Heterocyclic aromatic amines and their contribution to the bacterial mutagenicity of the particulate phase of cigarette smoke

Ewald Roemer\textsuperscript{a}, Thomas Meisgen\textsuperscript{b}, Joerg Diekmann\textsuperscript{b}, Lynda Conroy\textsuperscript{b}, Regina Stabbert\textsuperscript{a,*}

\textsuperscript{a}Philip Morris Products S.A., Philip Morris International R&D, Rue des Usines 90, 2000 Neuchâtel, Switzerland  
\textsuperscript{b}Philip Morris International R&D, Philip Morris Research Laboratories GmbH, Fuggersstr. 3, 51149 Cologne, Germany

**HIGHLIGHTS**

- A method for the quantification of 8 heterocyclic aromatic amines (HAAs) in cigarette smoke (CS) is reported.
- The mutagenic potency of these 8 HAAs and that of CS was determined in the Salmonella Reverse Mutation Assay.
- The 8 HAAs do not contribute significantly to the bacterial in vitro mutagenicity of CS.

**ARTICLE INFO**

Article history:  
Received 25 September 2015  
Received in revised form 18 December 2015  
Accepted 21 December 2015  
Available online 24 December 2015

Keywords:  
Heterocyclic aromatic amines  
Bacterial mutagenicity  
Cigarette smoke

**ABSTRACT**

Heterocyclic aromatic amines (HAAs) rank among the strongest known mutagens. Approximately 30 HAAs have been found in cooked foods (broiled, fried, and grilled) and several HAAs have been characterized as animal carcinogens. Nine HAAs have also been reported to be constituents of cigarette smoke (CS) raising concerns that HAAs might contribute significantly to the known carcinogenicity of CS. As HAAs are found predominantly in the total particulate matter (TPM) of CS, an improved method for the quantification of HAAs in TPM is reported allowing detection and quantification of 8 HAAs in a single run. The mutagenic potency of these HAAs and that of TPM from the reference cigarette 2R4F was determined in the Salmonella Reverse Mutation Assay (Ames assay) with tester strain TA98 and a metabolic activation system. The 8 HAAs, when applied together in the Ames assay, showed a clear sub-additive response. Likewise, the combination of HAAs and TPM, if at all, gave rise to a slight sub-additive response. In both cases, however, the sub-additive response in the Ames assay was observed at HAAs doses that are far above the amounts found in CS. The contribution of the individual HAAs to the total mutagenic activity of TPM was calculated and experimentally confirmed to be approximately 1% of the total mutagenic activity. Thus, HAAs do not contribute significantly to the bacterial in vitro mutagenicity of CS TPM.

© 2015 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Since the detection of substances with very high bacterial mutagenicity in cooked/broiled meat and fish and their identification as heterocyclic aromatic amines (HAAs), (Commoner et al., 1978; Sugimura et al., 1977a,b) this class of compounds has initiated extensive research that is still ongoing. HAAs are formed typically at higher temperatures (140–165 °C) as the result of Maillard reactions involving creatinine, free amino acids (especially tryptophan and glutamic acid), and sugars (Wakabayashi et al., 1993). Special attention is given in the literature to the following carboline type of HAAs: MeAuc, AucC, AucP, Trp-P, Trp-P-2, Glu-P, Glu-P-2, and the pyridazine type of HAAs: IQ, MelQ, MelQx, PHP. Harmar and norharman, although β-carbolines, are not considered as member of the HAA class in most publications as

http://dx.doi.org/10.1016/j.toxlet.2015.12.008  
0378-4274© 2015 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
they lack an exocyclic amine group. The exocyclic amine group of HAAs can undergo metabolic activation by N-hydroxylation producing an intermediate (aryl nitrenium ion) which has been implicated in general toxicity and DNA damage (Turesky and Le Marchand, 2011).

