Minimal amount of aflatoxin b_1 to produce a mutation in the Ames test with *salmonella thyphimurium* Ta-98

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Cantidad mínima de aflatoxina B_1 que produce una mutación en la prueba de Ames con *salmonella typhimurium* ta-98

Resumen. Se probaron diferentes concentraciones de aflatoxina B_1 (AFB_1) en la Prueba de Ames con *Salmonella typhimurium* TA 98 activada con fracción S_9 microsomal de hígado de rata y nicotinamida adenina dinucleótido 3´fosfato (NADP) como cofactor, en un rango desde 0.39 hasta 100 ng, con un promedio de 20 colonias mutantes espontáneas en los controles. Las diferentes concentraciones de AFB_1 provinieron de una solución estándar base de AFB_1 de 1 μg/mL que se cuantificó por cromatografía de líquidos de alta resolución (HPLC), con un Coeficiente de Correlación de r =0.99 en la curva de calibración. La cantidad mínima de AFB_1 capaz de producir una mutación fue de 10 ng/g (=10 ìg/kg). Es de suma utilidad tomar en cuenta esta cantidad como nivel máximo de tolerancia legal en la normatividad para alimentos de consumo humano. En este momento el nivel máximo de tolerancia que se usa para transacciones comerciales en el Tratado de Libre Comercio es de 20 μg/kg. La AFB_1 se une al ADN, formando aductos que son buenos biomarcadores de exposición, además hay un efecto acumulativo y los largos periodos de contacto con potentes cancerígenos dificulta su control en alimentos de consumo humano.

Palabras clave: Aflatoxinas, mustagénesis, lçimites legales.

Abstract. Different concentrations of aflatoxin B_1 (AFB_1) were tested in *Salmonella typhimurium* TA 98 Ames test activated by rat liver microsomal S_9 fraction and nicotinamide adenine dinucleotide 3´phosphate (NADP) as cofactor, at ranges from 0.39 to 100 ng, with an average of 20 spontaneous reversal colonies in the controls. The different AFB_1 concentrations derived from a stock AFB_1 standard solution of 1 μg/mL were quantified by liquid chromatography (HPLC), with a Correlation coefficient of r= 0.99 in the calibration curve. The minimum amount of AFB_1 that can produce a mutation was 10 ng/g (=10 μg/mL). This level can be useful to take into account for legal maximum tolerance levels in laws applied to foods for human consumption. At this moment the maximum tolerance level used for commercial transactions of NAFTA is of 20 ìg/kg. Nowadays legal maximum tolerance limits are used for commercial transactions but do not protect human health. The accumulative effect of AFB_1 in DNA, as adducts, and the long periods of exposure to these potent carcinogen makes its control more difficult in foods for human consumption.

Key Words: Aflatoxins, mutagenesis, legal limits.

Received 15 April 2004; accepted November 11 2004.
Recibido 15 abril 2004; aceptado 11 noviembre 2004.

Introduction

The Mexican population has maize as staple food, and this cereal is frequently contaminated with aflatoxins (AF) which are severe mutagens that pose a constant threat to the genome of an organism. Aflatoxins are secondary metabolites of the fungi *Aspergillus flavus* Link [10], *A. parasiticus* Speare [3]

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REVISTA MEXICANA DE MICOLOGÍA 19: 71-79, 2004 /

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and *A. nomius* [15] of well known chemical structure [2], biosynthesis [26], production conditions [6, 29] and toxic effects [23] such as carcinogenicity [20, 28], teratogenicity [18] and mutagenicity [17] among many other.

AF activate the proto-oncogene H ras to oncogene producing punctual mutations that cause substitutions G-C to T-A or G-C to A-T [30], most of the mutations are eliminated, but the carcinogen AFB_1 is accumulated in the DNA during lifetime and sometimes DNA cannot be repaired properly and these AF mutations can initiate a malignant neoplasia or cancer.

Aflatoxins are linked to DNA producing adducts (AFB_1-DNA) that are good biomarkers, there is a correlation of DNA adduct levels with tumor incidence [24].

Lee *et al.* [16] explained the molecular basis for the participation of mutation at codon 249 of the p53 gene (p53mt249) induced by the potent mutagen AFB_1 in the genesis of hepatocellular carcinoma (HCC).

