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Genotoxicity of Melanoidin Fractions Derived from a Standard Glucose/Glycine Model

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Genotoxic compounds can act at various levels in the cell (causing gene, chromosome, or genome mutations), necessitating the use of a range of genotoxicity assays designed to detect these different types of mutations. The production of melanoidins during the processing and cooking of foods is associated with changes in their nutritional character, and the discovery of mutagenic substances in pyrolyzed protein and amino acids has raised concern about the safety of these foods. The aim of this work was to test melanoidin fractions in three different in vitro assays (Ames test, Vitotox test, and micronucleus test). These melanoidin fractions were produced from the condensation of glucose with glycine and their separation was conducted by dialysis. The crude reaction mixture (before dialysis) and both the LMW and HMW fractions obtained by dialysis showed no genotoxicity in these assays, despite being tested at concentrations much higher than those naturally found in food products. The LMW fraction, however, showed toxicity at these high concentrations. The volatile fraction produced in this reaction showed genotoxicity only in the Vitotox test, at high concentrations.

KEYWORDS: Melanoidins; Maillard reaction products; genotoxicity; Ames test; Vitotox test; micronucleus test

INTRODUCTION

The Maillard reaction between free amino groups and carbonyl compounds occurs during the processing, cooking, and storage of foods, causing changes in flavor, color, and taste, and a loss of nutritional value (*1*). The latter is related to the destruction of essential amino acids, a decrease in digestibility, and the inhibition of proteolytic and glycolytic enzymes, as well as the production of anti-nutritional and toxic compounds (*2*). The presence of mutagenic substances was first observed in pyrolyzed proteins and amino acids (*3*). The Maillard reaction produces a complex mixture of products, the composition of which varies greatly with reaction conditions (e.g., temperature and incubation time) and includes melanoidins. Melanoidins are poorly defined in terms of structure, and the separation and identification of individual melanoidins is difficult, due to the complexity of the mixtures and the number of closely related structures present.

The frying or grilling of meat and fish is reported to generate low levels (in the range of ppb) of mutagenic or carcinogenic compounds, mainly classified as heterocyclic amines (*4*). Many of these are formed via the Maillard reaction from creatine, free amino acids, and monosaccharides. Some of the compounds produced via the Maillard reaction are reported to induce chromosome aberrations in Chinese hamster ovary cells, gene conversion in yeast, and mutagenesis in the Ames test (*1*).

Previous studies reported in the literature have produced differing results with respect to the mutagenicity of the Maillard (and melanoidin) reaction mixture and the separated fractions of this mixture. This is further complicated by the lack of standardized reaction conditions, because temperature and heating time, for example, have been shown to be vital factors in determining product composition and characteristics (*1*).

Vagnarelli et al. (*5*) tested the mutagenicity of a heated ribose/lysine mixture, whereas Yen et al. (*6*) investigated the anti-mutagenic effects of Maillard reaction products prepared from

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heating glucose, fructose, or xylose with arginine, glycine, lysine, or tryptophan. Yen et al. (*7*) also reacted xylose and lysine (1:2 molar ratio) dissolved in a phosphate buffer and refluxed at 100 °C for 10 h. The mixture was then fractionated with membrane filters, and the anti-mutagenic activity of the fractions was investigated.

Kitts et al. (*8*) used the Ames test to examine the mutagenicity of both the reaction mixture and fractionated components obtained from heating glucose and lysine. Tested without S9, the reaction mixture was mutagenic to both bacterial strains TA98 and TA100, but on the addition of S9, no mutagenicity was observed for TA98, and very little mutagenicity for TA100.

More recently, Brands et al. (*1*) studied the mutagenicity of heated sugar−protein model systems, using casein (3% w/w) and various sugars (150 mM). Their findings state that the mutagenicity of the reaction mixture increased with heating time (20, 40, and 60 min at 120 °C). Sugars tested were as follows (with decreasing mutagenicity): tagatose > fructose > galactose > lactulose > glucose > lactose. The mutagenic activity of these compounds was weak relative to that of chemical mutagens such as 4-nitroquinoline-*N*-oxide (4-NQO), and the mutagens formed were direct acting mutagens that were deactivated by the addition of S9.

