Inhibitory Mechanism of Naringenin against Carcinogenic Acrylamide Formation and Nonenzymatic Browning in Maillard Model Reactions

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Chemical model reactions were carried out to investigate the effect of a citrus flavonoid, naringenin, on the formation of acrylamide under mild heating conditions. Results showed that naringenin significantly and dose dependently inhibited the formation of acrylamide (20−50% relative to the control), although not in a linear manner. Moreover, the presence of naringenin in acrylamide-producing models effectively reduced the extent of browning. Careful comparison of the HPLC chromatograms of samples from the chemical model reactions revealed that naringenin likely reacted with Maillard intermediates, giving rise to new derivatives. Subsequent LC-MS analyses suggested that the proposed derivatives have a predicted molecular mass of 341 Da. Eventually, two derivatives were purified and characterized with LC-MS/MS and NMR spectroscopy as 8-*C*-(*E*-propenamide)naringenin and 6-*C*-(*E*-propenamide)naringenin, respectively. In other words, naringenin, a rather weak antioxidant, strongly inhibited acrylamide formation probably by directly reacting with acrylamide precursors, thus diverting them from the pathways that lead to acrylamide formation.

Introduction

Acrylamide (AA) has been classified by the IARC as a probable human carcinogen (*1*). Reports on the presence of AA in widely consumed dietary components, especially starch-rich food products such as fried potatoes and crispy bread (*2, 3*), have raised global concerns because these data imply that humans could be exposed to significant quantities of AA in the long term. Extensive research efforts have been carried out aiming to develop strategies that could effectively inhibit the formation of AA during thermal food processing. Reaction time and temperature were found to play important roles in the formation of AA. At high temperatures (e.g., 160 °C), AA could be formed in much shorter times than under mild thermal conditions such as 120 °C (*4*). Therefore, an appropriate combination of cooking temperature and time may help to effectively lower the AA content in foods. On the other hand, some studies showed that manipulating the contents of certain food constituents, such as by the addition of lysine or glycine (*5*) or pretreatment of potato with asparaginase to reduce asparagine content (*6*), could lead to reduced AA levels in food products. The addition of mono (e.g., Na^+) and divalent (e.g., Ca^{2+}) cations in equal molar quantities as the precursors was also reported to strongly reduce AA contents in chemical models (*7*).

It is proposed that AA formation is closely associated with the Maillard reaction (*6, 8, 9*). Among the different pathways, thermal degradation of asparagine in the presence of reducing sugars has been suggested to be the major route (*6, 8*). In addition to reducing sugars, a variety of carbonyl compounds, especially α-hydroxy carbonyls, could also enhance the formation of AA from asparagine (*6, 10*). More in-depth mechanistic studies have recently suggested 3-aminopropionamide (3-APA) as a transient intermediate on the pathway to AA formation (*10, 11*). The heating of 3-APA under aqueous or low water conditions at temperatures between 100 and 180 °C in model systems generated more AA than in the same reaction of asparagine, thereby pointing to 3-APA as a very effective precursor of AA (*12*). It was suggested that the addition of certain chemical agents such as pyridoxamine, which have strong dicarbonyl-trapping capabilities, could lead to effective reduction of AA formation by out-competing 3-APA for reactive dicarbonyl species (e.g., methyl glyoxal) (*4*). These studies have provided valuable information for the design of more targeted strategies to effectively reduce or prevent AA formation by interrupting the critical steps or scavenging key intermediates on the pathways to the formation of AA.

Our group has been extensively working with the development of effective strategies to attenuate health risk associated with food-borne genotoxic substances including heterocyclic amines (HAs) and AA. Recently, naringenin, a flavonoid that widely occurs in citrus and tomato, was recognized by our group as a potent inhibitor of the formation of genotoxic HAs (*13*). The effect was especially prominent with respect to the formation of PhIP, the most abundant HA found in food products. In addition, further mechanistic investigation suggested that naringenin likely suppresses PhIP formation via direct scavenging of the reactive carbonyl intermediate, phenylacetaldehyde (*14*). As carbonyl scavenging has been proposed to reduce AA formation, the present study aimed to investigate whether naringenin could also effectively suppress the formation of AA. Considering the fact that the majority of previous studies has focused on investigating high-temperature (≥160 °C) models

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Table 1. Aqueous Chemical Model Reactions[a]

	reactant concentration (mM)								
reactant	A	B	C	D	E	F	G	H	I
asparagine	25			25	25	25	25	25	
glucose		25		25	25	25	25		25
naringenin			10		5	10	25	10	10

[a] Model reactions A−I were carried out in phosphate buffer (0.1 M, pH 7.0) and heated at 128 °C for 1 h.

(chemical and food models) (*15, 16*) and that mild thermal conditions like those between 120 and 150 °C were capable of generating significant quantities of AA (*17*), the present study was conducted in chemical models subjected to mild heat treatment. Finally, the capability of naringenin to inhibit browning reactions in AA-producing models was examined because Maillard browning has been proposed to correlate with AA formation (*4*) and prevention of nonenzymatic browning could also be of significance to the food industry.

