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Degradation of Free Tryptophan in a Cookie Model System and Its Application in Commercial Samples

FRANCISCO J. MORALES,*,† ÖZGE Ç. AÇAR,‡ ARDA SERPEN,‡ GEMA ARRIBAS-LORENZO,† AND VURAL GÖKMEN‡

Instituto del Frío, Consejo Superior de Investigaciones Científicas, 28040 Madrid, Spain, and Department of Food Engineering, Hacettepe University, 06800 Beytepe, Ankara, Turkey

The stability of free tryptophan (Trp) was examined in five cookie-resembling models at varying baking temperatures and durations. Trp was measured by HPLC coupled with a fluorescent detector. Trp degradation was significantly greater in cookies formulated with glucose compared with sucrose, regardless of the temperatures and durations of baking. A lag period was clearly observed in cookies formulated with sucrose. The type of sugar used in the dough formulation affected not only the thermal destruction kinetics but also the degree of degradation of free Trp. However, the type of leavening agent (ammonium bicarbonate versus sodium bicarbonate) did not affect the rate of Trp destruction as happens in Maillard-driven reactions. In addition, the free Trp content was analyzed in nine different flours and sixty-two commercial cookies, and it was found that free Trp varied from 0.4 to 1287.9 mg/kg for rice and wheat bran, respectively. It was found that free Trp was significantly higher in dietetic commercial samples formulated with wheat bran compared with other flours.

KEYWORDS: Tryptophan; cookie; leavening agents; sugars; baking

INTRODUCTION

Tryptophan (Trp) is an essential amino acid and plays an important role in brain functions, neuronal regulation, relief of chronic pain, and the growth and development of infants (*1*, *2*). Trp is a substrate for not only synthesis of serotonin (neurotransmitter) and melatonin (hormone) but also niacin synthesis (coenzymes NAD and NADP) (*3*). In contrast, an excessive intake of Trp is reported to exert some atherogenic effect (*4*). Physiological concentrations of Trp are directly associated with its amount in the diet (*5*).

Thermal treatment of foods affects the content and bioavailability of essential amino acids (*6*). During baking of cookies, the dough undergoes physical and chemical reactions, leading to changes in the structure, taste, color, and volume. Trp is a limiting amino acid in cereals. Processing or cooking practices might reduce Trp bioavailability (*6*, *7*). In general, the nutritional quality of processed cereals is reduced due to various chemical reactions including the Maillard reaction (MR), protein cross-linking, protein denaturation, and oxidation/degradation of essential amino acids (*8*).

Trp degradation may be caused directly by oxygen free radical species generated in a Fenton-driven system and the MR (*9*). The role of transition metals, which induce both activation of the MR and site-specific oxidation (*10*), is well documented for dairy products (*11*). Furthermore, Trp oxidative degradation products have both toxicological and technological impact since they have been reported to trigger some potential toxic activity (*12*, *13*) and generate off-flavors such as 2-aminoacetophenone (*14*). However, studies described in the literature on destruction of Trp in foods (*11*) particularly in cereals have mainly been carried out on protein-bound Trp (*15*). In these cases, a partial protection of Trp residues embedded in the hydrophobic core of the protein aggregates formed during heat treatment is expected (*16*). The early MR stage produces radical species, followed by formation of reactive intermediates, and oxygen radicals during the transformation of the Amadori products into the end MR products (*17*, *18*). In turn, free radicals produced during glycation could be responsible for Trp degradation. Simat and Steinhart (*19*) showed that the pyrrole moiety was more susceptible to oxidation than the phenyl moiety. Apart from the degradation of Trp via oxidation (*18*), direct glycation of the Trp indole group with sugar during heating could also be responsible for some loss of Trp (*20*).

There is very limited information on the free Trp content in flours. In addition, the stability of free Trp during thermal processing of cereals is poorly understood. The purpose of the present study was to (i) investigate the effect of sugars, leavening agents, and baking conditions on the degradation of free Trp in different cookie model systems and (ii) analyze the Trp content in different flours and commercial cookies.

