Detection of Food-Derived Damaged Nucleosides with Possible Adverse Effects on Human Health Using a Global Adductomics Approach

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A range of damaged nucleosides, also found in digested dietary DNA, appear to be taken up by cells and incorporated into the cells' own DNA. Most incorporated damaged nucleosides will be repaired by cellular DNA repair systems. However, a small fraction of these will escape repair and thus ultimately create mutations. Over the long human lifespan this could be a mechanism that contributes to disease, cancer, and aging. This study analyzed damaged nucleosides derived from dietary DNA in a commercially successful fungus-based novel food, Quorn, and in two fungus-based food items with a history of safe use, button mushroom (*Agaricus bisporus*) and dried powdered brewers yeast (*Saccharomyces cerevisiae*). By using liquid chromatography combined with tandem mass spectrometry more than 90 putative DNA adducts were measured, showing that foods do contain a range of different DNA damages.

KEYWORDS: DNA damage; DNA adducts; adductomics; mass spectrometry; mutation

INTRODUCTION

It has long been assumed that the DNA component in foods does not cause adverse effects in humans. Recent results, however, indicate that the issue might not be that straightforward. A number of observations in the literature have led us to hypothesize that damaged deoxyribonucleosides and bases from food DNA can be taken up by cells in the gastrointestinal tract and, via imperfections in DNA synthesis, become incorporated into DNA (*1*) and ultimately create mutations (**Table 1**). This would imply that damaged nucleosides from foods could potentially be genotoxic and could act as mutagens and, over the long human lifespan, contribute to mutations, aging, and disease. Several damaged nucleosides/nucleotides have been demonstrated in vitro to be incorporated into DNA and most incorporated damaged nucleosides are also mutagenic (**Table 1**). The first results were obtained 10 years ago using sister chromatid exchange in human lymphocytes and Ames tests. Oxidized nucleosides, such as 8-oxo-2′-deoxyguanosine (8oxodG), 8-oxoguanosine, 2-oxo-2′-deoxyadenosine (2oxodA), 2-oxoadenosine, 5-hydroxy-2′-deoxycytidine (5OHdC), and 5-hydroxycytidine, were demonstrated to be genotoxic in both experimental systems (*2*). In *Escherichia coli* the triphosphates of 8oxodG and 2oxodA were shown to be directly incorporated into DNA and to induce mutations (*3*). 5-Formyl-2′-deoxyuridine has been reported to induce mutations in CHO cells (*4*), and deoxyuridine has been reported to be incorporated into mouse fibroblast DNA (*5*). These in vitro results

demonstrate that damaged nucleosides are not only taken up by cells but also incorporated into DNA, creating mutations. The damaged nucleosides used in these studies and a range of others may be expected to be present in human foods in low but potentially significant quantities.

A number of nucleoside analogues act by halting DNA synthesis after incorporation (*6*). It can be argued that these molecules are designed to do just that, but on the other hand these compounds have clearly demonstrated that the DNA synthesis machinery does indeed allow incorporation of altered nucleosides. Both *E. coli* and humans have specific enzymes to clear the nucleotide pool of oxidized nucleotides. The *E. coli* MutT protein and the human homologue MTH1 hydrolyze 8-oxodeoxyguanosine triphosphate to the monophosphate (*7, 8*). The diphosphate is hydrolyzed by NUDT5 (*9*). Deoxyuridine-triphosphate is hydrolyzed by deoxyuridine triphosphatase (*10*). These specialized enzymes have been reported to protect against mutations, indicating that damaged nucleosides can be a significant source of mutations.