Experimental Procedures

Solvents and Reagents. Naringenin, asparagine, and glucose were purchased from Sigma-Aldrich Co. (St. Louis, MO). Disodium hydrogen phosphate and all solvents used were obtained from BDH Chemicals Ltd. (Poole, United Kingdom). The Reacti-Therm III heating module (model 18840) and the screw cap Tuf-Bond Teflon-fitted glass reaction vials were purchased from Pierce (Rockford, IL).

Model Maillard Reactions. Effects of naringenin on AA formation and Maillard browning were investigated in chemical model systems. Compositions of the models are listed in Table 1. The reaction mixtures were dissolved in phosphate buffer (0.1 M, pH 7.0, 10 mL) in screw cap Tuf-Bond Teflon-fitted glass reaction vials (40 mL capacity) and heated in a Reacti-Therm III heating module at 128 °C for 60−180 min.

Sample Preparation. HPLC−diode array detection (DAD) was used for qualitative and quantitative analysis of AA. The reaction mixtures were immediately cooled in an ice−water mixture at designated time points. One milliliter of each chilled reaction mixture was transferred into an eppendorf and centrifuged at 14000 rpm for 10 min. The clear supernatant was subjected to HPLC-DAD analysis for the determination of AA. For analyzing the profiles of reaction products formed in different chemical models and changes in the contents of analytes proposed to arise from the reactions between naringenin and asparagine-derived reactive fragments, 500 μL of supernatant was diluted with 500 μL of methanol to prevent precipitation of the target analytes in HPLC vials.

For LC-MS characterization of the structures of analytes of interest, 8 mL of the chilled reaction mixture from each chemical model was extracted with 10 mL of ethyl acetate. The clear supernatant was transferred to a pear-shaped 50 mL flask and dried on a rotary evaporator under vacuum. The residue was redissolved in 1 mL of methanol for subsequent LC-MS analysis. For analysis of the antibrowning activity of naringenin in asparagine−glucose models, the reaction mixtures (10 mL) were transferred to Falcon centrifuge tubes and centrifuged (8000*g*) for 10 min. The clear supernatant was diluted (2×) with Milli-Q water before measuring the L^*, a^*, and b^* values with a Minolta Chorma meter (CR-400, Japan), where L^* value indicates lightness, a^* indicates redness, and b^* indicates greenness. An increase in L^*, a^*, and b^* values indicates an increase in lightness, redness, and greenness, respectively. The Chroma meter was calibrated right before each analysis. Triplicate experiments were performed.

Liquid Chromatography−DAD. All filtrates were analyzed on a Shimadzu HPLC system with a separation module (LC-20AT), an autosampler (SIL-20A), a degasser (DGU-20A3), and a photodiode array detector (SPD-M20A). For the analysis of AA, a prepacked Sunfire C18 column (250 mm × 4.6 mm, 5 μm, Waters Corp., Ireland) was used. The mobile phase was composed of water (solvent A) and acetonitrile (B) of the following gradients: 0−10 min, 100% A:0% B; 12 min, 20% A:80% B; 25 min, 20% A:80% B; 30 min, 100% A:0% B; and 46 min, 100% A:0% B. The injection volume was 25 μL, and AA was monitored at 205 nm. For comparison of the profiles of Maillard reaction products in different model reactions, separation was on an YMC-Pack Pro C-18 column (5 μm, 150 mm × 4.6 mm), and the mobile phase was 0.1% formic acid in water (A) and acetonitrile (B) of the following gradients: 0 min, 95% A:5% B; 45 min, 20% A:80% B; 48 min, 95% A:5% B; and 58 min, 95% A:5% B.

LC-MS. LC conditions were the same as those applied in the LC-DAD analysis of profile changes in Maillard reaction products described above. Effluent from the UV detector was split (1:4) with one part (200 μL/min) directed to the MS (QTrap 2000, Applied Biosystems) for spectrometric analysis and the remaining to waste. The MS conditions were as follows: negative ion mode; source temperature, 300 °C; curtain gas, 20 psi; spray voltage, −4500 V; collision gas, medium; declustering potential, −70 V; and scan range, 100−1000 Da. MS/MS analysis of the postulated naringenin adducts was set at m/z 340, and the collision energy was set at −40 V.

Isolation, Purification, and Structural Elucidation of Naringenin Adducts. To facilitate the subsequent isolation process, liquid−liquid extraction with ethyl acetate (3×) was used to obtain a concentrated sample. The extraction process was monitored using HPLC-PDA. The clear supernatant was pooled and concentrated on a rotary evaporator under vacuum. The extract thus obtained was redissolved in methanol and then loaded onto a Sephadex LH-20 column (40 cm × 4 cm i.d.). Elution was performed with 70% methanol (methanol−H_2O, 70:30, v/v), and eluate was collected using an automatic fraction collector. The profile of the fractions was checked by HPLC-DAD on a Shimadzu HPLC system. Similar fractions were combined. This open-column chromatographic process eventually led to two fractions with an enhanced content of the target adducts and a much lower level of interfering compounds. The fractions were finally separated by semipreparative HPLC on a Waters HPLC system to obtain two adducts in high purity (>95% by HPLC-PDA). Structures of the adducts were determined by one-dimensional (1D) and two-dimensional (2D) NMR spectroscopy on a 600 MHz spectrometer (Bruker, AVANCE 600).