MATERIALS AND METHODS

Chemicals. L-Tryptophan as a hydrochloride (Trp), glucose, and sucrose were purchased from Sigma (Diesenhofen, Germany). Methanol, acetonitrile, glacial acetic acid, and sulfuric acid (all Analar grades)

* To whom correspondence should be addressed. Phone: +34915492300. Fax: +34915493627. E-mail: fjmorales@if.csic.es.
† Consejo Superior de Investigaciones Científicas.
‡ Hacettepe University.

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were purchased from Merck (Darmstadt, Germany). Ultrapure water was used throughout the entire experiments (Milli-Q system, Millipore, Bedford, MA).

Dough Preparation and Baking Conditions. Flour and shortening were supplied by local producers, and other ingredients were purchased from local supermarkets. Five model cookies were prepared with 80 g of wheat flour, 1 g of salt, 17.6 g of deionized water, and different proportions of sugar and leavening agent as follows: (recipe 1) 35.0 g of sucrose, 0.8 g of sodium bicarbonate, and 0.4 g of ammonium bicarbonate; (recipe 2) 35.0 g of sucrose and 1.2 g of ammonium bicarbonate; (recipe 3) 35.0 g of sucrose and 1.2 g of sodium bicarbonate; (recipe 4) 35.0 g of glucose and 1.2 g of ammonium bicarbonate; (recipe 5) 35.0 g of glucose and 1.2 g of sodium bicarbonate. The dough pH value was 8.21, 8.14, 7.83, 8.41, and 8.42 for recipe 1, recipe 2, recipe 3, recipe 4, and recipe 5, respectively. The average protein content was 4.53% (w/w). The ingredients were thoroughly mixed, and the dough was rolled out to disks with a diameter of 5.5 cm and a thickness of 2 mm and baked at 180, 200, 210, and 220 °C for 10, 15, 20, and 25 min in a natural convection oven (Simsek Laborteknik, Turkey).

Commercial Samples. Sixty-two commercial cookies marketed in Spain and from 15 different producers were purchased from supermarkets. Products containing chocolate, dried fruits, or cream were excluded. The cookie in each package was powdered in a grinder, homogenized, and stored in polyethylene containers under vacuum at 4 °C until analysis. Twenty-five flours were obtained either from national producers or from local stores. The flours analyzed were wheat (*Triticum aestivun*), rice (*Oryza sativa*), maize (*Zea mays*), oat (*Avena sativa*), rye (*Secale cereale*), sorghum (*Shorgum vulgarae*), Indian vetch (*Lathyvus sativus*), soya (*Glycine max*), and chickpea (*Cicer arietinum*).

Analysis of Tryptophan. The cookie samples were ground and stored at −20 °C in high-density polyethylene bottles with plastic screw-capped lids prior to analysis. A finely ground sample (1 g) was weighed into a 10 mL polypropylene centrifuge tube with a cap, and 5 mL of mobile phase (10% acetonitrile in 0.1% aqueous acetic acid solution) was added. The sample was vigorously vortexed for 3 min and allowed to stand for 5 min at room temperature. The sample was centrifuged at 10000*g* for 10 min, and the supernatant was collected. A second extraction was performed with another 5 mL of mobile phase, and both supernatants were pooled. A fraction (1.5 mL) was centrifuged at 10000*g* for 10 min at 4 °C. After appropriate dilution in the mobile phase, the sample (10 μL) was filtrated into Amberlite vials. A Shimadzu HPLC system (Kyoto, Japan) equipped with an LC-20AD pump, an LC-20AD/AT low-pressure gradient former, an SIL-10ADvp autosampler, a CTO-10ASVP oven, and an RF-10AxL fluorescence detector controlled by a CBM-10A communication bus module was used. The chromatographic separations were performed on a Mediterranea-Sea-C18 analytical column (25 × 0.40 cm, 5 μm, Tecknokroma, Barcelona, Spain) using a gradient elution of 0.1% aqueous acetic acid solution (phase A) and acetonitrile (phase B) at a flow rate of 1.0 mL/min at 40 °C. Data acquisition was performed by acquiring chromatograms at an excitation wavelength of 274 nm and an emission wavelength of 352 nm. The column was equilibrated in 90% phase A and 10% phase B. The gradient was as follows: time 0−5 min, 10% B; time 10 min, 30% B; time 11−18 min, 10% B. The quantification of Trp was performed using a calibration curve. Stock solution of Trp was prepared at a concentration of 1000 μg/L. Working standards were freshly prepared by diluting the stock solution to concentrations of 1.25, 6.25, 12.5, 25, 50, and 75 μg/L. Each sample was analyzed in duplicate for Trp, and the mean of the two measurements was reported. Calculation of Trp destruction was as follows:

