Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 802 (2004) 211–215
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Effect of intestinal microfloras from vegetarians and meat eaters on the genotoxicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline, a carcinogenic heterocyclic amine

F. Kassie [a], E.F. Lhoste [b], A. Bruneau [b], M. Zsivkovits [a], F. Ferk [a], M. Uhl [a], T. Zidek [a], S. Knasmüller [a,*]

[a] *Institute of Cancer Research, University of Vienna, Borschkegasse 8A A-1090 Vienna, Austria*
[b] *Unité d'Ecologie et de Physiologie du Système Digestif, Institut National de la Recherche Argonomique (INRA) Domaine de Vilvert, 78350 Jouy-en-Josas, France*

Abstract

Aim of this study was to investigate the impact of intestinal microfloras from vegetarians and non-vegetarians on the DNA-damaging activity of 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline (IQ), a carcinogenic heterocyclic amine that is found in fried meats. Floras from four vegetarians (Seventh Day Adventists) and from four individuals who consumed high amounts of meats were collected and inoculated into germfree F344 rats. The rats were kept on isocaloric diets that either contained animal derived protein and fat (meat consumers group) or proteins and fat of plant origin (vegetarian groups). IQ (90 mg/kg bw) was administered orally, after 4 h the extent of DNA-damage in colon and liver cells was determined in single cell gel electrophoresis assays. In all groups, the IQ induced DNA-migration was in the liver substantially higher than in the colon. In animals harbouring floras of vegetarians, the extent of damage was in both organs significantly (69.2% in the liver, $P < 0.016$ and 64.7%, $P < 0.042$ in the colon, respectively) lower than in the meat consumer groups. Our findings show that diet related differences in the microfloras have a strong impact on the genotoxic effects of IQ and suggest that heterocyclic amines are less genotoxic and carcinogenic in individuals that consume mainly plant derived foods.

Keywords: Microflora; Genotoxicity; 2-Amino-3-methylimidazo[4,5-*f*]quinoline

1. Introduction

A number of investigations show that the diet has a strong impact on the intestinal microflora and its metabolic activities [1,2] and it is known that changes in the composition of the gut microflora affect the metabolism of DNA-reactive carcinogens [3,4].

We recently reviewed the available literature on the impact of intestinal bacteria on the genotoxic and carcinogenic properties of heterocyclic aromatic amines (HAs) that are formed during cooking and are considered to be involved in the aetiology of human colon cancer [5]. It is well documented that probiotic lactobacilli protect against the genotoxic and carcinogenic effects of HAs [6,7], whereas other representatives of the microflora, e.g. Bacteroides strains appear to contribute to the conversion of HAs to DNA-reactive carcinogens [5]. We found recently that DNA-damage induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in the colon and liver of normal rats is several-fold higher than in germfree animals [8]. In the same study, we also included a group of rats harbouring human flora (HFA rats), and in these animals the extent of DNA-damage in livers and colons was significantly lower than that seen in conventional animals. According to our knowledge, this is the only study in that the effects of complex microfloras on the genotoxic effects of HAs have been investigated in vivo in target organs of tumour induction by these compounds. Our observation indicates that the intestinal microflora has a strong impact on the DNA-damaging and carcinogenic properties of HAs that has been underestimated in the past. As a matter of fact,

Abbreviations: HA, heterocyclic aromatic amine; HFA, human flora associated; IQ, 2-amino-3-methyimidazo[4,5-*f*]quinoline; MHFA, meat consumer flora associated; SCGE, single cell gel electrophoresis assay; VHFA, vegetarian flora associated

* Corresponding author. Tel.: +43-1-427765142; fax: +43-1-42779651.
E-mail address: siegfried.knasmueller@univie.ac.at (S. Knasmüller).

1570-0232/$ – see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2003.10.045

most of the previous studies concentrated on hepatic xenobiotic drug metabolising enzymes that catalyse activation and detoxification of HAs [9].

In the present study, we compared the impact of intestinal floras from vegetarians (Seventh Day Adventists) and non-vegetarians on the genotoxicity of IQ in HFA rats. It is known that the composition, as well as the metabolic activities of human faecal flora is preserved in HFA animals [10–14]. To investigate differences in IQ induced DNA-damage in livers and colons, the single cell gel electrophoresis (SCGE) technique was used. We, as well as other groups, used SCGE-assays to study chemically induced DNA-damage of amines in inner organs of laboratory rodents [15–18]. It is assumed that the DNA-damaging properties of the HAs are responsible for their carcinogenic effects and combination experiments with antimutagens and IQ indicated that reduction of DNA-damage measured in hepatic and colonic tissue in SCGE-assays leads to the inhibition to formation of preneoplastic foci which develop into tumours [7,16,19,20].

