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Mutation Research 539 (2003) 195–201
Genetic Toxicology and Environmental Mutagenesis
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Mutagenic activity and heterocyclic amine carcinogens in commercial pet foods

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Received 23 April 2003; received in revised form 2 July 2003; accepted 2 July 2003

Abstract

Twenty-five commercial pet foods were analyzed for mutagenic activity using the Ames/*Salmonella* test with strain TA98 and added metabolic activation. All but one gave a positive mutagenic response. Fourteen of these samples were analyzed for heterocyclic amine mutagens/carcinogens and all but one contained 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 10 of 14 contained 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) as analyzed by HPLC and confirmed by photodiode array peak matching. From these findings it is hypothesized that there is a connection between dietary heterocyclic amines and cancer in animals consuming these foods.

Keywords: Heterocyclic amine; Mutagen; Carcinogen; Diet; Pet food; Companion animal

1. Introduction

Mutagenic substances that are animal carcinogens have been found in foods and beverages [1]. The genotoxic carcinogens are found in some plant and animal products, from fungal spoilage products to compounds produced during food preparation. Despite over three decades of research, connecting these to human cancers is elusive due to the low dose seen in the human diet and difficulty in determining accurate intakes over a lifetime [2].

It is well-established that potent genotoxic heterocyclic amines are produced by the heating of natural precursors in meat, the creatine, amino acids and sugars, during cooking at high temperatures [3–6]. The demonstrated mutagenicity of these compounds in bacteria, cells in culture [7,8], and mice [9,10] support the many studies of carcinogenicity in mice and rats [11,12]. DNA adducts are formed in rodents and humans consuming these compounds at low doses [13]. Two heterocyclic amines, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) have a specificity for mammary gland tumors in rats [14], and the prostate is a target for PhIP-induced cancer in the rat [12].

For environmental exposures, dogs have been suggested as sentinels for human disease because they share the habitat and sometimes even the food of their human companions [15] and are not influenced by workplace exposures. Cancer is a leading cause of pet animal deaths. For studies of the exposure to household indoor air pollutants, radon and environmental tobacco smoke, it was suggested by Bukowski and Wartenberg [16] that pet epidemiology be used for the advantage of shorter life spans and cancer

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doi:10.1016/S1383-5718(03)00164-5

latency periods, as well as the lack of confounding occupational exposures in pets. In one study, with no age adjustment, 23% of animal patients presented at autopsy died of cancer [17].

In this paper, we describe the analysis of commercial pet foods for mutagenic activity using the Ames/*Salmonella* test and the finding of heterocyclic amine carcinogens in selected samples. Like the human diet, the dog's diet contains cooked meat. The finding of carcinogens in pet foods and the similarities in cancer occurrence at sites in humans and sexually intact dogs suggest that dogs may be a good model for studying the relationship between dietary heterocyclic amines and cancer.

2. Materials and methods

2.1. Salmonella mutagenicity assay

The mutagenic activity of the sample extracts was determined using the standard plate incorporation assay described by Ames et al. [18], with *S. typhimurium* strain TA98 (a gift of Professor Bruce Ames, University of California, Berkeley) with 2 mg of Aroclor-induced rat liver S9 protein per plate for metabolic activation, and tested in 5, 10, 25, 50, and 100 μl volumes. A positive control, IQ, gave 1200–1500 revertants per 5 nanogram dose. Dimethylsulfoxide was the negative control (spontaneous revertant counts) and gave TA98 values of 30–100 revertant colonies per plate.

Dose–response curves of the mutagenic activity were calculated using the method of Moore and Felton [19]. A minimum of four dose points from duplicate platings was used, and the linear portion of the curve was used to calculate the number of revertants per gram of original sample extracted. The standard error of the linear fits for all samples averaged 9.6% and was less than 22% in all cases.

2.2. Samples and chemicals

Pet foods were purchased from local stores or were small samples packaged by the manufacturer and distributed as samples. Heterocyclic amine standards included 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*] quinoxaline (DiMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), all purchased from Toronto Research Chemicals (Downsview, Ont., Canada), and 2-amino-(1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*])pyridine (IFP) was purified from a natural product [20]. Concentrations of the standards were determined using established molar extinction coefficients of 41,100 at 273 nm for MeIQx, 40,000 at 275 nm for DiMeIQx, 19,400 at 316 nm for PhIP, and 19,400 at 323 nm for IFP. Absorbances were measured using a Shimadzu 2100 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). All solvents were HPLC grade.