AFB_1 induced mutation p53mt249 is critical during the formation of HCC following hepatitis B virus infection. P53mt249 markedly increases insulin-like growth factor II transcription from promoter 4, accumulating the fetal form of IGF-II. The blocking of apoptosis through enhanced production of IGF-II should provide a favorable opportunity for the selection of transformed hepatocytes.

In the ras gene superfamily, codon 12 (-TGGTG-) of the K-ras gene is the most frequently mutated codon in human cancers. AFB1 targeted carcinogen-DNA adduct formation is a major reason for the observed high mutation frequency at codon 12 of the K-ras gene in human cancers [12].

One of the highest incidence rates of HCC is found in China where chronic infection with hepatitis B virus (HBV) and exposure to aflatoxins in foodstuffs are the main risk factors [25]. HCC is the most common type of liver cancer, the major risk factors being hepatitis B and C viruses and AF; other factors such as alcohol are also of importance in some populations. Aflatoxins exposure biomarkers include urinary aflatoxin metabolites and aflatoxin-albumin adducts in peripheral blood [21].

AFB_1 causes chromosomal aberrations and DNA ruptures in animal and plant cells [22], as well as mutations in bacterial genes when they are activated with rat microsomal fraction [31]. The Ames Test is a reliable model to check mutagenicity of AF. The mutated TA98 strain of *Salmonella typhimurium* lacks the enzymatic machinery to synthesize its own histidine and can not grow in minimal culture medium. The presence of a mutagen allows revertant (mutated) colonies to rise in proportion to the mutagenic strength of the sample tested.

It was interesting to note that caffeic acid and glutathione [14] and the plant *Maytenus ilicifolia* [11] had an effective antimutagenic effect against AFB_1 in the Ames Test.

Commercial treaties between Mexico and United States have developed similar legislations in both countries with a maximum tolerance level of 20 µg/kg of total AF, in maize for human consumption, to be practical and keep these commercial transactions easy. This 20 µg/kg level is based on studies about the amount of AF that produced proliferation of biliary channels in ducks of one day of age, that was 21 µg/kg, so 20 µg/kg was considered "safe".

The purpose of this work is to know the minimal amount of aflatoxin B_1 (AFB_1) necessary to produce a mutation, in order to understand if the AF contamination accepted by governmental tolerance limits in maize represents a health risk, and therefore to determine if the present legislations of Mexico and USA protect the human health.

Materials and methods

Tests strains for the Ames Test

Salmonella typhymurium TA98 strain [*hisD3052, gal,* ▲ *(chl, uvrB, bio) rfa,* pKM101(MucA/B Amp)] were donated by Dr. Ames, in filter paper disks in agar. Disks were reactivated in 5 mL of nutritive broth Oxoid N°2 (nutritive broth 1.25 g dissolved in 50 mL of distilled water and sterilized at 121 °C for 15 minutes, and disposed in tubes with 5 mL each added with 5 ìL of ampicillin trihydrated from Sigma-Aldrich, Cat. A 6140), incubated 16 hours at 37°C in a soft shaker (LabLine). The presence of the genetic markers, the frequency of spontaneous reversion and their sensitivity to known mutagens were tested. Extra stock cultures were obtained by adding 0.8 mL of bacterial suspension, incubated 16 hours, in dimethyl sulphoxide (DMSO from J.T.Baker), frozen quickly with dry ice and stored at 80 °C (Forma Scientific Freezer). Disposable Petri dishes 10 x 15 mm of high transparency polyestirene sterilized with gamma radiation (Vecton Dickinson) with Vogel-Bonner minimum media, complemented with an excess of L-histidine (Sigma-Aldrich, Cat.H 6034), were prepared. A sterile solution of 0.1 mL of ampicillin trihydrated (Sigma-Aldrich, Cat. A6140), at a concentration of 8 mg/mL, was added to the media with the bacterial strain TA98.

Bacterial cultures for mutagenicity tests were obtained taking a sample of the bacterial strains stock in Petri dishes, and sowing it in 5 mL of nutritive broth, incubating at 37°C with overnight agitation. For each experiment we used fresh bacterial cultures. All samples were tested in 3 replicated plates.

New bacterial cultures were prepared for each new experiment, they were taken from the stock of bacterial TA-98 strain and never from the disposed Petri dishes, because these last could have lost the plasmid uvr Bt marker. All samples were tested in 3 replications.