Most food products derive from slow-growing organisms that contain on the order of 0.1% DNA (*11*). However, a few novel food products derive from fast-growing organisms and contain significantly more nucleic acids. It is therefore of particular interest to study the amount of DNA damage in novel foods and compare it to traditional foods. In addition, it cannot be excluded that novel foods could contain DNA adducts that have not been found in traditional foods. In this study we have focused on Quorn, which is a meat replacement product produced from the microfungus *Fusarium venenatum* (*12, 13*). The product was

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Article

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Table 1. Damaged Nucleosides That Are Incorporated into DNA or Are Mutagenic

name	experimental system	ref
8-oxo-2′-deoxyguanosine	sister chromatid exchange (SCE) in human lymphocytes	2
	primer extension/DNA pol I/DNA pol α	29
	direct incorporation into *E. coli* genome	3
	direct incorporation into the genome of MCF-7 cells	30
2-oxo-2′-deoxyadenosine	SCE in human lymphocytes	2
	in vitro DNA replication in HeLa extract followed by transformation in *E. coli*	31
	direct incorporation into *E. coli* genome	3
5-hydroxy-2′-deoxycytosine	SCE in human lymphocytes	2
5-formyl-2′-deoxyuridine	in vitro DNA replication with Pol I (Kf)	32
	phosphoribosyltransferase	4
deoxyuridine	alkaline sucrose density gradient sedimentation (*E. coli*)	33
	mouse	5
N2-ethyl-2′-deoxyguanosine	primer extension/DNA pol α	34

placed on the British marked in 1985 and is thus not considered as a novel food by European Union (EU) legislation (regulation 258/97) (*14*). However, in a more semantic meaning of the word, we clearly consider Quorn as a novel food because there is no history of safe use of *F. venenatum*. This particular strain was selected on the basis of its meat-like structure, nutrient content, and other parameters after a large screen. Quorn is maybe the most successful product of the many novel food products developed during the past 50 years (*15*). Quorn is now sold at health food and vegetarian markets in the United States and most European countries. Quorn is produced in continuous flow airlift reactors with a high growth speed (doubling time ∼ 4 h). As a fast-growing organism *F. venenatum* has a high nucleic acid content. The amount of RNA in the final product is reduced from 10 to 2% by autolysis, and the DNA amount is on the order of 1% (*16*). Fruit bodies of large fungi and colonies of microfungi including molds and fermenting yeasts have traditionally been consumed in many parts of the world. As food products with a history of safe use we have used button mushroom (*Agaricus bisporus*) and dried powdered brewer's yeast (*Saccharomyces cerevisiae*) for comparison with Quorn. Brewer's yeast is one of the most important cultured organisms, and yeasts have been used for fermentation and baking throughout human history. For more than a century, brewer's yeast has been widely used as a dietary supplement due to its high amounts of protein, vitamins, and minerals (*17*). In addition, it is common to use yeast and yeast-containing waste products from the fermentation industry as animal feed (*18*). Yeast products are commonly used as control for single-cell protein novel food products. Button mushroom (*A. bisporus*) has been collected and consumed probably for several thousand years and cultivated commercially since the 17th century. It is now commonly sold as white button mushroom, champignon, table mushroom, or portobello mushroom. *A. bisporus* is the most important commercial mushroom with an annual production of 1 million tons (*19, 20*).

To obtain experimental data to characterize our hypothesis, we have chosen to investigate the occurrence of damaged nucleosides in novel and conventional food DNA. On the order of 200 different physiologically relevant non-normal nucleosides are known (*21*), and many more could exist. To be able to detect the broadest range of damaged nucleosides we have chosen to use an adductomics approach (*22*). The adductomics approach is based on chromatographic separation of a nucleoside mixture combined with detection with tandem mass spectrometry (MS/MS). Deoxyribonucleosides can conveniently be detected by MS/MS by a characteristic neutral loss of deoxyribose (116 Da). By using multiple reaction monitoring (MRM) programs we could semiquantify each putative damaged nucleoside in a relevant molecular mass range (240−406 Da) by observing transitions in 1 Da increments, 240 → 124, 241 → 125, and so forth.

MATERIALS AND METHODS

Chromatographic reference standards of 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, thymidine, and 8-hydroxy-2′-deoxyguanosine were from Sigma Chemical Co. (St Louis, MO), and those of 5-methyl-2′-deoxycytidine and 5-hydroxy-2′-deoxycytidine were from Berry & Associates (Dexter, MI). All other chemicals were obtained from Sigma Chemical Co., unless otherwise stated.