Spectral Data of Compound 1. ^{1}H NMR (600 MHz, CD_3OD, 25 °C, TMS): δ = 7.75 (d, J = 16.0 Hz, 1H; H-2″), 7.27 (d, J = 8.5 Hz, 2H; H-2′ and H-6′), 6.84 (d, J = 16.0 Hz, 1H; H-1″), 6.74 (d, J = 8.5 Hz, 2H; H-3′ and H-5′), 5.91 (s, 1H; H-6), 5.40 (dd, J = 3.0 Hz and J = 12.6 Hz, 1H; H-2), 3.10 (dd, J = 17.1 Hz and J = 12.6 Hz, 1H; H-3a), 2.71 ppm (dd, J = 17.1 Hz and J = 3.0 Hz, 1H; H-3b). ^{13}C NMR (150 MHz, CD_3OD, 25 °C, TMS): δ = 196.6 (C-4), 167.9 (C-7), 166.2 (C-3″), 163.3 (C-5), 161.8 (C-9), 157.4 (C-4′), 130.0 (C-1′), 128.5 (C-1″), 127.6 (C-2′ and C-6′), 122.2 (C-2″), 115.2 (C-3′ and C-5′), 103.4 (C-8), 101.8 (C-10), 95.7 (C-6), 78.9 (C-2), 41.6 ppm (C-3).

Spectral Data of Compound 2. ^{1}H NMR (600 MHz, CD_3OD, 25 °C, TMS): δ = 7.83 (d, J = 16.0 Hz, 1H; H-2″), 7.22 (d, J = 8.5 Hz, 2H; H-2′ and H-6′), 6.93 (d, J = 16.0 Hz, 1H; H-1″), 6.72 (d, J = 8.5 Hz, 2H; H-3′ and H-5′), 5.87 (s, 1H; H-8), 5.29 (dd, J = 3.0 Hz and J = 12.7 Hz, 1H; H-2), 3.10 (m, 1H; H-3a), 2.64 ppm (dd, J = 17.1 Hz and J = 3.0 Hz, 1H; H-3b). ^{13}C NMR (150 MHz, (CD_3OD-DMSO-d_6, 80:20, v/v) 25 °C, TMS): δ = 196.1 (C-4), 168.6 (C-7), 167.9 (C-3″), 164.2 (C-5), 163.5 (C-9), 158.0 (C-4′), 131.3 (C-1″), 129.6 (C-1′), 128.2 (C-2′ and C-6′), 119.8 (C-2″), 115.3 (C-3′ and C-5′), 104.5 (C-8), 101.1 (C-10), 96.2 (C-6), 79.1 (C-2), 41.8 ppm (C-3).

Statistical Analysis. Statistical analyses were performed using the SPSS statistical package (SPSS, Inc., Chicago, IL). A paired samples t test was applied to determine whether a particular treatment of the sample would result in a significantly different level of a marker as compared with the control. Treatment differences with $P < 0.05$ were preset to indicate whether means were significantly different.

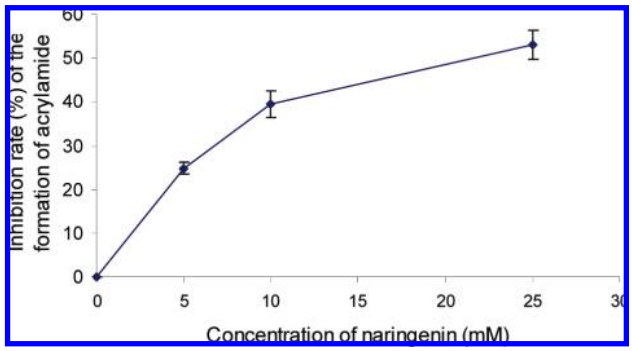

Figure 1. Effect of different concentrations of naringenin on the formation of AA in chemical models containing 25 mM each of asparagine and glucose and subjected to mild heat treatment (128 °C, 1 h).

