69.3%, where myricetin and kaempferol-3-O-glucoside exerted the maximal and minimal reduction efficiencies, respectively (Table 3). Similarly, the number and position of phenolic hydroxyls largely depend on the reduction efficiency by considering the effect of flavonols with all addition levels (Fig. S5). Similar to flavones, flavonols, such as the well-known quercetin, can inhibit the formation of reactive carbonyl species (Zhang, Chen, & Wang, 2014), which are important intermediates for the formation of acrylamide. Moreover, the reduction effect of flavonols with their optimized addition level on the formation of acrylamide seems better than the effect of isoflavones. These findings may be ascribed to the presence of 3-hydroxyl, which is absent in the structure of isoflavones. Besides, these results indicated that the position of B-cycle connecting with the C cycle contributes to the reduction effect of flavonoids.

3.5. Reduction of acrylamide using flavanols and their derivatives

Following the optimal addition level of flavanols and derivatives, the reduction effect ranged from 50.1% to 91.9%, where epigallocatechin gallate (EGCG) and epicatechin (EC) exerted

maximal and minimal reduction efficiencies, respectively (Table 4). Flavanols and derivatives reduce the formation of acrylamide presumably by trapping carbohydrates and/or preventing lipid oxidation (Cheng et al., 2014). Considering the effect of flavanols and derivatives with all additional levels, the inhibitory rates of acrylamide by EGCG improved by 23.4–39.4% compared with the effect of epigallocatechin (EGC), whereas the inhibitory rates of acrylamide by epicatechin gallate (ECG) improved by 21.0–29.8% compared with the effect of EC (Fig. S6). This improvement may be ascribed to the substitution of 3-hydroxyl with a 3-gallate group in the C-ring of the flavanol structure, which introduces an increase of 3 phenolic hydroxyls. These results verified that the number of phenolic hydroxyls as a primary factor contributes to the reduction effect of flavonoids.

3.6. Multiplex quantitative structure–activity relationship (QSAR)

The experimental data allowed us to more precisely evaluate the parameters for the reduction effect of flavonoids on a structural basis. To further investigate the effect of structural discrepancy of flavonoids on the reduction of acrylamide, three aspects of structural discrepancy were particularly attractive: (i) the difference in number and position of phenolic and alcoholic hydroxyls, (ii) the difference between the presence of flavone glycosides and aglycones, and (iii) the difference between flavone C-glycosides

Table 5
Multiplex QSAR models and cross-validation results of acrylamide reduction by flavonoids.

Addition level of flavonoids (mol/L)	Multiplex QSAR equation ^a	R ²	Q ²	s	F	RMS _{LOO} ²
10 ⁻⁴	IR = 0.175 GGI5 + 0.083 IDDE – 0.260 IC1 + 0.063 O-057 + 0.344	0.876	0.912	0.039	74.84	0.042
10 ⁻⁵	IR = 0.207 GGI5 + 0.068 IDDE – 0.326 IC1 + 0.067 nArOH + 0.627	0.900	0.917	0.039	83.36	0.042
10 ⁻⁶	IR = 0.169 MAXDN – 0.425 EEig07x – 2.939 MSD + 0.095 nArOH + 1.528	0.913	0.888	0.049	63.87	0.054
10 ⁻⁷	IR = 0.105 MAXDN – 0.319 EEig07x – 2.798 MSD + 0.106 nArOH + 1.354	0.935	0.921	0.047	83.33	0.049
10 ⁻⁸	IR = 0.115 MAXDN – 0.339 EEig07x – 2.795 MSD + 0.101 nArOH + 1.441	0.938	0.938	0.039	109.47	0.041
10 ⁻⁹	IR = 0.021 MLOGP2 + 0.145 GGI5 – 0.068 T12 + 0.093 O-057 + 0.166	0.927	0.955	0.031	174.93	0.035
10 ⁻¹⁰	IR = 0.154 MAXDN – 0.384 EEig07x – 3.433 MSD + 0.090 nArOH + 1.569	0.873	0.874	0.052	55.56	0.057
10 ⁻¹¹	IR = 0.338 Jhetp – 3.730 MSD + 0.037 nHDon + 0.043 nArOH + 0.276	0.835	0.905	0.045	74.49	0.049