$$\text{Trp destruction (\%)} = 100 - ([\text{Trp}]_t \times 100/[\text{Trp}]_o)$$

where $[\text{Trp}]_t$ = final Trp content in the sample (mg/kg) and $[\text{Trp}]_o$ = initial Trp content in the unprocessed dough (mg/kg).

Measurement of Water Activity. The water activity values of cookies were measured by an AquaLAB CX-2 (Decagon Devices Inc., Pullman, WA). The ground cookie sample was placed into the specimen holder of the device to record its water activity. The mean of two measurements was reported.

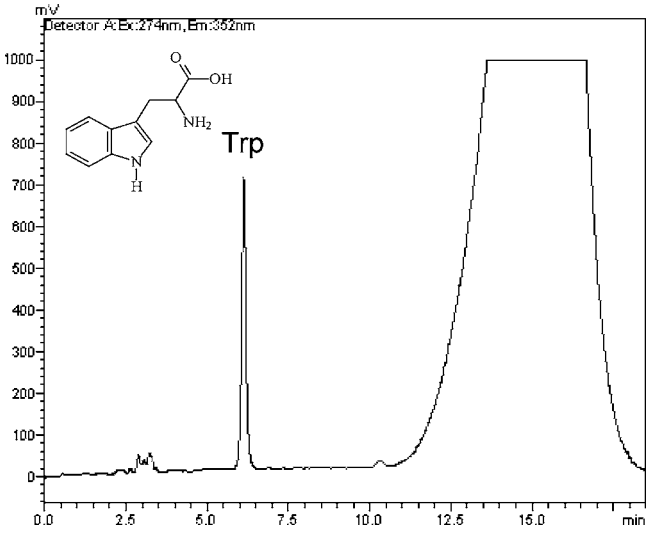

Figure 1. HPLC–fluorescence chromatogram of free Trp in a commercial cookie containing about 30 mg of free Trp/kg.

Total Protein Content. The nitrogen contents of the samples were determined using the Dumas method. Samples (0.800−1.000 g) were burned at 1050 °C following AOAC 992.15 (*21*) in a Leco model FP-2000 (Leco Instruments, Madrid, Spain) protein/nitrogen analyzer calibrated with EDTA. The nitrogen-to-protein conversion factor was 6.38%. The results were expressed as grams of protein/100 grams of the products.

Statistical Analysis of the Data. Statistical analysis of the data was performed by multiple ANOVA following a simple, balanced one-way model. The normality and constant variance of the residuals were checked. The Duncan *t* test was used to compare the means, and the level of significance was set at 95%. Statgraphic v.2.0 software (Statistical Graphics Corp., Rockville, MD) was used.