Some investigations into the mutagenicity of Maillard reaction products in food have shown a net mutagenic effect in foods that have been dried, fried, roasted, baked, and broiled (*9*). The water-insoluble fraction of the melanoidin reaction has been shown to inhibit the mutagenicity of known carcinogens by acting as effective binding agents (*9*).

Mutagenicity assays are an essential part of testing new compounds and products before they can be released commercially. Testing regularly consumed foodstuffs for potential mutagens is an important aspect of ensuring the safety of products for consumption. With the discovery of mutagenic substances in pyrolyzed protein and amino acids, concern was raised as to the safety of foods in terms of potential carcinogenic activity. Testing melanoidin fractions for potential genotoxic activity is thus of practical importance to the food industry.

Because the composition of Maillard reaction products varies greatly with reaction conditions, it is important to use a standard protocol in the production of the melanoidin fractions to facilitate comparison. Direct comparison of results from the testing of melanoidins for both mutagenic and anti-mutagenic properties has been complicated in the past by varying reaction times, products, and temperature. The establishment of the COST Action 919 (Cooperation in Science and Technology) protocol for the preparation of standard melanoidins should make future comparisons easier. The aim of the current paper is to shed more light on the mutagenicity of standard melanoidins prepared according to the COST protocol, using three different in vitro assays, namely the Ames test, the micronucleus test, and a more recent genotoxicity test, namely the Vitotox assay.

MATERIALS AND METHODS

Materials. Glycine (>99%) and D-glucose (99%) were obtained from Sigma (Bornem, Belgium). Dialysis tubing with a flat width of 33 mm was purchased from Sigma (Bornem, Belgium). This cellulose membrane retains >90% cytochrome c (MW 12,400) in solution over a 10 h period. The dialysis tubing was prepared according to the manufacturer's instructions.

Preparation of Melanoidins. The procedure outlined in the COST proposal was followed in the production of the melanoidins tested in this study (*10*−*12*). A 1:1 mixture of glycine (0.05 mol) and glucose (0.05 mol) was placed in a 300-mL Christ filter bottle and dissolved in 20 mL of distilled water and freeze-dried to constant weight. This mixture was then heated at 125 °C (in a preheated convection oven) for 2 h in an open container. This crude reaction mixture was allowed to cool in a desiccator and ground to a fine powder using a pestle and mortar. A 5-g aliquot of the ground material was added to 200 mL of distilled water, and the solution was stirred for 12 h at 4 °C to dissolve as much material as possible. This suspension was filtered through Whatman No. 4 filter paper, and the filtrate, which contained the water-soluble melanoidins, was collected. The residue on the filter paper was washed with 2 × 20 mL of distilled water, and the liquid obtained after washing was mixed with the original filtrate. This solution (solution A) was made up to 250 mL with distilled water in a volumetric flask. The residue obtained, the so-called water-insoluble fraction of the melanoidins, was frozen, freeze-dried, and stored at −32 °C until further use. This solution formed the crude reaction mixture and was separated using dialysis (6 × 12 h) into low and high molecular weight fractions. The HMW melanoidins were dissolved in water at a concentration of 20 000 ppm. A dilution series was prepared and tested in the assays. Storage of melanoidins was initially at −10 °C, then at −32 °C until further use.

For the preparation of the volatile fractions, a mixture of glycine (0.05 mol) and glucose (0.05 mol) was heated in a two-necked flask provided with a reflux condenser and a mechanical stirrer. The top of the reflux condenser was connected to a liquid nitrogen trap to catch all the volatiles. After heating this mixture at 125 °C during 1 h, the black brown cake was dissolved as much as possible in 200 mL of 1 M NaOH and extracted with Et_2O (5 × 60 mL). These extracts combined with the contents of the trap were consecutively dried ($MgSO_4$) and evaporated in vacuo (0 °C), after which a crude "volatile" fraction was obtained (0.5−0.8 g).

