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Sugar Loss Attributed to Non-Enzymatic Browning Corresponds to Reduce Calories Recovered in Low-Molecular-Weight Fraction

Ningjian Liang[1], Xiu-Min Chen[2] and David D Kitts[1*]

[1]*Food, Nutrition and Health Faculty of Land and Food Systems, University of British Columbia, Canada*
[2]*School of Food and Biological Engineering, Jiangsu University, P.R. China*

Abstract

Health agencies state that total dietary energy intake should not exceed a maximal of 25% of calories derived from added sugars. Bakery products are major food sources that contribute to the added sugars intake; however potential sugars losses due to Maillard reaction and caramelization, occur at typical baking temperatures. In this study we employed markers associated with non-enzymatic browning, that corresponded to loss of free sugars, generation of α-dicarbonyl compounds with changes in caloric content of digestible constituents in sugar-amino acid model and cake formulations. Sugars losses in simple model systems that reached 100 percent after 40 min baking at baking temperatures of 150°C and 180°C corresponded to reduced calorie content, in fractions with a MW<3000 (p<0.05). In comparison, less gross energy calories from sucrose were lost after a similar heat treatment in sucrose-amino acid mixtures. Model cakes baked at 150°C and 180°C, respectively followed this trend with invert sugar, having greater losses losses (p<0.01) than cakes containing sucrose. We conclude that thermal temperatures typical of baking that result in non-enzymatic browning reactions, reduce both the total sugars and corresponding calories due to conversation to non-bioavailable high molecular weight browning products.

Keywords: Added sugars; Non-enzymatic browning; Calories

Introduction

Carbohydrates are important nutrients in the human diet that contribute to energy metabolism, but there is relatively less recognition that sugars also provide many functional properties in foods that are vital for preservation and organoleptic appeal; central to ensure food quality [1]. The term sugars refer to monosaccharides (e.g. glucose, fructose and galactose) and disaccharides (sucrose, lactose, maltose). Sugar, on the hand refers specifically to sucrose (99.8%), derived from sugar cane or sugar beets. Added sugar is defined as those sugars, sweeteners and syrups that are eaten as such, or used as ingredients in processed and prepared foods [2,3]. A diet high in added sugars has been linked to a variety of health problems, including obesity [4], cardiovascular disease [5] and type 2 diabetes [6]. Despite the causal relationship reported between total and added sugars intake and a variety of chronic diseases, there exists some uncertainties as to the accuracy of these conclusions [7,8]. Research prioritization for studies that link dietary sugars with potentially related health outcomes continues to be an important area of investigation [9].

Health agencies state that total dietary energy should not exceed 25% of calories that are derived from added sugars. Recent estimates are that added sugars average 11%-13% of total energy intake in the Canadian diet [10]. The content of sugars in foods and related calories that appear on nutrition food labels are often derived from the summation of recipe ingredients rather than from actual measurements after processing [11]. Heating is commonly used in many food processing and preparation systems and chemical reactions result with added or naturally containing sugars transformed to non-enzymatic browning products by way of the Maillard reaction or caramelization. These reactions are potentially relevant in accurately reporting both the amount of sugar, and related calories which remain available for metabolism.

Bakery products are a major source of added sugars. Table sugar (sucrose) and invert sugars (glucose and fructose in equal proportions) are commonly used in bakery products. Inverted sugars have a lower tendency to crystallize compared to sucrose and therefore is used in food industry to minimize crystallization [12]. However, these reducing sugars, e.g. glucose and fructose react with amino acids when heated and thus participate in non-enzymatic Maillard browning reactions. These reactions generate components important for the taste, aroma, texture and appearance qualities of baked goods. The reactions also occur at the cost of losses of bioavailability of both reducing sugars and some amino acids. Baking times and temperatures are similarly important for caramelization of non-reducing (sucrose) and reducing sugars to produce brown color polymers such as caramelans, caramelens, and caramelins, as well as volatile chemicals which produce characteristic caramel flavor. Previous studies have demonstrated that beneficial properties, such as antioxidant capacity of browning products and generation of reactive α-dicarbonyl compounds parallel a reduction of free sugars in simple sugars-amino acid models heated at temperatures commonly used for baking [13,14]. In this study, we measured a number of chemical indices associated with the formation of non-enzymatic browning products in a model bakery product (e.g. cake formulation) using invert sugar as the added sugars compared to cakes with sucrose as the added sugar. We relate, our findings on markers of browning with changes in caloric density of the recovered available digestible energy remaining in the heated product.