With the exception of harman and norharman, the mentioned HAAs exhibit a clear in vitro activity inducing reverse mutations in Salmonella typhimurium ( Ames assay), morphological transformation in mouse fibroblasts, micronucleus induction in human cells, and DNA strand breaks ( Comet Assay) in human cells (Pflau et al., 1999; Dumont et al., 2010; Jagerstad and Skog, 2005; Sorensen et al., 1996; Viegas et al., 2012; Haza and Morales, 2011; Platt et al., 2010), and genotoxic effects in vivo as DNA adducts (Arimoto-Kobayashi et al., 2006; Dingley et al., 2003). Micronuclei formation could be demonstrated for PhIP in mice but not for MelQ and IQ (Durling and Abramsson-Zetterberg, 2005). The carcinogenicity of PhIP, MelQ, and IQ in mice and rats in various organs, like liver, pancreas, colon, mammary gland, and prostate is well established at doses around 10 mg/kg/day (Ohgaki et al., 1986; Schut and Snyderwine, 1999; Wakabayashi et al., 1993). Carcinogenicity studies in nonhuman primates with PhIP, MelQ, and IQ could only demonstrate a carcinogenic action of IQ in the liver at doses of 10 and 20 mg/kg/day (Takayama et al., 2008). Differences in metabolism between rodents and primates account for the observed differences in the carcinogenic effects (Turesky, 2005). In humans, no occupational exposures to pure HAAs have been reported. However, since the detection of HAAs in food, there are concerns that their presence in food might cause tumors in man (Commoner et al., 1978; Sugimura et al., 1977a,b). Several epidemiological studies have tried to find an association between the intake of, e.g., cooked meat, cooked fish, or fried potatoes and several tumor types, especially those of the colon (WCRF/AICR, 1997). These studies are mainly based on questionnaires exploring the diet of the participants. For colorectal adenomas or carcinomas there are more studies that showed an association (although not always statistically significant) than those that gave a negative result (Kim et al., 2013; Berlau et al., 2004). Associations between HAAs and tumors at other sites are in summary even more inconclusive. Considering these uncertainties, insufficient evidence exists to establish a definite conclusion on the role of HAAs in the genesis of human tumors (Alaejos et al., 2008; Santarelli et al., 2008). This conclusion is consistent with the assessments of the International Agency for Research on Cancer that has not listed any of the HAAs as a definite human carcinogen (IARC, 2015); IQ was classified as a ‘probable human carcinogen’ (Group 2A) and other assessed HAAs (AcC, Glu-P-1, Glu-P-2, MeAcC, MeIQ, MelQx, Trp-P-1, Trp-P-2) as ‘possible human carcinogens’ (Group 2B). Comparing the doses that gave rise to a distinct tumor development in rodents and monkeys with the estimated daily oral intake of HAAs by humans shows that the estimated human exposure is more than 1000 times lower. As such, a not yet identified mechanism would be needed to explain a link between HAAs and human tumorigenicity (Wakabayashi et al., 1993).

Maillard reactions are well-known to occur in the burning cigarette and as all components necessary to form HAAs are present in tobacco, it has been suggested that HAAs should also be found in cigarette smoke. Three years after the initial studies of Sugimura et al. at the National Cancer Center Research Institute in Japan where HAAs in the diet could be identified (Sugimura et al., 1977a,b) the first HAAs, AcC and MeAcC, were identified and quantified in TPM of cigarette smoke (Yoshida and Matsumoto, 1980; Matsumoto et al., 1981). Further studies by several different laboratories using different analytical methodologies identified additional HAAs in TPM (Yamashita et al., 1986; Kanai et al., 1990; Sasaki et al., 2001a; Manabe et al., 1991, 1990; Manabe and Wada, 1990; Wakabayashi et al., 1995; Kataoka et al., 1998; Smith et al., 2004; Turesky et al., 2005; Saha et al., 2009; Zhang et al., 2011).

Despite some concerns regarding the biological activity of HAAs in TPM, it was only in 1997 that several HAAs were included in a revised list of “carcinogens in tobacco and cigarette smoke” issued by Hoffmann and Hoffmann (1997). More recently, the U.S. Food and Drug Administration (FDA) has included 8 HAAs in their list of 93 “Harmful and Potentially Harmful Constituents (HPHCs) in tobacco products and tobacco smoke” (FDA, 2012). A more recent list of 39 priority toxic contents and emissions of tobacco products does not include HAAs (WHO, 2015).

As data on the mutagenicity of HAAs in the context of TPM are scarce and, regarding interactions, nearly non-existent, the research presented here was targeted to corroborate the existing potency data on single HAAs, their occurrence in TPM, their contribution to the overall mutagenicity of TPM, and their interactions with TPM or between the HAAs themselves. Hereby, an improved analytical method for the quantification of the HAAs in TPM was applied.

