ELSEVIER

Journal of Chromatography A, 763 (1997) 179–185

JOURNAL OF CHROMATOGRAPHY A

Analysis of foods for heterocyclic aromatic amine carcinogens by solid-phase extraction and high-performance liquid chromatography

Mark G. Knize*, Cynthia P. Salmon, Ellen C. Hopmans, James S. Felton

Biology and Biotechnology Research Program, L-452, Lawrence Livermore National Laboratory, Livermore, CA 94550, USA

Abstract

Carcinogenic and mutagenic heterocyclic aromatic amines (HAA) are natural products often present at ng/g levels in muscle meats when they are cooked at temperatures over 150°C. Using solid-phase extraction and high performance liquid chromatography (HPLC) with photodiode array UV detection, samples were analyzed for the following heterocyclic amines: DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline); IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline); MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline); and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine). Quality control samples, analyzed periodically over two years in a blind study, show relative standard deviations ranging from 22 to 38% for the compounds found, variations typical for analysis at ng/g levels. Amounts range from undetectable levels (less than 0.1 ng/g) to hundreds of ng/g of PhIP for frying or grilling at high meat surface temperatures. Beef, chicken, pork and lamb can all have greater than 10 ng/g of PhIP. Ground chicken breast meat has lower amounts of heterocyclic amines than intact muscle pieces of the same size cooked identically. Restaurant prepared samples that we analyzed contained undetectable levels up to 14 ng/g total heterocyclic amines for a beef steak sample. Not extracted with the above method are related mutagenic heterocyclic amines, which have been reported in cooked foods in our laboratory and others. Method development using ion exchange on an SCX solid-phase extraction cartridge shows promise in providing a method for the quantitation of these mutagenic dimethyl-, trimethyl- and furo-imidazopyridines where a practical analysis method is needed.

Keywords: Food analysis; Aromatic amines, heterocyclic; Amines

1. Introduction

The search for carcinogenic agents in foods is directed towards explaining differences in cancer occurrence in humans. Analysis of the health risks of dietary chemicals has focused either on exogenous chemicals added to foods, such as pesticides and artificial sweeteners, or endogenous substances naturally present or formed within foods, such as fats and fungal metabolites.

The finding that extracts of cooked muscle meats produce a potent response in the Ames/*Salmonella* mutation test led to studies using analytical chemistry methods to isolate and identify the chemicals responsible for the mutagenic activity. These potent mutagens in bacterial test systems have been shown to be carcinogenic in animal test systems and have been the subject of hundreds of research articles in the last 17 years [1–5].

The formation of mutagenic chemicals in meats has been explained by the condensation of creatine or creatinine with amino acids and sugars or their thermal decomposition products in model systems and meats [6,7].

Many heterocyclic aromatic amines have been isolated and identified from cooked meats or model systems, but four compounds are frequently reported in food surveys [8–12]. These are: DiMeIQx (2-

*Corresponding author.

PII S0021-9673(96)00720-0

amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline); IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline); MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline); and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine). We analyzed meat samples according to the PRS solid-phase extraction and HPLC method of Gross [8,13].

An additional group of compounds, that have been studied less, have been found in various meats and meat extracts by purification guided by the Ames/*Salmonella* mutagenicity assay [14]. Among these are amino-dimethyl- and amino-trimethyl-imidazo pyridines, DMIP and TMIP, respectively. They have been reported in only a few instances in foods or food extracts, because analytical standards have not been available for method development. For some of these, like TMIP, the exact structure is not known but several isomers are available. We believe some of these compounds may be important because early results suggest their mass amounts are in the range of the commonly found MeIQx. Thus, their analysis in foods needs to be undertaken. Complete analysis of the heterocyclic amine carcinogens in the human diet requires quantification of this whole group of compounds.

We report here food analysis results with the PRS solid-phase extraction method for heterocyclic amine analysis of foods and preliminary results for a new solid-phase extraction technique for the analysis of aromatic amine mutagens in foods for which no method is currently available.