DNA Extraction. Genomic DNA from the food matrices was isolated using a SDS-based protocol. A 2 g sample was dissolved in 20 mL of 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 0.1 M EDTA, 0.5% SDS, and 20 μg/mL ribonuclease A (Sigma Chemical Co.) and incubated for 30 min with gentle mixing before 100 μg/mL proteinase K (Sigma Chemical Co.) was added. The samples were then incubated for another 30 min. Each tube was centrifuged for 30 min at 12000*g* at 4 °C, and the supernatant was transferred to a fresh tube containing 20 mL of chloroform/isoamyl alcohol 24:1 (CI), gently shaken, and centrifuged for 30 min at 12000*g* at 4 °C. The supernatant was transferred to a fresh tube and centrifuged for 60 min at 30000*g* at 4 °C before extraction with CI was repeated. One milliliter of 5 M NaCl and 15 mL of ice-cold isopropanol were added to the supernatant and incubated for 60 min or overnight at −20 °C before the sample was centrifuged for 30 min at 12000*g* at 4 °C. The pellet was washed twice with 5 mL of a solution containing 70% ethanol, dissolved in 10 mM Tris, 1 mM EDTA, 1 μg/mL ribonuclease A (Roche, Mannheim, Germany), and 200 U/μL ribonuclease T1 (Roche) and incubated for 60 min with gentle mixing. Twenty milliliters of CI was added to the tube, and the contents of the tube were gently mixed before centrifugation for 10 min at 12000*g* at 4 °C. The supernatant was transferred to a clean tube, and 7.5 mL of 7.5 M ammonium acetate and 15 mL of ice-cold isopropanol were added before incubation for 60 min at −20 °C. The tube was centrifuged for 30 min at 12000*g* at 4 °C, and the pellet was washed twice with 10 mL of a solution containing 70% ethanol, air-dried at room temperature, and dissolved in ultrapure water. At this stage, the *A. bisporus* DNA was still contaminated by a black substance we assumed to be melanin. The *A. bisporus* DNA was therefore further purified on a Zymo-spin column (DNA Clean & Concentrator 500, Zymo Research, Orange, CA) according to the supplier's instructions. DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Enzymatic Hydrolysis of DNA and Sample Preparation. Each 1 mL DNA sample from the DNA extracts (concentration adjusted to 1200 ng/μL) was added to 50 μL of 0.2 M MgCl₂, 10 μL of 1.0 M Tris-HCl, pH 7.5, and 40 μL of 10 U/μL DNase I (Roche) and incubated for 60 min at 37 °C with gentle mixing. Five microliters of 1 U/μL alkaline phosphatase (Roche) was added and the incubation continued for 60 min. Ten microliters of 15 U/mL phosphodiesterase I from *Crotalus adamanteus* venom and 10 μL of 0.01 U/μL phosphodiesterase II from bovine spleen were added, and the incubation was continued for 60 min. Each sample was filtered with a Microcon YM-3 spin filter (Millipore, Bedford, MA) at 14000*g* overnight at 4 °C. The filtrate was added to formic acid to a final concentration of 1% and analyzed by LC-MS/MS.