2. Materials and methods

2.1. Cigarettes, mainstream smoke (MS) generation and trapping

The American blended reference cigarettes 2R4F were obtained from the University of Kentucky, Kentucky Tobacco Research and Development Center (Davis and Vaught, 1990). The cigarettes were conditioned unpacked in open containers according to International Organization for Standardization (ISO) standard 3402 (ISO, 1999), i.e., at least 48 h at target conditions of 22 ± 1°C and a relative humidity of 60% ± 3%. MS was generated on a 20-port Borgwaldt smoking machine (RM20H, Hamburg, Germany) according to ISO Standard 3308 (ISO, 1991). In brief, puff volume, puff duration, and puff frequency were 35 ml, 2 s, and 1/min, respectively.

The yields per cigarette (means ± SE, N = 4) obtained under these conditions were 9.77 ± 0.04 mg TPM, 0.71 ± 0.01 mg nicotine, 1.08 ± 0.04 mg water, and 11.2 ± 0.1 mg carbon monoxide. The TPM of 5 cigarettes/sample was trapped on a glass fiber filter (Cambridge filters; Filtrona Instruments, Milton Keynes, UK) for chemical analyses and TPM from 20 cigarettes (2 filters) for mutagenicity determination.

2.2. Sample preparation and chemical analyses

The analytical method used for the identification and quantification of HAAs was based on a previously published method (Sasaki et al., 2001b) with modification as follows (major differences to Sasaki et al. were triple quadrupole technology in both PCI and NCI modes instead of single quadrupole technology only in the NCI mode): TPM trapped on glass fiber filters was extracted with 11 ml hydrochloric acid (0.1 N) containing 100 µl calibration standard solution (d₃-IQ; Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada)) for 30 min in an ultrasonic bath. The extract was washed two times by shortly shaking with approximately 10 ml dichloromethane which was removed and discarded. Then the pH was adjusted by the addition of 15 ml of K₂CO₃ solution (saturated) and washed two times again with 10 ml dichloromethane, each. After each washing step, the dichloromethane extracts were concentrated under a nitrogen flow to 2 ml. The sample was derivatized for 30 min at 70°C by addition of 20 µl pyridine and 4 µl pentfluoropropionic anhydride (PFAA) prior to addition of 4 µl N,N-dimethylformamide acetal-methyl-8 (Sigma-Aldrich, Buchs, Germany). The solution was concentrated to dryness under nitrogen and the residue dissolved in 0.1 ml
n-butyl acetate for analysis by gas-chromatography coupled to a tandem mass spectrometer (GC–MS/MS).

The GC–MS/MS system consisted of a 5890 GC (Agilent Technologies, Waldbronn, Germany), equipped with an A200S autosampler (CTC Analytics, Zwingen, Switzerland) and coupled to a TQF 700 triple quadrupole mass spectrometer (Finnigan MAT; Thermo Fisher, MA, USA); the system was controlled by an ALPHA station 255/233 (Digital Equipment). The chromatographic column was a DB17–MS (30 m × 0.25 mm, 0.25 pm film thickness; J&W Scientific—Agilent Technologies, Waldbronn, Germany). The carrier gas was helium at a head pressure of 200 kPa. The GC oven temperature program was 1 min at 100 °C, then ramped to 300 °C at 20 °C/min. The sample injection was kept at 270 °C and the transfer line at 250 °C. The sample injection was made in splitless mode using a focus liner (SGE—Agilent Technologies, Waldbronn, Germany). The ion source temperature was 200 °C. The analytes were ionized in the PCI and NCI mode with isobutane as CI gas (3500 mtorr). The tandem mass spectrometer was operated in the Single Ion Monitoring (SIM) mode, monitoring the molecular ions of the HAAs derivatives and their deuterated analogues. SIM masses for quantitation were: Act = 330.1, MeActC = 358.1, Trp–P–2 = 343.1, Glu–P–1 = 344.1, Glu–P–2 = 330.1, PhIP = 385.1, IQ = 359.1, MeIQ = 373.1.

All HAA standards (reference and internal standards) were purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada) and had a purity of ≥97%.

2.3. Determination of bacterial mutagenicity

Mutagenicity of TPM was assessed in the Salmonella Reverse Mutation Assay (Maron and Ames, 1983) according to the Organization for Economic co-operation and Development (OECD) guideline 471 (OECD, 1997) as previously described (Roemer et al., 2012). Determinations were performed in the plate incorporation assay version with the tester strain TA98, in the presence of a metabolic activation system consisting of the post mitochondrial fraction (S9) of liver cells, i.e., those assay conditions giving rise to the most pronounced effects.