Materials and Methods

Materials

Glucose (>99.5%), fructose (>99%), sucrose (>99%); lysine, glycine glyoxal (40% in water), methylglyoxal (40% in water), 3,4-hexanedione

***Corresponding author:** David D. Kitts, Food, Nutrition and Health Faculty of Land and Food Systems, University of British Columbia, 2205 East Mall, Vancouver, BC, V6T 1Z4, Canada, Tel: 6048225560; E-mail: ddkitts@mail.ubc.ca

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(>95%), 2,3-diaminonaphthalene, Trolox, pepsin, trypsin and chymotrypsin were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucosone (>98%) and 3-deoxyglucosone (>75%) were purchased from Toronto Research Chemicals (Ontario, Canada). Ultrapure water was obtained by using a Serial Mili-Q system form Milipore. Other chemicals were all analytical grade unless stated otherwise.

Preparation of sugar-amino acid baking models

An equal-molar glucose, fructose, sucrose and amino acid (lysine and glycine) mixture was baked in an oven (IVP 8580, Inglis® Home Appliances, ON, Canada) at temperatures of 150°C and 180°C, respectively, as reported previously [13,14]. Samples were then free-dried and ground into powder using a mortar and pestle and stored at 4°C for further analysis. Each model consisted of three batches and subsamples were taken for analysis of sugar loss and gross energy in triplicate.

Preparation of model cakes

Two model cakes were made in this study. The formulation of model cake A was: 80.0 g invert sugar, 90.0 wheat flour, 1.5 g baking soda, 50.0 g egg, 40 mL sunflower oil and 30 mL of water. The formulation of model cake B was the same as model cake A, except that 80.0 g of invert sugar was used to replace the 80 g sucrose. The ingredients were thoroughly mixed, and the dough was divided into three batches prepared in a Muffin Pan (4 cm internal diameter and 3 cm height) to give 40 g of dough per mug. Cake dough containing either sucrose or invert sugar were oven baked (IVP 8580, Inglis® Home Appliances, ON, Canada) at 150°C for 40 min, or at 180°C for 30 min.

Measurement of the color characteristics

Color differences were determined using the Hunter Labscan 600 spectrocolorimeter, using luminosity or brightness (L^*=0 black and L^*=100 white), a^* (red-green: negative value represents greenness and positive value represents redness) and b^* (yellow-blue: negative value represents blueness and positive value represents yellowness). The color difference between a processed sample and a reference was expressed as ΔE (Equation (1), according to [14].

$$\Delta E = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2]^{0.5} \quad \text{Equation (1)}$$

L^*, a^*, and b^* are values of a processed sample and L^*_0, a^*_0, and b^*_0 are values of a reference sample, which is the unbaked dough.

In vitro digestion of model and cake samples

Model sugar-amino acid and freeze-dried cake samples were mixed and suspended in deionized water at a concentration of 20 mg/mL to conduct the *in vitro* digestibility analysis [15]. Suspensions were adjusted to pH 2 and a two-step proteolysis procedure was initiated using pepsin (porcine stomach mucosa; EC 3.4.23.1;) added to sample (sample: enzyme ratio = 25:1) for 1 h, using a shaker (100 rpm) held at 37°C. The mixture was then adjusted to pH 8 using $NaHCO_3$ and re-incubated with a trypsin chymotrypsin mixture at a sample to enzyme ratio of 200:1 for another 2 h incubation, held at 37°C. After digestion, samples were boiled to inactivate enzymes.

