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MUTAGENICITY OF SANGAK BREAD WITH EMPHASIS ON GASOLINE AS A BURNING FUEL

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ABSTRACT

In this research, the first batch of Sangak bread was collected from those bakeries in Shiraz that use gasoline as a burning fuel. The Felton *et al.* method (3) was used to prepare acidic extract (containing both acidic & neutral components) and basic extract of bread samples, gasoline soot, and artificially contaminated bread. *Salmonella*/mammalian-microsome assay (Ames test) was used to detect mutagens in collected bread samples. The mutagenicity of acidic and basic extracts of different samples was assayed on TA98 and TA100 tester strains in the presence or absence of rat liver extract (S9). The results of soot and artificially contaminated bread with soot indicate that: (i) the mutagenic compounds in these samples are extracted in acidic fraction and this fraction shows mutagenicity on both TA98 and TA100, with higher activity on TA100. (ii) The mutagenic activity of acidic fraction increases evidently in the presence of S9 mix. (iii) The mutagenicity of basic fraction is negligible. The results of analysis of bread samples reveal that the basic extract in the presence or absence of S9 mix on TA98 and in the presence of S9 mix on TA100 has mutagenic activity. Furthermore, this study indicates that the acidic extract of bread samples possesses mutagenicity in the presence and absence of S9 mix on TA100 and in the absence of S9 mix on TA98. The mutagenicity of acidic extract indicates that bread may be contaminated with soot during the thermal processing.

Key words: Ames test, Gasoline soot, Mutagens, *Salmonella*/mammalian-microsome assay, Sangak bread.

1. Former Graduate Student (now Instructor) and Assistant Professor, respectively.

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جهش زایی نان سنگک تهیه شده باسوخت گازوییل

رقیه رمضانی و احمد کرباسی
به ترتیب مربی واستادیار بخش علوم وصنایع غذائی دانشکده کشاورزی دانشگاه شیراز، شیراز، جمهوری اسلامی ایران.

چکیده

در این بررسی اولین پخت نان سنگک از نانوایی هایی که در سطح شیراز سوخت گازوییل رامصرف می کردند جمع آ وری گردید و با استفاده از روش فلتون و همکاران (۳) فازهای اسیدی (شامل ترکیبات اسیدی وخنثی) و بازی از نمونه های نان ، دوده گازوییل ونان آلوده شده به دوده، تهیه شد. با بکارگیری تست زیست شناسی Ames وبا استفاده از باکتری های سالمونلا گونه های TA98 وTA100 در حضور وعدم حضور عصاره کبد موش (S9) اثرجهش زا بودن فازهای استخراج شده از نمونه ها بررسی گردید .نتایج حاصل از نمونه دوده و نانی که بطورعمد به دوده گازوییل آلوده شده بود نشان داد که: (الف) فاز اسیدی بر باکتری های مورد آ زمایش (TA98 و TA100) اثرجهش زایی نشان می دهد و باکتری TA100 حساسیت بیشتری را بروز می دهد. (ب) افزایش جهش زا بودن فاز اسیدی در حضور S9 دیده شد و (ج) اثر فاز بازی ناچیز است. نتایج به دست آ مده از اثر جهش زایی مواد استخراج شده از نان نشان می دهد که فاز بازی بر باکتری TA98 در حضور وعدم حضورS9 ، و بر باکتری TA100 درحضورS9 اثر جهش زایی دارد. همچنین فاز اسیدی، در حضور و عدم حضورS9 بر باکتری TA100 و در غیابS9 بر باکتری TA98 اثر جهش زایی دارد. نتایج نشان می دهد که جهش زا بودن فاز اسیدی به دلیل آ لودگی نان به دوده، در طی فرآ یند حرارتی است.

