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Chlorogenic Acid Oxidation-Induced Greening of Sunflower Butter Cookies as a Function of Different Sweeteners and Storage Conditions

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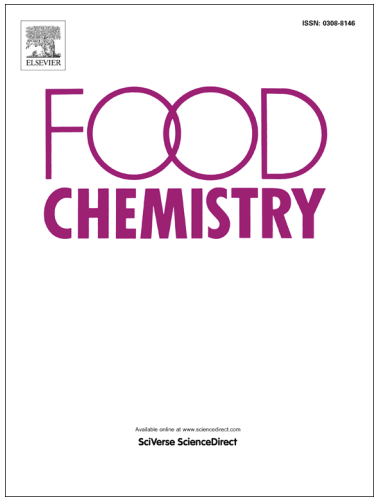

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Highlights:

- Sweeteners with higher moisture and/or pH enhanced greening in cookies
- Storage at higher relative humidity resulted in higher surface greening
- Chlorogenic acid-lysine adduct content was inversely correlated to greening reactants
- Lowering the pH, moisture and a_w would inhibit greening when it is not desired.

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ABSTRACT

Sunflower butter use as an allergen-free alternative to tree and legume nut butter in baking is limited by chlorogenic acid induced greening that occurs at alkaline pH. Limited information is available on controlling this greening in a food matrix. This study examined how different liquid sweeteners and relative humidity influenced greening of sunflower butter cookies. Doughs had similar initial pH (7.52-7.66) which increased to 8.44-9.13 after baking as ranked: xylitol>maple syrup>corn syrup>honey>agave syrup. Cookies made with maple syrup had the highest moisture and greening corresponding with lowest free chlorogenic acid. The % greening followed the same trend as greening intensity, and was positively correlated (r=0.9101) with chlorogenic-lysine adduct content. Our findings provide an ingredient solution to controlling greening, as results demonstrate that greening can be promoted with high relative humidity storage, and use of high moisture and pH ingredients. Unwanted greening can be inhibited by simply changing the liquid sweetener.

Keywords: chlorogenic acid, greening, moisture, pH, sunflower butter

Chemical Compounds Studied in This Article

Chlorogenic acid (PubChem CID: 1794427); L-lysine (PubChem CID: 5962)

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1. Introduction

Sunflower seed butter can act as an alternative to legume and tree nut based plant butters, which are members of the "big 8" allergens affecting an estimated 0.6-1.3 and 0.4-0.6 % people in USA who suffer from peanut and tree nut allergies respectively (FDA, 2016; Peabody, 2016). Sunflower nut butter contains a higher phenolic content (1-5%) than other nut butters. Of the total phenols, 50-70% is chlorogenic acid (CGA), a substrate in both browning and greening reactions with sunflower protein during aqueous processing or under alkaline conditions (Bekedam, Schols, Van Boekel, & Smit, 2008; Weisz, Kammerer, & Carle, 2009; Yabuta, Koizumi, Namiki, Hida, & Namiki, 2001).

Use of sunflower seed butter can cause bitterness and firmness, and it is difficult to spread compared to peanut butter, which can make it less acceptable to consumers (Lima & Guraya, 2005). Due to the bitterness, sweetened versions of sunflower butter are commercially available (NPI, 2014). These sweeteners differ in pH, moisture, and phenolic content and this could affect the visual appeal of sunflower butter bakery products. For instance, the higher pH of maple syrup compared to honey could impact post-baking color reactions (Ball, 2007) such as the greening reaction in sunflower seed based products. Texture and taste are challenges the industry has overcome, but the green color remains a problem when using sunflower butter in bakery applications when greening is considered undesirable. The oxidation product of CGA dimer: *o*-quinone reacts with amino acids and side chains of proteins to form green trihydroxy benzacridine (TBA) derivatives under alkaline conditions. Yabuta et al. (2001) showed that pH influences the binding of CGA to sunflower protein. Increasing pH from 5 to 9 results in a color change from yellow to blue-green. The role of pH and moisture in polyphenoloxidase induced

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greening has been determined (Vaintraub & Kratch, 1989), however, the role of moisture in non-enzymatic greening reactions has not been fully investigated in a food matrix.

2. Materials and methods

2.1. Cookie Formulation and Experimental Design

Two batches of sunflower butter cookie dough with different sweeteners were prepared separately and baked at 149 °C (300 °F). After mixing flour (39.7%), baking soda (0.6%) and salt (0.6%), egg (13%), sweeteners (21.2%), sunflower butter (24.3%) and vanilla extract (0.6%) were then added. The doughs (0.5±0.2 cm thick) were cut with a 4.5 cm diameter cutter. Baking was carried out using a convection oven (JA12SL, Doyon, Inc. Saint-Côme-Linière, Canada) at 149 °C for 7 min, with the temperature monitored using a thermocouple. Three desiccator cabinets (Fisherbrand™) containing NaOH, K_2CO_3, and $(NH_4)_2SO_4$ solutions were prepared, and had RH of 75, 79, and 84% RH respectively measured using a LogTag® humidity and temperature recorder.

2.2. pH and °Brix Index of Sweeteners

A xylitol solution was prepared by dissolving xylitol granules (4.4 g) in 5 ml nano-filtered water at 75°C (88% w/v) to obtain a similar moisture content as honey. The same ratio of sweeteners in cookies were used to make sweetener-water solutions by weighing 0.1 g of the liquid sweeteners and adding 5 ml nano filtered water. Sweetener solutions (2% w/v) were vortexed for 30 s and then incubated at room temperature for 3 h on a shaker (Rocker II, 260350, Boekel Industries, Inc. PA) at a speed of 1.5*g. The pH was tested after incubation using a LabQuest 2® pH meter (Vernier Software & Technology, OR, USA). The °Brix index of 0.3 ml

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of each sweetener was tested using a PAL-α, ATAGO refractometer (Nova Tech International, Inc. TX, USA).

2.3. Physical Tests of Cookie and Dough

2.3.1. pH and Water Activity (a_w)

Dough and cookie sample solutions (10%, w/v) were prepared with nano filtered water (Miller, Graf, & Hoseney, 1994), and incubated for 3 h on a shaker at a speed of 1.5*g. After incubation, cookie and dough samples were centrifuged (AccuSpin 1R-75003449, Thermo Fisher Scientific, Inc. CA) at 9000*g at 4 °C for 15 min and the supernatant was used for testing (AACCI, 1999a). Water activity of cookies was measured right after each color testing (section 2.5) using a water activity meter (Model 3ET, Aqua Lab Technologies, Inc. CA, USA) according to manufacturer's instructions.