^a **MAXDN**, maximal electrotopological negative variation; **MSD**, mean square distance index (Balaban); **MLOGP2**, squared Moriguchi octanol–water partition coefficient; **GGI5**, topological charge index of order 5; **T12**, second Mohar index; **Jhetp**, Balaban-type index from polarizability weighted distance matrix; **EEig07x**, eigenvalue 07 from edge adjacency matrix weighted by edge degrees; **IDDE**, mean information content on the distance degree equality; **IC1**, information content index (neighborhood symmetry of 1-order); **O-057**, phenol/enol/carboxyl OH; **nHDon**, number of donor atoms for H-bonds (N and O); **nArOH**, number of phenolic hydroxyls. IR, inhibitory rate; R², squared correlation coefficient; Q², cross validation coefficient; s, standard deviation; F, F value; RMS_{LOO}², predictive error sum of squares.

and O-glycosides. Based on the evaluation of the QSAR method, 33 descriptors regarding the structural parameters of selected flavonoids were optimized from 1664 descriptor using the genetic algorithm method, which is a useful technique to search the large probability space with many descriptors for a small number of molecules (Shahlaei, 2013). The values and meanings of 33 optimized descriptors of all 24 selected flavonoids are shown in Table S1. As a rule of thumb, for reliable results, the data set should be approximately 5 times larger than the number of selected descriptors (Shahlaei, 2013). As a result, 8 MLR equations were established to describe the structural–activity relationship when flavonoids were added within the range of 10⁻¹¹–10⁻⁴ mol/L (Table 5). For a variable selection, a maximal number of four variables could be selected to reduce the risk of chance correlation in the present study. A key parameter, **nArOH**, which represents the number of phenolic hydroxyls, was present in most optimized multiplex QSAR equations when the addition levels of flavonoids were 10⁻¹¹, 10⁻¹⁰, 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ mol/L, whereas **O-057**, which indicates the number of phenol/enol/carboxyl hydroxyls, was present in the equation when the addition levels of flavonoids were 10⁻⁹ and 10⁻⁴ mol/L (Table 5). Considering the structures of selected flavonoids, except flavonols, the value and meaning of **O-057** are equal to **nArOH** because there are no enol/carboxyl hydroxyls in the current selected flavonoids. Furthermore, the topological structures of flavonoids also significantly contribute to the reduction effect. The two topological descriptors, **MAXDN** and **MSD**, which represent the maximal electrotopological negative variation and Balaban mean square distance index, respectively, are the most predominant factors. Other descriptors that contributed to the reduction effect are indicated in Table 5. Overall, the multiplex QSAR study demonstrated that phenolic hydroxyls instead of alcoholic hydroxyls in the chemical structure of flavonoids contribute to the reduction of acrylamide (Fig. S7). Therefore, the reduction of acrylamide could be robustly predicted using the multiplex QSAR models. As a result, the relationship between the number and position of phenolic hydroxyls of flavonoids and inhibitory rates of acrylamide by flavonoids with the optimal addition levels is shown in Table S2. The reduction of acrylamide and its correlation with the phenolic hydroxyls of flavonoids with other addition levels is shown in Table S3. We found a good correlation (R²: 0.835–0.938) between the experimental and predicted inhibitory rates based on all of the multiplex QSAR model equations.

The results in Tables 1–5 show that phenolic hydroxyls instead of alcoholic hydroxyls play an important role in the reduction of acrylamide using flavonoids. A chemometric parameter, **nArOH** or **O-057**, was selected as the variable in all 8 multiplex QSAR equations. For the flavonoids, **nArOH** represents the phenolic hydroxyl number of A and B rings in the original nucleus of