2. Experimental

2.1. Chemicals

DiMeIQx, MeIQx, IQ and PhIP were purchased from Toronto Research Chemicals, Downsview, Ontario. DMIP (2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine) and IQx (2-amino-3-methylimidazo[4,5-*f*]quinoxaline) were synthesized at LLNL as reported [15,16]. TMIP isomers (1,5,6-trimethylimidazopyridine and 3,5,6-trimethylimidazopyridine) were the kind gift of Dr. Mary Tanga, SRI International [17]. The structures of these compounds are shown in Fig. 1.

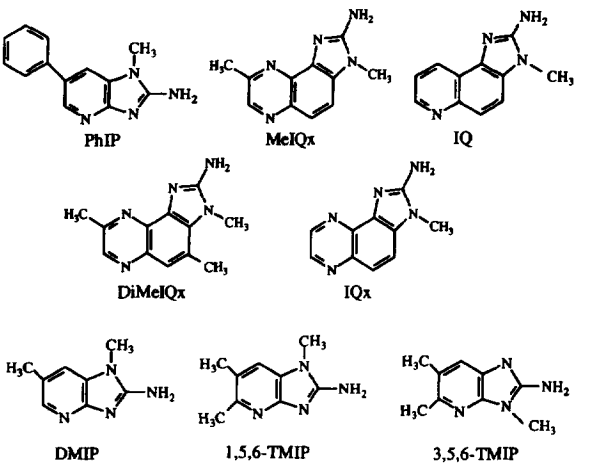

Fig. 1. Heterocyclic amine mutagens used in this study.

2.2. Food samples

Laboratory-cooked samples were fried in a pan or grilled over charcoal or propane to a well-done but edible condition in our laboratory or those of collaborators. The commercially pre-cooked meat samples were purchased from restaurants in the San Francisco Bay area. The cooking technique used for some commercially cooked samples remains unknown. The degree of cooking of restaurant-cooked meats was unspecified or "well-done" by our request. All meat samples with bones were deboned before homogenization. All samples included every part of the meat normally eaten and were homogenized in a food blender to create a uniform sample.

2.3. Extraction and purification

Extraction for HPLC analysis was done as previously described [18] using methods developed by Dr. Gian Gross, referred to here as the PRS method [8,13]. Briefly, 4-g samples were homogenized in 1 *M* NaOH and mixed with Chem Elut diatomaceous earth (Varian Sample Preparation Products, Harbor City, CA, USA) to form a free-flowing powder. The mixtures were poured into extraction columns coupled to Bond Elut PRS (propylsulfonic acid–silica gel, 500 mg) solid-phase extraction columns (Varian Sample Preparation Products). Heterocyclic amines were extracted to the PRS columns with 40 ml of 5% toluene in dichloromethane. The sample retained by

the PRS cartridge was washed with 40% 0.1 *M* HCl–methanol and transferred with 20 ml of 0.5 *M* ammonium acetate and concentrated on a 100 mg Bond Elut C_{18} extraction column. After elution, the samples were evaporated to dryness and resuspended with an internal standard solution.

An acid/ion-exchange solid-phase extraction scheme (SCX method) allowing extraction and purification of a group of heterocyclic amines not extracted by the PRS method (TMIP, DMIP) is under development. Briefly, cooked meat was homogenized in 0.1 *M* HCl–methanol (3:2), centrifuged, and the supernatant diluted with 0.1 *M* HCl to a 20% methanol content. The resulting extract was applied to an endcapped C_{18} SPE cartridge (International Sorbent Technology, Hengoed, UK) coupled to a Bond Elut SCX cartridge (Benzenesulphonic acid, 500 mg; Varian Sample Preparation Products). The C_{18} cartridge trapped contaminants while allowing the heterocyclic amines to pass to the SCX cartridge where they were retained. The C_{18} cartridge was discarded and the SCX column was washed successively with 0.1 *M* HCl–methanol (3:2), MeOH, water and 1.0 *M* ammonium acetate, pH 8. The analytes were eluted with 1.0 *M* ammonium acetate (pH 8)–methanol (1:1) and after removal of the MeOH from the eluent this fraction was concentrated on a Bond Elut C_{18} cartridge. After elution with methanol–ammonium hydroxide (9:1), the samples were evaporated to dryness and resuspended in mobile phase. This represents current progress and all steps and possible additional ones are being optimized to maximize analyte recovery and minimize co-extracted interferences.