LC-MS/MS Analysis. Liquid chromatography was performed on an Atlantis T3 column (3.5 μm, 150 × 2.1 mm; Waters, Milford, MA), using a Waters 2670 ultrahigh-performance liquid chromatography (UPLC) module. Separation was achieved using a linear gradient elution at 0.25 mL/min starting with 100% aqueous buffer (H₂O containing 0.1% vol/vol formic acid), rising to 22.5% methanol (containing 0.1% formic acid) over 18 min. The column was washed with 100% methanol for
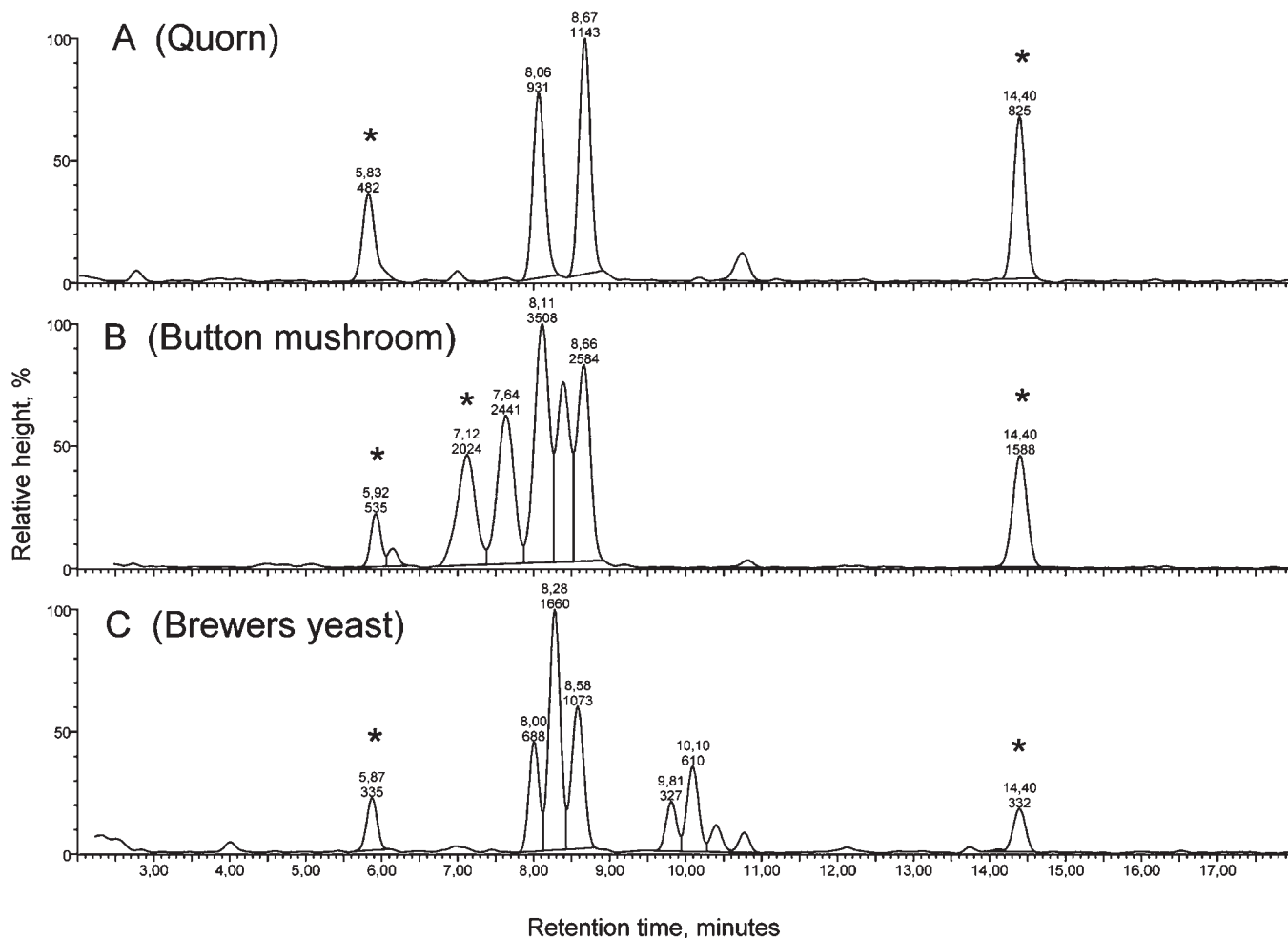
Figure 1. Example of a chromatographic trace. The transition 300.1 → 184.05 is plotted for Quorn (**A**), button mushroom (**B**), and brewer's yeast (**C**). The traces were processed as described under Materials and Methods. The retention times are aligned to the peak at 14.4 to allow easy comparison. The adjustments were <4%. The peaks labeled with an asterisk (∗) were excluded due to similarity to cytosine, 2′-deoxycytosine, and 2′-deoxyguanosine, respectively.