2.3.2. Moisture Content and Spread Factor

Moisture content was determined using a vacuum oven as outlined in AOAC method 925.09 (AOAC, 2005) with modifications. Samples (3 g) were placed into pre-dried aluminum pans and put in a vacuum oven (Model 281, Thermo Fisher Scientific, Inc. CA), maintained at 60 °C and pressure of -70 kPa for 24 h. After drying, samples were cooled in a desiccator for 6h. The weights of samples were recorded before and after drying.

The spread factor was measured following AACCI Method 10-50.05 (1999b) by randomly selecting 6 cookies (three from each batch). A vernier caliper (Mecanic Type 6911. KWB, Inc. Switzerland) was used to measure the width and thickness of cookies. The spread factor was calculated by dividing the width by the thickness of the cookies.

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2.4. Protein Content

Supernatants (250 µl) from section 2.3 were prepared following the DNPH assay as outlined by Hawkins, Morgan, and Davies (2009), and protein content was determined at 280 nm using a spectrophotometer (Vernier Software & Technology, OR, USA).

2.5. Color Changes

Color changes were measured after 0.25, 1, 4, 7, 11, and 24 h post-baking under uncovered storage and 1, 4, and 7 days after three relative humidity/RH conditions (75, 79, and 84%). Greening changes were measured using a spectrophotometer (CM-2500d, Konica Minolta, Inc. Japan) and analyzed using SpectraMagic NX color data software. The spectrophotometer aperture size was 8 mm and the scan number was twice per sample. The illuminant was D_{65} and the radius of illumination area was 8 mm, with an observation angle of 10°. Data was collected as L* [darkness to lightness (0-100)], a* [greenness (-a*) to redness (a*)], and b* [blueness (-b*) to yellowness (b*)]. Two different surface locations (upper and bottom) of whole cookie samples were placed under a 2 mm cylinder probe and L*, a*, and b* was recorded. The cookie samples were then sliced to measure internal greening.

The percent internal greening of cookies was measured using an image analyzer CV-X422A and CA-H1DB VisionDatabase Ver 1.2 (Keyence America, Corp. CA, USA). Samples were placed under a camera with 2M pixels with 16 speed color change-coupled devices (CA-HX200C) and the image was captured for analysis. The distance from the camera to cookie was 28 cm. The image size was set as 1600x1200, shutter speed was 1/30, and the sensitivity was 6.3. The binary luminosity was 180 for greening and 85 for browning. The radius of the cookies averaged 2.3 cm and the preset area on the image was kept at a radius of 1.5 cm. The mouse

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pointer was used for selecting green areas on cookies. The percent greening was calculated by dividing the green area by the whole selected cookie area (Ishak & Hudzari, 2010).

Cookie samples (0.6 g in 20 ml) were then homogenized (Multi-prep Homogenizer, PRO Scientific Inc., Oxford, CT, USA) at $1.3x10^3$*g for 1 min. The solutions were filtered using Double Rings® No. 102 filter papers and then through a 0.45 µm nylon filter. Green and brown color of solutions were determined using a SpectroVis® Plus spectrophotometer (Vernier Software & Technology, OR, USA) at λ_{680} and λ_{420}, respectively.

2.6. Chlorogenic Acid and Trihydroxy Benzacridine Derivatives Content

Dough or cookies (0.9g) were dissolved in 30 ml HPLC water. After homogenization (Multi-prep Homogenizer, PRO Scientific Inc., Oxford, CT, USA) at $1.3x10^3$*g for 1 min, sample solutions were centrifuged at $9x10^3$*g (AccuSpin 1R-75003449, Thermo Fisher Scientific, Inc. CA, USA) for 20 min at 4 °C. The supernatant was first filtered through Double Rings® No. 102 filter paper and then filtered through a 0.45 µm nylon filter for HPLC analysis. Chlorogenic acid purchased from Sigma-Aldrich (Saint Louis, MO, USA) was used to make a standard curve (0-0.060 mg/ml in HPLC water).

Chlorogenic acid quantification was carried out on an Agilent 1100 series HPLC (Agilent Technologies, Inc. Santa Clara, CA, USA) with a Phenomenex® Luna 5µ C8 (2) 100 Å (150 x 2 mm, 1.5 µm particle size) column using a modified method from Jully, Toto & Were (2016). The UV-Vis detector was operating at 320 nm. Mobile phases were 0.1% glacial acetic acid/water (A) and 0.1% glacial acetic acid/acetonitrile (B). The gradient used was 0 min, 6.0% B; 2 min, 7.0% B; 2.5 min, 7.2% B; 3 min, 7.3% B; 4 min, 7.4 % B; 5 min, 7.5 % B; 5.5 min, 7.7 %; 6 min, 10 % B; 7 min, 6.0 % B; 8 min 6.0 % B at a flow rate of 0.8 ml/min with column temperature at 30 °C.

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Chlorogenic acid-lysine standard was prepared by mixing 5 ml of 112 mM lysine with 5 ml of 28 mM CGA solutions. The pH of CGA-lysine solution was adjusted to 9.0 and stirred for 20 h at room temperature for greening reaction (Bongartz, Brandt, Gehrmann, Zimmermann, Schulze-Kaysers, & Schieber, 2016; Prigent, Voragen, Li, Visser, van Koningsveld, & Gruppen, 2008). The original adduct solution was diluted 1:2, 1:4, 1:6, 1:8 and 1:10 (v:v) for quantifying CGA-lysine adducts. The CGA control solution was diluted 1:1 with HPLC water, and was also stirred for 20 h without adjusting pH. Adducts were determined using a Phenomenex® Luna 5µ C8 (2) 100 Å (150 x 2 mm, 1.5 µm particle size) column with LC/MS (LC: Ultimate 3000 series, Thermo Fisher Scientific, Inc. CA; MS: Impact II, Bruker, CA, USA) according to the method of Bongartz et al. (2016) with modification: the gradient program was 0-20 min, 2% B; 20-20.5 min, 17.7% B; 20.5-22.5 min, 100% B and 22.5-36 min 2% B. MS detection used Electrospray Ionization source (ESI) with positive polarity, while the end plate offset and capillary voltage was kept at -500 and 4500 V, respectively. The ion and collision energy were 4.0 and 25.0 eV. For ion cooler, the transfer and prepulse storage time were 220.0 and 20.0 µs, respectively. The dry gas temperature was kept at 180 °C with a flow rate of 4 l/min. The UV-Vis detector was monitored at 280, 320, and 631 nm. The mass spectra of the column eluate for positive ion ranged from *m/z* 50 to 1200.