2.3. Sample extraction

Extraction was done using a modification of the solid phase extraction described by Gross and Grüter [21]. Briefly, each sample was ground in a blender, homogenized in 1 M NaOH, mixed with diatomaceous earth (Hydromatrix, Varian, Harbor City, CA, USA), and extracted with 50 ml ethyl acetate onto propylsulfonic acid (PRS) silica cartridges (500 mg, Varian, Harbor City, CA, USA). For mutagenicity testing, the PRS cartridge was eluted with 2 ml methanol/ammonium hydroxide, (9:1, v:v), evaporated to dryness, re-dissolved in DMSO, and tested in duplicate plates.

2.4. Heterocyclic amine analysis

For heterocyclic amine analysis, samples were extracted as detailed above, with further purification by washing the PRS cartridge with HCl and transferring to C18 cartridges (100 mg, Varian, Harbor City, CA, USA) with ammonium acetate, and the extract was analyzed by high performance liquid chromatography as reported [22]. HPLC separation was done using a 250 mm × 4.6 mm i.d. TSK gel ODS80-TM column (TosoHaas, Montgomeryville, PA) with a mobile phase of triethylamine phosphate 0.01M pH 3.2 (A solvent) and acetonitrile (B solvent). A linear gradient (5–15% B from 0 to 10 min; 15–25% B from 10 to 20 min; 25–55% B from 20 to 30 min) was used. Samples were analyzed on a Millennium 2010 HPLC system (Millipore Corp., Milford, MA) with a WISP autosampler, model 996 photodiode array detector, and

a Hewlett-Packard 1046A programmable fluorescence detector.

Heterocyclic amines were identified by concurrence of their retention times and ultraviolet (UV) spectral shapes with established library spectra for each of the compounds. PhIP and IFP were further confirmed by the presence of fluorescence peaks corresponding to UV peaks having the correct spectral shape and retention times. Correction for incomplete recovery of analytes was determined by spiking separate samples with known amounts of each of the four heterocyclic amines prior to ethyl acetate extraction. Recoveries typically ranged from about 20% for PhIP and IFP to 60% for MeIQx and DiMeIQx. Nanograms of analyte per gram of pet food sample were calculated from the resulting chromatogram peak areas, with correction for incomplete recoveries. Fluorescence peak areas were used for quantifying PhIP and IFP. The limit of detection for the heterocyclic amines was about 0.05 ng/g depending on the presence or absence of co-extracted interferences.

3. Results and conclusions

Fig. 1 shows the dose–response curves for four of the pet food samples numbered 13, 16, 17, and 22 as examples of analysis of the mutation data. The revertant colonies per Petri plate are plotted against the gram-equivalents of original pet food that was extracted. The slope of the line was used to determine the mutagenic response for 25 brands of pet foods shown in Table 1. All but one sample, number 8, gave a positive response (a positive slope for the dose–response curve) in the mutagenicity test. Potencies per gram of food ranged from 16 revertant colonies per gram for sample 9 to 992 revertant colonies for sample 18. The standard error of the line was used to give the uncertainty of each mutagenic activity measurement. For three foods, 15, 19, and 22, new samples were obtained several months after the original as a check on the consistency of the sample batches. In each case the new samples were 3–6-fold different from the original, suggesting general variability in the manufacture