Analysis of sugar content

The sugar present in amino acid-sugar model products was determined by methods reported previously [13,14]. Standard calibration curves for glucose, fructose and sucrose were constructed with a concentration range consisting of 0.625 to 20 mg/mL the sugar content in cake samples was analyzed by HPLC after performing sample clean-up. Briefly, water was added to 5 g of ground cake powder and 1 g of ribose, as the internal standard, was added to form a mixture having a total volume of 33.3 ml. The mixture was vortexed, centrifuged at 1600´ g for 10 min and the supernatant collected; this extraction was repeated two more times (2×33.3 mL) with water. A 10 mL aliquot was recovered from the pooled supernatant and fractionated by size exclusion using a 3000 Molecular Weight Cut Off regenerated cellulose membrane (Millipore Corporation, Billerica, MA, USA). The portion with <3000 MW was collected and prepared for solid phase extraction. Superlclean™ LC-18 SPE tube (Suplelco Analytical, Bellefonte, PA, USA), first conditioned with 2 ml of acetonitrile and then 2 ml of water. The sample was passed through the cartridge tube and collected for sugar analysis using the HPLC conditions described above.

Preparation of α-dicarbonyl derivatives

α-dicarbonyl compounds recovered from sugar-amino acid models and baking models were first derivatized with 2,3-diaminonaphthalene (DAN) as described previously [16]. Briefly, 1 mL of 10 mg/mL lyophilized sample mixtures, or varying concentrations of standards glyoxal (GO), methylglyoxal (MGO), glucosone and 3-deoxylgucosone (3-DG) dissolved in 10 mmol/L phosphate buffer (pH 7.4) were incubated with DAN (50 µL; 2 mg/mL) in the presence of 25 µL of 0.001% 3,4-hexanedione at 4°C for 24 h. HD was used as an internal standard.

The extraction procedures used for α-dicarbonyl recovery in cakes were as follows: 2 g of cake powder was mixed with 10 ml phosphate buffer (10 mM, pH 7.4) containing 0.001% HD as internal standard. The mixture was vortex at highest speed for 10 min followed by centrifugation at 4000 x g for 10 min. The supernatant was collected, and the pellet was extracted two more times with 2 × 15 ml phosphate buffer (10 mM, pH=7.4). The supernatant from 3 times extraction was pulled together and 15 ml transferred to a 3000 Molecular Weight Cut Off regenerated cellulose membrane tube (Millipore Corporation, Billerica, MA) and centrifuged at 4000 g for 30 min at 25°C. The portion with <3000 MW was collected and underwent derivatization. Briefly, 1 mL collected liquid, or varying concentrations of standards GO, MGO, glucosone and 3-DG dissolved or internal standard [16] in phosphate buffer (10 mM, pH=7.4) were incubated with DAN (50 µL; 2 mg/mL) at 4°C for 24 h.

Quantification of α-dicarbonyl compounds

α-dicarbonyl benzoquinoxaline derivatives in baking model and cakes were quantified using Agilent 1100 series HPLC system equipped with a Sphereclone ODS (2) column (5 µm, 4.6 mm × 150 mm) and fluorescence detector (Ex at 267 nm and Em at 503 nm). The column temperature was controlled at 30°C. The mobile phase A was 0.2% formic acid and mobile phase B was 100% acetonitrile. The sample was eluted with a gradient fluid of 28%-45% B from 0 to 13 min, 45%-85% B from 13 to 20 min, 85%-18% B from 20 to 23 min, and kept at 18% from 23 to 28 min at a flow rate of 1 ml/min. The injection volume was 10 µL. Calibration curves of known standard benzoquinoxaline derivatives were used to calculate the α-dicarbonyl content in the sugar-amino acid model and cake samples. The amount of α-dicarbonyl compounds was expressed as microgram/g dry matter (d.m.).

Measurement of calories (gross energy)

Gross energy of sugar-amino acid model samples and digested cake model samples were measured using an IKA Oxygen Bomb Calorimeter. Initial cake samples, powdered extracts of crude digestion

and recovered MW<3000 fractions, respectively, were pressed into a tablet to a final weight of 1.0 gram. An igniter wire was used to carry the flame to the tablet and the vessel was filled with oxygen. The sealed bomb was placed in a water bath with temperatures recorded before and after ignition. The rise in water temperature after ignition was used to calculate the gross energy of the sample. Benzoic acid (having heat of combustion 6319 cal/g) was used as a standard material to test the accuracy of the Bomb Calorimeter every day before running samples.

Statistical analysis

Data were subjected to one-way variance analysis (one-way ANOVA), and post-hoc analysis using the Tukey's test, with a 95% confidence interval for the comparison of the averages (SPSS 19.0, LEAD Technologies, Inc., Chicago, IL, USA).