2.7. Statistical Analysis

The effect of liquid sweetener type, storage conditions, storage time and their interactions were evaluated in a mixed model two- and three-way analysis of variance (ANOVA): two fixed effects (sweeteners and storage conditions) as variables were repeated at 9 different time points. Where a significant effect of treatment was detected, the Student T-test was used to determine the levels of significance between dependent variables and two and three-way interactions of independent variables. All values were reported as the mean ± standard deviation from two

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batches of dough made on the same day. Differences were considered significant when $P<0.05$. Correlations between variables were also calculated using Statistics Analysis Software (SAS institute Inc. NC, USA).

3. Results and Discussion

3.1. Compositional and Physical Analysis of Sweeteners, Cookies and Dough

3.1.1. °Brix and pH of Sweeteners

The °Brix index (soluble solid content) of each liquid sweetener (2% solution) were ranked as: honey (81.23)>corn syrup (77.48)>agave syrup (76.85)>xylitol (75.05)>maple syrup (65.80).

Honey solutions had significantly lower pH ($P<0.05$), while maple syrup solutions had the highest pH (Table 1). The lower pH of honey is attributed to higher organic acids (0.57%,) formed during fermentation from nectar into honey where glucose is converted to hydrogen peroxide and gluconic acid by glucose oxidase, in addition to having formic, citric, acetic, and malic acids (Silva, Gauche, Gonzaga, Costa, & Fett, 2016).

3.1.2. pH, Water Activity (a_w), Moisture and Spread Factor of Dough and Cookies

The pH of 10% dough solutions ranged from 7.52-7.66. Dough made from honey had the lowest pH of 7.52 compared to other dough solutions. After baking, pH of all cookies increased (Table 1). For instance, pH of cookie solutions made with maple syrup and xylitol increased from 7.57 and 7.66 to 8.99 and 9.13, respectively. Higher pH of cookies after baking was possibly related to the sodium bicarbonate's leavening action during baking to produce sodium carbonate, which is more alkaline than sodium bicarbonate (Gokmen, Acar, Serpen, & Morales, 2008). The pH of cookie solutions made with xylitol was highest, followed by maple syrup, corn syrup, honey and agave syrup, correlated with the initial pH in sweeteners (Table 1).

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Cookies made with maple syrup had the highest a_w, while cookies made with xylitol had the lowest a_w after storage (Table 2; $P<0.05$), consistent with differences in moisture content of the sweeteners (Table 1). As expected the a_w was inversely related to °Brix with a correlation coefficient of -0.6229.

Cookies made with maple syrup had the highest moisture content ($P<0.05$). The moisture content of cookies was positively correlated (r=0.8701) with the moisture content in sweeteners (Table 1; Fradinho, Cristiana Nunes, & Raymundo, 2015). The difference in moisture and humectancy of sugars affected the water loss during baking (Cauvain & Young, 2008), as observed in cookies made with honey which had the lowest percent moisture loss (26%) compared to water loss in other cookies that ranged from 30-42%.

The spread factor (SF) of cookies was negatively correlated (r=-0.6780) with moisture content of the different sweeteners (Fradinho, Cristiana Nunes, & Raymundo, 2015). The SF of dough was similar except for dough made with maple syrup which had the lowest SF. The lowest SF of maple syrup dough was due to the highest moisture content of maple syrup, which made the dough easier to spread (Suas, 2009). After baking, the SF of cookies decreased possibly due to the leavening effect of baking soda (Serna-Saldivar, 2012; Zhang, Nishizu, Kishigami, Kato, & Goto, 2013). During baking, the moisture content affected SF, because when moisture content is low, the transition from rubbery to glassy phase occurs which then stops the shrinkage of dough during baking (Table 1).

*3.2. Hunter L*a*b* and Image Analysis*

Cookies made with maple syrup and xylitol both turned green within 4 h when left uncovered at room temperature storage, whereas cookies made with other sweeteners did not show surface greening until after 24 h (Fig. 1a-b). Internal greening commenced in cookies made with maple syrup and xylitol right after baking (Fig. 1c). Higher greening in cookies made with

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xylitol and maple syrup was attributed to these sweeteners both having the highest initial pH of the sweeteners tested, in addition to highest initial moisture in maple syrup (Table 1). In contrast, the delayed greening that occurred after 4 h in cookies made using honey and agave syrup, was attributed to lower initial pH and moisture content in these cookies. Cookies made with agave syrup greened faster than cookies made with honey (1.7 and 0.4 % per hour during 24 h of uncovered storage, respectively), despite having similar pH after 24 h, which indicated initial moisture content of sweeteners affected the greening reaction more than pH during storage (Table 1, Fig. 1d).

The % internal greening in cookies that were stored uncovered at room temperature increased with time during 24 h due to continual CGA-amino acid reactions (Fig. 1d, Yabuta, et al., 2001).

Cookies in the chambers with highest RH (84%) had higher greening, due to the higher moisture in the environment (Yabuta, et al., 2001). Within each chamber, cookies made with maple syrup and xylitol showed the highest greening, consistent with results in uncovered cookie samples, where sweeteners with higher initial moisture and/or pH were more green (Fig.1, Fig. S1 - supplementary material). Under alkaline conditions, the hydroxyl groups on the *o*-quinone loose protons and the negatively charged oxygen atoms oxidize easily which then react with amino groups to form the green pigments in sunflower butter cookies (Yabuta, et al., 2001). Besides storage conditions, time also affected color. The increased a* value after 4 and 7 days in some cookies made with maple syrup and xylitol was caused by the continued greening reaction which resulted in green to blue-green pigments formed (Yabuta, et al., 2001), which resulted in lower b* but higher a* value. The % internal greening of cookies shown in Fig. 1, followed a

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similar trend to that of the greening intensity, where cookies made with maple syrup showed the highest % greening followed by cookies made with xylitol and corn syrup.