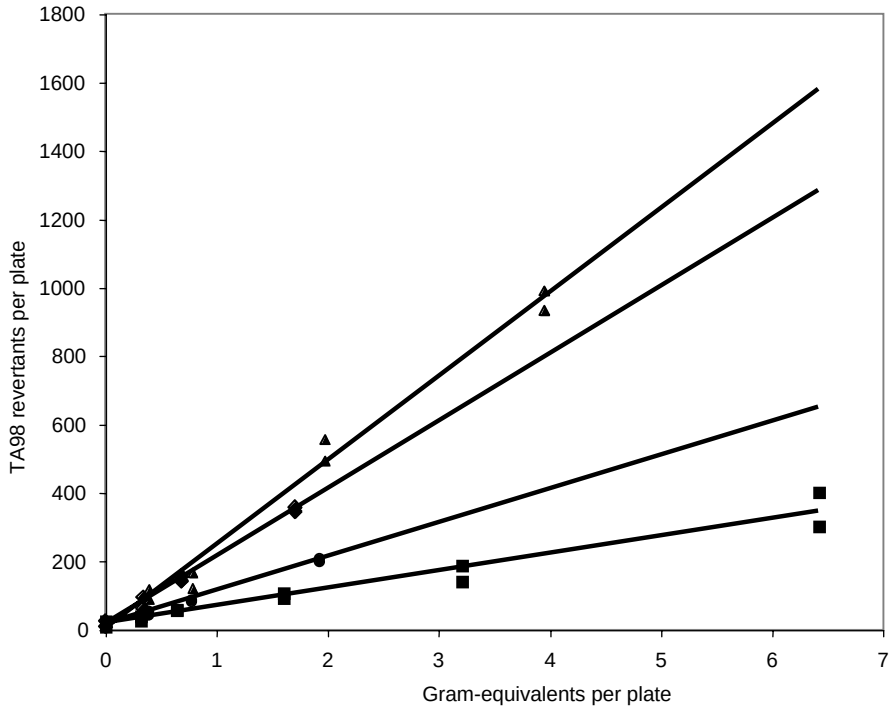

Fig. 1. Graph of mutagenic potency of pet food samples 13 (diamond symbol), 16 (circle), 17 (triangle), and 22A (square). Gram-equivalents are the amount used for each dose calculated to the unextracted food purchased.

Table 1
Mutagenic activity and heterocyclic amines in pet foods

Pet food	Mutagenic activity	Heterocyclic amines (ng/g)		
	Revertants/g	MeIQx	PhIP	DiMeIQx
1 (cat)	117 ± 13	0.53	1.7	ND
2 (dog)	104 ± 3	0.17	0.8	ND
3 (dog)	36 ± 3	ND	ND	ND
4 (dog)	34 ± 5	0.04	ND	ND
5 (dog)	203 ± 8	0.86	ND	ND
6 (cat)	183 ± 11	–	–	–
7 (dog)	91 ± 3	–	–	–
8 (dog)	Not positive	–	–	–
9 (dog)	16 ± 3	–	–	–
10 (dog)	34 ± 4	–	–	–
11 (puppy)	130 ± 8	0.9	1.6	ND
12 (dog)	44 ± 4	–	–	–
13 (dog)	198 ± 6	–	–	–
14 (cat)	36 ± 8	–	–	–
15A (puppy)	161 ± 10	–	–	–
15B (puppy)	48 ± 3	1.1	2.4	ND
16 (cat)	99 ± 3	–	–	–
17 (cat)	246 ± 10	–	–	–
18 (dog)	992 ± 60	0.8	70	ND
19A (dog)	357 ± 14	1.4	14	0.17
19B (dog)	59 ± 11	3.3	14	0.11
20 (dog)	30 ± 5	–	–	–
21 (dog)	172 ± 9	–	–	–
22A (dog)	51 ± 4	–	–	–
22B (dog)	195 ± 16	0.26	18	ND
23 (dog)	174 ± 16	1.4	21	0.12
24 (dog)	115 ± 7	0.34	ND	0.02
25 (kitten)	209 ± 19	0.57	3.5	ND

ND: not detected in the sample; –: not done. Two different samples of the same food (A and B) for pet foods 15, 19, and 22 were tested.

of the products. No consistent differences in mutagenic activity between dog, cat, puppy, or kitten foods were seen in this limited sampling. Differences might be expected if the percentage of meat products or differences in heating of meat products varied with the type of food. Details of the manufacture of the pet foods and sources of ingredients appear to be proprietary information.

A subset of samples covering a range of mutagenic potencies of the samples was analyzed for heterocyclic amines of the kind formed during the cooking of meat. Fig. 2 shows the chromatograms and confirming UV absorbance spectra of a typical sample. MeIQx was identified in 13 samples, PhIP was identified in 10, and DiMeIQx was found in three foods. These ratios are typical for cooked meats, with PhIP and MeIQx most abundant and always less DiMeIQx found. Interestingly, PhIP was more abundant than MeIQx in all samples in which it was found. IFP was not found in any sample, although IFP is frequently found in well-done meats [20]. The heterocyclic amines identified generally account for only one-fourth to one-half of the measured mutagenic activity, using the potencies of MeIQx, PhIP, and DiMeIQx of 100, 2, and 320 TA98 revertants per nanogram, respectively. In initial studies several samples were spiked with nine heterocyclic amines and recoveries were determined, yet none of the heterocyclic amines other than the three reported here were detected to explain the higher than expected mutagenic activity that we measured. This suggests other known or unknown mutagens are responsible for some of the activity measured.

The levels of mutagenic activity in pet foods are comparable to levels reported in the human diet. For meats cooked by professional chefs and served in restaurants, levels ranged from undetectable levels for one sample of 22 tested, up to 720 TA98 revertants per gram of meat [23]. The pet foods and meats for human consumption are nearly identical in the range of mutagenic potencies.

