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# Formation and elimination reactions of 5-hydroxymethylfurfural during *in vitro* digestion of biscuits

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ABSTRACT

This study investigated the possible formation and elimination reactions of 5-hydroxymethylfurfural (HMF) with amino and sulfhydryl groups in commercial biscuits during simulated *in vitro* gastrointestinal digestion. At the end of gastric phase, significant increase was observed in HMF contents of biscuits ($p < 0.05$). By high-resolution mass spectrometry (HRMS) analysis, it was confirmed that sugar dehydration products such as 3-deoxyglucosone and 3,4-dideoxyglucosone accumulated in biscuits during baking were converted to HMF under gastric conditions. However, reactions of HMF with amino acids proceeded with the progress of digestion. HRMS analysis in both HMF-amino acid model systems and in biscuits confirmed that formed HMF reacted with amino and sulfhydryl groups through Michael type addition. In addition, formation of Schiff base during intestinal phases led to a significant decrease in the concentrations of HMF ($p < 0.05$).

## 1. Introduction

5-Hydroxymethylfurfural (HMF) is a furanic aldehyde that is formed in food during thermal process *via* the Maillard reaction and from dehydration of sugars under acidic conditions (Ames, 1992; Kroh, 1994). Concentration of HMF in foods is mainly related with thermal process applied thus it is used as a marker for monitoring the thermal process (Ibarz, Pagan, & Garza, 2000; Morales & Jimenez-Perez, 2001; Rada-Mendoza, Sanz, Olano, & Villamiel, 2004). Coffee, dried fruits, caramel, bread and bakery products are the most important sources of dietary HMF (Murkovic & Pichler, 2006; Ramirez-Jimenez, Garcia-Villanova, & Guerra-Hernandez, 2000). Cookies from bakery products were reported to be containing 0.5–74.5 mg/kg of HMF depending on heating load applied (Ameur, Mathieu, Lalanne, Trystram, & Birlouez-Aragon, 2007). Twice baked biscuits having excessive thermal treatment may have higher amounts of HMF.

According to the data reported in literature, it is still not clear if human exposure to HMF possesses potential health risks. It was reported that HMF was rapidly absorbed in gastrointestinal tract when rats were orally fed by different concentrations of HMF (0.08–500 mg/kg body mass) (Capuano & Fogliano, 2011). Delgado-Andrade, Seiquer, Navarro, and Morales (2008) reported that both absorption and transport of HMF was promoted while the cells were subjected to higher concentrations of HMF. The results from these studies suggest low or no mutagenic effect of HMF. But the major concern about dietary HMF is based on its conversion to 5-sulfoxymethyfurfural (SMF) (Capuano & Fogliano, 2011). Mutagenicity or genotoxicity of SMF, which is converted from HMF, was confirmed by both *in vitro* and *in vivo* studies. In a recent *in vivo* study carried out with animals and humans, it was reported that HMF is metabolized to highly electrophilic SMF that reacts with DNA. They also confirmed the formation of DNA adducts of SMF *in vivo* (De la Cueva et al., 2017). SMF has also been reported to initiate tumor formation in mice skin (Lee, Shlyankevich, Jeong, Douglas, & Surh, 1995; Surh & Tannenbaum, 1994). Moreover, HMF can be converted to acrylamide and furan through Maillard reaction, which are classified as “possibly carcinogenic to humans” (Cai et al., 2014; Gokmen, Kocadagli, Goncuoglu, & Mogol, 2012; Mesias-Garcia, Guerra-Hernandez, & Garcia-Villanova, 2010).