Control treatments for Ames test

The following 4 negative control treatments were included:

a) Without microsomal S9 fraction, without phosphate buffer solution (PBS) nor AFB_1.

b) With 500 µL of microsomal S9 fraction (from Molecular Toxicology Incorporated Moltox induced with Aroclor 1254), without PBS nor AFB_1

c) Without microsomal S9 fraction, with 500 µL PBS, without AFB_1

d) Without microsomal S9 fraction, with no PBS, and with either 100 ng or 50 ng AFB_1.The stock AFB_1 (Sigma-Aldrich, Cat. A 6636) concentration of 1µg/mL was dried at 45°C and resuspended in 500 µL of DMSO for Ames test.

Agar medium preparation

1. TopAgar

Contained 0.6% agar (Merck Co.) and 0.5 % NaCl (Merck Co.) heated at boiling temperature until it looked translucent, when this mixture was at room temperature, 10 mL of a 0.5 mM solution of histidine-biotin that had 0.077 g of L-histidine and 0.0122 g of biotin (Sigma-Aldrich) in 100 mL of sterile distilled water) was added. The histidine-biotin solution was stored at 4°C in a dark glass bottle. Later, all the components were mixed and aliquotes of 2.5 mL were done in sterile tubes and stored in the freezer. When the tubes with top agar were needed, they were previously melted in boiling water.

2. Agar plates

Petri dishes (Vecton Dickinson) contained 30 mL of minimal agar (2% glucose, 1.5% of agar) (Merck Co) in Vogel-Bonner E medium.

The minimal medium E of Vogel-Bonner included 7.5 g of Bacto Agar (Difco) or purified Agar-Agar (Merck Co.) without inhibitors, diluted in 300 mL of distilled water and sterilized at 121°C, during 15 minutes. Ten grams of dry dextrose (Merck Co.) diluted in 100 mL distilled water were sterilized at 121 °C for 15 minutes.

The concentrated Voguel-Bonner solution was done with 10 grams of magnesium sulphate heptahydrated, 100 g of citric acid monohydrated, 500 g of dibasic anhydrous

potassium phosphate, 175 g of tetrahydrated amonium sodium phosphate (SigmaAldrich) and 600 mL of distilled water. Salts were added slowly in a thermo shaker (LabLine) at 35 °C, once dissolved they were taken to 1 L , which was vacuum filtered, and one mL of chloroform (Merck Co.) was added and they were stored in a dark glass bottle at room temperature, it can be stored in this way for 6 months. Ten milliliters of concentrated Voguel-Bonner solution and 90 mL of distilled water were mixed and sterilized at 121°C for 15 minutes. Once sterilized, the dextrose and the agar were mixed with minimum media E of Vogel-Bonner and dispensed in Petri dishes.

3. Nutrient broth

Bacterial culture for the experiments was obtained by inoculating TA98 strain in Nutrient broth (1.25 g Nutrient Broth diluted in 50 mL of distilled water, and sterilized at 121°C for 15 minutes) complemented with ampicillin 5 mL and incubated with soft shaking for 16 hours at 37°C.

4. Mixture of S_9 microsomal concentrate fraction, salt solution and NADP.

S_9 fraction of 0.02 µL microsomal concentrate of rat liver (Molecular Toxicology Inc.) induced by Arochlor was used together with a volume of 0.02 mL of a mixture of magnesium chloride (MgCl) 0.4 M (Merck Chemical Co.), and potassium chloride (KCl) (Merck Chemical Co.) 1.65 M. This solution was prepared dissolving 8.1332 g of Mg Cl hexahydrated and 12.3019 g of KCl in 100 mL of distilled water, with 0.0013 g Glucose 6 phosphate (Merck Chemical Co.), 0.0030 g NADP (Sigma-Aldrich) as cofactor and 0.9 mL of 0.2 M phosphate bufffer PBS pH 7.4.

Salts and PBS solutions were added to the glucose and â Nicotinamide adenine dinucleotide 3´phosphate sodium salt (NADP) (Sigma-Aldrich, Cat. N5881), they were filtrated and at last the S_9 fraction of rat liver microsomal concentrate was added. PBS buffer solution was done at 7.4 pH.