TPM was extracted from the glass fiber filters with DMSO and then the TPM extract/suspension was separated from the glass fibers by centrifugation. The TPM-DMSO suspensions were stored immediately after preparation at −75 ± 1.5 °C until use. For further details on smoke generation see Section 2.1.

The bacteria were derived from stock cultures maintained at −196 °C with 0.09 ml DMSO/ml cell suspension. They were prepared from overnight cultures inoculated with the first cultivation of the master copies originally received (Bruce N. Ames, Berkeley, CA). An aliquot (10 μl) of the thawed stock culture was used as inoculum cultivated in 30 ml of nutrient broth at 36 ± 1 °C in a shaking incubator for approximately 11 h before harvest. The bacteria were then in a late logarithmic/early stationary growth phase.

For plating and exposure, bacteria suspended in culture medium, TPM dissolved in DMSO or DMSO alone, 59 mix (supernatant after centrifugation of liver homogenate from Aroclor 1254–induced rats with a NADPH-regenerating system in 0.1 mol/l phosphate buffer, pH 7.4 (Cytostet Cell Research, Roosdorp, Germany)), were added to the top agar supplemented with histidine (His) and biotin (0.05 nmol each). The components were mixed and spread evenly on minimal glucose agar plates. After the top agar hardened, the plates were incubated in the dark at 36 ± 1 °C for 44 to 48 h. The number of His+ revertant colonies was determined with an automatic colony counter. Negative and positive strain-specific and S9-specific control substances (DMSO, daunomycin, 2-aminoanthracene, 2-amino-5-murolone) were assayed in parallel. Control results were always within the historical range.

2.4. Test strategy

Each HAA identified and quantified in TPM by the method described above was tested in the Ames assay for mutagenicity alone, in combination with TPM from the standard reference cigarette 2R4F, and in combination with the other identified and quantified HAAs.

Based on the dose/response data published in the literature for the testing of HAAs in the Ames assay, dose ranges were expanded far beyond the yields that could be expected to be found in TPM.

2.5. Statistics

Data are presented as arithmetic means and standard deviations (SD) or standard errors of the means (SE). The normal mutagenic response for TPM is characterized by a linear increase in the number of revertants as the amount of TPM is increased. At higher doses the linearity vanishes (Bernstein et al., 1982). The mutagenic response reported here was calculated as the slope (revertants per plate/mg or ng TPM) of the linear portion of the dose–response curve fitted with Poisson-weights to the data, often referred in the literature as specific mutagenicity. Likewise, the HAAs, at lower doses, showed a linear mutagenic response as a function of the dose. For these, curve fits were done in the same way. The spontaneous revertants were subtracted in all calculations.

For the assessment of additivity, it was assumed, that the addition of a single dose of TPM should shift the linear dose/response curve of the HAA upward by an amount equal to the response of the TPM alone. Likewise, for the combination of all HAAs the sum of all single responses at a given dose was assumed as the expected response under the assumption of additivity. This simplified approach does not account for, e.g., slight deviations from linearity already starting at low doses or that the fit of the linear dose/response line does not start exactly at the origin (x = 0, y = 0). However, this approach is considered to be adequate to conclude, if there are biologically relevant deviations from additivity or not.

3. Results

3.1. Chemical analyses

Eight of the 9 HAAs reported to be cigarette smoke constituents could be detected and quantified. Trp–P–1 could not be detected. MeActC, PhIP, IQ, and MelQ could be derivatized twice (PCI mode). ActC, Glu–P–1, Glu–P–2, and Trp–P–2 could only be derivatized with PFBA (NCI mode).

The yields per cigarette varied among the 8 HAAs considerably. From the HAA with the lowest yield (Glu–P–1) to the highest yield (ActC) there was a factor of 660. With the exception of MelQ, the

| HAA | Yield (ng/cigarette) | Quantification limit (ng/cigarette) |
|-----|---------------------|-----------------------------------|
|     | Mean | SD  | Mean | SD   |
| ActC | 28.4 | 0.8 | 3.0  | 0.09 |
| MeActC | 7.57 | 0.11 | 0.81 | 0.03 |
| Trp–P–2 | 4.40 | 0.22 | 0.47 | 0.02 |
| Glu–P–1 | 0.043 | 0.003 | 0.015 | 0.003 |
| Glu–P–2 | 0.211 | 0.008 | 0.023 | 0.001 |
| PhIP | 0.086 | 0.004 | 0.009 | 0.0004 |
| IQ | 0.324 | 0.016 | 0.035 | 0.002 |
| MelQ | 0.338 | 0.027 | 0.036 | 0.003 | 0.32 |
quantification limits were well below the yields from the reference cigarette 2R4F. The associated relative standard errors were around 5% (see Table 1). The optimized method showed high selectivity and less interferences of co-eluting compounds, good signal-to-noise ratios (resulting in low limits of quantification), a wide linear range of quantitation, and good accuracy and reproducibility.