In summary, the different sweeteners, storage conditions, storage time, and interaction between sweeteners and time had a significant effect on greening ($P \leq 0.0001$, F test; Table 3). There was also a significant interaction ($P < 0.0001$) between liquid sweeteners used and storage time with greening intensity, indicating the use of different sweeteners and storage time can both be manipulated to alter greening during storage. Although different RH conditions significantly affected the greening intensity, their interaction with time and different sweeteners had no effect on greening intensity (Table 3).

3.3. Changes in Color Reactants and Products in Sunflower Dough and Cookies

3.3.1. Protein Content

Dough and cookies made with corn syrup had the highest free protein content, possibly due to less color reactions (Maillard and greening combined) occurring during dough preparation and baking (Table 1). Corn syrup has less reducing sugar compared to honey and agave sryup (St-Pierre, et al., 2014), so cookies with corn syrup would be expected to have lower browning. In addition, cookies made with corn syrup had a lower pH compared to cookies made with maple syrup and xylitol. These differences accounted for reduced browning and greening and thus lowered the consumption of protein (Table 1; Ames, 1998; Wang, Qian, & Yao, 2011). The lower protein content in dough made with honey and agave syrup was likely due to the higher fructose and associated protein fructosylation that occurred during preparation and baking (Dills, 1993).

After baking, the water losses in dough were 42, 37, 32, 30, and 26% in cookies made with xylitol, corn, agave, maple syrups and honey respectively, resulting in increased protein

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content in cookies compared to dough on the same gram basis. The lower protein content in cookie solutions made with maple syrup and xylitol could be due to the dominant greening reaction in addition to Maillard (Table 1; Dills, 1993; Yabuta, et al., 2001).

3.3.2 Chlorogenic Acid

Chlorogenic acid content was similar in doughs. The similar CGA content is because despite CGA existing naturally in honey, maple and agave syrups (Abou-Zaid, Nozzolillo, Tonon, Coppens, & Lombardo, 2008; Nayik & Nanda, 2016), it is in lower quantities than that present in the sunflower butter, which was the main contributor of CGA in cookie dough. The CGA decreased in all cookie treatments after baking compared to dough. Chlorogenic acid can be a reactant in Maillard and greening reactions or hydrolyze into caffeic and quinic acids, other phenols and catechol products (Billaud, Roux, Brun-Merimee, Maraschin, & Nicolas, 2003), causing further losses of free CGA.

Cookies made with xylitol and maple syrup had the lowest CGA content, while cookies made with agave syrup and honey had higher CGA content (Table 4). During baking, free CGA binds with amino groups to form TBA derivatives under alkaline conditions, which explained why cookies made with higher pH sweeteners (maple syrup and xylitol) turned green soon after baking (Yabuta, et al., 2001).

The decrease in CGA in cookies made with xylitol, maple and agave syrups during 24 h storage could be caused by free chlorogenic acid continually reacting with primary amino acids and proteins to form TBA derivatives, producing the green color in cookies (Yabuta, et al., 2001). Cookies made with xylitol had the highest pH and lowest moisture (P<0.0001), but had higher free CGA after 7 days under 84% RH storage compared to cookies made with maple

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syrup. Xylitol, being a non reducing sugar does not participate in Maillard reactions during baking and this could have accounted for some of the higher free CGA.

The higher moisture content of maple syrup also had a greater effect on greening than the higher pH of xylitol (Table 1, Fig. 1). The increased greening caused by higher initial moisture content can likewise explain why cookies made with honey had a higher pH and less % greening (P<0.01) compared to agave syrup cookies, which had the lowest pH but more greening (Table 1, Fig. 1).

3.3.3. Chlorogenic Acid-lysine Adducts and Melanoidins

The CGA loss due to the different colored reactions were distinguished by quantifying green pigments at 680 nm and melanoidins at 420 nm. Bongartz et al. (2016) found that when lysine binds to *o*-quinone formed from oxidized chlorogenic acid dimer, the mixture resulted in the highest greening among 20 different amino acids. Lysine's positively charged ε-amino group with a high pKa around 10.5 in polypeptides makes it highly reactive (Mendoza & Vachet, 2009). The CGA-lysine adduct solutions were thus used to monitor the green pigment production in the present study. Higher moisture and pH ingredients increased greening in sunflower butter cookies resulting in lower free CGA in cookies made with maple syrup (Fig. 1, Table 1, Table 4). The predominant interaction between *o*-quinone with primary amino acids or side chains from proteins are hydrogen and covalent bonds at alkaline pH. The free CGA, CGA-lysine adducts and greening pigment intensity had correlation coefficient of -0.9241, 0.8738 and 0.9501, respectively with greening (-a*) in cookies after 0.25h of uncovered storage. However, formation of CGA-lysine adducts was not significantly affected by time (0.25 h to 24 h uncovered storage condition) for all treatments (Table 3), so once adducts formed, the differences over time were minimal.

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Heat accelerates the molecular movement of free CGA and amino groups, and enhances the greening reaction and formation of melanoidins (Bekedam, et al., 2008). Maillard reaction between carbonyls (reducing sugar and CGA) and amino groups from sunflower butter, flour and eggs forms brown melanoidin pigments (Bekedam, et al., 2008). Melanoidin in cookies made with xylitol, corn and agave syrups were not significantly different. Browning was mostly related to the type of sugar in the various formulations Higher glucose content in honey (~49%) could explain the higher A_{420} of cookies made with honey than cookies made with corn (31%) and agave (10%) syrups (Fig. S2- supplementary material; St-Pierre, et al., 2014). The high A_{420}, indicative of the Maillard reaction in cookies made with maple syrup, may have been due to sucrose inversion to glucose and fructose to form melanoidins under alkaline conditions or interference from green color of sample solutions (Wang, Qian, & Yao, 2011). Higher pH may have provided the alkaline conditions to favour binding between anions and nucleophilic carbonyl compounds (Hayase, Kim, & Kato, 1984).

After baking, cookies had decreased A_{294} compared to the dough caused by polymerization of Schiff bases to form Amadori products and then browning (Table S1-supplementary material; Ajandouz, Tchiakpe, Dalle Ore, Benajiba, & Puigserver, 2001). Increased A_{294} from 0.25 to 24 h after baking indicated that formation of Schiff bases which are intermediate Maillard reaction product continually formed during storage. The higher A_{294} of dough and cookies made with honey and agave syrup during the 24 h storage, was due to their higher reducing sugar content. The lowest ratio of A_{294}/A_{420} in cookies made with maple syrup, was indicative of formation of the least brown colored polymers during baking (Table S1 - supplementary material; Chen & Kitts, 2011) in favour of the green pigments.