flavonoids (potential position: 5-, 6-, 7-, 8-, 2'-, 3'-, 4'-, 5'- and 6'-OH) and phenolic hydroxyls of 3-gallate in the structure of flavanol derivatives; **O-057** represents the enolic hydroxyl number of C rings (3-hydroxyl) in addition to the hydroxyls that **nArOH** indicates but does not include the alcoholic hydroxyls in the glycoside structures. Based on the results in Figs. S2–S5, the inhibitory rate of acrylamide increases with the increase in the number of phenolic hydroxyls in most of cases. For instance, a maximal reduction effect (91.9%) of EGCG (8 phenolic hydroxyls) was observed when the addition level was 10⁻⁹ mol/L. Furthermore, the effect of phenolic hydroxyls on the reduction of acrylamide was investigated, and some valuable information on a structural basis was obtained: (i) both 3',4'(ortho)-di-hydroxyls in the B cycle and 5,7(meta)-di-hydroxyls in the A cycle of the flavonoid molecule greatly contribute to the reduction of acrylamide; (ii) the reduction effect of flavone C-glycosides is more significant than flavone O-glycosides despite sharing the identical flavone aglycone skeletons (e.g., orientin > luteolin > luteolin-7-O-glucoside). This result may be ascribed to the number alternation of phenolic hydroxyls in flavone C-glycosides and O-glycosides compared to their aglycones. The insertion of C-glycoside maintains the original number of phenolic hydroxyls in its aglycone and increases the number of alcoholic hydroxyls. However, the substitution of O-glycoside reduces the number of phenolic hydroxyls. (iii) The reduction effect of flavones is better than isoflavones despite sharing an identical number of hydroxyls in the flavonoid skeletons.

This structural basis study using chemometrics provides an experimental means to ascertain the most likely reduction mechanism of acrylamide by adding flavonoids. Few studies have elucidated the effect of structures and related functional groups of natural product chemicals on the decrease of acrylamide formation via the structure–activity relationship investigation. A positive correlation between carbonyl values of phenolic chemicals and the formation of acrylamide was found in a model system (Ou et al., 2010). Thus, the contribution of bioactive carbonyl compounds and their antioxidant properties to the conversion of asparagine into acrylamide has been concerned. Phenolic chemicals may compete with the carbonyl groups of reducing sugars in Maillard reactions and then affect the generation of acrylamide (Hamzalıoğlu & Gökmen, 2012). Unfortunately, it is unclear how the hydroxyl groups of the chemicals contribute to the reduction of acrylamide formation in the Maillard reaction to date. Plant-derived polyphenols such as flavonoids are typical antioxidants bearing aromatic phenolic structures that are widely present in natural herbs (Denev, Kratchanov, Ciz, Lojek, & Kratchanova, 2012; Perron & Brumaghim, 2009). It is interest to investigate the role of aromatic rings with hydroxyl groups of flavonoid antioxidants in the reduction of acrylamide formation. Current work provides pioneering evidence that both number and position of phenolic hydroxyls

contribute to the reduction of acrylamide generation. Moreover, the application of QSAR tools provides an alternative way to the chemical elucidation of acrylamide formation and reduction by antioxidant chemicals.

4. Conclusions

The present study demonstrates how flavonoids are likely to reduce acrylamide formation and shows the contribution of phenolic hydroxyls instead of alcoholic hydroxyls on this reduction (Fig. S7). The investigation of the dose-dependent reduction of acrylamide by flavonoids shows a close correlation between the number of phenolic hydroxyls instead of alcoholic hydroxyls in different types of flavonoids and the reduction effect. Using multiplex QSAR approaches, we quantitatively demonstrated the role of phenolic hydroxyls in the reduction of acrylamide using flavonoids. 3',4'(ortho)-dihydroxyls in the B cycle, 3-hydroxyl or 3-gallate in the C cycle, and 5,7(meta)-dihydroxyls in the A cycle of the flavonoid molecular significantly contribute to the reduction of acrylamide. The reduction effect of flavone C-glycosides is more significant than flavone O-glycosides despite sharing identical flavone aglycone skeletons. The reduction effect of flavones is more significant than isoflavones despite sharing an identical number of miscellaneous hydroxyls in the flavonoid skeletons. The current multiplex QSAR equations effectively predict the inhibitory rates of acrylamide via the addition of flavonoids. This study can be considered to be a pioneer study on the reduction mechanism of acrylamide and introduces a broader thought on controlling the acrylamide contaminant using chemical agents on a structural basis.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.12.038>.

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