INTRODUCTION

The possible relationship between diet and cancer has attracted interest in recent years. Gastric cancer is a very typical cancer related to nutrition and dietary condition (15). High incidence of colon and breast cancer in human are related to intake of high level of calories and fat (1, 16). A positive association was reported between intake of fried meat and the risk of female-hormone related

cancer (8). Concern about the role of diet in human cancer has prompted the search for mutagenic-carcinogenic compounds in common foods. A growing amount of evidence indicates that mutagenic substances are formed during the heating of various foods and food components. Three kinds of mutagenic and carcinogenic compounds are produced during the cooking process, (i) polycyclic aromatic hydrocarbons, (ii) nitropyrene and (iii) heterocyclic amines (18). The reports clearly indicate that during the thermal process of baking some carcinogenic hydrocarbons are formed (17). 3, 4-benzo pyrene and some other polynuclear aromatic compounds have been detected in flour and bread. The presence of benzo(a)pyrene in smoked food products, toasted bread (11) and burnt bread (4) was reported.

In Iran, baking depends on using unrefined residual oil, gasoline and natural gas as burning fuel. In this research, we were concerned about finding how direct flame produces mutagenic compounds in baking products. Sangak bread is baked in a special baking oven (Tanur) which is covered with small gravel that is heated directly by the burning fuel. At the beginning of baking, due to incomplete combustion of fuel, large amount of soot is produced that can contaminate the surface of bread. Therefore, the mutagenicity in first batch of Sangak bread which is baked using gasoline as burning fuel was determined. We also investigated the effects of direct flame in producing mutagenic compounds in bread during baking.

MATERIALS AND METHODS

Materials

Chemicals. Chemicals such as sodium azide, 4-nitroquinoline N-oxide, benzo(a)pyrene, 2-amino fluorene, 4-nitro-o-phenylene diamine, ampicillin trihydrate, D-biotin, L-histidine chloride, D-glucose-6-phosphate (mono sodium salt), and NADP (sodium salt), were obtained from Sigma Chemical Company (St. Louis, MO 63160, U.S.A.) and the other chemicals from E. Merck Chemical Co. (Frankfurter Strãsse 250,6100 Darmstadt, Federal Republic of Germany). Microbiological media were purchased from Difco Laboratories (Detroit Michigan, 48232, U.S.A.) and oxoid nutrient broth No. 2 from Oxoid Ltd. (Wade Road, Basing Stoke, Hants, RG 24 OPW,

England), rat liver S9 from Microbiological Associates Inc. (9900 Blackwell Rd. Rock Ville, MD 20850, U.S.A.), tester strains TA98 and TA100 from Ames Laboratory (Biochemistry Department, University of California, Berkeley, CA 94720, U.S.A.).

Methods

Extraction of mutagenic compounds. The acetone extraction method (3) was used to extract mutagenic compounds.

Sangak bread samples. The first batches of baked breads were collected from ten bakeries which used gasoline as burning fuel in Shiraz. The samples were weighed (50 kg fresh samples with average moisture of 26.7%) and dried in open air for 48 hr at room temperature (5-6% moisture), then ground and stored in air tight plastic bags. For extraction, 1000 g of each bread sample in portions of 100 g each were added to 200 ml of acetone. After shaking at 300 rpm for 2 hr using Gyrotory waterbath shaker (Model G76, New Brunswick Scientific Co. Inc., N.J., U.S.A.), the resulting homogenate was vacuum-filtered through a Buchner funnel. The solids were again homogenized in acetone (100 ml) and shaking was continued for another hour and then vacuum-filtered. The filtrates were combined and stored at -18° C for 18 hr to induce protein precipitation, then they were filtered through Whatman No.1 filter paper. The clear yellow filtrate was concentrated to near dryness and treated as follows to obtain extracts.

In a 500-ml separatory funnel, 200 ml of 0.01 N HCl was added to the concentrated filtrate and extracted 3 times with 100, 50, 50 ml CH_2Cl_2, respectively, to obtain acidic extracts (containing acidic and neutral components). The aqueous phase was used for the following fractionation. The pH of aqueous phase was adjusted to 12 with 1N NaOH and extracted twice with 100 and 50 ml CH_2Cl_2, respectively. The basic extract was obtained by collecting the CH_2Cl_2 phase. The aqueous phase was discarded.

The methylene chloride extracts were dried over sodium sulfate, and evaporated to a small volume under nitrogen stream at 40° C. Different aliquots were combined to obtain organic extract based on 500 g bread samples (5-6% moisture) in duplicate for each bakery. Further reduction in volume was followed under nitrogen gas at 40° C. The weights of crude

extracts were determined. The average wt. of acidic and basic extracts per 500 g bread samples (n=20) were 1.47 and 0.07 g, respectively. Extracted samples were stored at -18° C. In mutagenicity assay, the extracts were dissolved in 1 ml ethylene glycol.