RESULTS AND DISCUSSION

HPLC Analysis of Free Trp. Figure 1 shows a chromatogram profile of Trp separation in cookies. The analysis was completed within 17 min, and Trp was eluted at 6.08 min without interference. However, it was necessary to apply a short washing step up to 30% acetonitrile due to the appearance of less polar fluorescent compounds in some processed samples. Those compounds could be related to aqueous soluble peptides containing Trp. It was found that the detection limit was 0.01 mg/kg, and the limit of quantification was set as 0.05 mg/kg for a dilution factor applied of 100. The effective range of measurement varied between 0.05 and 75 mg/kg. If a sample had a Trp content of more than 75 mg/kg, it was diluted by 10-fold and then reanalyzed. The relative standard deviations for reproducibility and repeatability in commercial samples were 5.51% and 1.30%, respectively ($n = 8$).

Cookies Baked at Controlled Conditions. Free Trp was analyzed in the different model systems, and the results are expressed as the rate of disappearance or destruction. Baking was performed at different temperatures (180, 200, 210, and 220 °C) for different times (10, 15, 20, and 25 min). The initial free Trp content in the dough ($[\text{Trp}]_o$) was 25.3 ± 0.55, 26.1 ± 0.81, 27.4 ± 1.64, 23.8 ± 0.30, and 24.1 ± 2.10 mg/kg for recipes 1, 2, 3, 4, and 5, respectively.

Figure 2 depicts the thermal destruction kinetics of Trp in recipe 1, which was used as a reference formulation. The baking time and temperature showed a strong influence on the stability of free Trp. At 180 °C, 1.9%, 9.2%, 18.7%, and 29.1% Trp was destroyed within a baking time of 5, 10, 15, and 20 min, respectively, showing that destruction of Trp was time-depend-

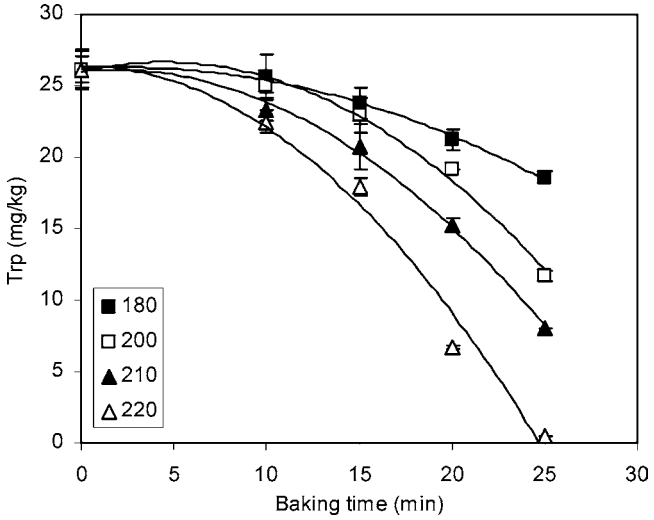

Figure 2. Effect of baking temperatures and durations on the destruction of free Trp (mg/kg) in recipe 1 (control).

ent. From a toxicological point of view, Trp degradation is particularly important because its degradation products are toxic (*13*). The results are in agreement with those reported on the cereal products (*22*, *23*). Horvatic and Vedrina-Dagojevic (*24*) found that the availability of Trp and methionine during industrial production of flakes was significantly reduced and positively correlated with formation of lipid oxidation products.

Destruction of Trp was a function of the temperature and duration of baking when the free Trp content was expressed as a percentage of the initial amount among the five recipes used (**Figure 3**). Recipe 1 was used as the control since it was prepared according to a recipe described in AACC (American Association of Cereal Chemists) method 10-54 (*25*) with some modifications. Some ingredients were not added such as vegetable oils, butter, honey, eggs (dried), or various powder dairy-based ingredients to avoid some misinterpretation of results. Thus, the effect of fat oxidation products on the stability of free Trp was not examined in this study. Similarly, milk proteins were not added because they are rich in Trp and residual proteolysis could cause some interference in the model system.