For heterocyclic amines, total levels in restaurant-cooked meats ranged from 0.5 to 20.2 ng/g for a char-broiled steak [23]. Pet foods had a similar range of heterocyclic amines: five samples of the 14 tested had combined levels over 15 ng/g. We estimate that the dose of heterocyclic amines that pets receive to be 5-fold higher than the human dose, since the pet food is the exclusive diet of many pets, and we estimate cooked meat to be about 17% of the percentage by weight of the solid human diet based on USDA food surveys [24].

It appears that the heterocyclic amines are the products of cooking, but a systematic study of pet food ingredients and their manufacture would identify the source and perhaps suggest ways to prevent their occurrence in the foods. Cooking meat to microbiological sterility can be done without the formation of heterocyclic amines [25], which are formed at high temperatures or moderate temperatures combined with long cooking times, factors which could be controlled during pet food manufacturing [3,4,26].

Since the occurrence of mammary and prostate carcinomas is high in sexually intact dogs, it is plausible that the dietary heterocyclic amines may be involved

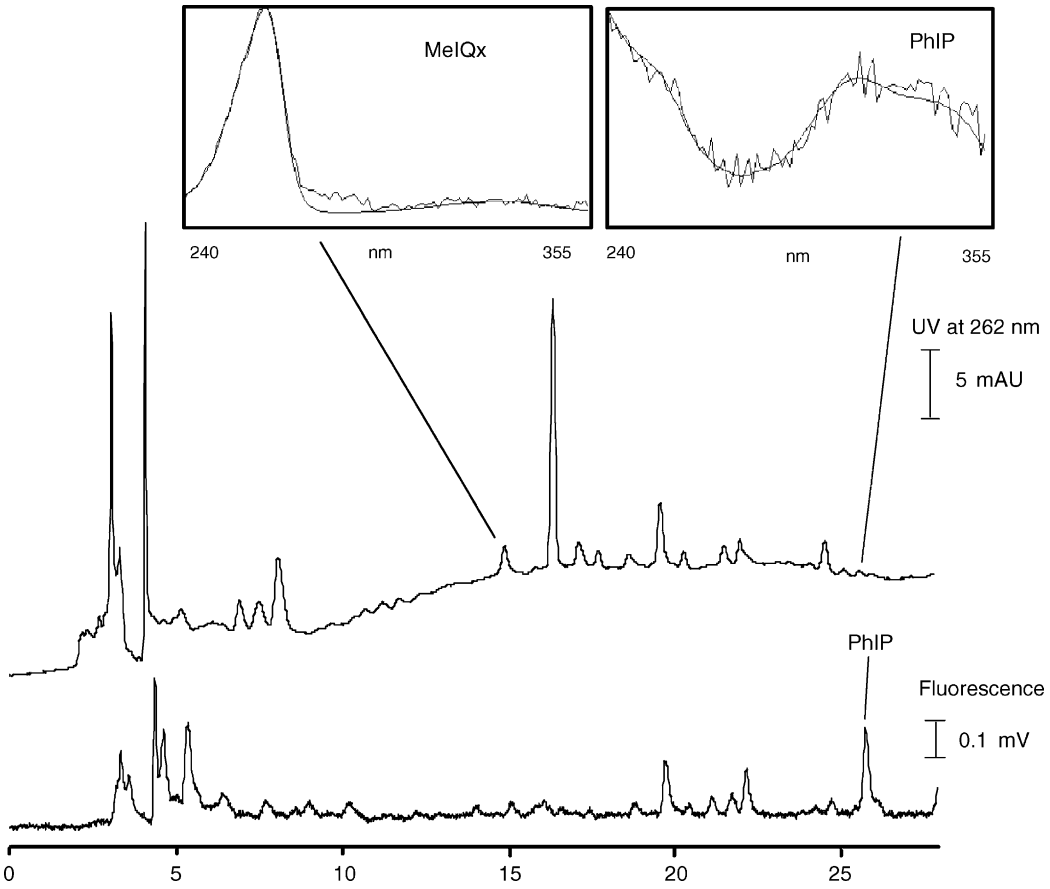

Fig. 2. HPLC chromatograms and UV absorbance spectra of sample 11. A reversed-phase HPLC separation was done with a linear gradient. See Section 2 for details.

in this cancer initiation. A study of the factors influencing canine cancer identified a high intake of red meat as associated with mammary tumors in a case/control study in dogs [27]. Breast cancer in dogs has been investigated and believed to be "caused by nutritional factors acting early in life" [28]. Colon and rectal cancers, which are putative endpoints of heterocyclic amines exposures, are much lower in dogs than in humans [29]. Still, exposure of pets to heterocyclic amines may provide relevant insight to the effects of these same heterocyclic amines in humans.