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3.3.4. LC-MS Analysis of Cookie Solutions

After cyclization of CGA quinone and amino groups, a trihydroxy benzacridine derivative (TBA) core structure forms which has a *m/z* of 700. When the TBA core structure binds with lysine, the m/z is 829. Trihydroxy benzacridine derivatives and TBA-lysine both result in green color. Cookies made with maple syrup had a higher mass spectrum intensity at *m/z* 700 than cookies made with honey when compared at the same storage time (Fig. 2b-e). The higher intensity of TBA compounds (*m/z* 700) in cookies made with maple syrup than cookies made with honey was due to more greening adducts formed due to the higher initial moisture and pH of the maple sweetener used (Fig. 2, Yabuta, et al., 2001).

The CGA-lysine standards formed several ions such as: *m/z* 147, 191, 353, 371, 527, and 700 some of which correspond to lysine (146 g/mol), quinic acid (192 g/mol) , CGA (354 g/mol), CGA with a water molecule attached (371), and TBA derivative-dehydrated quinic acid (526 g/mol) (Fig. 2, Table S2-supplementary material, Bongartz et al. 2016). Amongst cookie solutions, besides the greening adducts (*m/z* 700) and CGA+ H_2O (*m/z* 371), there were several ions with *m/z* 119, 143, 184, 445, 548, and 559, which could correspond to threonine (119 g/mol), or other fragments from the Maillard reaction (Schwarzenbolz, Hofmann, Sparmann, & Henle, 2016; Cerny & Guntz-Dubini, 2013).

The fragmentation of CGA-lysine adducts (*m/z* 829 $[M+H]^+$) and the greening TBA derivative (*m/z* 700 $[M+H]^+$) produced several ions: *m/z* 130 (36.51%), 147 (100%), 191 (37.11%), 293 (40.63%), 527 (8.37%) and 611 (8.31%) (Table S2-supplementary material). The cookies made with honey had more fragments than cookies made with maple syrup. The main fragments in cookies with honey had *m/z* 127, 163, 259, 289, 325, 343, 487, 505 and 738, while cookies made with maple syrup had 143, 262, 308, 355, 559 and 682 (Fig. S3, Table S2-

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supplementary material). The *m/z* 163, 487 and 738 fragments in cookies made with honey were possibly Maillard reaction products (Golon, Kropf, Vockenroth, & Kuhnert, 2014; Zhang, Ames, Smith, Baynes, & Metz, 2009). The greater Maillard reaction in cookies made with honey was attributed to the higher reducing sugar in honey compared to the greening reaction products found in cookies made with maple syrup due to maple syrup's higher initial moisture and pH (Yabuta, et al, 2001). The cookies made with maple syrup however had more fragments from TBA derivatives, such as *m/z* 262 and 308 similar to masses of CGA-lysine adducts found by Bongartz et al.(2016).

Chromatograms of CGA-lysine adduct standard, CGA control, and cookies made with maple syrup and honey, representing highest and lowest greening intensity amongst treatments with two storage conditions (24 h uncovered and 84% RH for 7 days storage; Fig. 1, Fig. S1) are presented in Fig. S3. Quinic, chlorogenic, and caffeic acid had retention time of 7.43, 10.38, and 11.22 min respectively (Fig. S3b-supplementary material). The quinic and caffeic acid were hydrolysis products from CGA formed during incubation (Bekedam, Schols, Van Boekel, & Smit, 2008). The CGA content was higher in the cookies made with honey compared to those made with maple syrup, consistent with results in section 3.3.2. (Table 4). Higher CGA in cookies made with honey was attributed to lower moisture content and pH which slowed down the color reaction and left more reactants (Prigent, et al., 2008; Yabuta, et al., 2001). With time, CGA content decreased. Cookies made with maple syrup and honey after 7 days storage under 84% RH condition had less CGA but higher CGA-lysine adducts content than those of 24 h uncovered storage, which indicated that as CGA decreased, the CGA-lysine adducts, a marker of the greening reaction increased during storage (Table 4, Fig.S3-supplementary material). The lower CGA content in cookies made with maple syrup than cookies made with honey indicated

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that greening in cookies regardless of storage conditons may have occurred from the higher initial moisture and pH when maple syrup was used (Yabuta, et al., 2001).

4. Conclusions

Use of sunflower butter as a potential replacement for plant based butters can be hampered by the greening that forms when high pH conditions are used in baking. The present study shows that selection of sweeteners should be considered when using sunflower butter in baking due to their effect on greening induced by CGA-protein interactions. The higher CGA in cookies made with honey stemmed from the lower pH values that decreased CGA-lysine adduct formation compared to those made with maple syrup. The cookies made with sweeteeners that had higher initial moisture and pH had less free CGA and CGA-lysine adducts formed due to the consumption of CGA in the greening reaction. The higher moisture and a_w may have increased the molecular movement of reactants resulting in higher % greening and greening intensity. Our findings thus suggest that lower greening can be obtained by use of low pH and low moisture sweeteners such as honey, while higher greening can be achieved by use of high pH and moisture sweeteners such as maple syrup. Given that consumer acceptance of foods is influenced by color, controlling this greening would enable the use of sunflower butter as a replacement for peanut and other tree nut butters in baking for those with allergies to these nuts. Selection of sweeteners used for baking in addition to control of humidity conditions is suggested as a way to control surface greening. Further studies should focus on quantifying how much chlorogenic acid acts as a reactant in browning versus greening reactions and their associated effects on nutrition.

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Conflict of Interest

Authors do not have any conflict of interest

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Figure Captions

Fig. 1. Greening changes in cookies made with sunflower butter as a function of time and sweetener used. Hunter "a" greening (-a*) of upper (a), bottom (b), internal (c) and % internal greening (d) of cookies during 24 h of uncovered storage.

Fig. 2. Mass spectrometric fragments of standards (chlorogenic acid-lysine adducts, chlorogenic acid) and cookies made with maple syrup and honey after 24 h of uncovered storage and 7 days under 84% RH condition. TBA: trihydroxy benzacridine derivatives, MRPs: Maillard reaction products fragments.