Isolation of Nondialyzable Melanoidins by Dialysis. Solution A (50 mL) was brought in 21 cm of dialysis tubing and was dialyzed against 1 L of distilled water for 24 h at 4 °C with two changes of the surrounding water. At the end of the dialysis, the contents of the dialysis tubing with the high molecular weight fraction (HMW), or so-called nondialyzable melanoidins, were transferred to a 500-mL round-bottom flask, frozen in a liquid nitrogen bath, and freeze-dried until all the water had been removed. The HMW water-soluble melanoidins were stored in a desiccator in a freezer at −32 °C until further use.

Ames Test. *Salmonella/Microsome Assay.* The Ames test, commonly used to identify mutagenic substances, utilizes different bacterial strains of *Salmonella typhimurium* to identify different types of mutations (*13*). Strain TA98 gives an indication of frame-shift mutations, while a positive response from strain TA100 indicates base-pair substitution. Other strains may also be used. The classical Ames test (*13*, *14*) was used in this study, with the addition of a preincubation step of 20 min in a water bath (37 °C). This allows closer contact between the potential mutagen and the bacteria, before the agar is added and the mixture spread on plates for incubation (*15*).

For a substance to be considered genotoxic in the Ames test, the number of revertant colonies on the plates containing the test compounds must be more than twice the number of colonies produced on the solvent control plates (i.e., a ratio above 2.0). This is because a certain level of spontaneous mutations occurs in the Ames bacteria, and this must be factored into the calculations. In addition, a dose response should be evident for the various concentrations of the mutagen tested.

The crude reaction mixture, and volatile fraction obtained from the Maillard reaction between glucose and glycine, as well as the LMW and HMW fractions obtained from the dialysis of the Maillard reaction product was tested in the Ames test in a concentration range of 50−10 000 ppm. Aliquots of the melanoidin fractions (100 uL) were added to 100 uL of an overnight culture of histidine-requiring strains of TA 98 or TA100 (*originally* obtained from B. Ames Laboratory, University of California at Berkeley, CA). This was incubated with 500 uL of the S_9 mix (with 0.1 M phosphate buffer) for 20 min at 37 °C. Molten top agar (2 mL) was added to the mixture, which was then spread over a minimum agar plate (Oxoid-GmbH, Wesel, Germany). The His^+-revertant colonies were counted after 48 h incubation at 37 °C. Three replicate plates were prepared per concentration and the experiments were performed twice.

Table 1. Ratio of His+ Revertants Induced in *Salmonella typhimurium* Bacteria by Melanoidin Fractions, Obtained from the Condensation of Glucose and Glycine, Compared to Spontaneous Revertants (Carried Out with the Ames Test)[a]

	TA98								TA100							
melanoidin fraction ↓ and	−S9				+S9				−S9				+S9			
concentration (ppm) →	80	400	2000	10000	80	400	2000	10000	80	400	2000	10000	80	400	2000	10000
crude reaction mixture	1.4	1.1	1.0	1.1	1.4	1.2	1.0	1.1	1.6	1.4	1.3	1.4	1.3	1.3	0.9	1.1
volatiles	1.1	0.9	1.1	1.1	1.6	1.7	1.4	1.4	1.1	1.1	1.1	0.9	1.2	1.1	1.1	1.2
LMW fraction	0.8	0.7	0.8	0.7	1.3	1.4	1.3	1.3	0.9	0.9	1.0	1.0	0.7	0.7	0.8	0.7
HMW fraction	0.6	0.6	0.7	0.5	1.5	1.1	1.1	1.5	0.9	0.9	1.0	1.0	0.8	0.7	0.7	0.8

[a] Positive Controls, carried out with the Ames test (ratio revertants over spontaneous frequency): 4-NQO = 4-nitroquinoline-*N*-oxide (4 ppb): resulted in an average ratio of 6.0–10.0 (TA98) and 4.0–10.0 (TA100). B(*a*)p = benzo[*a*]pyrene (8 ppm): resulted in an average ratio of 6.0–10.0 (TA98) and 3.5–11.0 (TA100).