The present study found that the type of sugars affected significantly the stability of Trp (**Figure 4**). Recipes 4 and 5 formulated with glucose showed greater destruction of Trp than recipes 2 and 3 formulated with sucrose for a given water activity. In addition, destruction of Trp was greater in glucose recipes at most temperatures and baking durations, except for baking conditions at 220 °C with a duration of longer than 20 min. At more severe baking conditions, the rate of sucrose hydrolysis could be an important source of reducing sugars, and it was plausible that destruction of Trp mediated by glycoxidation was favored. Recently, Dragojevic et al. (*15*) reported that Trp was stable in dietetic cookies (high wheat bran and soy flour) in which the sugar content was lower or it was replaced by an artificial sweetener. Maillard reaction and sugar caramelization are important reactions taking place in the models which are influenced by the pH. However, the present study found that the type of leavening agent used had no or little effect on the degradation of free Trp.

Regardless of the recipe composition and baking temperatures, there was a lag period of ~10 min before the destruction of Trp in sucrose recipes had an exponential increase. In contrast, the destruction of Trp was very rapid and linear in glucose recipes without having a lag period. Evaporative cooling is one of the physical factors leading to the structural change

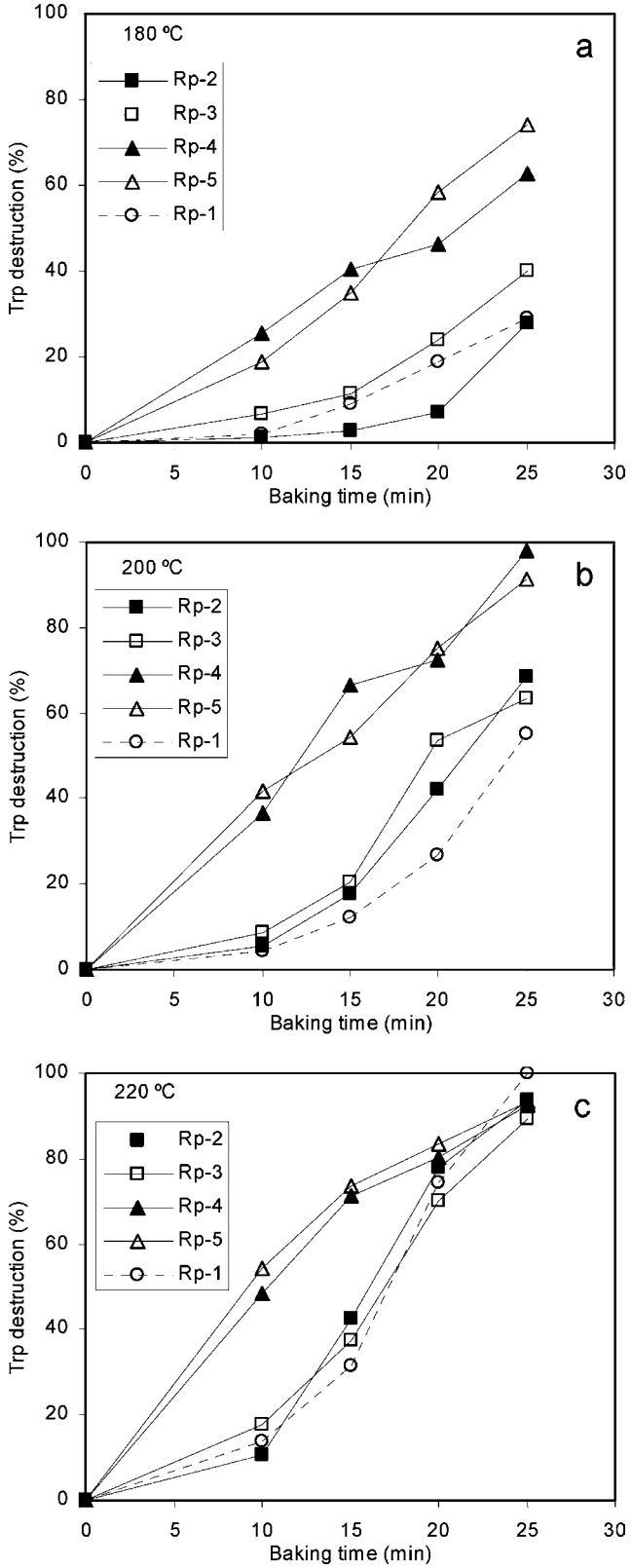

Figure 3. Effect of baking temperatures on the destruction of free Trp at 180 °C (a), 200 °C (b), and 220 °C (c).