Results and Discussion

Inhibitory Activity of Naringenin against the Formation of AA in Chemical Model Systems. Chemical model reactions were carried out to investigate the potential of naringenin in reducing the formation of AA under mild thermal conditions (128 °C, 60 min). Because the samples in this study were not in the form of complex matrices that might hinder the analysis of AA, LC-DAD was selected for quantitative and qualitative analyses. Results showed that naringenin dose dependently reduced the content of AA, although not in a linear manner (Figure 1). Moreover, an obvious significant ($P < 0.05$) inhibitory effect was observed even at a level of addition as low as one-fifth (molar ratio) that of the AA precursors, asparagine/glucose. These data demonstrated naringenin as an effective inhibitor of AA formation under mild heating conditions. Although naringenin mainly occurs in conjugated forms, especially glycosides in citrus fruits, the conjugates could be converted into naringenin via hydrolysis of the crude natural extracts (*18*). The inhibitory effect produced with 10 mM naringenin (~40%) was double that with 5 mM naringenin (~20%), but a further increase in the level of addition to 25 mM was not paralleled with a comparable promotion of inhibition (~60%). Apart from the possibility of a nonlinear relationship between concentrations and naringenin's activities to inhibit AA formation, the observation that a 25 mM level of addition actually caused some of the naringenin to form precipitate might help explain the lower than expected inhibitory effect that resulted. Therefore, for further experiments on evaluating the antibrowning potential of naringenin and characterizing the mechanism of inhibition of AA formation in AA-producing models, only low levels of addition (5 and 10 mM) were applied.

Influence of Naringenin on Color Development in Asparagine−Glucose Chemical Models. Color is one of the most important parameters that affect customers' perception for foods. Therefore, experiments were also carried out to evaluate the capacity of naringenin to influence color development in the asparagine−glucose Maillard model. Because visual perception of color is an integration of different dimensions of the color space, color change is better reflected by monitoring parameters that are indicative of the different dimensions, not merely by measuring the "antibrowning" activity, which in some previous studies has usually been determined by spectrophotometric measurements taken at a single wavelength (*19*). Results from colorimetric analysis showed that the addition of naringenin to the asparagine−glucose model affected all of the three coordinates of the color space, as reflected by changes in the *L** (lightness), *a** (redness), and *b** (greenness) values. Moreover, the effects were in a concentration- and time-dependent manner.

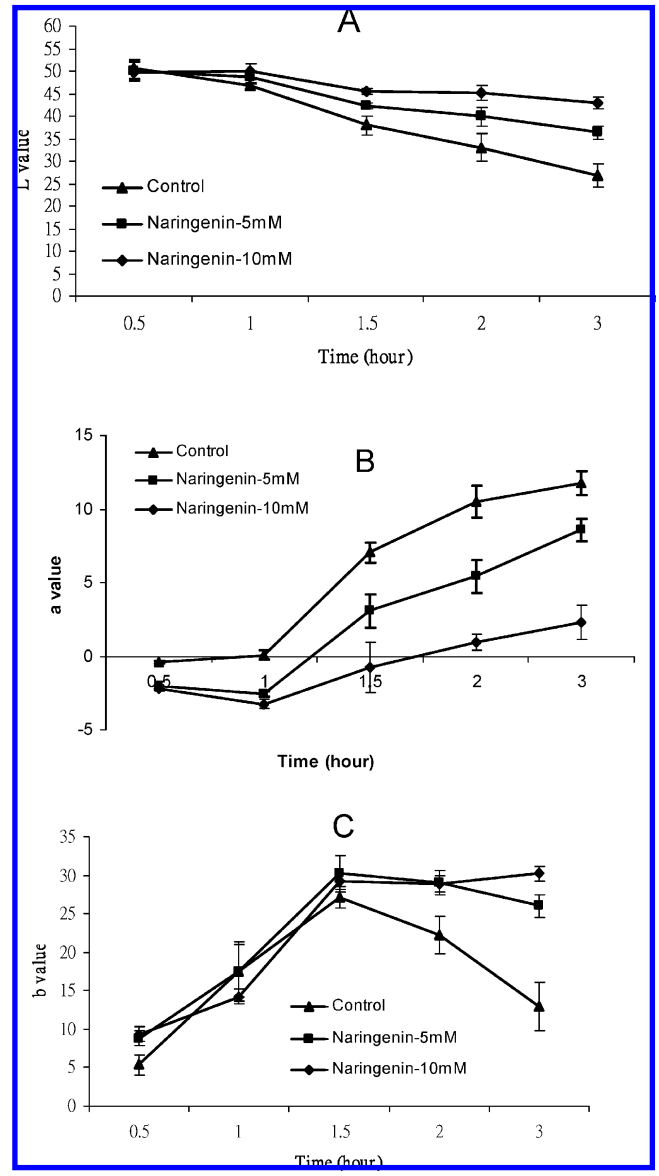

Figure 2. Time−course study on the effect of naringenin on color development in AA-producing chemical models. (A) Changes in *L** value, (B) changes in *a** value, and (C) changes in *b** value.