1 min before it was reequilibrated with aqueous buffer. The UPLC system was coupled to a Quattro Ultima Pt triple-quadrupole mass spectrometer operating with an ESI interface (Waters Micromass, Manchester, U.K.). ESI parameters were a spray voltage of 3.5 kV, desolvation temperature at 250 °C, source temperature at 150 °C, and cone gas and desolvation gas at 60 and 600 L/h of N_2, respectively. The mass spectrometer was operated in MS/MS mode with argon as collision cell gas at 1×10^{-3} Torr. MS/MS collision energy was set to 10 eV for all observed transitions. Quantification of the nucleosides was performed with MRM in positive ionization mode, observing a neutral loss of 116.05 Da (corresponding to deoxyribose) starting at 240.1 → 124.05, observing seven transitions per injection, and ending at 406.1 → 290.05. Each injection contained nucleosides derived from 5 μg of DNA.

Quantification of LC-MS/MS Chromatograms and Data Analysis. Each chromatographic peak was integrated using MassLynx 4.1 software (Waters) with automatic noise reduction and mean smoothing (smoothing parameters were window size = ±3 and number of smooths = 3). A signal-to-noise ratio of >9 was used to select peaks. The four normal nucleosides in each sample were used as an internal standard to normalize all areas to allow direct comparison between the samples. At the retention time of each normal nucleoside we observed a variety of isotope peaks and adduct ions. A ribonucleoside that loses water on the sugar during ionization will be erroneously detected as a deoxyribonucleoside. To avoid detecting such false peaks, we have excluded all peaks with the same retention times as the four ribonucleosides, the four deoxyribonucleosides, and 5-methyl-2′-deoxycytidine. All chromatograms were inspected manually to exclude spikes from

electrical noise. The data were log transformed before plotting as bubble charts.