of cookies during baking. This is prevented when the cooling temperature is over 100 °C (*26*). On the other hand, reducing sugars are more efficient for glycoxidation of Trp. In sucrose recipes, a certain heat load is necessary to hydrolyze the glycoside bound between glucose and fructose which would participate in the glycoxidation of Trp. Then the type of sugar

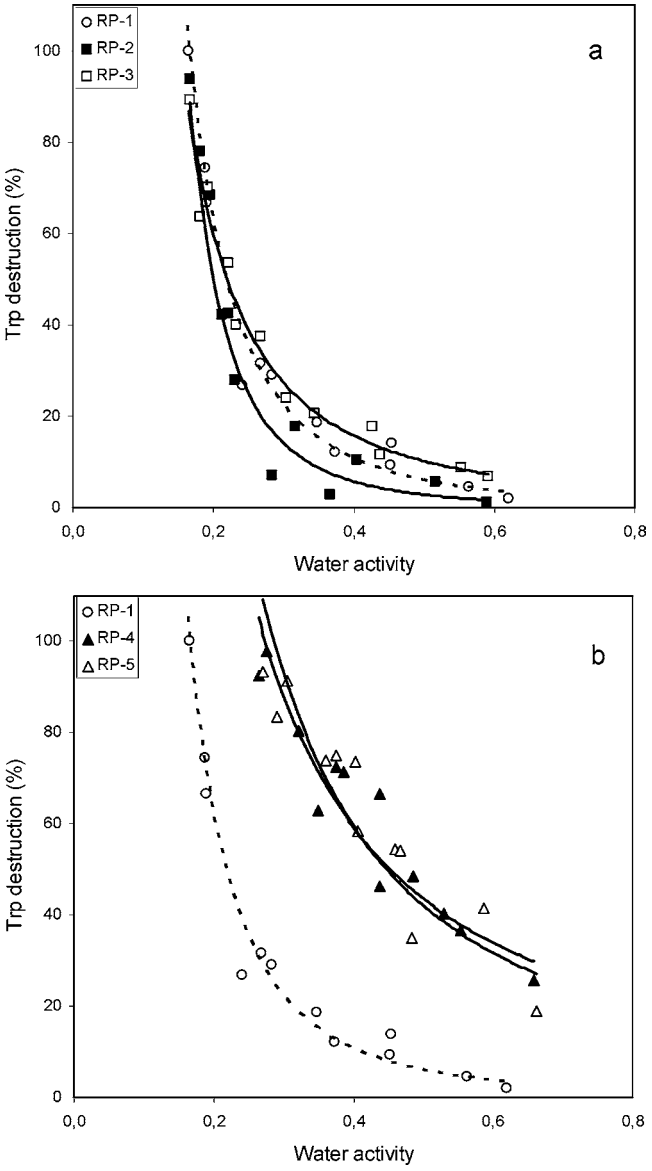

Figure 4. Relationship between water activity and destruction of free Trp in receipe 1 (open circles and dashed line), sucrose recipes (a), and glucose recipes (b).

affects not only the thermal destruction kinetics of free Trp but also the extent of destruction.

The final moisture content and surface color are commonly used to determine the end point of the baking process. Increasing the baking temperature also increased the rate of drying, regardless of the recipe composition. **Figure 4** represents the correlation between water activity and destruction of Trp for sucrose (**Figure 4a**) and glucose (**Figure 4b**) recipes, compared with the control at all temperatures and baking durations. Water activity of the dough varied between 0.780 and 0.814 in the recipes. Cookies containing the same sugar in the formulation showed a similar exponential trend, regardless of the leavening agent used. However, free Trp destruction in glucose recipes was remarkably greater at a given water activity. Fifty percent of the initial amount of free Trp was destroyed at a water activity between 0.20−0.22 and 0.45 for sucrose and glucose recipes, respectively.