2. Experimental

2.1. Chemicals

2-Amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) was purchased from Toronto Research Chemicals (Toronto, Canada). Proteinase K and (−)-1,4-dithio-L-threitol for the isolation of colon cells and collagenase for the isolation of the liver cells were from Sigma (St. Louis, USA). Normal- and low-melting point agarose and RPMI medium were obtained from Gibco (Paisly, UK). Inorganic salts for SCGE-assays and ethidium bromide were from Merck (Darmstadt, Germany).

2.2. Human donors and collection of faeces

The faeces were collected from eight volunteers (four females and four males). All participants were healthy and did not consume pharmaceutical drugs and filled out questionnaires concerning their nutritional habits. Four individuals were members of the Seventh Day Adventists (France) and all were strictly vegetarian, the meat eaters group consisted of persons that consumed: 100 g meat per day. Freshly passed stools were collected in plastic bags under anaerobic conditions (GENbag anaer Biomérieux, Marcy l'Etoile, 69, France) and transported immediately to the laboratories.

2.3. Animals and diet

Germfree male Fischer F344 rats were bred in the germfree breeding unit of the animal facility of INRA. The rats were reared in Trexler-type isolators (Ingénia, Villejuif, France) fitted with a rapid transfer system (La Calhène, Vélizy, France). They had free access to food and drinking water and were kept under standard conditions (21°C; 12-h, light-dark cycle). The germfree status of the rats was confirmed before inoculation of the human floras.

2.4. Experimental design

At 10 weeks of age, 32 rats (261 ± 3 g) were randomly divided into two isolators (vegetarian and meat group). The rats from each cage were administered diluted faecal flora suspensions from either a meat eater or from a vegetarian donor by gavage as described previously [21,22]. After inoculation, the rats were fed with chows mimicking the donors diets in terms of quality of protein and fat. Animals with vegetarian floras received proteins and fats of plant origin, whereas the diet of the "meat eaters group" contained animal derived proteins and part of the fats were of animal origin. A detailed description of the composition of the two diets which were isocaloric and isoproteic is given in Table 1. The pelleted food was packed in double vacuum bags and sterilised by γ-irradiation at 45 kGy (UAR, Villemoisson-sur-Orge, France). Four weeks after the inoculation of the faeces, the rats were treated orally with IQ (90 mg/kg bw suspended in corn oil). Four hours later, liver and colon cells were isolated as described by Bradley et al. [23] and Brendler et al. [24], respectively. It was found in earlier experiments that the optimal exposure time for

Table 1
Composition of the experimental diets (g/kg)

	MHFA diet	VHFA diet
Corn starch	289.85	286.90
Saccharose	50.00	50.00
Mashed potatoes	290.00	260.00
Casein	50.00	0.00
Isolated soy protein[a]	120.00	170.00
Corn oil	30.00	30.00
Soybean oil	00.00	30.00
Lard	30.00	0.00
Cellulose (Durieux)	60.00	60.00
Cholesterol	0.15	0.10
Methionine	0.00	3.00
Mineral additive[b]	70.00	70.00
Vitamin additive[c]	10.00	10.00
Protein ($N \times 6.25$)[d]	170	170
Gross energy (MJ/kg)	18	18

MHFA, meat consumer flora associated; VHFA, vegetarian flora associated.

[a] Isolated soy protein PRO FAM 646 (Société Industrielle des Oléagineux).

[b] In g/kg diet: $CaHPO_4$ 30.1, KCl 7.0, NaCl 7.0, MgO 0.735, $MgSO_4$ 3.5, Fe_2O_3 0.210, $FeSO_4{\cdot}7H_2O$ 0.35, $ZnSO_4{\cdot}7H_2O$ 0.141, $MnSO_4{\cdot}H_2O$ 0.17, $CuSO_4{\cdot}5H_2O$ 0.035, $CoSO_4{\cdot}7H_2O$ 0.28, KI 0.56.

[c] In mg or IU/kg diet: retinol 19800 IU, cholecalciferol 2500 IU, thiamine 20 mg, riboflavine 15 mg, pantothenate 70 mg, pyridoxine 10 mg, myoinositol 150 mg, cyanocobalamine 0.05 mg, ascorbate 800 mg, tocopherol 170 mg, menadione 40 mg, niacine 100 mg, choline 1360 mg, folate 5 mg, *p*-aminobenzoic acid 50 mg, biotine 0.3 mg.