Results and Discussion

Caramelization and Maillard reactions are examples of non-enzymatic browning that collectively result in loss of sugar(s); however, the extent that non-enzymatic browning equates to reduction of available calories from sugars has not fully been appreciated. Sucrose exposed to high temperature treatments will hydrolyze into glucose and fructose, which further participates in both caramelization and the Maillard reaction; the latter dominates when amino acids are also present. Formation of browning pigments occurs during the final stages of sugar degradation during baking. Figure 1a shows the percentage of sugar losses in sugar-amino acid mixtures, at 150°C and 180°C, respectively which approached or reached 100% after 40 min heating compared to only 21.53 ± 3.28% for the sucrose-amino acid mixture when heating at 180°C. The relative degree of sugars lost was also interestingly affected by the type of amino acid present in the simple sugar-amino acaid mixture. A similar result has been reported in a study on the reactivity of glucose, fructose compared to sucrose with glycine (1:1 molar ratio) at 60°C in aqueous solution [17] and also at higher temperatures [16], confirming that sucrose takes longer than fructose or glucose to react with amino acids when heated. Explanation for this is given by the different melting points of D-glucose, D-fructose, and D-sucrose at specific heating rate (1°C min^{-1}); which are 146°C, 103°C, and 179°C, respectively [18]. Glucose and fructose will melt quickly at 150°C and then react with amino acid faster than sucrose, which is poorly melted at 150°C and mostly remains in the system. At higher temperature (e.g. 185°C) the glycosidic linkage connecting glucose with fructose in sucrose will break producing free reducing monosaccharide [19]. This degradation product participates in the Maillard reaction with amino acids at thermal conditions typical of baking. Both the rate and degree of loss of sucrose compared to glucose and fructose can be explained by the thermal conditions where sucrose is first hydrolyzed to corresponding monosaccharide reducing sugars.

In the present study, the sugar retained in cakes made from invert sugar compared to those made from the same amount of sucrose revealed that 17.94 ± 8.19 and 21.74 ± 8.19% of invert sugar in model cake A was lost after baking at 150°C and 180°C, respectively (Figure 1b). In comparison, cakes containing sucrose lost only 6.39 ± 2.54%, when baked at 180°C. Hence, cakes formulated with invert sugar contained less sugar after baking compared to those containing the same amount of sucrose.

We expressed color changes in model cakes made from sucrose and invert sugar using the Total Color Difference (ΔE), which is the overall color difference of a heated sample compared to an unprocessed sample (Figure 2). In simple sugar-amino acid model systems, greater ΔE values

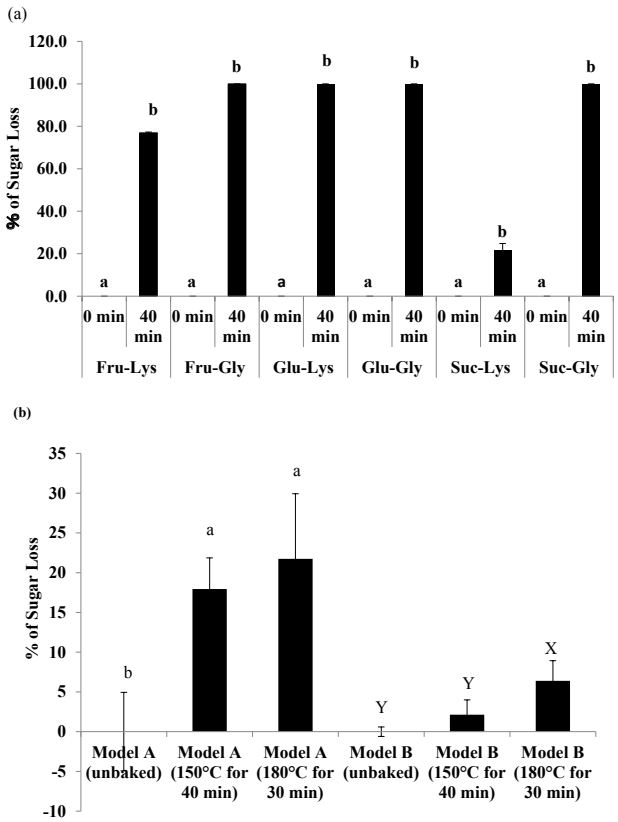

Figure 1a: Percentage (%) of sugar loss in Fru-Lys, Fru-Gly, Glu-Lys, Glu-Gly, Suc-Lys, Suc-Gly models baked at 180°C for 0-40 min. Data are shown as mean ± standard deviation from three independent experiments performed in triplicate. Different letters in this figure indicate significant ($p < 0.05$) differences in % of sugar loss between 0 min and 40 min within the same sugar-amino acid model by using Tukey's test.