2.4. High-performance liquid chromatography

HPLC separation conditions are given in the legends to Figs. 2 and 3. Samples were analyzed on a Millennium 2010 HPLC system (Millipore, Milford, MA, USA) with a WISP autosampler, Model 996 photodiode array detector and a Hewlett-Packard 1046A programmable fluorescence detector (excitation 307 nm; emission 370 nm). The identities of chromatographic peaks were confirmed by comparing the peaks' UV absorbance spectrum to library spectra acquired from standard solutions. Results were corrected for losses determined from spiked samples.

3. Results and discussion

Fig. 2 shows chromatograms from the extract of a fried beef sample, spiked with four heterocyclic amines (1, upper chromatogram) or unspiked (1, lower chromatogram). UV absorbance at 262 nm shows MeIQx, DiMeIQx and PhIP peaks in the unspiked samples, which are confirmed by the sample and library spectra, shown on the right of Fig. 2. Fluorescence chromatograms (2) show a much larger signal over the background peaks for PhIP, the only heterocyclic amine of these three that fluoresces. The confirmation by comparing UV spectra is essential, because even with the extensive solid-phase purification, many unknown, potentially interfering peaks are present in each chromatogram.

Quality control of the reproducibility of our work with the solid-phase extraction and HPLC procedures was evaluated, with no trends or sample misclassifications seen. Relative standard deviations ranged from 22 to 37% for individual compounds for a high temperature sample analyzed 13 times over the course of the study (data not shown).

Table 1 shows the average of duplicate extractions of restaurant or laboratory-cooked samples analyzed by the PRS method. In general, PhIP is the most abundant heterocyclic amine, followed by MeIQx, but the ratios vary greatly. Bacon seems to form MeIQx and PhIP equally, chicken and beef generally form more PhIP. These restaurant-cooked and laboratory-cooked samples show higher amounts of MeIQx, DiMeIQx and PhIP than fast-food meat products which in all cases studied had less than 1 ng/g total combined heterocyclic amines [18]. A large study of chicken cooked in a variety of ways showed some samples, those broiled or cooked on a propane grill, to have unusually high amounts of PhIP with several samples containing 50 to 450 ng/g [19]. Our values (Table 1) are in general agreement with other food surveys but cooking conditions are shown to be an important variable in heterocyclic amine formation.

Fig. 4 shows the effect of grilling time on the formation of PhIP in chicken breast meat, either