[d] *N*: actual nitrogen content, 6.25: global standard industry factor.

experiments with IQ is 4 h [16,25]. The exposure time is also within the time frame suggested in guidelines for in vivo comet assays [26].

2.5. Single cell gel electrophoresis (SCGE) assay

Phosphate buffered saline, alkali (electrophoresis) buffer, lysis solution, neutralisation buffer and ethidium bromide stain were composed as described by Singh et al. [27]. Agarose coated slides were made with 1.5% normal melting agarose according to the protocol of Klaude et al. [28].

Microgel electrophoresis was performed according to Singh et al. [27], with some modifications [16]. Analysis of DNA-damage was made by measuring the comet tail lengths of the indicator cells with a fluorescence microscope (Nikon, EFD-3, 125-fold magnification) connected to a monitor with a specific macro for the NIH-public domain image analysis program [29]. From each organ, three slides were prepared, and from each slide 50 cells were analysed.

2.6. Statistical analysis

Comet results were analysed using multiple ANOVA. All statistical evaluations were done using the Proc Mixed of SAS vers 8.12. (SAS, SAS/STAT User's Guide, 1999). A P-value <0.05 was considered statistically significant.

3. Results and discussion

The comet lengths measured in colonocytes of rats harbouring vegetarian intestinal floras (VHFA) and microfloras from meat consumers (MHFA) are depicted in Fig. 1a–b. Each bar represents the mean ± S.D. of the comet lengths measured in three animals which had been inoculated with the flora of an individual donor. It can be seen that the extent of damage varied over a broad range, the lowest effect in the vegetarian group was seen in donor #V2 (tail length: 5.2 μm), the strongest migration was seen with donor #V4 (9.2 μm). Similar inter-individual variations were seen in the meat consumer groups (highest value 14.4 μm, #M2; lowest value 8.5 μm #M1). The mean values and standard deviations of all VHFA and MHFA groups are summarised in Fig. 1c. It is evident that the extent of IQ induced DNA-damage was significantly (approximately 30%) higher in the animals harbouring floras of meat eaters than that seen in the vegetarian groups.

The DNA-migration seen in the liver cells of the different groups are shown in Fig. 2a (MHFA animals) and Fig. 2b (VHFA animals). The samples of one group (donor #M4) could not be evaluated due to failure of the electrophoresis. The extent of DNA-damage was distinctively higher than that seen in the colon cells. The inter-individual differences between the individual donors were approximately 25% and again the overall extent of DNA-migration was significantly lower in the VHFA groups than in the MHFA groups (Fig. 2c).

We analysed the extent of DNA-migration in untreated conventional, germfree and HFA animals in a number of studies [6,8,16]. The average DNA-migration was in these experiments consistently between 4 and 7 μm. In order to save animals, we used in the present study only one untreated rat from each individual group as a control; the extent of DNA-damage was in all animals in liver and in colon cells