The finding of mutagenic activity and rodent carcinogens in pet food may also have implications for animal studies of carcinogens, particularly in dogs or cats. Diets for control and experimental animals may have contained low levels of heterocyclic amine carcinogens, perhaps affecting experimental results, for example the 38% of female beagles with malignant complex adenocarcinomas in a control population [30].

Pets may be good models for some human cancers, particularly if a biomarker of exposure or effect is discovered. Recent discovery of signature gene losses for PhIP might be such a marker in pets [31]. Breed differences in response to these heterocyclic amines may shed light on cancer susceptibility genes utilizing existing pet populations.

There are likely to be multiple causes for cancer in humans and pets. A plausible contributing cause may be lifelong exposure to heterocyclic amines that

are genotoxic rodent carcinogens in the diets of each. Pets may not be sentinels of human heterocyclic amine exposure, since human and pet diets differ, but pets may be sentinels of heterocyclic amine effects.

Acknowledgements

We thank Elizabeth A. McNiel, University of Minnesota, for review of this manuscript. This work was performed under the auspices of the US Department of Energy by the University of California, Lawrence Livermore National Laboratory under contract no. W-7405-Eng-48.

References

[1] W.K. Lutz, J. Slater, Chemical carcinogens and overnutrition in diet-related cancer, Carcinogenesis 13 (1992) 2211–2216.
[2] R. Sinha, An epidemiologic approach to studying heterocyclic amines, Mutat. Res. Fundam. Mol. Mech. Mutagen. 506 (2002) 197–204.
[3] T. Sugimura, M. Nagao, K. Wakabayashi, Mutagenic heterocyclic amines in cooked food, in: L. Fishbein, I.K. O'Neill, M. Castegnaro, H. Bartsch (Eds.), Environmental Carcinogens: Selected Methods of Analysis IV, IARC Scientific Publication, Lyon, 1981, pp. 251–267.
[4] K. Skog, G. Steineck, K. Augustsson, M. Jägerstad, Effect of cooking temperature on the formation of heterocyclic amines in fried meat products and pan residues, Carcinogenesis 16 (1995) 861–867.
[5] M.G. Knize, N.H. Shen, J.S. Felton, The Production of Mutagens in Foods, Air Pollution Control Association, 1988, pp. 1–8.
[6] J.S. Felton, M. Jägerstad, M.G. Knize, K. Skog, K. Wakabayashi, Contents in foods, beverages and tobacco, in: M. Nagao, T. Sugimura (Eds.), Food Borne Carcinogens: Heterocyclic Amines, Wiley, West Sussex, 2000, pp. 31–71.
[7] L.H. Thompson, J.D. Tucker, S.A. Stewart, M.L. Christensen, E.P. Salazar, A.V. Carrano, J.S. Felton, Genotoxicity of compounds from cooked beef in repair-deficient CHO cells versus *Salmonella* mutagenicity, Mutagenesis 2 (1987) 483–487.
[8] J.A. Holme, J.K. Hingslo, E. Soderlund, G. Brunborg, T. Christensen, J. Alexander, E. Dybing, Comparative genotoxic effects of IQ and MeIQ in *Salmonella typhimurium* and cultured mammalian cells, Mutat. Res. 187 (1987) 181–190.
[9] K. Masumura, K. Matsui, M. Yamada, M. Horiguchi, K. Ishida, M. Watanabe, O. Ueda, H. Suzuki, Y. Kanke, K.R. Tindall, K. Wakabayashi, T. Sofuni, T. Nohmi, Mutagenicity of 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP) in the new gpt delta transgenic mouse, Cancer Lett. 143 (1999) 241–244.
[10] A.M. Lynch, N.J. Gooderham, D.S. Davies, A.R. Boobis, Genetic analysis of PHIP intestinal mutations in MutaMouse, Mutagenesis 13 (1998) 601–605.
[11] T. Sugimura, Overview of carcinogenic heterocyclic amines, Mutat. Res. 376 (1997) 211–219.