Soot sample. Gasoline soot was collected on the bottom of a water cooled flask placed directly above the gasoline flame and stored in a dark bottle at room temperature. Two gasoline soot samples, 12.5 mg and 25 mg were weighed in duplicate. The mutagenic compounds were extracted as described earlier for bread samples.

Artificially contaminated bread sample. One thousand grams of bread sample in 100 g portions was mixed well with 5 mg gasoline soot . Extracts were prepared in duplicate based on 500 g bread sample and 25 mg gasoline soot.

***Salmonella*/mammalian microsome mutagenicity assay.** The mutagenic activity of samples was assayed according to the Ames test on *Salmonella typhimurium* TA98 and TA100 (12). Prior to the assay of the mutagenicity of samples, dichloromethane was evaporated thoroughly at 40-45 °C with a stream of nitrogen. The samples were then redissolved in 1 ml ethylene glycol. The mutagenicity was determined in the presence of 0.1 ml of sample extract, 0.1 ml tester strains ($1\text{-}2\times10^9$ cells ml^{-1}) with 0.5 ml S9 mix (50 µl S9 $plate^{-1}$) and without S9 mix.

RESULTS

Standard Mutagens

The spontaneous reversion of tester strains was routinely determined. On the average (30 plates) 22 and 147 revertants per plate without S9 mix were obtained for TA98 and TA100, respectively. These data are in the acceptable range by Ames Laboratory (12). During the mutagenicity assay, the specificity of each strain and the efficiency of S9 mix were confirmed by testing the standard mutagens. The general view of these results (12 plates for each treatment using tester strains TA98 and TA100 with and without S9) are shown in Fig. 1.

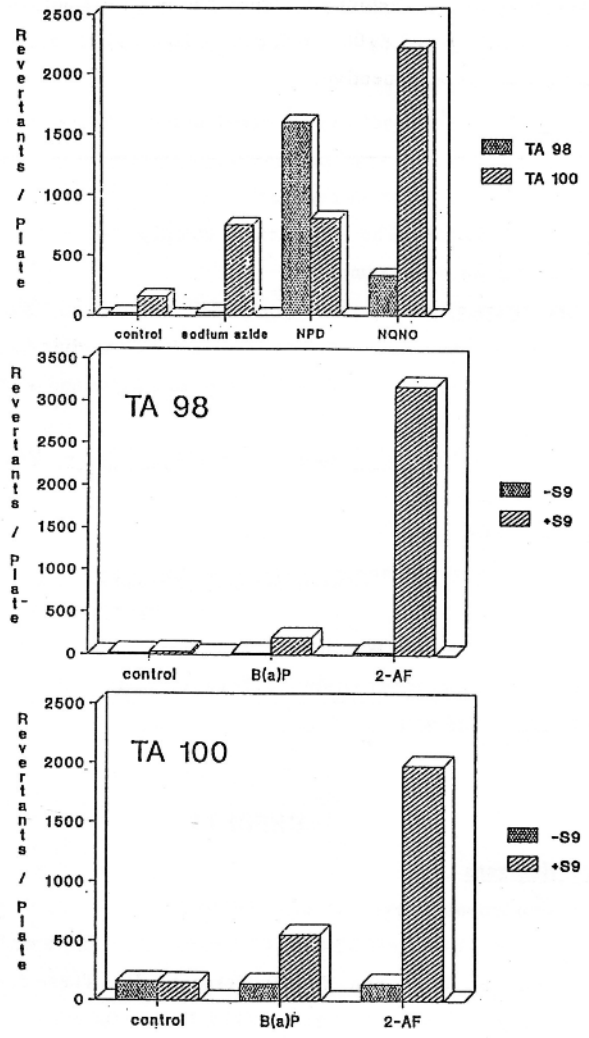

Fig. 1. Mutagenicity of standard mutagens in comparison with control on TA98 and TA100. Sodium azide (1.5 µg plate^{-1}). NPD=4,nitro-o-phenylenediamine (20 µg plate^{-1}). NQNO=4-nitro quinoline-N-oxide (0.5µg plate^{-1}). B(a) P= Benzo (a) Pyrene (1 µg plate^{-1}). 2AF= 2-Amino fluorene (10 µg plate^{-1}).