Free Trp Content in Flours. The amount of Trp in 25 flours of rice, maize, rye, oat, wheat, sorghum, whole wheat, Indian vetch, soya, white chickpea, and wheat bran were analyzed (**Figure 5**). Rice flour contained the least amount of free Trp

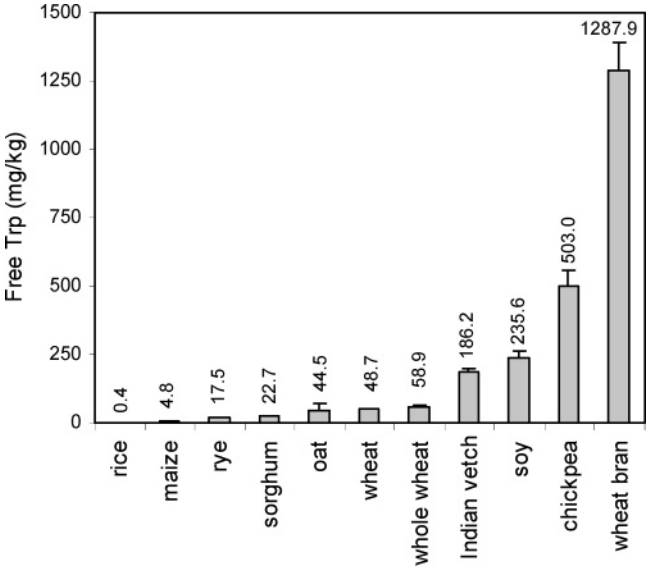

Figure 5. Distribution of free Trp (mg/kg) in different flours.

(0.4 mg/kg), whereas whole wheat bran had the most (1287.9 mg/kg). Wheat is the most common flour used in cookie formulation and contains an average of 48.7 mg/kg free Trp. In general, Trp did not vary significantly in the same type of flour (<10.5%), except for oat (44.5 ± 23.9 mg/kg, 53.6%, $n = 3$). In this regard, the contents of free and total Trp have been recently revised in different flours, highlighting the importance of nonprotein Trp (*27*).

Free Trp in Commercial Cookies. The distribution of free Trp in commercial digestive and semisweet cookies was studied, and it was found that it ranged from 2.7 to 118.8 mg/kg with a mean value of 37.0 mg/kg and a median of 32.7 mg/kg. It was found that one dietetic cookie was detected to have 118 mg/kg due to a high proportion of wheat bran used (64%), with fructose being the sugar source. It was expected that a greater amount of free Trp was present in wheat bran based cookies because wheat brain had the most Trp (**Figure 5**). A conventional cookie contains wheat flour, sugar, fat, and water, together with a number of commonly added ingredients in a minor proportion such as salt or leaving agent. Other cookies contain oat, rice, mal, maize, barley, and soy in combination with wheat flour. The least amount of free Trp was found in a cookie containing rice flour (2.7 mg/kg), simply because rice flour has the least free Trp (0.4 mg/kg).

In summary, degradation of Trp in processed foods has technological, nutritional, and toxicological implications. In foods, Trp degradation can be induced by oxygen radical species, oxidized lipids, hydrogen peroxide, reducing sugars, and carbonyl compounds formed during MR or caramelization (*19*, *24*, *27*, *29*). The present results emphasized the Trp degradation in five cookie model systems was temperature- and time-dependent. In addition, the present study found that glucose as a baking ingredient had a greater destructive effect than sucrose on Trp in cookies, most likely because a reducing sugar could undergo direct glycation with the Trp indole (*20*) or greater radical oxidation mediation during the Maillard reaction (*10*, *17*, *18*). It was further found that the leavening agents had little effect on the stability of Trp. It should be noticed that free Trp varies greatly with different flours when baking ingredients are formulated.

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