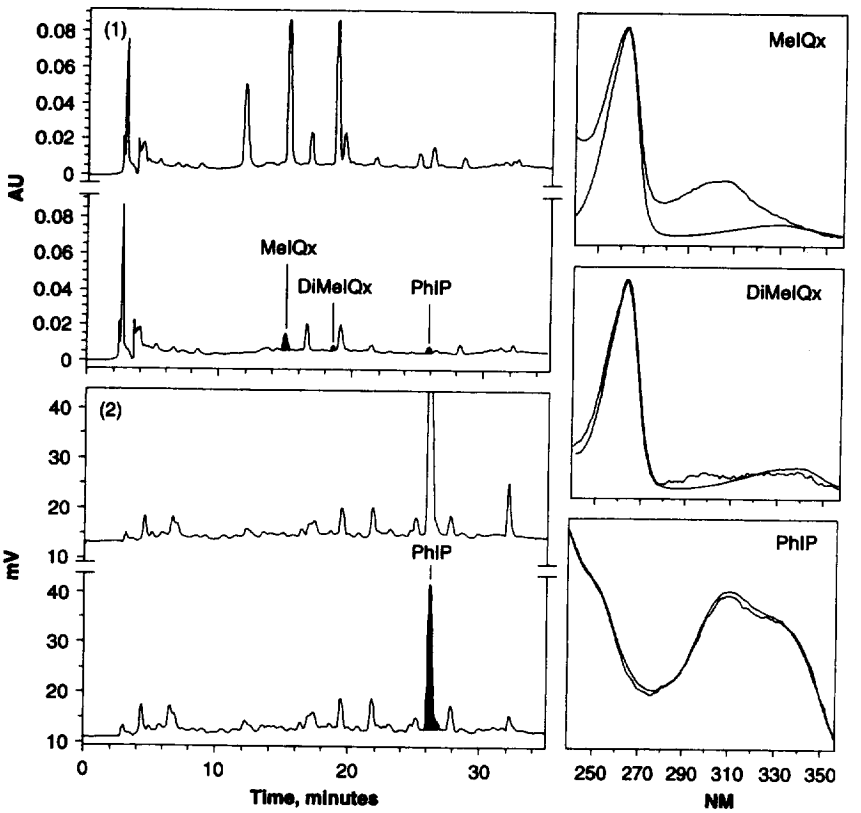

Fig. 2. HPLC chromatograms and photodiode array UV absorbance spectra of the PRS analysis of a fried beef sample. Upper left (1): spiked (upper) and unspiked chromatograms at 262 nm. Lower left (2): spiked (upper) and unspiked chromatograms from fluorescence detector, (excitation=307 nm, emission=370 nm). HPLC conditions: TSK gel ODS80-TM column (TosoHaas, 220 mm×4.6 mm I.D.), a mobile phase of triethylamine phosphate 0.01 *M*, pH 3.6 (A solvent) and acetonitrile (B solvent), flow 1 ml/min. A linear gradient (5–15% B from 0–10 min; 15–25% B from 10–20 min; 25–55% B from 20–30 min) was used.

ground and formed into patties or left intact. In all cases, PhIP was formed in higher amounts in the whole skinless, boneless chicken breast meat sample than in the ground sample. The reasons for this are not clear, but the temperature at the meat surface or the availability of precursors may be affected by grinding the muscle tissue.

A new method for the analysis of additional heterocyclic amines, those not recovered by the PRS method, is under development. The SCX solid-phase extraction columns used exhibit a dual mode of action, a cation-exchange and an apolar mechanism. Both modes are capable of retaining the heterocyclic amines. The heterocyclic amines pass through the endcapped C_{18} solid-phase extraction column due to the 20% methanol content of the extract, but are retained on the SCX column by the cation-exchange mechanism. The washes described in the extraction procedures were designed to make optimal use of this dual mode character by switching from retention of the amines by the cation-exchange mechanism to retention by the reversed-phase mechanism. Some samples, such as chicken grilled at high temperatures, may require further purification. Table 2 shows preliminary data comparing recovery in the PRS method and the newly developed SCX method for heterocyclic amines. Although in some cases the recovery of the compound is lower in the new acid/SCX method compared to the PRS method, it appears that the new method recovers the total set of heterocyclic amines we have tried. Fig. 3 shows chromatograms of a chicken extract spiked with