Bread Extracts

The results of mutagenicity of acidic and basic extracts (40 plates for each strain, TA98 and TA100, with and without S9) of bread samples are shown in Figs. 2 and 3, respectively.

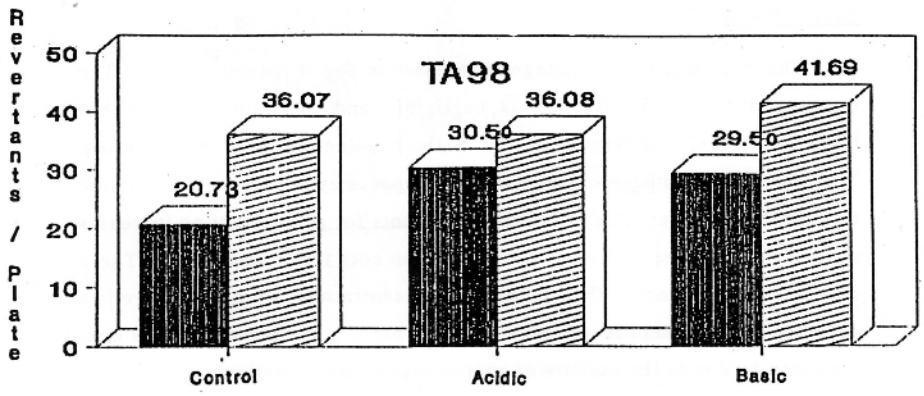

Fig. 2. Mutagenicity of acidic and basic extracts of bread samples on TA98. (Extracts of 50 g dried bread plate^{-1}).

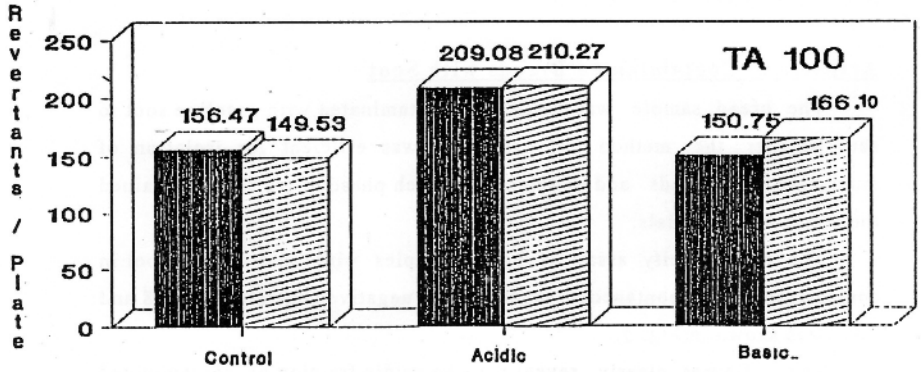

Fig. 3. Mutagenicity of acidic and basic extracts of bread samples on TA100. (Extracts of 50 g dried bread plate^{-1}).

The mutagenic activity of acidic fraction in comparison with control group on TA100 is relatively high (Fig. 3). The basic fraction of bread samples reveals mutagenic activity on TA98, both in the presence or absence of S9 mix (Fig. 2) and on TA100 in the presence of S9 mix (Fig. 3).

Soot Extracts

The results of soot mutagenicity assay in Fig. 4 indicate that: (i) The mutagenicity of basic fraction is negligible and the acidic fraction has higher mutagenic activity than the basic fraction on both tester strains. Therefore, the mutagenic compounds in soot may be extracted in acidic fraction (ii). The number of induced revertants for acidic fraction increases significantly in the presence of S9 mix on both tester strains (iii). These curves show distinctly that at higher concentrations of soot, there is a decrease in the number of revertants per plate in the presence of S9 mix. This may be due to the toxicity of soot at higher concentrations.

TA98 and TA100 tester strains detect various frame shift and base pair substitution mutagens, respectively. In soot mutagenicity assay, TA100 shows higher sensitivity than TA98 to acidic fraction. Therefore, base pair substitution mutagens exist more predominantly than frame shift mutagens in acidic extract of gasoline soot.