HPLC chemical analysis

1. AFB_1 standard for the calibration curve

AFB_1, (Sigma Cat. A6636) stock concentration of 1 µg/mL in methanol (J.T.Baker) was prepared using a Perkin Elmer Spectrophotometer Lamba 3A UV/Vis calibrated with $K_2 Cr_2 O_7$. The calibration curve was done with four dilutions (0.25, 0.5, 1.0 and 2.0 µg) of the AFB_1 standard stock concentration in methanol HPLC. Derivatization was done with 100 µL AFB_1 added with 200 µL hexane (J. Baker) and 200 µL of trifluoroacetic acid (Sigma-Aldrich, Cat. T6508) mixed thoroughly in vortex heated at 40°C in a closed vial for 10 minutes, dried under gaseous N and redissolved in 200 µL of acetonitrile/ deionized water (1:3 v/v), then 30 µL of each dilution were injected individually into the chromatograph. A linear regression analysis was performed. The calibration curve was analyzed with Excel software.

2. HPLC conditions

Series 400 liquid chromatograph pumping system, connected to a LC-10 fluorescence detector, controlled by a LCI-100 computing Data integrator all from Perkin Elmer and a C_{18} column from Prodigy 5 ODS-2 column 250 mm x 4.6 mm x 5 µm from Phenomenex. Mobile phase was 60% water, 22% acetonitrile (J.T. Baker) and 18% methanol, mixed, filtered and degasified. Fluid speed 1 mL/min.

3. Quantitation of AFB1 by spectrophotometer.

Methanol was applied in two quartz cells and a screening at 750 nanometers (nm) was run to know the absorbance of the solvent methanol. The liquid chromatograph (HPLC) makes the adaptations to substract this background absorbance so it does not interfere with the mycotoxin´s one. Later the problem solution of AFB1 was placed in one quartz cell and read at different UV light wave lengths from 355 to 365 nm, because the range where this mycotoxin is detected is between 360 and 362 nm.

The following formula was applied:

$$\frac{\text{Highest absorbance (at 355-365 nm)} \times \text{AFB1 molecular weight (mw = 312)}}{\text{Extinction coefficient (= 21800)}} = \text{AFB1 concentration}$$

To prepare a AFB1 standard solution (stock) of 1 µg/mL (= 1000 ng/mL) we divided one between the AFB1 concentration obtained, and the result was subtracted from 1000 (because one mL = 1000 µL) in order to know how many µL we had to use from the standard and from methanol to make the stock dilution.

Ames Test

To establish the mutagenicity assay an aliquote of surface agar, melted at 45°C, was the substrate of 0.1 mL of TA-98 strain of *Salmonella typhimurium* incubated overnight in nutrition broth, the AFB_1 dilution to test, and 0.5 mL of S_9 microsomal rat liver concentrate to facilitate the metabolic activation of the treatments. Bacteria can survive some minutes at 45°C, but S_9 fraction cannot, so it was added when the media was just warm, shaking the mixture and putting it in Petri dishes that already had minimum agar with glucose (Merck Chemical Co.), spreading in a uniform way in less than 20 seconds and letting it to solidify.

Plates were placed at room temperature before applying the different concentrations of the mutagen AFB_1 by triplicate. In each one of the two experiments with AFB_1, the first applying a range from 10 to 100 ng of AFB_1, and in the second, with a range from 0.39 to 50 ng as positive controls. Also negative controls were included to verify the behavior of the bacterial strains in contact with the mutagen (AFB_1). Plates were closed quickly to avoid the effect of light on the photosensitivity of the reactives. The plates, from the treatment and control plates were incubated 48 hours at 37 °C. Finally, the revertant colonies were counted after one hour.

Results interpretation

A positive result is defined as an increase in the number of revertant (mutated) colonies that should be at least twice the frequency of spontaneous reversion shown when the colonies of the negative controls are counted. This fact shows an increment of the number of mutants in relation to the concentration of the tested compound. It is recommended to test concentrations in an ample range (2, 20 and 500 µg) per plate in the presence and absence of S_9 microsomal fraction. A positive result must have a clear dose-response using a narrow range of concentrations that must have a linear response [19].

Results and discussion

Calibration curve

The area results of the HPLC calibration curve (Table 1) of AFB_1 standard are presented. The coefficient of correlation of AFB_1 ($r = 0.99210447$) shows the direct relation between the standard concentration and the chromatographic area. With the calibration curve and the area presented in the HPLC the concentration and purity of AFB_1 standard were determined.

Ames Test

The Ames experiments with a range of concentrations from 0.39 to 100 ng of AFB_1 , are presented in Tables 2 and 3, with 10.0 ng as the minimal detection limit of AFB_1 to produce a mutation. There were revertant colonies also in negative controls.