Vitotox Assay. The Vitotox test was originally developed for the detection of genotoxic and toxic effects of pure chemicals (*16*). It is a short-term assay (4 h), based on SOS response-dependent induction in genetically modified *Salmonella typhimurium* bacteria. The test utilizes two bacterial strains to test for both genotoxicity and toxicity. The test is based on bacteria that contain the *lux* operon of *Vibrio fischeri* under the transcriptional control of the *rec*N gene, which is part of the SOS-system. When the bacteria are incubated with a genotoxic compound, the *rec*N promoter is depressed initiating expression of the *lux* operon. This expression results in light production, which is measured and expressed as a function of genotoxicity (*16*). The protocol described by Verschaeve et al. (*17*) was followed.

Certain compounds cause false positive results by acting directly on the light production (e.g., aldehydes) or by enhancing the bacterial metabolism. A constitutive light-producing bacterial strain with a *lux* operon under the control of the strong promoter *pr1* is thus used as an internal control system. This second strain also serves as a measure of toxicity. This test correlates well with the Ames test, but is much more sensitive, requiring much lower concentrations of the test compound to produce a response (*18*).

The S/N (signal/noise) ratio represents the light production of the exposed cells divided by the light production of nonexposed cells. A substance is considered genotoxic when the S/N is higher than 1.5 for at least two of the concentrations tested and when a clear dose-dependent relationship is observed (*17*). In addition, the ratio of S/N for *rec*/*pr1* must be greater than 1.5. If the *pr1* strain is strongly induced, no conclusions may be drawn about the genotoxicity of the compound, even if the ratio of *rec*/*pr1* exceeds 1.5. Where the S/N ratio drops below 0.8, a toxic response is indicated.

Micronucleus Test. The micronucleus test is a eukaryotic cell-based test for genotoxicity. The use of peripheral human lymphocytes in this test means that the results are directly applicable to humans, and no extrapolation, as in the case of bacterial-based tests, is necessary. The assay detects breakage to chromosomes and chromosome loss. The protocol described by Fenech and Morley (*19*) was followed.

Broken chromosomal material, or entire chromosomes that were damaged or incorrectly aligned (mispairing) at metaphase/anaphase become surrounded by nuclear membranes forming micronuclei. The potential of a possible mutagen to be clastogenic or to disrupt cell division and cause aneuploidy is measured by scoring the number of micronuclei in a population of dividing cells. It is, however, not possible to distinguish whether the mutagen causes aneuploidy or clastogenesis by the methods used in this study (FISH staining is necessary). The addition of cytochalasine B halts the process of cell division in telophase. After the cell culture has been fixed and spread on microscope slides, up to 1000 binucleate cells are investigated and the number of cells with one or more micronuclei is counted. The normal frequency of micronuclei is typically 0−5%, with only one micronucleus per cell.

RESULTS AND DISCUSSION

Positive Control Tests. Positive controls were carried out parallel with the test samples, using the Ames test and the Vitotox assay. As positive controls 4-nitroquinoline-*N*-oxide (4-NQO) was used in the absence of a metabolising S9 fraction, whereas benzo[*a*]pyrene (B(*a*)p) was used in the presence of S9. Applied concentrations were 4 and 8 ppm. Results obtained showed that these positive controls behave as expected (*20*) and that consequently all tests can be considered valid. Depending on the experiment and the *Salmonella* strain used, 4-NQO induced from 4 up to 10 times more revertants in the Ames test compared to the spontaneous (negative control) frequency. For benzo(*a*)pyrene, the increase was from 3.5 up to 11 times the spontaneous frequency (**Table 1**). **Figure 1** gives a typical example of a dose−response relationship for both positive controls in the VITOTOX test. The concentrations of the positive controls 4 and 8 ppm, were chosen on the basis of this kind of dose−response relationship.