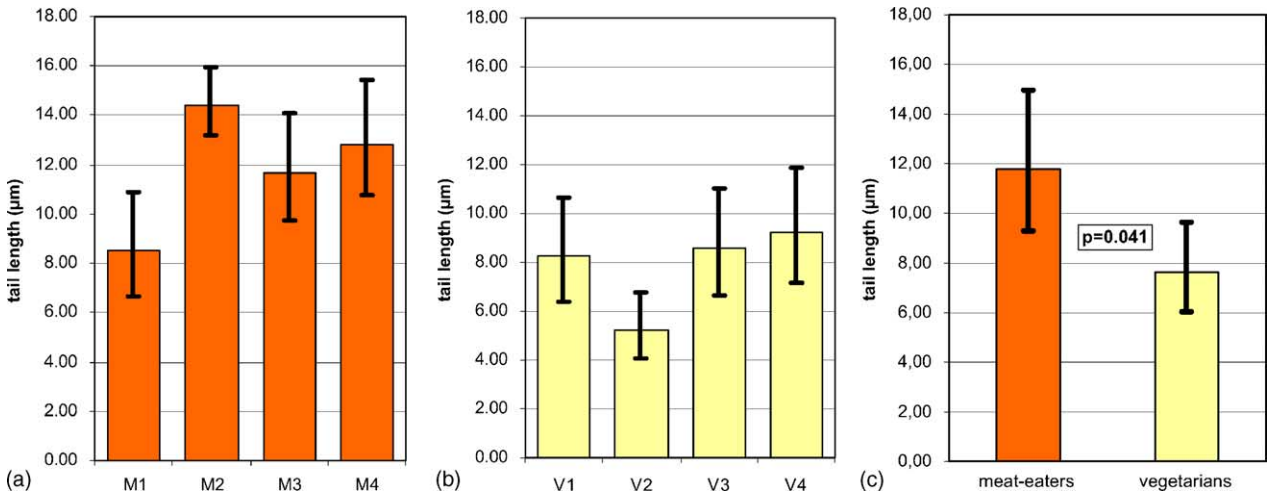

Fig. 1. (a–c) Induction of DNA-migration by IQ in colonocytes of animals harbouring intestinal floras from meat consumers (M1–4) and vegetarians (V1–4). Intestinal microfloras were inoculated into germfree animals, subsequently, the animals were kept on diets that contained either animal or plant derived protein and fat. The animals received a single dose of IQ by gavage (90 mg/kg bw in corn oil). After 4 h, the rats were killed, livers and colons removed and the comet lengths determined as described further in materials and methods. From each organ, three slides were prepared and from each slide, 50 cells were evaluated. Fig. 1 a shows the results with animals harbouring floras from meat consumers, Fig. 1b depicts the effects in colon cells of animals harbouring the intestinal flora from vegetarians. The bars represent means ± S.D. of data from three animals per donor. Fig. 1c depicts the means ± S.D. of all vegetarian and non-vegetarian groups.

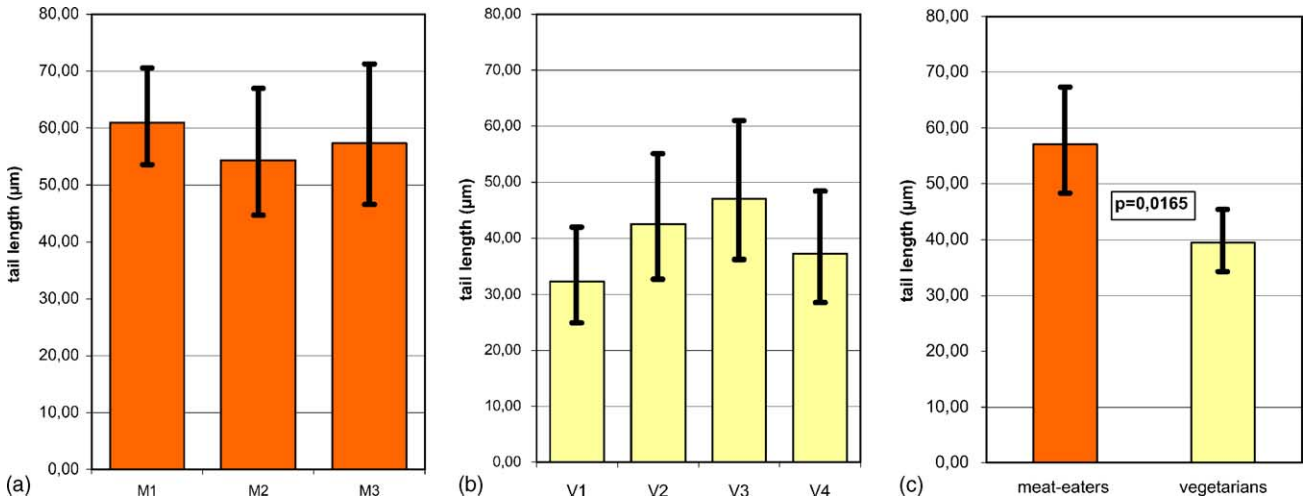

Fig. 2. (a–c) Induction of DNA-migration by IQ in hepatocytes of animals harbouring intestinal floras from meat consumers and vegetarians. The experiments were carried out as described in the legend of Fig. 1.

consistently equal to 6 μm and no significant differences were seen between vegetarians and meat consumers (data not shown).

A possible explanation for the differences seen in the VHFA and MHFA animals are alterations in the faecal bacterial β-glucuronidase activity. Reddy and Wynder [30] reported that Americans on a standard Western diet had two to five times higher activity of this enzyme than vegetarians and ethnic groups which consume less meat and higher amounts of vegetables. This observation is supported by the results of human intervention studies which showed that shifts from normal (meat containing) to vegetarian diets lead to a decrease of the β-glucuronidase activity [31,32]; also in a rat model, a significant increase of this enzyme was found when the animals received a high meat diet [33]. It is well known that glucuronidation is a major detoxification pathway for IQ and other HAs [9,16] and its is conceivable that cleavage of HA glucuronides by the intestinal microflora results in release of breakdown products which are converted to DNA-reactive carcinogenic metabolites [5,9,34].