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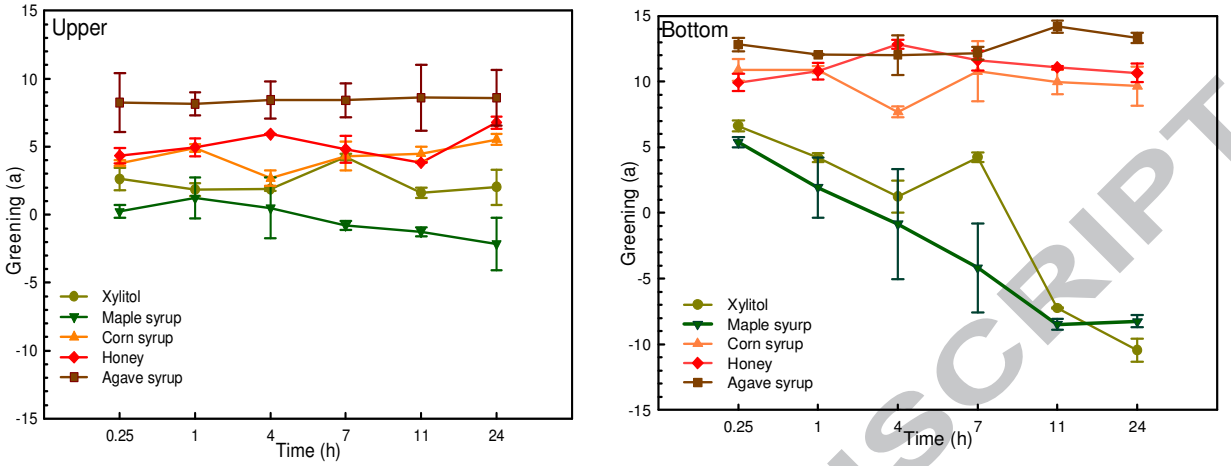
Figures

a) Upper greening in cookies

b) Bottom greening in cookies



c) Internal greening of cookies

d) % Internal greening of cookies

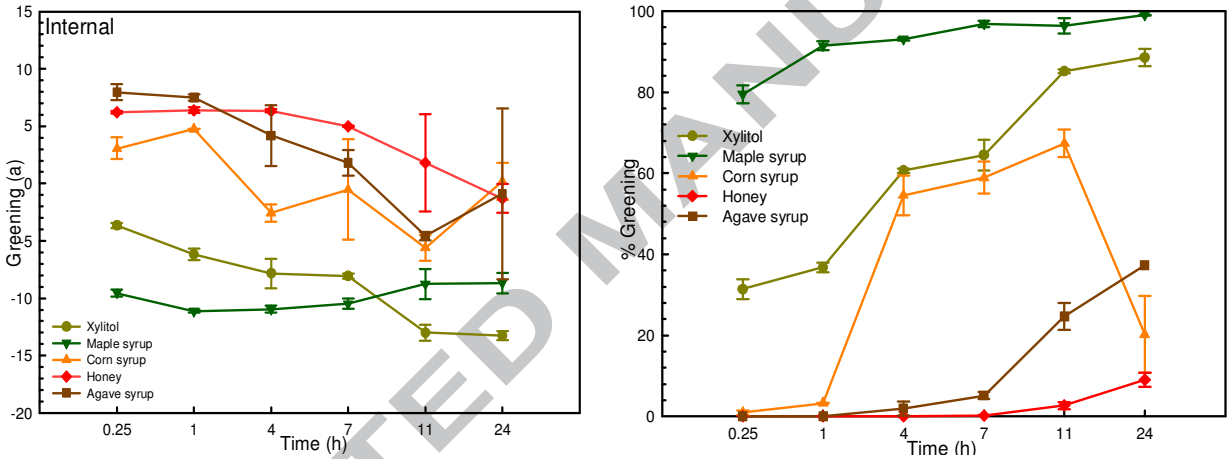


Fig. 1.

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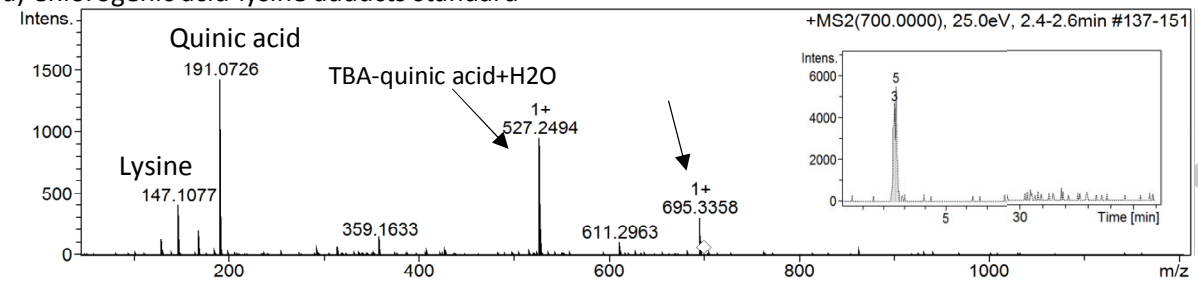
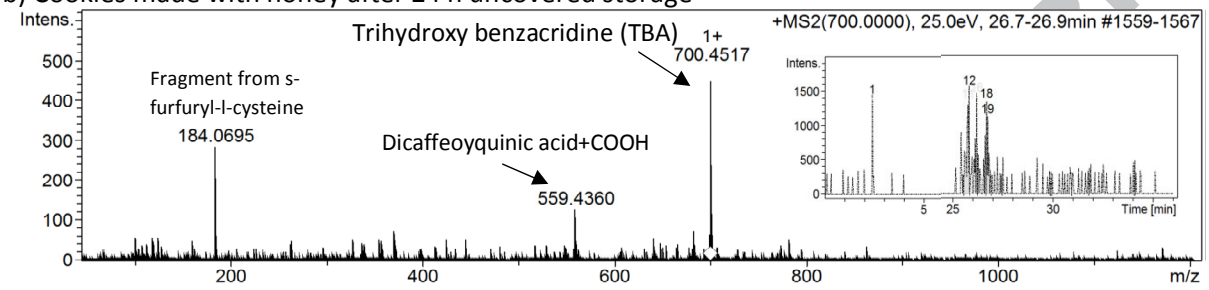
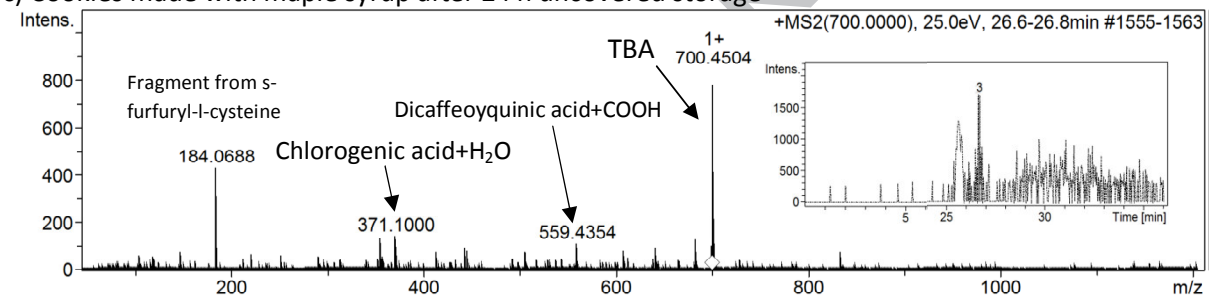
a) Chlorogenic acid-lysine adducts standard