Ames Test with *S. typhimurium* strain TA98 negative control alone, gave a spontaneous reversion average of 20 revertant colonies and 10.0 ng of AFB_1 , as treatment, gave 68 colonies that were 3 times higher than the controls, and can be taken as a positive result.

The number of reverted colonies is directly proportional to the mutagen effect. The results are compared

Table 1: AFB_1 calibration curve.

N° of dilutions	Concentrations of AFB_1 standard µg/mL	Chromatografic Areas
1	0.25	450059
2	0.5	548085
3	1	4142473
4	2	9371459
Regression line	y = -1415569 + 53798272 x	
Coefficient of Correlation	r = 0.99210447	

with the control treatments and the Ames test is considered positive if the number of the reverted (mutated) colonies triplicates the number of the control treatments.

The IARC determines the carcinogenicity of a toxin not its minimal quantity to produce a mutation, this agency reports studies done in different animals and the way the ingested mycotoxin causes cancer in each species. They do not measure the minimal amount to cause a mutation, but the carcinogenic effect itself. There is a distance between causing a mutation to the fact of developing a cancer. Mutations are being produced frequently by different factors, among which aflatoxins are common, until DNA cannot repair itself and begins a malignant process, if promoters and an inheritance factor are present. The advantage of Ames Test is that it can measure mathematically the reverted (mutated) bacterial colonies that is in fact the mutant potency of an carcinogenic agent and the origin of a cancer. Cancer is a very complicated process where many factors interact (age, sex, hormones, resistance-susceptibility, promoters, H-ras proto-oncogenes species, amount of ingested AF in a certain time, if it was injected or orally given, etc.) and to measure it numerically is not easy. Most of the studies report sick or dead animals and the time of the symptom appearance. The beauty of Ames Test is to make mutagenicity countable when counting the mutated colonies.

Ninety percent of the cancers are produced by mutations that can be quantified by Ames Test , and the fact of having mutations is a risk factor of disease.

In fact there is a relation between the amount of maize ingested by a person and the risk to develop a liver disease such as chronic hepatitis B and C, and viral cirrhosis, this was concluded from the correlation of the amount of AF contamination in the urine of 210 patients with 40 000 data of different kinds of "risk foods" ingested during the last week, month and year, established in a questionnaire that was applied to the patients [1].

Adducts (AFB1 link to DNA) are considered good biomarkers of a high risk of disease, they are in direct relation with the malignity of the tumor. This fact is well accepted by the international scientific community, there are studies [7, 8, 9] that testify that adducts are the authentic activated carcinogens that produce punctual mutations, that eventually can initiate the malignant tumor.

AF are present in maize of Mexico, there is a good survey with 12,000 samples and 60,000 analysis in 5 years [13]. Also milk is contaminated with AFM1 [4] and aflatoxicol [5], so this study becomes relevant because Mexico imports maize and milk and the maximum tolerance level of these foods in the legislation is the only protection for the Mexican population at this point.

If the maximum legal tolerance level of AFB_1 contamination for human foods in U.S.A., Canada and Mexico (NAFTA) is 20 µg/kg for totalAF, this amount means that the addition of the four most common AF (AFB_1 , AFB_2, AFG_1 , AFG_2) should be 20 ng at the most, but in maize that

Table 2. Higher quantities of AFB_1 tested by Ames test with the strain *Salmonella typhimurium* TA-98 (100 µL) to know minimal amount to produce a mutation.

N° of Dish	S_9 Arochlor induced (µL)	PBS Control (µL)	AFB1 (ng)	Ames test: Number of revertant colonies: Replitations 1	2	3	Average of revertant colonies
			Controls				
1	-	-	-	15	34	20	23
2	500	-	-	18	18	25	20
3	-	500	-	21	13	25	20
4	-	500	100	14	12	10	12
			Treatments				
5	500	-	20	235	257	255	249
6	500	-	40	270	290	259	273
7	500	-	60	706	804	900	803
8	500	-	80	920	970	846	912
9	500	-	100	1074	1038	922	1010

has mainly *A. flavus* that produces only AFB_1 and AFB_2 , 20 ng would be divided in the 2 AF that are 10 ng for AFB_1 and another 10 ng AFB_2 that is certainly producing a mutation. If the contaminated food is peanuts, then *A. parasiticus* is the common fungi producing the 4 mentioned AF (AFB_1 , AFB_2, AFG_1, AFG_2), if AF are present in equivalent amounts, the legal protection is "adequate" because the accepted contamination for AFB_1 should be 5 ng and legal maximum tolerance level of 20 µg/kg do protect the population, although peanuts and maize are frequently with higher amounts of AF contamination.