From **Figure 1A**, it can be concluded that 4-NQO in the *rec* strain (w/o S9) clearly results in a dose-dependent light induction, which means the compound is shown to be genotoxic (signal/noise ratio > 1.5), but not toxic, because no induction or decrease in light production is observed (S/N > 0.8 for *pr1* strain, see **Figure 1B**).

Test with Melanoidins. Table 1 represents the results for all melanoidin fractions tested in the Ames test for both the TA98 and TA100 bacterial strains, with and without S9 activation. None of the melanoidin fractions tested showed mutagenicity in this test in the concentration range applied. Glucose−lysine reactions have yielded substances inducing reverse mutations in TA100, without enzyme (S9) activation (*21*). Experiments conducted by Kitts et al. (*8*) again showed the mutagenicity of these products to TA100 and TA98 without S9. On addition of S9, the mutagenicity induced in TA100 decreased, and was eliminated in TA98. There was considerable variation in the active dose ratios for both intact and nonvolatile neutral and basic fractions of Maillard reaction products and melanoidin fractions in the different short-term tests used to estimate genotoxicity (*8*).

Figure 2 represents the results obtained in the Vitotox test for the LMW fraction (A) and HMW (B) fractions, while **Figure 3** shows the *rec*/*pr1* ratios for the crude fraction (before dialysis) (A) and the volatile fraction (B) from the Maillard reaction. The ratios for *rec*/*pr1* are approximately 1 for the crude, LMW, and HMW fractions indicating no genotoxic response. The volatile fraction produced a positive response, shown by the original graphs (**Figure 4**) where the *rec* (**Figure 4A**) bacterial strain was strongly induced. However, the highest concentrations show low signal-to-noise ratios suggesting toxicity. This is confirmed by the results shown in **Figure 4B** (*pr1* bacterial strain) where all concentrations indicated toxicity as the lines lie below 0.8.

While the Vitotox assay proved to be very successful in the testing of pure chemicals and drugs for mutagenic effects (*17*, *18*), it has not been exhaustively tested for complex mixtures of compounds. Therefore, this study presented a valuable