Figure 1b: Percent of sugar loss during baking process in cake Model A (with invert sugar) and cake Model B (with sucrose) before and after baking at two different conditions. Values are shown as mean ± standard deviation from three independent experiments performed in triplicate. Different letters in this table indicate significant ($p<0.05$) differences in % of sugar within the same model before and after baking loss by using Tukey's test.

for reducing sugars-amino acid mixtures were obtained for both glucose and lysine compared to the non-reducing sucrose-amino acid mixture at both 150°C and 180°C [13]. Cakes made from invert sugar in this study also yielded significantly higher ΔE values than those made with sucrose, denoting greater production of browning. At high baking temperatures, sucrose first undergoes pyrolysis to participate in caramelization, or, alternatively at lower pH conditions hydrolyzes to yield glucose and fructose; the latter in the presence of amine groups reacts in Maillard reaction [20]. Hence, to some degree both caramelization and Maillard reaction are contributing to total browning.

Prior to the formation of brown pigments, heat degradation of sugar(s) results in the formation of hydroxymethylfurfural (HMF), [21,22]. The relatively lower sucrose loss obtained in our baked cakes, compared to invert sugars equates with the corresponding ΔE values and refers to the hydrolysis step that precedes sucrose inversion into monosaccharides and subsequent conversion to HMF. A color/HMF relationship exists which explains the kinetics and formation mechanisms of HMF [23]. This transformation is another example of loss of sugar derived calories that otherwise could be used for energy metabolism.

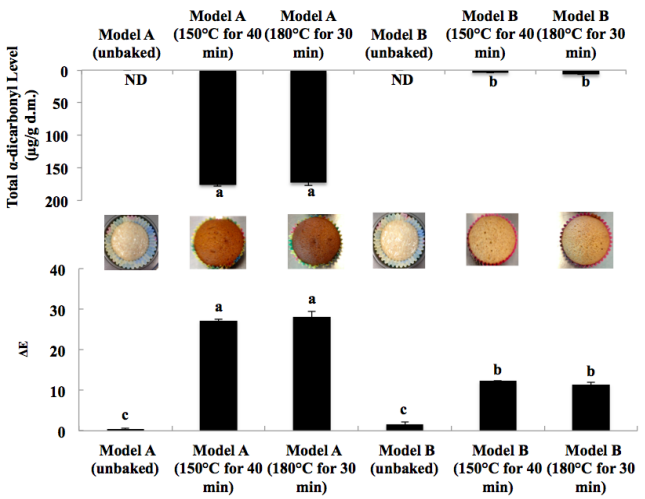

Figure 2: Total α-dicarbonyl compounds level and Color Difference (ΔE) of cake model A (with invert sugar) and cake model B (with sucrose) before and after baking at two different conditions. Data are shown as mean ± standard deviation from three independent experiments performed in triplicate. Different lower-case letters in this figure indicate significant differences (p<0.05) in the value of ΔE by using Tukey's test.