Also other mechanisms might account for the differences seen between the meat consumers and the vegetarian groups. In a comparative study with vegetarian Adventists and Non-Adventists (on a meat containing diet), significantly higher lactobacilli counts were found in the faeces of the vegetarians [35]. In another study with different ethnic groups, those who consumed low amounts of meat, had the highest count of lactobacilli in their faeces [36], also in a Chinese study the same phenomenon was observed [37]. A number of in vitro studies showed that lactobacilli inactivate HAs including IQ by direct binding (for review see [5]) and some experimental data with animals indicate that lactobacilli are also protective against the genotoxic and carcinogenic effects of these compounds in vivo [8,37,38]. It is possible that the inactivation of IQ by lactobacilli accounts at least partly for the effects seen in the present study. Moore and Moore [36] reported that the decrease of lactobacili counts in meat consumers was paralleled by an increase of Bacteroides species. In this context it is notable that we recently found in in vitro experiments that certain Bacteroides strains enhance the mutagenic activity of a HA containing meat extract, [5] and preliminary SCGE experiments with gnotobiotic rats harbouring Bacteroides strains showed that the extent of HA induced DNA-damage in colon and liver is increased in these animals [38].

In conclusion, the results of the present experiments support the assumption that the intestinal microflora has a strong impact on the DNA-damaging effects of IQ and show for the first time that differences exist in this regard between the floras from vegetarians and meat consumers. It is known from earlier studies that the decrease of DNA-migration measured in SCGE-assays in colons and livers is paralleled by a decline of the formation of preneoplastic foci in these organs [7,19,20], therefore our findings indicate that individuals who consume low amounts of meat are not only exposed to lower levels of HAs but are also protected against the health risks of these compounds by their microflora. Attempts to further elucidate the mechanisms which account for this phenomenon are in progress.

Acknowledgements

This study was sponsored by an EU project (HCAMINES, to SK and EL). The Authors thank M. Bensaada, S. Bruel, J. Durao, and R. Durao for their careful technical assistance. They also thank Dr. P. Tailliez for his help in the PLS analysis.

References

[1] I.R. Rowland, I.K. Mallett, A. Wise, Crit. Rev. Toxicol. 16 (1985) 31.
[2] R.J. Hambly, C.J. Rumney, M.E. Fletcher, P.J. Rijken, I.R. Rowland, Nutr. Canc. 27 (1997) 250.