RESULTS AND DISCUSSION

DNA from food sources can be expected to contain a range of DNA damages depending on the species, growth conditions, environmental parameters, processing, storage, and other factors. The most common DNA damages (e.g., 8oxodG and 5OHdC) can be expected to be found in all food products, whereas more rare damages can be expected in fewer, particular food products. To be able to detect in principle every DNA adduct, we have used liquid chromatography combined with tandem mass spectrometry and taken advantage of the fact that the glycosidic bond in nucleosides makes it possible to measure a characteristic neutral loss of deoxyribose (116.05 Da) to identify nucleoside adducts (*22*). We have measured every transition in a relevant mass range beginning with 240.1 → 124.05 and ending with 406.1 → 290.05. This approach generates an overall fingerprint of the number and amount of DNA damage but without identifying each adduct. These experiments require that the nucleoside sample is relatively clean to avoid contaminating molecules that could lose 116 Da by chance and thus be detected as a nucleoside adduct. As described in detail under Materials and Methods, we have devised an extensive DNA purification protocol to obtain
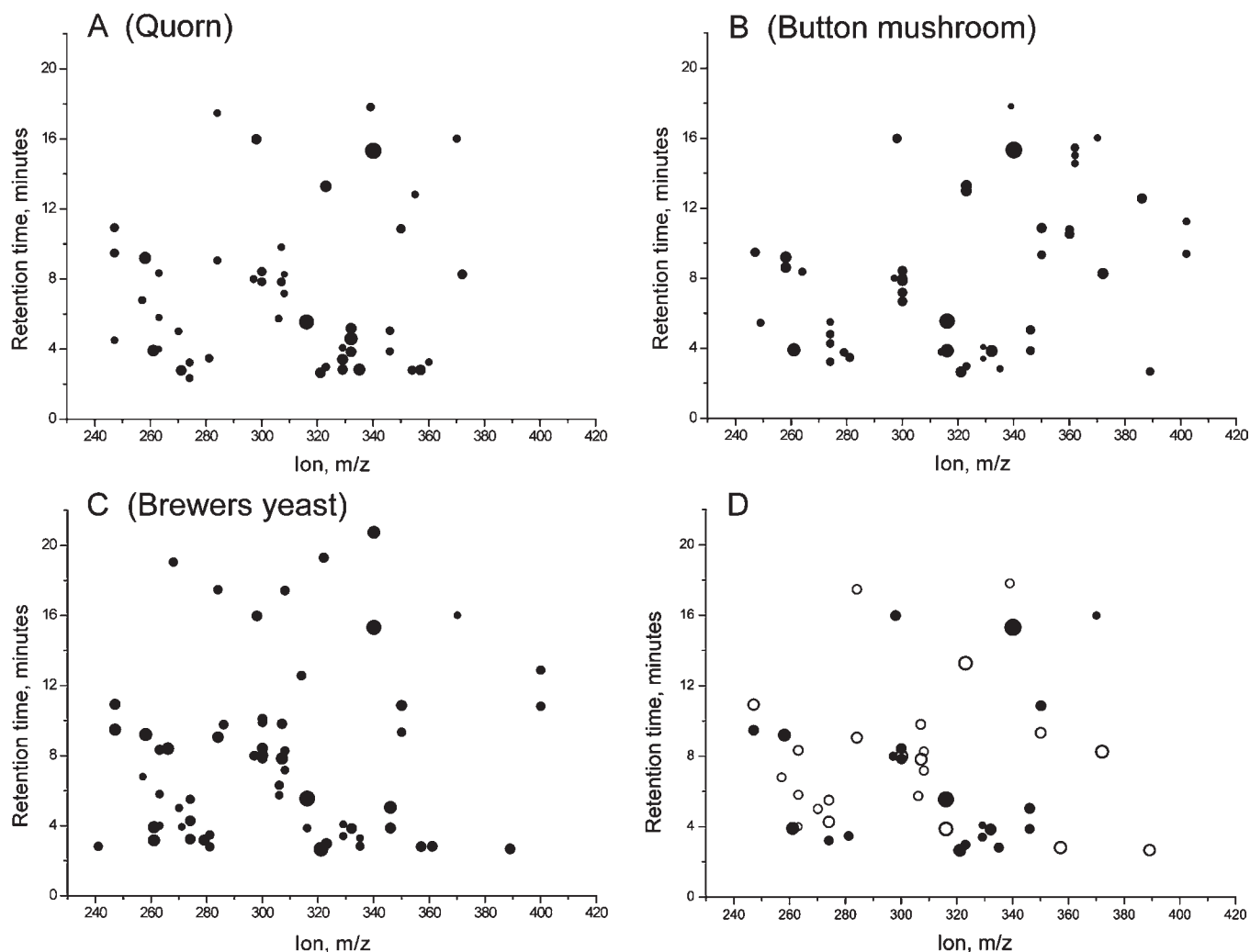
Figure 2. Bubble chart of normalized log transformed areas for Quorn (**A**), button mushroom (**B**), and brewer's yeast (**C**). Panel **D** shows ions that are common for all three samples (●) and in two of them (○). The areas were integrated as described under Materials and Methods.

Table 2. Non-normal Nucleosides Quantified with External Standards

name	retention time	transition	measured unit[a]	Quorn	*A. bisporus*	brewer's yeast
5-methyl-2′-deoxycytidine	9.2	242.1 → 126.05	% 5MedC/dC	0.0004	0.40	0.49
5-hydroxy-2′-deoxycytidine	7.5	244.1 → 128.05	5OHdC/10⁶dC	5.6	nd[b]	9.3
8-hydroxy-2′-deoxyguanosine	17.3	284.1 → 168.05	8oxodG/10⁶dG	11.2	1.4	57.4

[a] All ratios expressed in mol. [b] Not detected.

high-quality DNA. The method was designed to purify both high molecular weight and fragmented low molecular weight genomic DNA because fresh samples (*Agaricus bisporus*) could be expected to contain high molecular weight DNA, whereas the DNA of dried samples (brewer's yeast and Quorn) could be expected to be fragmented. Alcohol precipitation has been demonstrated to precipitate fragments as small as 26 base pairs (*23*). When the three samples were run on an agarose gel, we observed that *A. bisporus* DNA migrated as ∼10−50 kbp fragments, whereas brewer's yeast and Quorn DNA were much more fragmented and predominately migrated as a few hundred base pair long fragments (Supplementary Figure 1 of the Supporting Information). The DNA was enzymatically hydrolyzed to nucleosides to avoid formation of oxidized nucleosides. As an example of a chromatographic trace, the transition 300.1 → 184.05 is shown in **Figure 1**. The traces contain a total of nine peaks in the three samples. Ions with similar retention time as the four deoxyribonucleosides and the four ribonucleosides in addition to 5MedC