We measured the presence of α-dicarbonyl compounds in cakes since they are formed during Maillard reaction or are produced from degradation of monosaccharide by dehydration or oxidation process; typical for heat treated foods, such as cookies [24], honey [25] and coffee [26]. These compounds also represent important precursors for the formation of brown pigments and flavor in foods that result from Maillard reaction products. We recovered three α-dicarbonyl compounds (e.g. glucosone, 3-deoxylglucosone, and methylglyoxal) in the baked cake models (Figure 2), compared to four (e.g. glucosone, 3-deoxylglucosone, glyoxal, and methylglyoxal) reported previously in sugar-amino acid models [13]. In our former study, detectable concentrations of α-dicarbonyl compounds were observed as early as 5 minutes in Glu-Lys and Fru-Lys models heated at 150°C and 180°C, respectively, contrasted a significantly (p<0.05) lesser amount of α-dicarbonyl compounds generated from the Suc-Lys model observed early on with baking. However, a dramatic increase in α-dicarbonyl compounds occurred after 10 minutes heating. α-dicarbonyl compounds are key intermediates for the formation of carbohydrate-based melanoidins [27,28]. The content of α-dicarbonyl compounds in cakes made with sucrose (e.g. Model B) were found to be less than cakes containing invert sugar (Cake Model A). In model cake A, glucosone, methylglyoxal, and 3-deoxylglucosone were detected after baking at 180°C for 30 min, yielding 173 ± 4 μg/g of total α-dicarbonyl compounds, among which, glucosone and methylglyoxal accounted for 79 ± 2 μg/g and 34 μg/g, respectively. Previous studies have reported glucosone content in commercial cookies made using sucrose ranged from 4.8 to 26 μg/g and methylglyoxal content ranged from 3.7 to 81.4 μg/g [24]. In baked cake A, we also detected 3-deoxylglucosone content (60 ± 1 μg/g) at a concentration that was higher than that recovered from sucrose formulated cake (e.g. 7 ± 0 μg/g) when baking was done at 180°C for 30 min.

The gross energy contained in a food is defined as the heat produced from unit sample when it is completely combusted in a bomb calorimeter. Substances that are not digested and absorbed across the entire length of the gastrointestinal tract will not be available for metabolism, hence the calories associated with those substances are potentially lost. For the purpose of defining energy metabolism, digestible energy refers to the proportion of gross energy that is absorbed and is the difference between combustible or gross energy present in the food item, corrected for what is not available for metabolism, e.g. that amount lost from non-digestible components. In order to answer the question as to whether the loss of sugar will lead to a decreased energy density, thus reflecting a reduced total caloric content, we measured the caloric content in separate experiments both before and after heat treatment of sugars-amino acid models, or in the baked cakes. To account for this, we separated fractions following digestion that contained a molecular weight (MW) lower than 3000Da from those that had a MW>3000Da in each model sample. This enabled us to obtain an estimate of digestible energy from components such as sugars that had low molecular weight and thus were bioavailable, by correcting the energy in this fraction with energy contained in the MW<3000 per gram of sugar-amino model sample. The energy or caloric density from digested extracts derived from sugar-amino acid models before baking (0 min) and after baking at 180°C is presented in Figure 3. The results show significant losses in energy occurred in the MW<3000 Da fraction for every gram of sample after baking at 180°C, in reducing sugar-amino acid models compared to non-reducing sugar models. This result corresponded to the relative amount of sugar lost in these same model systems.

In relationship to these observations we determined that the energy of model cakes ranged from 5281 to 5575 Cal/g of dry weight (Figure 4), with no significant difference between model cake A (with invert sugar) and mode cake B (with sucrose). Following baking, the digestion of cakes with digestive enzymes and separation of fractions with low molecular weight (<3000 Da) from the portion with MW>3000 Da, enabled the measurement of energy density of the digestible portion of each cake mix. The energy of the aqueous portion with MW<3000 Da in every gram of cake Model A (with invert sugar) and cake Model B (with sucrose) after artificial digestion is shown in Figure 4. The energy from the portion with MW<3000 Da in every gram of cake powder decreased significantly after baking in model cake A and B. In cake A (with invert sugar), the energy from the portion with MW<3000 Da decreased around 36% after baking at 180°C, compared to cake B (with sucrose), where the energy from the MW<3000 Da fraction was lower, or about 12% after baking at 180°C. A significant negative correlation (r) between the % of sugar loss and the calorie content from the portion with MW<3000 Da (-0.79: p<0.001) was obtained from baked model cakes indicates that the loss of sugar contributed to the decrease in available energy from sugar.