3.2. Biological assessments

3.2.1. Mutagenicity of single HAAs, contribution to and interference with mutagenicity of TPM

The 8 HAAs which could be quantified in 2R4F cigarette smoke were tested as pure substances. In concentrations above those present in 2R4F cigarette smoke, all 8 HAAs showed clear

Fig. 1. Mutagenic response (M, SE) in Salmonella typhimurium tester strain TA98 + S9 metabolic activation towards HAAs present in cigarette smoke, dosed as pure substances (dashed lines; two experiments) and together with a constant dose of 100 μg TPM (solid lines). Standard errors are too small to show up in the graphs.
Table 2
Calculated contributions of the HAAs to the *in vitro* mutagenicity of cigarette smoke TPM in *Salmonella typhimurium* tester strain TA98 + S9 metabolic activation, based on their amount in TPM and their specific mutagenicity as near substances.

| Substance | Specific mutagenicity (revertants/ng) Mean | HAA-yield (ng/cig.) | Total mutagenicity (revertants/cig.) | Contribution to TPM mutagenicity (%) |
|-----------|------------------------------------------|---------------------|-------------------------------------|--------------------------------------|
| 2R4F-TPM  | 0.0037                                   | -                   | 43976                               | 100                                  |
| AcC       | 0.248                                    | 0.002               | 28.4                                | 7.05                                 | 0.016                                |
| MeAcC     | 0.399                                    | 0.058               | 7.57                                | 3.0                                  | 0.007                                |
| PhIP      | 1.41                                     | 0.20                | 0.086                               | 0.1                                  | 0.000                                |
| Glu-P-2   | 25.0                                     | 9.6                 | 0.211                               | 5.3                                  | 0.012                                |
| IQ        | 36.8                                     | 4.2                 | 0.324                               | 11.9                                 | 0.027                                |
| Glu-P-1   | 45.6                                     | 5.2                 | 0.0430                              | 2.0                                  | 0.004                                |
| Trp-P-2   | 46.7                                     | 0.11                | 4.40                                | 205.3                                | 0.467                                |
| MelQ      | 823.0                                    | 60.0                | 0.338                               | 278.2                                | 0.633                                |
| Sum       | 979                                      | -                   | -                                   | -                                    | 1.17                                 |

**Fig. 2.** (A) Mutagenicity of a mix of the 8 quantified HAAs in the ratio they occur in TPM. Dosing was performed as multiples of the concentration occurring in 1 μg TPM (TPM equivalents); (B) Mutagenicity of TPM of the reference cigarette 2R4F, mixes of the 8 HAAs 10 times and 100 times higher than in the respective TPM dose, alone (MIX10 and MIX100) and in combination with TPM of the reference cigarette 2R4F (MIX10/2R4F and MIX100/2R4F). Remarks: standard error in most cases too small to show up in the graphs.

Mutagenic activity with a distinct dose-response. The activity of single HAAs differed by 3 orders of magnitude (see Fig. 1).

TPM also showed a clear mutagenic response at 100 μg TPM/plate with nearly 400 revertants/plate, which was still within the linear dose-response range. Choosing this as a reference dose, the HAAs generally showed a linear response up to doses that were several 1000 times higher than the response from 100 μg TPM. Only Trp-P-2, and possibly Glu-P-2, showed a decline in the steepness of the dose-response curve at a dose 300 times higher than that present in 100 μg TPM (see Fig. 1).