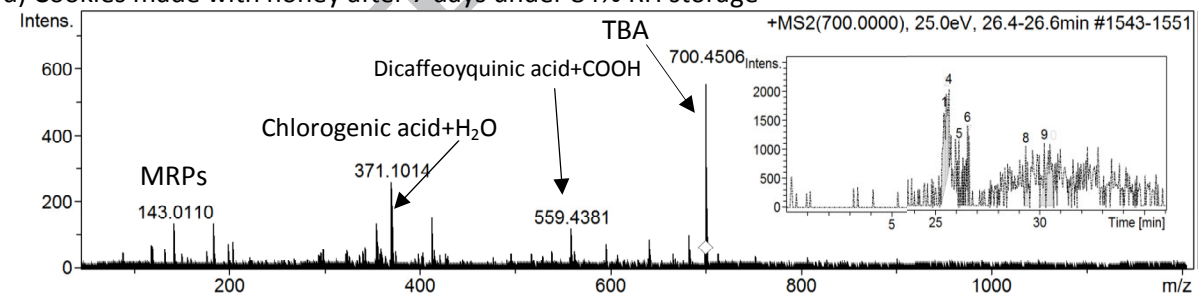
b) Cookies made with honey after 24 h uncovered storage



c) Cookies made with maple syrup after 24 h uncovered storage



d) Cookies made with honey after 7 days under 84% RH storage



e) Cookies made with maple syrup after 7 days under 84% RH storage (TIC+MS2: 700.0000)

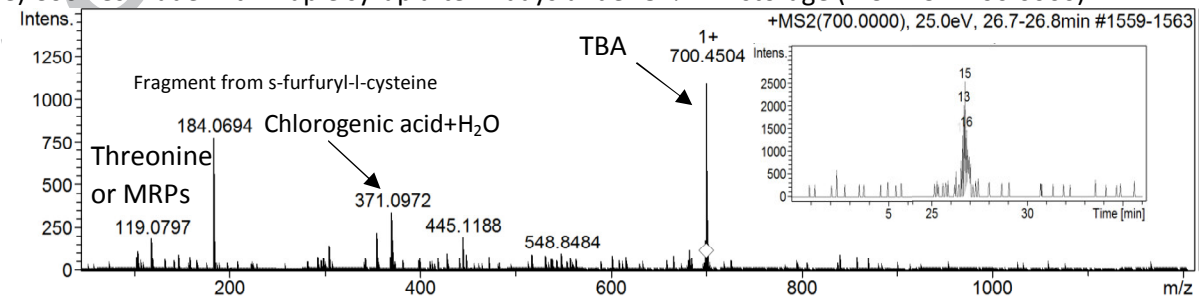


Fig. 2.

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Tables

Table 1. pH, spread factor, moisture and protein content of dough and cookies

Treatment	pH				Spread factor	
	Sweeteners solution†	Dough†	Cookie† (0.25 h)	Cookie† (24 h)	Dough	Cookie (0.25h)
Maple syrup	6.28±0.115[a†]	7.57±0.05[ab]	8.99±0.01[b]	8.94±0.02[ab]	3.05[b]	2.05[b]
Xylitol	6.12±0.045[a]	7.66±0.03[a]	9.13±0.01[a]	9.05±0.02[a]	5.08[a]	2.88[a]
Corn syrup	4.76±0.005[b]	7.62±0.01[ab]	8.69±0.05[c]	8.42±0.02[ab]	5.06[a]	2.59[ab]
Agave syrup	4.49±0.035[c]	7.55±0.03[ab]	8.44±0.04[d]	8.30±0.03[ab]	4.71[a]	3.00[ab]
Honey	3.93±0.035[d]	7.52±0.04[b]	8.63±0.02[c]	8.41±0.05[b]	4.40[a]	2.43[ab]

Treatment	Moisture (%)			Protein content (mg/g wet basis)		
	Sweeteners	Dough	Cookie	Dough	Cookie (0.25h)	Cookie (24h)
Maple syrup	32.40±0.017[a]	19.96±0.00[a]	14.06±0.00[a]	12.20±0.42[b]	11.82±1.11[bc]	26.10±2.93[bc]
Xylitol	12.05±0.000[d]	15.44±0.00[b]	8.97±0.00[d]	11.30±0.18[bc]	15.00±1.25[ab]	35.91±4.25[ab]
Corn syrup	22.06±0.008[b]	18.02±0.00[ab]	11.34±0.00[c]	21.93±1.34[a]	16.51±0.86[a]	37.64±2.03[a]
Agave syrup	23.45±0.015[b]	17.90±0.00[ab]	12.09±0.00[b]	8.95±0.26[c]	9.76±1.13[c]	23.85±0.19[c]
Honey	13.56±0.016[c]	15.49±0.02[b]	11.45±0.00[c]	9.28±0.31[c]	10.49±0.36[c]	34.38±2.08[ab]

Values are the average of four replicates from four different cookies ± standard deviation. The same letters in each column for the same variable are not significantly different (P>0.05).
† Concentrations of sweetener solutions, dough and cookie solutions for pH test were 2, 10, and 10 % (w/v), respectively.

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Table 2. Water activity (a_w) of cookies after baking and storage.