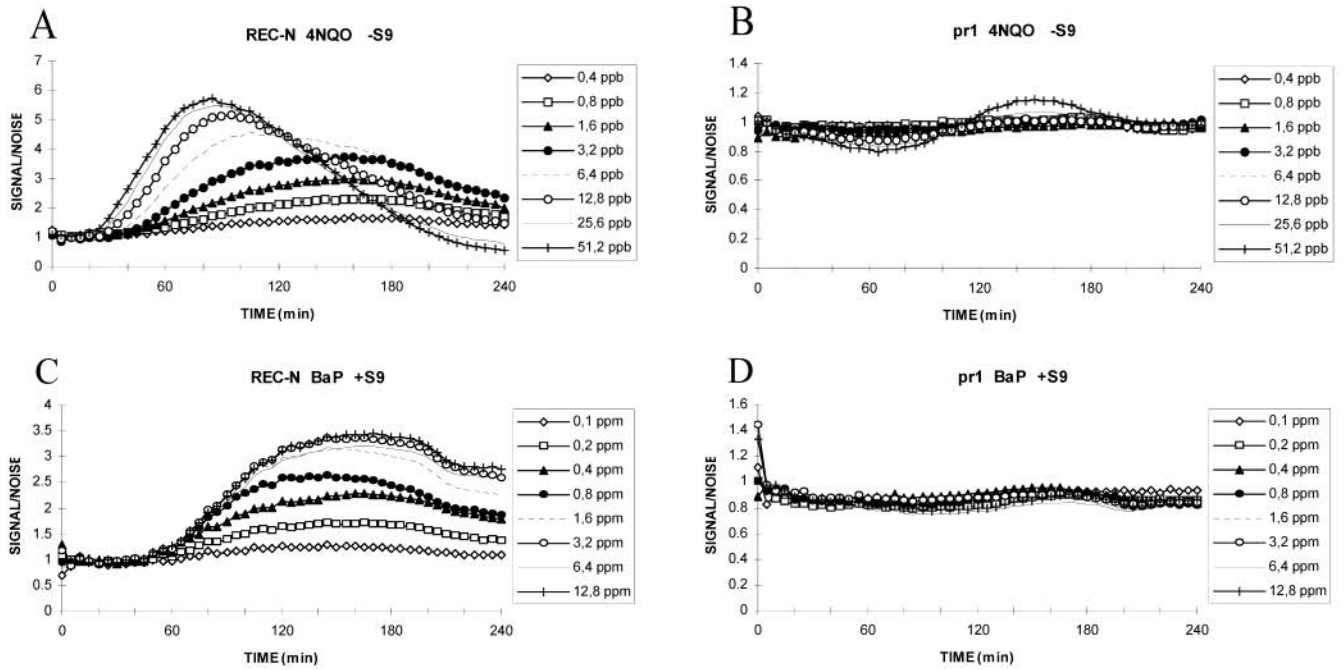

Figure 1. Results from the positive control and validation tests, using the Vitotox assay. (A) Mutagenicity of 4-nitroquinoline-*N*-oxide using *rec*-N strain (−S9), (B) toxicity of 4-nitroquinoline-*N*-oxide using *pr1* strain (−S9), (C) mutagenicity of benzo[*a*]pyrene using *rec*-N strain (+S9), and (D) toxicity of benzo[*a*]pyrene using *pr1* strain (+S9).

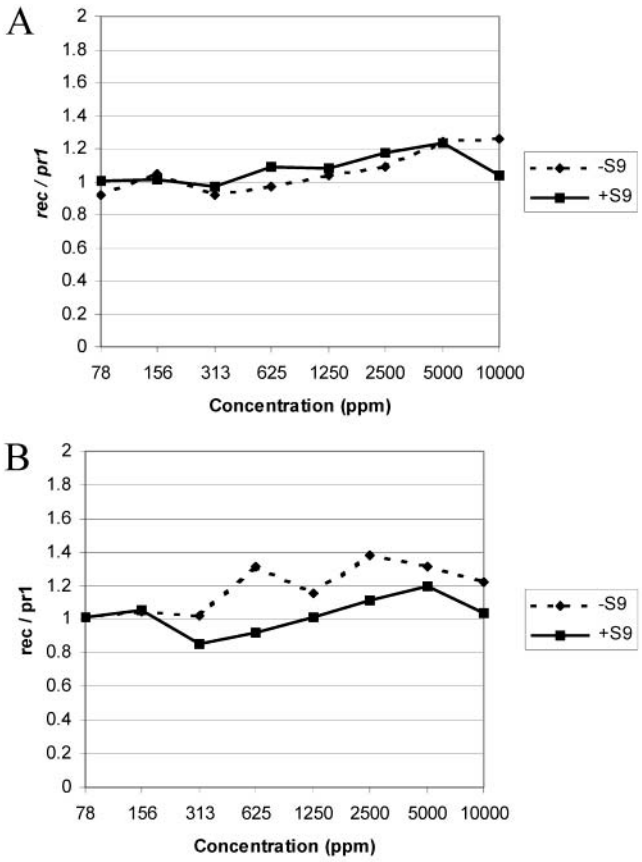

Figure 2. Results from the testing of (A) LMW and (B) HMW melanoidin fractions, obtained from the condensation of glucose and glycine, in the Vitotox assay.