In terms of lightness (*L** value) (Figure 2a), interestingly, the control and the naringenin-treated samples were almost at the same level at the 30 min time point. As the duration of heat treatment increased, lightness (*L** value) of the reaction systems decreased gradually but with different rates in different models. In particular, decrease in lightness was the most drastic in the control model, whereas the 10 mM naringenin-treated sample was the least drastic. The *a** value is the most commonly used parameter to monitor browning, with a lower value indicating a lower degree of browning. The addition of 5 and 10 mM naringenin to AA-producing models both significantly inhibited browning reactions throughout the time frame examined (30−180 min) (Figure 2b). As expected, 10 mM naringenin was more effective than 5 mM. Moreover, a greater degree of suppression of browning was observed toward the latter time points. Apart from an effect on organoleptic properties, it was recently reported that browning, which is associated with the advanced and final stages of the Maillard reaction, was significantly correlated with the content of AA in food models (*20*). Arribas-Lorenzo and Morales (*4*) found that browning was nearly 2-fold less when pyridoxamine was added to a glucose−asparagine

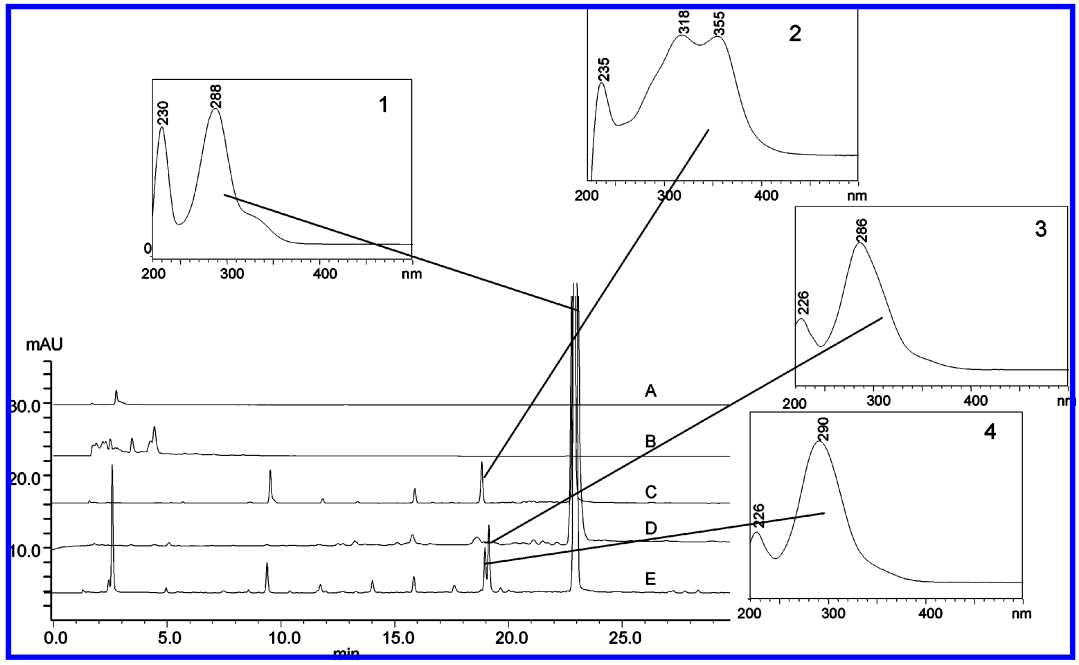

Figure 3. HPLC chromatograms (288 nm) of chemical model reactions carried out in 0.1 M phosphate buffer (pH 7) at 128 °C for 60 min: (A) 25 mM asparagine, (B) 25 mM asparagine + 25 mM glucose, (C) 10 mM naringenin, (D) 25 mM glucose + 10 mM naringenin, and (E) 25 mM asparagine + 10 mM naringenin. Spectrum 1, naringenin; spectrum 2, a naringenin derivative with nearly identical retention time as a postulated adduct of naringenin and asparagine fragment; and spectra 3 and 4, the two postulated adducts with higher polarities than naringenin.

model subjected to heat treatment at temperatures ranging from 120 to 180 °C. This antibrowning activity correlated significantly with the effect of pyridoxamine to inhibit AA formation in their study. A similar phenomenon was also found in our study. In particular, the more potent inhibition of AA formation by 10 mM than by 5 mM naringenin was accompanied with a higher antibrowning effect in the asparagine−glucose models.