Applying more rigid demands on the linearity and thus excluding doses that showed statistically significant (*p* < 0.05) deviations from linearity, the specific mutagenicity (revertants/ng HAA) of AcC showed the shallowest response slope with an activity about 50 times higher than that of TPM. MelQ showed the highest response being 200,000 times more active than TPM (Table 2). Calculations, based on the activity of individual HAAs as pure substances and their yields in TPM, revealed that the total contribution of HAAs was about 1% to the total mutagenicity of TPM.
3.2.2. Mutagenicity of HAA mixtures, contribution to and interference with mutagenicity of TPM

When applying mixtures of all 8 HAAs in the same concentration ratio as measured in TPM, and in doses up to approximately 1000 times the amount found in 1 μg TPM, the mutagenic responses were not different from background levels as one would expect under the assumption of simple additivity. At higher doses the responses were linear, but weak, and distinctly lower than expected. At a dose of 10,000 times the amount found in 1 μg TPM, a factor of approximately 5 between expected and measured responses could be calculated (Fig. 2A).

When TPM at doses of up to 160 μg/plate was fortified with mixes of the 8 HAAs at 10 and 100 times the amount already contained in the TPM, the mutagenic response was additive (Fig. 2B).

4. Discussion

4.1. Chemical analyses

Analytes occurring only in ng/g amounts in a matrix constitute a special challenge for the analytical chemist. This holds especially true for cigarette smoke which contains approximately 8700 identified constituents (Rodgman and Perfetti, 2013). For the analysis of HAAs in cigarette smoke reported in the current study, the method reported by Sasaki et al. (2001b) was further optimized by use of triple quadrupole technology in both PCI and NCI modes instead of using single quadrupole technology only in the NCI mode. This resulted in a higher selectivity, lower limits of quantification, and better accuracy and reproducibility.

With these modifications, it was possible to measure the yields of 8 of the 9 HAAs reported in cigarette smoke TPM (see Table 3). The presence of Trp-P-1 could not be confirmed. With one exception, the amounts of Trp-P-1 in TPM reported in the literature are below 1 ng/cigarette. There are no analytical methods in the available literature that report on the measurement of 8 HAAs in TPM in one run. Comparing the reported yields of HAAs in TPM of cigarettes from different markets which have been obtained using different analytical methodologies, the reported data in the present study are well within the reported yield ranges providing confidence that the reported results are not biased, e.g., by interference from other smoke constituents or other artefacts (see Table 3).

4.2. Biological assessments

_S. typhimurium_ mutagenicity (Ames assay) data reported in the literature show a rather high variation from laboratory to laboratory. From the data for the mutagenic response of HAAs in _S. typhimurium_ tester strain TA98 with metabolic activation reported in different laboratories and summarized by the International Agency for Research on Cancer (IARC, 2015), the following lowest dose (ng/ml) to exhibit a mutagenic effect can be calculated: PhIP 54.8 ± 86.2, IQ 50.3 ± 100, and MeIQ 2.6 ± 5.7 (means and standard deviations). The high variation is most probably due to differences in the competence of the metabolic activation systems, as the HAAs exert their mutagenic activity only after metabolic activation. It has been shown, that different S9 fractions for metabolic activation alter the number of revertants in the Ames assay by more than a factor of 10 (Kato, 1986). However, this high variation generally does not constitute a major drawback, when comparing mutagenic potencies of different test substances obtained under identical conditions. Similar to previous studies, _S. typhimurium_ tester strain TA98 with metabolic activation was used to determine the mutagenic activity of HAAs since it has been shown that this strain is the most responsive strain to the mutagenic activities of HAAs (Felton et al., 1995; Schwab et al., 2000) and cigarette smoke TPM (Roemer et al., 2012). Together with a low intra-study variability, this assay variant qualifies best for the detection of possible differences in mutagenicity between different test substances in the context of HAAs and TPM.

The bacterial mutagenicity results for the single HAAs are well within the reported ranges in literature. Considering the broad literature ranges, as pointed out above, this was expected. More importantly, however, the potencies of the single HAAs relative to each other obtained in this study fit to those reported in the literature (Shishu et al., 2003). Mixes of the 8 HAAs gave rise to responses that were distinctly below that expected under the assumption of additivity for the single HAAs. Considering the rather high doses applied, there are several probable explanations for this sub-additive effect: the metabolic activation system was overwhelmed, the metabolic products have some protecting properties, or the high amount of HAAs with rather low activity have gained a competitive advantage over the more active ones at the side of action within the cells. This phenomenon is deemed only of theoretical interest, because of the unrealistic high doses of HAAs applied. The same holds true for the sub-additive effects when combining TPM and HAAs.