Artificially Contaminated Breads with Soot

The bread sample was artificially contaminated with gasoline soot to see whether the method of extraction was efficient for isolation of mutagenic compounds and to determine which phase of fractions contained the mutagenic materials.

The mutagenicity assay of bread samples with and without soot in comparison with spontaneous reversion as negative controls on TA98 and TA100 is shown in Fig. 5.

These figures clearly reveal that the acidic fraction of contaminated bread with soot has higher mutagenic activity than the basic fraction, on both tester strains. The number of revertants in each extract increases in the presence of S9 mix. However, it is more noticeable in the acidic fraction of contaminated bread.

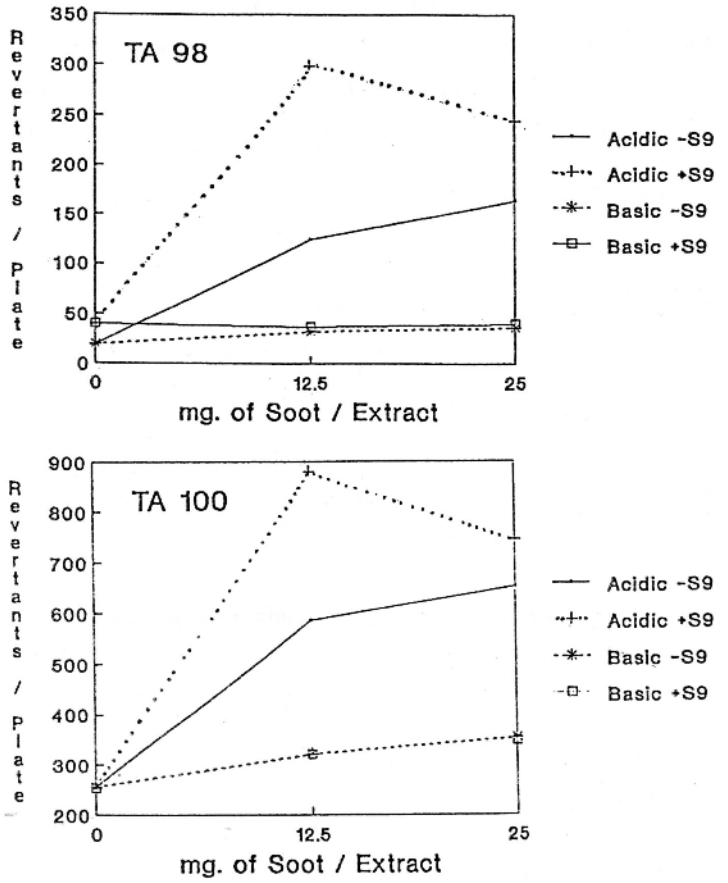

Fig 4. Mutagenicity assay of gasoline soot extracts on TA98 and TA100.

These results show that, the method of extraction is satisfactory for separating and extracting mutagenic compounds into acidic extract. Finally, the presence of activation enzymes in S9 mix increases the mutagenic activity of compounds in the acidic fraction. Therefore, the mutagenic activity of acidic fraction of bread contaminated with soot may be due to the presence of the compounds in the soot.

Statistical Analysis

Application of suitable statistical test (θ) is needed to determine the mutagenicity of bread extracts (6, 7). The value of θ for TA98 and TA100 are shown in Table 1. At 5% significance level if θ> 1.64, the chemical is considered to be mutagen.