To date, limited and controversial data about the fate of HMF during digestion was reported. Delgado-Andrade et al. (2008) studied the bioavailability of HMF in breakfast cereals and reported that some part of HMF retained in the non-soluble fraction after digestion affecting its availability depending on food matrix. They also reported that HMF degraded to some extent during gastrointestinal digestion. On the other hand, Rufian-Henares and Delgado-Andrade (2009) reported that most part of the Maillard reaction products and HMF showed stability and resistance to *in vitro* digestion. Since HMF contains a reactive carbonyl group that might be involved in interactions with amino and sulfhydryl groups, reactions of HMF during gastrointestinal tract is of importance. This study aimed to monitor the changes in HMF content of biscuits due

*Abbreviations:* HMF, 5-hydroxymethylfurfural; HRMS, high-resolution mass spectrometry

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to reactions of HMF with amino and sulfhydryl groups during *in vitro* multi-step enzymatic digestion.

## 2. Materials and methods

### 2.1. Chemicals and consumables

5-Hydroxymethyl furfural (HMF), L-cysteine, L-lysine were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium chloride, sodium chloride, magnesium chloride, ammonium bicarbonate, and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). The enzymes: pepsin (≥ 250 U/mg solid) from porcine gastric mucosa, pancreatin (4 × USP) from porcine pancreas, protease from *Streptomyces griseus* (≥ 3.5 U/mg solid) and Viscozyme L, bile extract, porcine were also purchased from Sigma Aldrich (Deisenhofen, Germany). Formic acid (98%), methanol and acetonitrile were from JT Baker (Deventer, Holland). Potassium hexacyanoferrate, zinc sulfate, disodium hydrogen phosphate anhydrous, and sodium dihydrogen phosphate dihydrate were purchased from Merck (Darmstadt, Germany). The Carrez I and Carrez II solutions were prepared by dissolving 15 g of potassium hexacyanoferrate and 30 g of zinc sulfate in 100 mL of water, respectively.

Syringe filters (nylon, 0.45 μm), Oasis HLB (1 mL, 30 mg) solid-phase extraction cartridges, Atlantis Hypersil-gold column (100 mm 2.1 mm i.d., 1.9 mm) and Atlantis dC18 (250 × 4.6 mm, 5 μm) column were supplied by Waters (Milford, MA).

### 2.2. Preparation of biscuits

Commercial biscuits including regular, twice baked and baby biscuits were used to determine the fate of HMF during *in vitro* digestion. These biscuits were chosen since they contain sugar degradation products that are formed during baking process. Even the consumers from all ages consume baby biscuits, they are really important due to higher consumption by babies and kids (ages ranging between 6 months and 7 years). In addition, twice-baked biscuits are highly heat-treated forms of regular biscuits. For these reasons, they were obtained from a local market and subjected to *in vitro* digestion. Main nutritional ingredients were given as 75.7 g carbohydrate (24.5 g sugar), 5.7 g protein and 11.4 g fat for 100 g regular biscuits. These values were given as 79.9 carbohydrate (22.9 g sugar), 7.4 g protein and 9.6 g fat for 100 g twice-baked and 74.1 carbohydrate (24.1 g sugar), 4.6 g protein and 14.9 g fat for 100 g baby biscuits. Biscuit samples were ground and freeze dried prior to the digestion process. In addition, they were analyzed to determine their initial HMF contents.

### 2.3. Preparation of model systems

Different model systems composed of HMF namely “HMF”, HMF and lysine “HMF-LYSINE”, HMF and cysteine “HMF-CYSTEINE” were prepared to determine the fate of HMF and its interactions with different amino acids during *in vitro* digestion. For the model systems, 10 μmol of each reactant were dissolved in 10 mL of deionized water, and directly subjected to digestion process.

To test the effect of higher amounts of cysteine on interactions between cysteine and HMF, model system containing 70 μmol of cysteine with 10 μmol of HMF (HMF-7CYSTEINE) was subjected to *in vitro* digestion.

For the subtraction of the interaction with the enzymatic proteins (HMF w/o ENZYMES), 10 μmol of HMF were dissolved in 10 mL of deionized water. They were not subjected to enzyme addition but the digestion procedure was also applied with the pH adjustment and the addition of simulating juices.