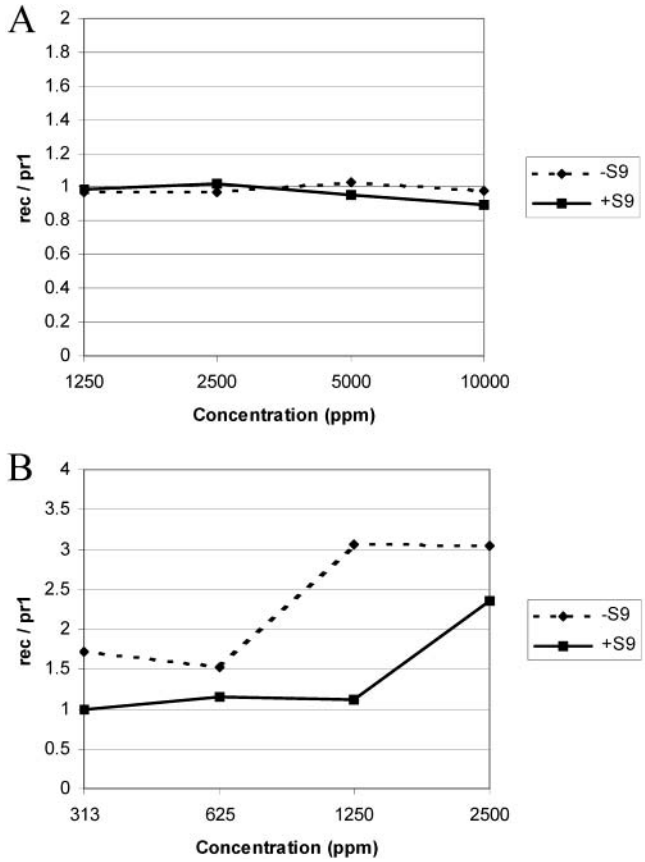

Figure 3. Results from the testing of (A) crude and (B) volatile melanoidin fractions, obtained from the condensation of glucose and glycine, in the Vitotox assay.

opportunity to use the assay in testing the different molecular weight fractions of melanoidins from the Maillard reaction. To date, overall, no positive results have been obtained for the samples tested, giving a positive indication of the safety of melanoidins derived from the model system. However, the volatile fraction does show some genotoxicity at subtoxic concentrations. Samples from the LMW (water-soluble) fraction

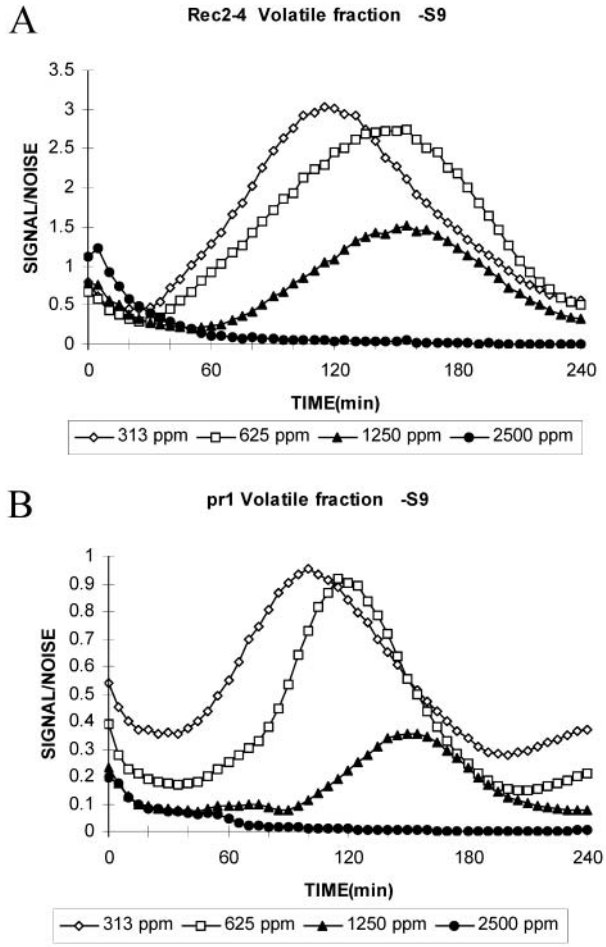

Figure 4. Results obtained for the (A) *rec* and (B) *pr1* bacterial strains from the testing of the volatile fraction, obtained from the condensation of glucose and glycine, in the Vitotox test.