Naringenin Probably Inhibited AA Formation via Trapping of Reactive AA-Associated Maillard Intermediates. It has been well-established that AA is formed from reactions closely associated with the Maillard reaction. Probably because a majority of chemical agents that have been tested and demonstrated to be capable of inhibiting the formation of genotoxic substances formed during heat treatment of foods are phenolic compounds (*13, 16, 21*), early studies tend to ascribe the inhibitory activity to the antioxidant capacities of these inhibitors. Besides a lack of conclusive evidence to support antioxidant activity as a major mechanism in mediating the inhibitory effects observed in these studies, more recent studies have pointed to the involvement of direct trapping of reactive Maillard species in bringing about the inhibition of genotoxic substances, including AA (*4*) and HAs (*14*). On the basis of these recent mechanistic studies and considering the fact that the weak antioxidant capacity of naringenin (*13*) could not possibly have contributed significantly to its strong inhibition of the formation of AA, an array of chemical model reactions was carried out to identify changes in the profile of chemical species generated in models with or without the presence of naringenin. Because the naringenin-derived adducts are expected to have a strong chromophore, LC-DAD was applied to analyze the chemical profiles of the above samples. LC-UV chromatograms were registered at different wavelengths. As shown in Figure 3, the model systems where both naringenin and asparagine were present with or without glucose contained a few distinct analytes (models E and H), which were absent from the control models (models A−D). Among them, the two denoted in Figure 3E (R_t 18.8 and 19.0 min, respectively) predominated. These two analytes have similar UV absorption spectra as naringenin (R_t 22.5 min) but have slightly higher polarity than naringenin as indicated by their shorter LC retention times. These data suggest that the unique analytes probably contain a naringenin-like chromophore. The higher polar nature could result from the introduction of one or more polar substituents. Of particular interest is the analyte with a retention time of 19.0 min. This analyte nearly coincides with an analyte from the model containing naringenin alone. Further examination revealed that these two analytes have different UV absorption spectra, which therefore suggest a high probability of different chemical species, despite their elution at nearly the same time point in the LC program. It is also clearly shown in the chromatograms (Figure 3) that only a small portion of the added naringenin was consumed during the reaction time frame (1 h). This is in agreement with our previous study (*14*), which demonstrated that the carbonyl-trapping capacity of naringenin in similar reactions systems was sustainable through prolonged heating processes, which could be several times longer than the timeframes examined in the present study.

It was reported that asparagine alone can undergo thermal degradation to form AA (*9*). Alternatively, asparagine can first react with a reducing sugar, followed by degradation of the resulting glycosylamine to form AA (*9*). Meanwhile, Strecker aldehydes were also suggested to react with asparagine to form AA (*6*). It was noticed that key adducts formed from naringenin and AA precursors/intermediates in the asparagine−naringenin (model H) and the asparagine−glucose−naringenin (model F) models have identical LC-UV behavior. However, it has to be pointed out that the levels of the adducts in model F were lower than those in model H. This reduced level of adduct formation could result from the consumption of asparagine or asparagine degradation products by glucose in the Maillard reaction in the latter model system.

Apart from comparison of LC-DAD chromatograms, LC-MS was also used to verify the absence of the analytes proposed to have arisen from the reactions between naringenin and asparagine/asparagine degradation products in the control models (models A−D). In addition, LC-MS analysis was done to