[3] B.R. Goldin, Ann. Rev. Microbiol. 40 (1986) 367.
[4] R.W. Cadwick, E. George, L.D. Claxton, Drug Metabol. Rev. 24 (1992) 425.
[5] S. Knasmüller, H. Steinkellner, A. Hirschl, S. Rabot, E.C. Nobis, F. Kassie, Mutat. Res. 480–481 (2001) 129.
[6] M. Zsivkovits, Masters Thesis, Vienna, Austria, 2002.
[7] E. Tavan, C. Cayuela, J.-M. Antoine, G. Trugan, C. Caugier, P. Cassand, Carcinogenesis 23 (2002) 477.
[8] F. Kassie, S. Rabot, M. Kundi, M. Chabikovski, H.M. Quin, S. Knasmüller, Carcinogenesis 22 (2001) 1721.
[9] M. Nagao, T. Sugimura (Eds.), Food Borne Carcinogens Heterocyclic Amines, Wiley, Chichester, New York, Weinheim, Brisbane, Singapoure, Toronto, 2000.
[10] A. Debure, J.F. Colombel, B. Flouri, M. Rautureau, J.C. Rambaud, Gastroenterol. Clin. Biol. 13 (1989) 25.
[11] Z. Djouzi, C. Andrieux, V. Pelene, S. Somarriba, F. Popot, F. Paul, P. Mionsan, O. Szylit, J. Appl. Bacteriol. 79 (1995) 117.
[12] P. Raibaud, R. Ducluzeau, F. Dubos, S. Hudault, H. Bewa, M.C. Muller, Am. J. Clin. Nutr. 33 (1980) 2440.
[13] E.F. Lhoste, I. Catala, M. Fiszlewicz, A.M. Gueugneau, F. Popot, P. Vaissade, T. Corring, O. Szylit, Br. J. Nutr. 75 (1996) 433.
[14] A.K. Mallett, C.A. Bearne, I.R. Rowland, M.J. Farthing, C.B. Cole, R. Fuller, J. Appl. Bacteriol. 63 (1987) 39.
[15] Y.F. Sasaki, K. Sekihashi, F. Izumiyama, E. Nishidate, A. Saga, K. Ishida, S. Tsuda, Crit. Rev. Toxicol. 30 (2000) 629.
[16] F. Kassie, S. Rabot, M. Uhl, W.W. Huber, H.M. Qin, C. Helma, R. Schulte-Hermann, S. Knasmüller, Carcinogenesis 23 (2002) 1155.
[17] B.L. Pool-Zobel, C. Neudecker, I. Domizlaff, S. Ji, U. Schillinger, C. Rumney, M. Moretti, I. Vilarini, R. Scassellati-Sforzolini, I. Rowland, Nutr. Canc. 26 (1996) 365.
[18] K. Sekihashi, A. Yamamoto, Y. Matsumura, S. Ueno, M. Watanabe-Akanuma, F. Kassie, S. Knasmuller, S. Tsuda, Y.F. Sasaki, Mutat. Res. 517 (2002) 53.
[19] F. Kassie, M. Uhl, S. Rabot, B. Grasl-Kraupp, R. Verkerk, M. Kundi, M. Chabicovsky, R. Schulte-Hermann, S. Knasmuller, Carcinogenesis 24 (2003) 255.
[20] F. Kassie, V. Mersch-Sundermann, R. Edenharder, K.L. Platt, F. Darroudi, E. Lhoste, C. Humbolt, E. Muckel, M. Uhl, M. Kundi, S. Knasmuller, Mutat. Res. 523–524 (2003) 183.
[21] S. Rabot, L. Nugon-Baudon, M.P. Raibaurd, O. Szylit, Brit. J. Nutr. 70 (1993) 323.
[22] N. Roland, D.S. Rabot, L. Nugon-Baudon, Food Chem. Toxicol. 34 (1996) 671.
[23] M.O. Bradley, G. Dysart, D. Fitzsimmons, P. Harbach, J. Lewin, G. Wolf, Canc. Res. 42 (1982) 2592.
[24] S. Brendler, A. Tompa, K.H. Hutter, R. Preussmann, B.L. Pool Zobel, Carcinogenesis 13 (1992) 2535.
[25] M. Uhl, Ph.D. Thesis, Vienna, Austria, 2002.
[26] A. Hartmann, E. Agurell, C. Beevers, S. Brendler-Schwaab, B. Burlinson, P. Clay, A. Collins, A. Smith, G. Speit, V. Thybaud, R.R. Tice, Mutagenesis 18 (1) (2003) 45.
[27] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, Exp. Cell Res. 157 (1) (1988) 184.
[28] M. Klaude, S. Eriksson, J. Nygren, G. Ahnstrom, Mutat. Res. 363 (1996) 89.
[29] C. Helma, M. Uhl, Mutat. Res. 466 (2000) 9.
[30] B.S. Reddy, E.L. Wynder, J. Natl. Canc. Inst. 50 (1973) 1437.
[31] B.S. Reddy, J.H. Weisburger, E.L. Wynder, Science 183 (1974) 416.
[32] G.K. Johansson, L. Ottova, J.A. Gustavsson, Nutr. Canc. 14 (1990) 239.
[33] B.R. Goldin, S.L. Gorbach, J. Natl. Canc. Inst. 57 (1976) 371.
[34] J. Alexander, H. Wallin, O.J. Rossland, K.E. Solberg, S. Grivas, Carcinogenesis 12 (1991) 2239.
[35] S.M. Finegold, V.L. Sutter, P.T. Sugihara, H.A. Elder, S.M. Lehmann, Z. Phillips, Am. J. Clin. Nutr. 30 (1977) 1781.
[36] W.C.E. Moore, L. Moore, Appl. Env. Microbiol. 61 (1995) 3202.
[37] K.T. Chung, C.T. Kuo, F.J. Chang, Zonghua Min Guo 22 (1989) 163.
[38] M. Zsivkovits, F. Kassie, G. Sontag, U. Nabinger, S. Knasmüller, in: Proceedings of the 2nd International Workshop on Analytical, Chemical, and Biological Relevance of Heterocyclic Aromatic Amines in May 2003, Graz, Austria, p. 32.