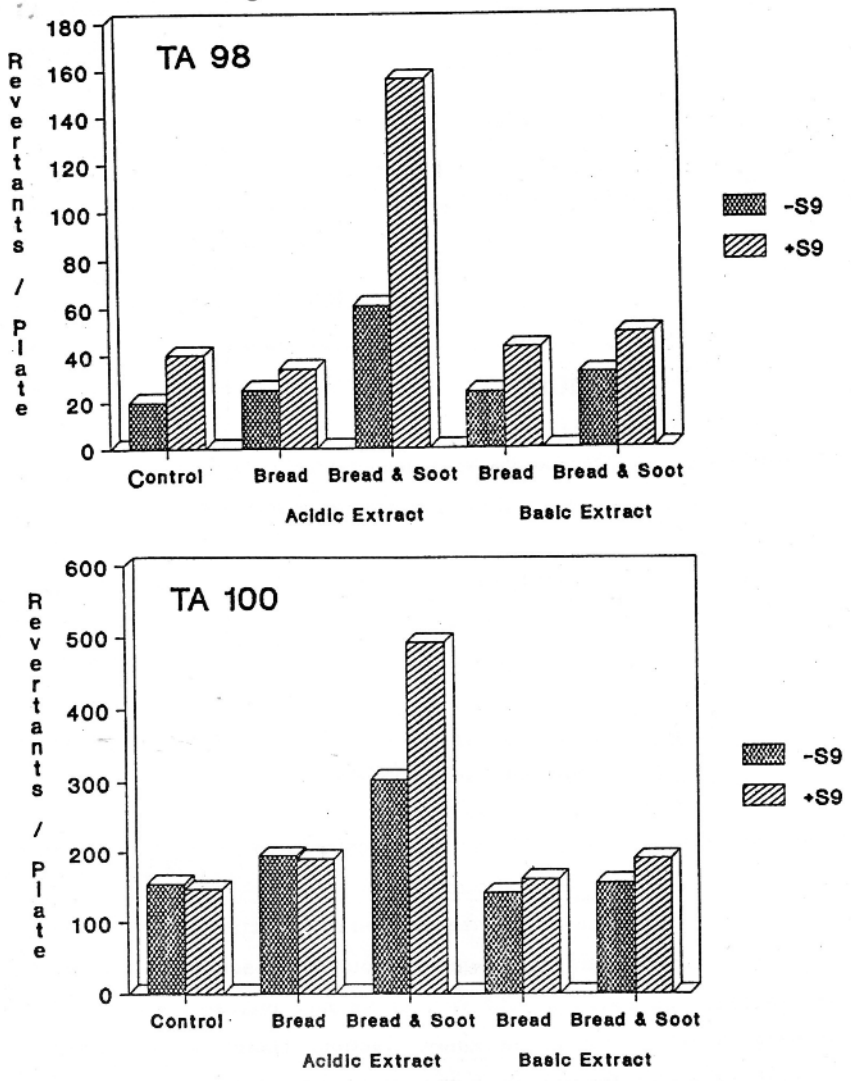

Fig. 5. Mutagenicity assay of bread sample extracts with soot (50 g dried bread and 2.5 mg soot plate^{-1}) and without soot (50 g dried bread plate^{-1}).

Table 1. Summary of mutagenicity data (plate incorporation test) for bread extracts with and without S9 on TA98 and TA100.

Treatments	Groups	θ† TA98	θ† TA100
-S9	Control Acidic extract	6.12§	12.33§
+S9	Control Acidic extract	-¶	14.32
-S9	Control Basic extract	5.55§	-¶
+S9	Control Basic extract	2.9§	4.3§

† $\theta = [(M-0.5) - b(m+0.5)]/ab(M+m)$

a, b = Σ Number of plates in each treatment between phase extract and control group, respectively.

M, m= Σ Number of revertant in each treatment between phase extract and control group, respectively.

§ Extracts are mutagens at 5% significance level ($\theta > 1.64$).

¶ θ is negative ($\theta < 1.64$) and extracts are non-mutagenic.

The results of Duncan's multiple range test (Table 2) indicate that at 5% probability level a significant difference exists between the means of revertants in control groups, basic and acidic extracts on TA98 and TA100.

Table 2. Comparison of means of number of revertants per plate in control groups, acidic and basic extracts of bread samples with and without S9 on TA98 and TA100.

Treatments	Groups	Revertants plate^{-1}† TA98	Revertants plate^{-1}† TA100
-S9	Control	20.73 d	156.47 bc
+S9	Control	36.07 b	149.53 c
-S9	Acidic extract	30.50 c	208.58 a
+S9	Acidic extract	36.08 b	210.24 a
-S9	Basic extract	29.53 c	150.75 c
+S9	Basic extract	41.69 a	166.10 b

† Means in each columm followed by the same letter are not significantly different at the 5% probability level (DMRT).

The results of t-test between each group are shown in Table 3. T-test analysis indicates that acidic fraction of bread samples on TA100 and basic fraction on TA98 are significantly different in comparison with the control group.