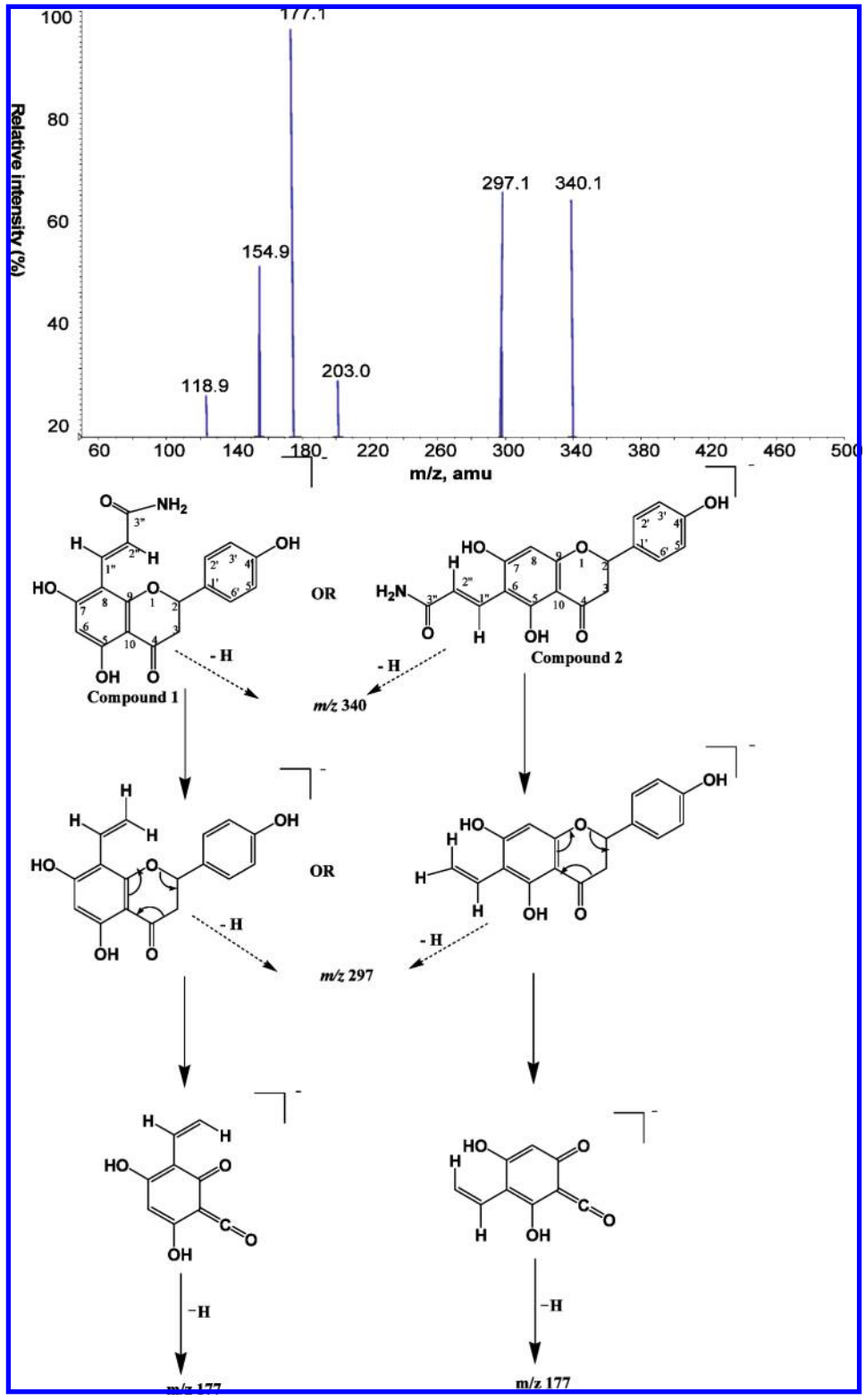

Figure 4. MS/MS spectrum of an analyte of *m/z* 340 and proposed fragmentation pathways.

determine the molecular weights of these analytes. Analysis was performed in both the positive and the negative ionization modes. Results showed that two of the analytes (retention times at 18.8 and 19.0 min) have a predicted molecular weight of 341, which suggests the presence of an odd number of nitrogen atom(s) in the molecules. In addition, this predicted molecular weight corresponds to a molecular composition of a naringenin plus an AA molecule. No adduct corresponding to an MW of 341 was detected in the control models (models A−D). As asparagine was the only source of nitrogen-containing reactant in the chemical models, the additional fragment introduced onto naringenin most likely originated from asparagine. MS/MS was subsequently performed for these analytes. Figure 4 shows a representative MS/MS spectrum registered with the negative ionization mode for one of the *m/z* 340 analytes together with the proposed fragmentation pathways. The fragment ion of *m/z* 297 might arise from the loss of an amide group, and that of *m/z* 177 might arise from retro-Diels−Alder degradation of the C ring of naringenin. MS/MS data therefore suggest that the adducts derived from the reaction between naringenin and

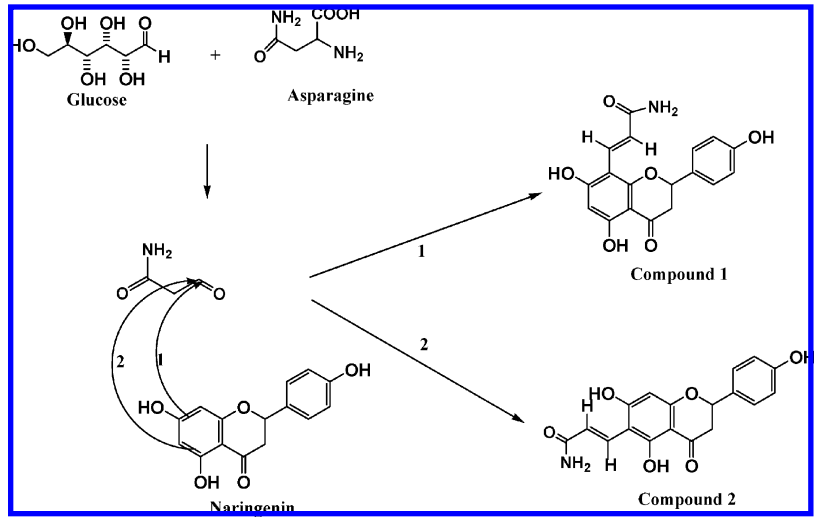

Figure 5. Proposed pathways for the scavenging of amide source to inhibit AA formation by naringenin.

asparagine (models E and H) probably have an AA substituent at either C-6 or C-8 on the A ring of naringenin.