Extrapolating our *in vitro* data to the human condition would not be possible without mentioning a potential role for the gut microbiome on releasing energy available to the host from non-digestible, browning products which enter the large intestine. The energy density of sugars absorbed from the small intestine, and which are not excreted in the urine provides approximately 4 Kcal/g after metabolism. Sugars reaching the large intestine intact and fermented will lose approximately 50% energy calories, some going to fermentation products as well as growth of bacteria, which in turn release the energy as heat, an event termed specific dynamic action. Moreover, subjects fed sugar containing foods that are thermally processed show a significant correlation between HMF ingestion and HMF urinary excretion [29], which is another source of irreversible loss of sugar after ingestion. Moreover, dicarbonyl and furfural compounds generated from sugar degradation also derive aldo-condenstation products that eventually lead to high molecular weight melanoidins. The fermentation capacity

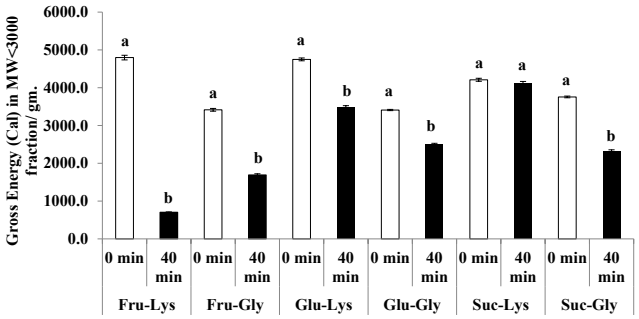

Figure 3: Gross energy (Calorie) from the portion of compounds with MW<3000 in every gram of the powder of models (Fru-Lys, Fru-Gly, Glu-Lys, Glu-Gly, Suc-Lys, Suc-Gly) without baking (0 min) and after baking at 180°C for 40 min. Values are shown as mean ± standard deviation from three independent experiments performed in triplicate. Different letters in this figure indicate significant ($p < 0.05$) differences in gross energy between 0 min and 40 min within the same sugar-amino acid model by using Tukey's test.

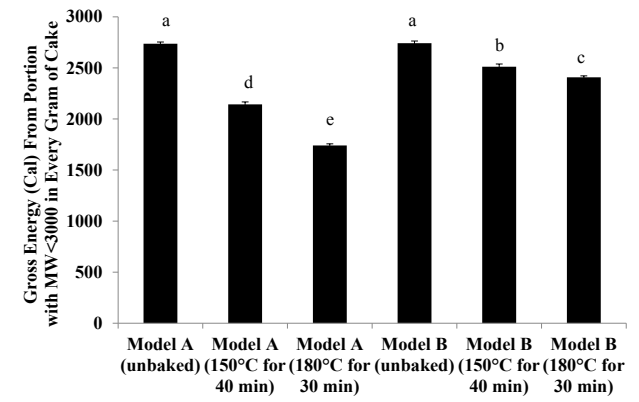

Figure 4: Gross energy (Calorie) from the portion of compounds with MW<3000 Da in every gram of cake Model A (with invert sugar) and cake Mode B (with sucrose) before and after baking at two different conditions after freeze-drying. Values are shown as mean ± standard deviation from three independent experiments performed in triplicate. Different lower-case letters in this table indicate significant ($p<0.05$) differences in gross energy from portion with MW<3000 in every gram of cake powder by using Tukey's test.

of these compounds is not completely known and there is suggestion that some Maillard reaction products have antimicrobial potential that would limit the amount of energy made available to the host [30]. More studies are needed to determine to what extent low and intermediate molecular weight melanoidins may contribute to the overall metabolic energy made available to consumers from sugar due to this symbiotic relationship.

Conclusion

In conclusion, thermal conditions used to produce bakery products result in non-enzymatic browning reactions that lead to significant reductions in content of sugars and potentially those related calories available for absorption and metabolism. Our data indicates that the intake of calories from added sugars from bakery products available for metabolism, especially products made using invert sugar, could be overestimated if the influence of the non-enzymatic browning reactions is not considered. The changes in color and generation of α-dicarbonyl compounds in model cakes confirmed that non-enzymatic browning takes place in baked goods depending on the sugar source. The reduced availability of sugars and calories from non-digestible high molecular weight fractions misleads the energy density information from sugars given on the food label. This was shown to more so the case with invert sugar, a mixture of reducing glucose and fructose, than sucrose a non-reducing disaccharide. Future whole-body digestibility and energy studies are in order to establish the significance of these findings especially on caloric control and glycemic responses attributed to dietary sugar intakes from baked products.

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