**Table 1**
Preparation of the digestion fluids.

| Solution | SSF | | SGF | | SDF | |
|---|---|---|---|---|---|---|
| | pH = 7.0 | | pH = 3.0 | | pH = 7.0 | |
| | Volume (mL) | Stock (g/L) | Volume (mL) | Stock (g/L) | Volume (mL) | Stock (g/L) |
| KCl | 10 | 46.7 | 28 | 46.7 | 5.4 | 46.7 |
| $KH_2PO_4$ | 20 | 68 | 0.9 | 68 | 0.8 | 68 |
| $NaHCO_3$ | 4 | 84 | 6.5 | 168 | 42.5 | 42.5 |
| NaCl | 1 | 120 | 10 | 120 | 8 | 120 |
| $MgCl_2$ $(H_2O)_6$ | 1 | 30 | 2 | 30 | 1.1 | 30 |

### 2.4. In vitro digestion process

An *in vitro* multi-step digestion procedure consisting oral, gastric, duodenal and colon phases was used. Concentrations of enzyme solutions were adapted from the procedure reported by Papillo, Vitaglione, Graziani, Gokmen, and Fogliano (2014). Digestion fluids simulating the saliva (simulated salivary fluid, SSF), gastric juice (simulated gastric fluid, SGF) and duodenal juice (simulated duodenal fluid, SDF) were used to mimic the conditions of gastrointestinal tract. Preparation of these digestion fluids is given in Table 1. 5 g of dry ground biscuit or 10 mL of the model system were transferred to a glass flask with screw cap. For biscuit samples, 5 mL of SSF was added and the flask was shaken for 2 min to simulate the oral passage. Liquid model system samples were not submitted to the oral phase; thus, they were put directly into the gastric phase. After 5 mL of pepsin solution (12.5 mg/mL in 0.1 M HCl) and 10 mL of SGF were added, the mixture was adjusted to pH 2.0. Then, the acidified mixture was incubated at 37 °C by shaking for 2 h at an agitation speed of 60 strokes per min to simulate the gastric phase. Bile salts were dissolved in the SDF solution to a concentration of 10 mg/mL. The pH was adjusted to 7.5 after the gastric phase. After that, 20 mL of the mixture of SDF with bile salts and 5 mL of pancreatin solution (10 mg/mL in water) were added to the flask. The mixture was incubated at 37 °C by shaking for 2 h at an agitation speed of 60 strokes per min to simulate the duodenal phase. The colon phase was simulated by a consecutive hydrolysis of proteins and polysaccharides in the sample. For this, 5 mL of protease solution (1 mg/mL, pH 8.0) was added, and the mixture was incubated at 37 °C by shaking for 1 h. Then, 150 μL of Viscozyme L was added, and the mixture was incubated at 37 °C by shaking for 16 h at an agitation speed of 30 strokes per min. Aliquots of samples were withdrawn from the flask at the end of simulated gastric, duodenal and colon phases for the analyses. All samples were digested in triplicate as described above.

### 2.5. Measurement of color

Color measurements (CIE L*a*b*) were acquired by using a computer vision-based image analysis technique as described previously (Mogol & Gökmen, 2014). The surface color of biscuits was given as average L* (lightness), a* (redness), and b* (yellowness) values. The colors of all three cookie replicates were individually determined, and data were reported as the average ± standard deviation.

### 2.6. Analysis of HMF in the digests by HPLC-PDA

Aliquots of the digests withdrawn from the samples of aqueous model systems were centrifuged at 11,180*g* for 5 min. Aliquots of the digests withdrawn from biscuits were lyophilized. Dried powder (500 mg) was triple extracted with water (5–2.5–2.5 mL) by vortexing for 3 min. The combined extract was clarified by Carrez clarification. The mixture was centrifuged at 10,000*g* for 5 min. 2 mL of supernatant was immediately filtered through 0.45 μm syringe filter into an auto