were excluded as possible ionization artifacts. The two first peaks at retention times of 5.9 and 7.1 in addition to the last at retention time 14.4 were excluded due to similarity in the retention times with C, dC, and dG, respectively. Two of the remaining six peaks are common to all three samples, whereas two can be found in both the button mushroom and brewer's yeast samples. Two peaks are unique to button mushroom and another two to brewer's yeast, respectively. All chromatographic traces were analyzed the same way to exclude ionization artifacts. A total of 91 ions were detected in the three samples (**Figure 2** and Supplementary Table 1 of the Supporting Information). Of these 91 ions 21 were common for all three samples and 23 were common for two of the samples (**Figure 2D**). Thus, these three samples have both a unique DNA damage pattern, because 47 peaks are unique to one of the samples, and extensive similarities due to their common peaks.

Three nucleosides were quantified with external standards (**Table 2**). Most fungi have targeted methylation mechanisms

producing 5MedC (*24*), and consistently we detected 5MedC in all three samples. The 5MedC value found for brewer's yeast (0.5 mol %) is in good agreement with literature values for *S. cerevisiae* (0.3−1 mol %) (*25*). We have not been able to find literature values for the amounts of 5MedC for Quorn or *A. bisporus*. Remarkably, the Quorn genome is practically unmethylated, having 1000-fold lower levels than button mushroom and brewer's yeast. This low level could invoke an immune response in the gastrointestinal tract after ingestion, as is seen with other unmethylated DNA in vivo in general (*26*). This issue might justify further investigation and might be an issue for food safety authorities in relation to food safety assessments of novel foods with low levels of 5MedC.

8oxodG is easily created during DNA extraction and sample preparation and has therefore been notoriously difficult to measure. A lot of work has been put into establishing a consensus, defining the background level of 8oxodG, which now seems to be established as 0.3−4.8 oxodG per 10^6 dG in fresh tissue (*27*). The button mushroom was the only fresh food product of the three samples, and for this sample we determined the concentration of 8oxodG to be 1.4 8oxodG per 10^6 dG, well within the normal range for fresh tissue. This indicates that the DNA extraction and sample preparation does not introduce artificially created 8oxodG.

Currently there is no generally accepted approach to assess the safety of novel foods (*28*), and safety assessments are done on a case-by-case basis. Normally, food safety assessments include taxonomic identification, characterization of macronutrients (protein, fat, carbohydrates, fiber), micronutrients (vitamins, minerals), primary metabolites, secondary metabolites (antimetabolites, toxins), uptake of environmental pollutants (selenium, cadmium, arsenic), allergy testing, history of use, animal feeding studies, metabolomics, and exposure (intake levels). DNA damages are currently not assessed at all in food safety considerations regarding novel foods (or any other foods). Our data show that food contains a broad range of damaged nucleosides and that many of these are unique to the food in question. Because it appears from the literature that damaged nucleosides from food to some extent will be incorporated into DNA, it is important to determine whether this mechanism produces mutations or cytotoxic effects that will contribute to disease. Both animal experiments and epidemiological studies will be needed to address this question. In addition, there is clearly a need to address the identity of damaged nucleosides from foods and their effect on the consuming organism.

Supporting Information Available: Supplementary Table S1 (integrated, normalized areas from the LC-MS/MS analysis used in **Figure 2**) and Supplementary Figure S1 [agarose gel electrophoresis analysis of purified DNA (purified DNA from Quorn (lane 2), button mushroom (lane 3), and brewer's yeast (lane 4)) was run on an 0.8% agarose gel in 1× TBE at 90 V for 45 min; lane 1 contains GeneRuler 1 kb DNA ladder (Fermentas) with DNA sizes indicated; 0.5 μg of DNA was loaded in each lane]. This material is available free of charge via the Internet at http://pubs.acs.org.

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