Isolation, Purification, and Characterization of Adducts Formed from Naringenin and AA-Associated Maillard Intermediates. To acquire more solid evidence about the structures of the adducts, and thus the chemistry of the inhibitory activity of naringenin against AA formation, the two adducts discussed above were purified following a series of solvent extraction and chromatographic procedures. Both compounds were in the form of a cloudy white solid. Their structures were characterized by 1D and 2D NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** showed the same pattern of hydrogen and carbon atoms. Comparison with reported data suggested the presence of a naringenin substructure (*22*). The ^{13}C NMR spectrum of compound **1** established the presence of 18 carbon signals, which matched well with a structure derived from the substitution of an AA on naringenin (Figure 4). Distortionless enhancement by polarization transfer (DEPT) spectra revealed the presence of a single methylene group in compound **1**, suggesting that the CH_2 group at the 3-position of naringenin substructure likely remains intact. Literature data reported nearly identical chemical shifts between H-6 and H-8 of naringenin (*22*). The occurrence of an aromatic singlet at δ H 5.91 (1H) in the ^{1}H spectrum of compound **1** suggested substitution at C-6 or C-8. The ^{13}C NMR spectrum of compound **1** showed that C-8 was shifted by about 5 ppm downfield, consistent with the substitution of a downfield-shifting group at this position. Further evidence for the full assignment of the structure of compound **1** was provided by DEPT and heteronuclear multiple bond correlation (HMBC) experiments (Figure 4). For compound **1**, the HMBC spectrum revealed three-bond couplings of H-1″ to C-7 and C-9 and that of H-2″ to C-8. Two-bond coupling was also observed for H-1″ to C-8. In addition, the DEPT spectrum of compound **1** revealed C-8 as a quaternary carbon, suggesting the occupation of C-8 by a substituent. On the basis of the ^{3}J (16.0 Hz) between H-1″ and H-2″, this compound was suggested to have a *trans* configuration. Its structure was therefore established as 8-*C*-(*E*-propenamide)naringenin. Compound **2** was accordingly elucidated as 6-*C*-(*E*-propenamide)naringenin. It was proposed that reactions between asparagine and a reducing sugar or a reactive carbonyl could lead to the formation of an azomethine ylide, which in turn could rearrange to form intermediates that might be hydrolyzed to 3-APA or 3-oxopropanamide (*23*). Although 3-APA, rather than 3-oxopropanamide, has been reported as a key intermediate on the pathway to the formation of AA, the depletion of the latter amide source might consequently promote the conversion of the azomethine ylide to 3-oxopropanamide and thus reduce the flux of azomethine ylide towards the 3-APA pathway. A schematic illustration of one of the probable reaction pathways, which might contribute to the scavenging of an amide source by naringenin in AA-producing model systems is given in Figure 5. This is in good agreement with our previous study (*14*), which identified C-6 and C-8 of the A ring of naringenin as active sites for trapping reactive Maillard species.

Taken together, the LC-UV, LC-MS, and NMR data, significant quantities of adducts were formed via trapping of asparagine-derived reactive species by naringenin. In this study, the end product, AA, rather than intermediates on the pathway to AA formation, was selected as the marker to assess the activity of naringenin against the formation of AA. To examine the correlation of the proposed inhibitory mechanism, namely, scavenging of asparagine-derived reactive fragment(s), with the observed inhibitory effect on AA formation by naringenin, changes in the content of the postulated adducts in the models were determined based on the total integrated peak areas of the two analytes (compounds **1** and **2**). It was found that the 10 mM naringenin-treated sample had a much higher content of these adducts than the 5 mM-treated sample. Because these adducts were a result from the trapping of asparagine-derived fragment(s), this phenomenon indicates a link between adduct formation and inhibition of AA generation by naringenin.

With the use of isotope-labeled asparagine and glucose, a previous study showed that the amide group from asparagine was the source of AA nitrogen and that all three AA carbon atoms were derived from asparagine (*6*). On the other hand, it was reported recently that pyridoxamine suppressed AA formation probably via the scavenging of reactive carbonyl intermediates of the Maillard reaction, forming stable adducts (*4*). The inhibitory mechanism of this later study was grounded on the findings that carbonyls (α-dicarbonyls and α-hydroxycarbonyls) can enhance the conversion of asparagine into AA (*10, 24*). In other words, the carbonyl species could be regarded as "accelerators" for the reaction. Therefore, from a mechanistic point of view, the most effective inhibition of AA formation could possibly be accomplished with the application of chemical agents that both trap the essential amide source of AA and scavenge the carbonyl accelerators from the reaction system. The former could be considered as having greater significance

than the latter because in the reaction system of the present study, the moiety scavenged and introduced onto the A ring of naringenin is a key amide source, whereas for the reactive carbonyls that act like accelerators, when one is scavenged by a carbonyl scavenger, its role could possibly be taken up by a new carbonyl species generated through the Maillard reaction. Nevertheless, in the present study, no major adducts corresponding to the trapping of reactive glucose-derived carbonyl species, such as methyl glyoxal by naringenin, were identified in the chemical models. This is in agreement with our previous mechanistic study on the inhibition of HA formation by naringenin (*14*). It was proposed that antioxidant polyphenols might undergo oxidation to form quinones that could react with intermediates of AA such as 3-APA by means of quinone−amine interaction (*21*). In contrast, the present study found that naringenin was capable of directly trapping the amide source of AA. To the best of our knowledge, this mechanism of action with respect to the inhibition of AA formation by phytochemicals has been reported for the first time.

Conclusion

In conclusion, naringenin effectively reduced the content of AA in asparagine−glucose chemical models subjected to mild heat treatment. LC-DAD and LC-MS analyses showed that naringenin was capable of forming adducts with asparagine degradation products. The findings of the present study not only identify a strong inhibitor of AA formation but also provide insights into the mechanism(s) by means of which weak antioxidant polyphenols inhibit the formation of AA.

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