Table 3. Comparison of number of revertants in control groups and bread extracts on TA98 and TA100 using t-test analysis.

Treatments	Groups	TA98	TA100
-S9	Control Acidic extract	t= -4.17 p= 0.0001†	t= -5.7 p< 0.0005†
+S9	Control Acidic extract	t= -0.00 p= 1.0	t= -8.51 p< 0.0005†
-S9	Control Basic extract	t= -3.17 p= 0.0025†	t= 1.14 p= 0.26
+S9	Control Basic extract	t= -2.65 p= 0.011†	t= -2.68 p= 0.0098†

† The numbers of revertants in these groups show significant differences.

DISCUSSION

Comparison of bread mutagenicity in this study with those from other studies (13, 14) indicates that the mutagenicity of basic fraction of bread samples (Tables 1, 2 and 3) is in agreement with those reported in the literature. Basic fraction of toasted bread showed mutagenicity in *Salmonella*/microsome assay (13, 14). The latter authors prepared bread samples by toasting in an electric stove and the browned or charred portions of the bread were used for extract preparation. But in our study Sangak bread samples were used without any further treatment. Moreover, a different method of extraction was applied. The mutagenicity of heterocyclic amines in the basic fraction of some cooked foods has been well demonstrated. Also the mutagenicity of the basic fraction of bread is related to the presence of heterocyclic amines that may be produced during the browning reaction in baking (14). On the other hand, the higher sensitivity of TA98 to the mutagenic compounds in the basic fraction is in agreement with those reported by some investigators (3). The basic fraction

of bread samples revealed mutagenic activity on TA98 rather than on TA100 (Figs. 2 and 3).

This study also indicates that the presence of mutagenic compounds in the acidic fraction of bread samples (Tables 1, 2 and 3) might possibly be due to the contamination of bread surface with soot during incomplete combustion of gasoline fuel. The mutagenicity of basic extract of bread samples has been investigated by some investigators (13, 14). However, these authors ignored the mutagenicity assay of the acidic fraction of bakery products. The following reasons concerning the nature of mutagenic compounds in the acidic extract of bread samples may be outlined:

(i)-The presence of polycyclic aromatic hydrocarbons (PAHs) in smoked and grilled foods has been demonstrated very well. In fact, the incomplete combustion of the fuel used as source of heat could contaminate the surface of food with the PAH containing fumes (10). It has long been recognized that products from incomplete combustion are carcinogenic to human. Benzo(a)pyrene has been identified as one of the active mutagenic/carcinogenic compounds of soot (5). Evidence from the experiment on soot suggests that most mutagenic compounds in soot are extracted into the acidic fraction. The mutagenicity of the acidic extract of soot sample with and without S9 mix on TA98 and TA100 was clearly demonstrated (Fig. 4). Moreover, the results of artificially contaminated breads with soot confirmed its mutagenic activity (Fig. 5). The occurrence of PAHs, nitro-PAH and oxygenated PAH has been reported in the neutral/acidic subfractions (10). There is, however, no doubt about the mutagenicity of acidic extract of bread samples that may be related to the contamination of bread with polycyclic aromatic hydrocarbon compounds in soot during baking. The higher risk of lung cancer in bakeries using modern baking ovens that produce some PAH has been emphasized (17).

(ii)- Some investigators reported the high sensitivity of *S. typhimurium* strain TA100 to polycyclic aromatic hydrocarbons (2, 9) which is in full agreement with our data. The ability of benzo(a)pyrene to induce His^+ reversion is higher on TA100 than on TA98 tester strain (Fig. 1). Furthermore, the results of mutagenicity assay of the acidic extract of soot sample and artificially contaminated bread showed higher activity on

TA100. Indeed, the increase of mutagenic activity on TA100 rather than TA98, by acidic extract of bread samples may be explained by the presence of PHAs in this phase.

In conclusion, the results of this study indicate that it is necessary to modify the burning system to minimize the formation of mutagens during thermal treatment of bread. The foregoing results give rise to a number of questions which require further investigation on other types of bread, especially those that are baked in bakeries using unrefined oil as an energy source. Purification of mutagenic compounds and determination of the amount of PAHs in the acidic fraction is of further interest.

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