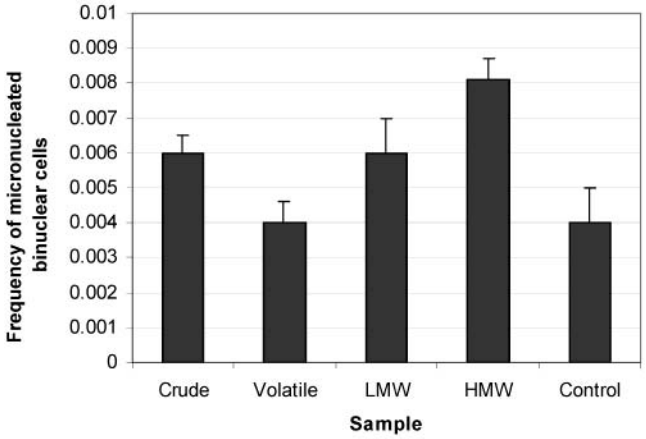

Figure 5. Frequency of micronuclei induced in binucleate human lymphocyte cells by melanoidin fractions obtained from the condensation of glucose and glycine.

tested in the Vitotox assay have shown no indication of mutagenicity, but some toxicity is evident above 5000 ppm.

Figure 5 represents the results from all 4 melanoidin fractions tested in the micronucleus test. None of the samples produced significantly elevated numbers of micronuclei relative to the control.

The condensation of glucose and glycine under the standard COST 919 protocol produced melanoidin fractions, with the exception of the volatile fraction, that showed no genotoxicity in the tests described above. This fraction showed both toxicity and genotoxicity in the Vitotox test, and should be investigated further. This product was, however, tested at concentrations far exceeding those found in food. Hence, it can be concluded that no genotoxicity was observed using our standardized protocol.

In effect, many compounds are formed in the Maillard reaction, some mutagenic and some anti-mutagenic. These cannot be differentiated, and it is thus the net influence of the combined mutagenic and anti-mutagenic compounds that is tested in the assays (*1*). The effect of genotoxic and mutagenic substances on bacteria can be strongly correlated to the mutagenic and tumor initiation effects of those substances in mammals (*16*).

Testing very high concentrations of the melanoidins can be misleading as these compounds are not present in such high concentrations in food. In the current study, we have shown that low concentrations are harmless. The potential hazard of Maillard reaction products is thought to be relatively small, and in relation to the well documented anti-oxidant properties of the Maillard reaction products, the mixtures seem to be more beneficial than harmful (*3*).

This work provides a preliminary investigation in this field and utilizes a variety of genotoxicity tests. This enables the detection of DNA damage as well as the loss and/or damage of entire chromosomes. It is necessary to use a battery of tests, based on different mechanisms, to fully test for genotoxicity. The Ames test detects induction of gene mutations, whereas the micronucleus test allows detection of chromosome and/or genome mutations. The Vitotox assay detects DNA damage that may or may not result in true mutations. Both eukaryotic-cell-based assays and bacterial tests should be used in conjunction to investigate compounds or mixtures suspected of being carcinogenic.

It can be concluded that the crude reaction mixture (before dialysis), and both the LMW and HMW fractions obtained by dialysis showed no genotoxicity in these assays, despite being tested at concentrations much higher than those naturally found in food products. The LMW fraction, however, showed toxicity at these high concentrations. The volatile fraction produced in this reaction showed genotoxicity only in the Vitotox test, at subtoxic concentrations.

The melanoidins investigated in this study were produced from the condensation of glucose and glycine, according to the COST Action 919 protocol. However, it seems very worthwhile to study the mutagenicity of other melanoidins, such as those obtained from the glucose−lysine reaction as well, using the three in vitro assays discussed in this paper. For further validation of the method and to investigate the possible antimutagenic activity of the melanoidins, the melanoidin fractions could then be spiked with mutagens to test for inhibition of the mutagenic response.

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