In contrast to the results reported here, a profound sub-additive effect resulting from the combination of increasing amounts of TPM added to a fixed dose of a single HAA has been reported using the same test system (TA98 + S9) and 6 HAAs investigated here (Lee et al., 1994). The response of nearly all HAAs could be reduced up to approximately 50%. For three of the HAAs tested here (MeIQ, Glu-P-1, Trp-P-2), the observed strong reduction reported by Lee et al.
(1994) could not be reproduced. For the other three HAAs (IQ, Glu-P-2, Trp-P-1) the applied doses by Lee et al. were distinctly higher than the high doses applied in the current study, and thus, do not allow a comparison of the data.

In conclusion, toxicity data on HAAs provided in the literature and the results on bacterial mutagenicity obtained in the present study under test conditions described suggest that the minute amounts of HAAs in cigarette smoke TPM play a negligible role in the in vitro mutagenic activity of cigarette smoke.

Acknowledgement

The authors are grateful to the technical staff at the late Philip Morris Research Laboratories in Cologne, Germany, for their excellent work and endurance to conduct this study.

References

Alaejos, M.S., Gonzalez, V., Afonso, A.M., 2008. Exposure to heterocyclic aromatic amines from the consumption of cooked red meat and its effect on human cancer risk: a review. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 25, 2–24.

Arimoto-Sebagayashi, S., Ishida, R., Nakai, Y., Idei, C., Takata, J., Takahashi, E., Okamoto, K., Negishi, T., Konuma, T., 2006. Inhibitory effects of beer on mutation in the Ames test and DNA adduct formation in mouse organs induced by 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Biol. Pharm. Bull. 29, 67–70.

Berau, J., Glei, M., Zoll, B.L., 2004. Colonic cancer risk factors from nutrition. Anal. Bioanal. Chem. 378, 737–743.

Kato, R., 1986. Metabolic activation of mutagenic heterocyclic aromatic amines from protein hydrolysates. Crit. Rev. Toxicol. 16, 307–348.

Kim, E., Coelho, D., Blachier, F., 2013. Review of the association between meat consumption and risk of colorectal cancer. Nutr. Res. 33, 983–994.

Lee, C.K., Munoz, J.A., Fulp, C., Chang, K.M., Rogers, J.C., Borgerding, M.F., Doolittle, D. J., 1994. Inhibitory activity of cigarette-smoke condensate on the mutagenicity of heterocyclic amines. Mutat. Res. 322, 21–32.
aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells. Food Chem. Toxicol. 50, 949–955.
Wakabayashi, K., Kim, I.S., Kurosaka, R., Yamaizumi, Z., Ushiyama, H., Takahashi, M., Koyota, S., Tada, A., Nukaya, H., Goto, S., et al., 1995. Identification of new mutagenic heterocyclic amines and quantification of known heterocyclic amines. Princess Takamatsu Symp 23, 39–49.
Wakabayashi, K., Ushiyama, H., Takahashi, M., Nukaya, H., Kim, S.B., Hirose, M., Ochiai, M., Sugimura, T., Nagao, M., 1993. Exposure to heterocyclic amines. Environ. Health Perspect. 99, 129–134.
WCRF/AICR, 1997. Nutrition and the prevention of cancer: a global perspective. World Cancer Research Fund in Association with American Institute for Cancer Research, Washington DC. available at http://www.dietandcancerreport.org/cancer_resource_center/downloads/Second_Expert_Report_full.pdf.
WHO, 2015. WHO Technical Report Series 989—Report on the Scientific Basis of Tobacco Product Regulations: Fifth Report of a WHO Study Group. Geneva, Switzerland.
Yamashita, M., Wakabayashi, K., Nagao, M., Sato, S., Yamaizumi, Z., Takahashi, M., Kinae, N., Tomita, I., Sugimura, T., 1986. Detection of 2-amino-3-methylimidazo[4,5-f]quinoline in cigarette smoke condensate. Jpn. J. Cancer Res. 77, 419–422.
Yoshida, D., Marumoto, T., 1980. Amino-alpha-carbolines as mutagenic agents in cigarette smoke condensate. Cancer Lett. 10, 141–149.
Zhang, L., Ashley, D.L., Watson, C.H., 2011. Quantitative analysis of six heterocyclic aromatic amines in mainstream cigarette smoke condensate using isotope dilution liquid chromatography-electrospray ionization tandem mass spectrometry. Nicotine Tob. Res. 13, 120–126.