[12] T. Shirai, M. Sano, S. Tamano, S. Takahashi, T. Hirose, M. Futakuchi, R. Hasegawa, K. Imaida, K.-I. Matsumoto, K. Wakabayashi, T. Sugimura, N. Ito, The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-imidazo[4,5-*b*] pyridine, Cancer Res. 57 (1997) 195–198.
[13] K. Dingley, K. Curtis, S. Nowell, J. Felton, N. Lang, K. Turteltaub, DNA and protein adduct formation in the colon and blood of humans after exposure to a dietary-relevant dose of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, Cancer Epidemiol. Biomark. Prevent. 8 (1999) 507–512.
[14] E.G. Snyderwine, M. Venugopal, M. Yu, Mammary gland carcinogenesis by food-derived heterocyclic amines and studies on the mechanisms of carcinogenesis of 2-amino-1-methyl-6-phenlyimidazo[4,5-*b*]pyridine (PhIP), Mutat. Res. 506–507 (2002) 145–152.
[15] D.W. Knapp, D.J. Waters, Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy in humans, Mol. Med. Today 3 (1997) 8–11.
[16] J.A. Bukowski, D. Wartenberg, An alternative approach for investigating the Carcinogencity of indoor air pollution: pets as sentinels of environmental cancer risk, Environ. Health Perspect. 105 (1997) 105–112.
[17] S.J. Withrow, Why worry about cancer in Pet Animals? in: S.J. Withrow, E.G. MacEwen (Eds.), Small Animal Clinical Oncology, second ed., Saunders, Philadelphia, 1996, pp. 1–3.
[18] B.N. Ames, J. McCann, E. Yamasaki, Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsomal mutagenicity test, Mutat. Res. 31 (1975) 347–364.
[19] D. Moore, J.S. Felton, A microcomputer program for analyzing Ames test data, Mutat. Res. 119 (1983) 95–102.
[20] P. Pais, M.J. Tanga, C.P. Salmon, M.G. Knize, Formation of the mutagen IFP in model systems and detection in restaurant meats, J. Agric. Fd. Chem. 48 (2000) 1721–1726.
[21] G.A. Gross, A. Grüter, Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products, J. Chromatogr. 592 (1992) 271–278.
[22] M.G. Knize, R. Sinha, N. Rothman, E.D. Brown, C.P. Salmon, O.A. Levander, P.L. Cunningham, J.S. Felton, Fast-food meat products have relatively low heterocyclic amine content, Fd. Chem. Tox. 33 (1995) 545–551.
[23] M.G. Knize, R. Sinha, E.D. Brown, C.P. Salmon, O.A. Levander, J.S. Felton, N. Rothman, Heterocyclic amine content in restaurant—cooked hamburgers, steaks and ribs, J. Agric. Food Chem. 46 (1998) 4648–4651.
[24] A.R.S., US Department of Agriculture Data Tables: Food and Nutrient Intakes by Region, 1994–1996, 1998.
[25] C.P. Salmon, M.G. Knize, F.N. Panteleakos, R. Wu, D.O. Nelson, J.S. Felton, Minimization of heterocyclic amines and thermal inactivation of *Escherichia coli* in fried ground beef, J. Natl. Cancer Inst. 92 (2000) 1773–1778.
[26] M.G. Knize, B.D. Andresen, S.K. Healy, N.H. Shen, P.R. Lewis, L.F. Bjeldanes, F.T. Hatch, J.S. Felton, Effect of

temperature, patty thickness and fat content on the production of mutagens in fried ground beef, Food Chem. Toxicol. 23 (1985) 1035–1040.
[27] D.P. Alenza, G.R. Rutteman, L. Pena, A.C. Beynen, P. Cesta, Relation between habitual diet and canine mammary tumors in a case-control study, J. Vet. Intern. Med. 12 (1998) 132–139.
[28] E.G. Sonnenschein, L.T. Glickman, M.H. Goldschmidt, L.J. McKee, Body conformation, diet, and risk of breast cancer in pet dogs. A case-control study, Am. J. Epidemiol. 133 (1991) 694–703.
[29] J.L. Kelsey, A.S. Moore, L.T. Glickman, Epidemiologic studies of risk factors for cancer in pet dogs, Epidemiol. Rev. 20 (1998) 204–217.
[30] S.A. Benjamin, A.C. Lee, W.J. Saunders, Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles, Vet. Path. 36 (1999) 423–436.
[31] A.T. Christian, E.G. Snyderwine, J.D. Tucker, Comparative genomic hybridization analysis of PhIP-induced mammary carcinomas in rats reveals a cytogenetic signature, Mutat. Res. 506 (2003) 113–119.