Codex Alimentarius has a maximum tolerance limit of 10 µg/kg that is more strict than NAFTA (Canada, U.S.A. and Mexico) 20 µg/kg level, and can "protect" the health of the human beings, but the frequent ingestion of these amounts can trigger the cancer mechanism in susceptible persons.

The problem is that the present experiment represents a single dose per triplicate and the question is what happens with the ingestion of several of these doses that a person ingests in maize, dairy products, wheat, rice, species, raisins, figs, etc. daily during years and that accumulate in DNA of all the cells.

The population is accumulating these carcinogens and no legal limit can prevent this fact. So these legal limits are helpful for commercial transactions only but do not represent a real protection to the health of the human population. We have to remember that we cannot know the AF contamination of all the food that we eat, because the analyzed samples are few and if they are chemically extracted with organic solvents (methanol, acetonitrile, etc.), they cannot be eaten.

Prokaryotic or eukaryotic organisms are exposed to a multitude of DNA damaging agents such as AFB_1 , as a result, organisms have evolved important mechanisms to repair DNA damage and systems (cell cycle checkpoints) that delay the resumption of the cell cycle after DNA damage, to allow more time for these accurate processes to occur. The difficulty to repair DNA damage can let a mutagenic event to occur. Most bacteria, including *Escherichia coli*, have evolved a coordinated response to these challenges to the integrity of their genomes and this inducible system is named SOS response and it controls both accurate and potentially mutagenic DNA repair functions [27].

Table 3: Low quantities of AFB_1 tested by Ames test with the strain *Salmonella typhimurium* TA-98 (100 µL) to know the minimal detection limit that produces mutagenicity.

N° of Dish	S_9 Arochlor induced (µL)	PBS Control (µL)	AFB1 (ng)	Ames test Number of revertant colonies Replications 1	2	3	Average of revertant colonies
			Controls				
1	-	-	-	26	26	27	26
2	500	-	-	26	26	22	23
3	-	500	-	20	14	16	17
4	-	500	50	13	25	17	18
			Treatments				
5	500	-	0.39	27	21	20	23
6	500	-	0.78	19	16	25	20
7	500	-	1.6	40	32	35	37
8	500	-	3.8	40	30	30	33
9	500	-	6.25	29	40	35	35
10	500	-	**10.0 ***	**68**	**69**	**67**	**68 ****
11	500	-	12.5	82	87	87	85
12	500	-	25	202	160	153	172
13	500	-	50	337	370	285	331

* Minimum amount o f AFB_1 that produced a mutation.
** Amount of reverse colonies that triplicate the average spontaneous control number .

The problem is starvation, because most of the cereals and maize [13], dairy products [4, 5] , oilseeds, spices, etc. have AF contamination and if legal standards are very strict, there will be no enough amount of food for humans. The problem has no easy solution, is either to eat risky food or starvation. Only governmental or industrial check-ups made by each food company, of statistically useful number of samples of food for humans and feed for animals, can prevent the ingestion of AF that are very frequent and dangerous contaminants, and can give some security to protect human and animal health. But again, the price of food will increase by the additional cost of these extra analysis, and economically low income people will suffer the consequences.

Conclusions

The minimal quantity of AFB_1 that can produce a mutation was 10 ng which gave 68 colony revertants, 3 times higher than the controls that had an average of 20 revertant colonies. Maximum tolerance legal limits applied in Canada, Mexico and United States (NAFTA) are useful for commercial transactions, but do not protect human health against these potent mutagens and carcinogens.

Acknowledgements

To the Institute of Biology where the study was done and that supplied the lab, materials and equipment. To Institute of Research in Biomedical Sciences, UNAM for providing an adequate lab for Ames test.To the Programs Supera and Promep of the Autonomous University of San Luis Potosi for the PhD Scholarship of Maria de Guadalupe Moctezuma. To Felipe Villegas for the drawing of the Figure. To Celina Bernal, Armando Luqueño, Joel Villavicencio, Jorge Gerardo López, Julio César Montero and Alfredo Wong for computational help.

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