Storage conditions	Sweeteners / Time (h)	Maple syrup	Xylitol	Corn syrup	Agave syrup	Honey
Uncovered	0.25	0.798±0.01^{a}	0.535±0.00^{d}	0.672±0.00^{c}	0.694±0.01^{bc}	0.712±0.01^{b}
	1	0.827±0.01^{a}	0.579±0.01^{c}	0.695±0.02^{b}	0.687±0.00^{b}	0.666±0.02^{b}
	4	0.708±0.01^{a}	0.526±0.00^{c}	0.657±0.02^{b}	0.637±0.01^{b}	0.630±0.01^{b}
	7	0.731±0.00^{a}	0.535±0.01^{d}	0.620±0.01^{c}	0.642±0.00^{b}	0.643±0.00^{b}
	11	0.719±0.01^{a}	0.539±0.00^{c}	0.611±0.00^{b}	0.621±0.02^{b}	0.608±0.00^{b}
	24	0.664±0.00^{a}	0.520±0.01^{d}	0.532±0.01^{cd}	0.597±0.03^{b}	0.583±0.01^{bc}
	Time (day)					
75% RH	1	0.802±0.00^{a}	0.666±0.00^{d}	0.754±0.00^{c}	0.759±0.02^{b}	0.761±0.00^{b}
	4	0.749±0.02^{a}	0.629±0.01^{e}	0.663±0.02^{d}	0.740±0.00^{b}	0.737±0.01^{c}
	7	0.714±0.02^{a}	0.597±0.02^{c}	0.589±0.02^{d}	0.663±0.00^{b}	0.715±0.00^{a}
79% RH	1	0.833±0.00^{a}	0.693±0.01^{d}	0.751±0.02^{c}	0.751±0.01^{c}	0.770±0.01^{b}
	4	0.793±0.01^{a}	0.668±0.00^{e}	0.715±0.02^{d}	0.756±0.01^{b}	0.738±0.01^{c}
	7	0.737±0.04^{a}	0.643±0.03^{d}	0.739±0.02^{a}	0.681±0.02^{b}	0.675±0.01^{c}
84% RH	1	0.815±0.01^{a}	0.708±0.01^{d}	0.810±0.01^{b}	0.745±0.01^{c}	0.746±0.01^{c}
	4	0.794±0.01^{a}	0.695±0.01^{e}	0.756±0.01^{b}	0.722±0.00^{d}	0.727±0.01^{c}
	7	0.741±0.00^{a}	0.695±0.01^{e}	0.737±0.00^{b}	0.719±0.01^{c}	0.714±0.01^{d}

Values are the average of four replicates ± standard deviation.

Same letters in each row are not significantly different (P>0.05).

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Table 3. General linear model effect and interaction of different sweeteners, humidity conditions, and storage time on greening intensity and greening reaction reactants and products

Independent variables		Greening	Chlorogenic acid	Protein	Chlorogenic acid-lysine adducts
Liquid sweeteners (L)	df	4	4	4	4
	ms	669.26	6.27	28.82	2.79
	P	<0.0001	<0.0001	=0.0001	<0.0001
	S	****	****	****	****
Humidity conditions (H)	df	3	3	3	3
	ms	227.05	4.60	1559.81	27.04
	P	<0.0001	<0.0001	<0.0001	<0.0001
	S	****	****	****	****
Time (T)	df	7	1	1	1
	ms	112.14	0.01	1778.86	0.0006
	P	<0.0001	=0.5793	<0.0001	=0.8910
	S	****	*NS*	****	*NS*
Interaction (LxT)	df	28	8	8	8
	ms	33.07	0.35	32.70	0.85
	P	<0.0001	<0.0001	<0.0001	<0.0001
	S	****	****	****	****
Interaction (TxH)	df	4	--	--	--
	ms	14.27			
	P	=0.2687			
	S	*NS*			
Interaction (LxH)	df	12	8	8	8
	ms	14.90	0.45	0.034	0.077
	P	=0.1932	<0.0001	1.0000	=0.0458
	S	*NS*	****	*NS*	*
Interaction (LxHxT)	df	16	--	--	--
	ms	8.307			
	P	=0.7122			
	S	*NS*			

df refers to degrees of freedom, ms refers to mean square, *P* refers to p value, *S* refers to the significant level: **** refers to very significant; * refers to significant (*P*<0.5), *NS* refers to not significant.

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Table 3. Chlorogenic acid (mg/g) and chlorogenic acid-lysine adducts (mg/g) in dough and cookies made with various sweeteners.

Sweeteners	Dough (2 h)	Cookie				
		After baking (0.25 h)	After baking (24 h)	75% RH (7 days)	79% RH (7 days)	84% RH (7 days)
Chlorogenic acid (mg/g) quantified using HPLC						
Maple syrup	2.60±0.046[a]	0.49±0.032[c]	0.28±0.003[c]	0.85±0.098[c]	0.58±0.022[c]	0.37±0.000[c]
Xylitol	2.60±0.068[a]	0.44±0.070[c]	0.34±0.105[c]	0.91±0.003[c]	0.77±0.093[c]	0.78±0.049[b]
Corn syrup	2.65±0.016[a]	0.99±0.048[b]	1.00±0.063[b]	2.31±0.057[b]	1.64±0.247[b]	1.05±0.061[b]
Agave syrup	2.68±0.041[a]	1.39±0.017[a]	1.27±0.065[a]	3.08±0.594[b]	2.40±0.047[a]	1.95±0.205[a]
Honey	2.72±0.037[a]	1.31±0.136[a]	1.45±0.054[a]	4.35±0.028[a]	2.44±0.136[a]	1.88±0.008[a]
Chlorogenic acid-lysine adducts (mg/g) quantified using Absorbance at 680 nm						
Maple syrup	0.40±0.013	2.40±0.054[a]	3.21±0.393[a]	3.71±0.036[a]	4.04±0.151[a]	4.08±0.110[a]
Xylitol	bdl	2.20±0.071[ab]	2.82±0.079[ab]	2.94±0.183[b]	2.83±0.088[b]	3.46±0.141[ab]
Corn syrup	bdl	2.06±0.088[b]	2.00±0.135[bc]	2.26±0.083[c]	2.64±0.040[b]	2.59±0.194[bc]
Agave syrup	bdl	1.56±0.111[c]	1.79±0.191[cd]	2.02±0.160[cd]	1.71±0.109[c]	1.96±0.070[c]
Honey	bdl	1.35±0.172[c]	1.16±0.358[d]	1.72±0.222[d]	2.05±0.621[d]	1.96±0.522[c]

bdl refers to below detection limit.
Values are the average of four replicates from four different cookies ± standard deviation.
Same letters in each column of chlorogenic acid and chlorogenic acid-lysine adducts data are not significantly different (P>0.05).

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