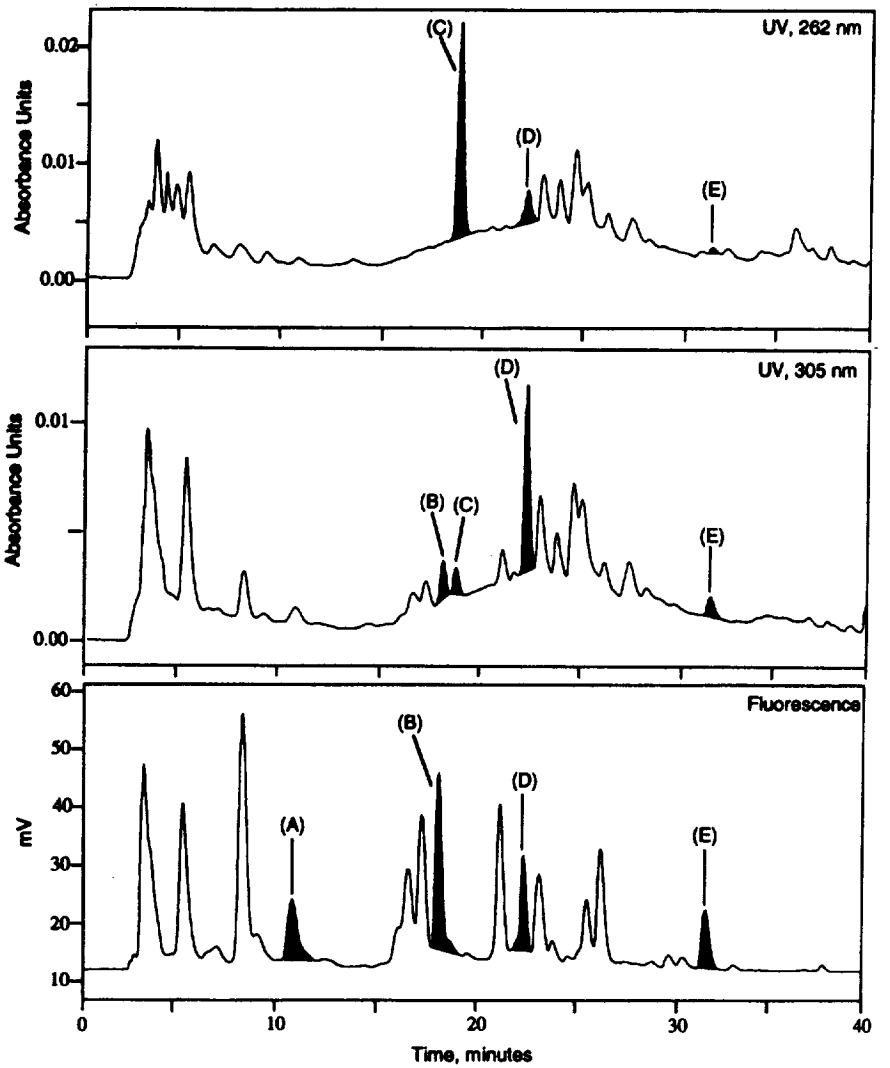

Fig. 3. Chromatograms (upper panel: UV absorbance at 262 nm; middle panel: UV absorbance at 305 nm; bottom panel: fluorescence with excitation=307 nm and emission=370 nm) of a grilled chicken sample spiked with DMIP (A), 1,5,6-TMIP (B), IQx (C) and 3,5,6-TMIP (D) and processed using the newly developed SCX method. Naturally present in this sample was PhIP (E). HPLC conditions: TSK gel ODS80-TM column, a mobile phase of triethylamine phosphate 0.025 *M*, pH 3.6 (A solvent) and acetonitrile (B solvent), flow 1 ml/min. After running isocratically at 5% B for 5 min, the following linear gradients were started: 5–17% B from 5–15 min; 17–25% B from 15–25 min; 25–55% B from 25–35 min; 55–80% B from 35–40 min.

DMIP, IQx, 1,5,6- and 3,5,6-TMIP and purified using the new method. PhIP occurs in large amounts in grilled chicken and was detected in addition to the spiked heterocyclic amines. Many interfering peaks are still present and are likely obscuring the MeIQx and DiMeIQx found in this sample by the PRS method. Work is currently underway to optimize the washing of the SPE columns to remove contaminants, and to optimize the HPLC separation.

There are additional heterocyclic amines known in foods. An imidazofuro pyridine (IFP) was purified from meat cooked with added creatine [20], congeners of DiMeIQx [21,22] and PhIP [23] were isolated from meats or meat-derived products. These

Table 1
Heterocyclic amine content of cooked meat samples, restaurant and laboratory cooked, analyzed by the PRS method

Type of sample	Source[a]	MeIQx(ng/g)	DiMeIQx (ng/g)	PhIP(ng/g)
Pork, blackened, Cajun style	R	0.53[b]	ND[c]	3.0
Bacon A	L	11	2.0	1.5
Bacon B	L	27	9.3	36
Bacon C	L	1.0	ND	ND
Ham, grilled	L	2.3	0.2	1.5
Lard, 160°C	L	ND	ND	ND
Chicken Fajita, Mexican style	R	0.54	ND	6.4
Chicken breast A, grilled	L	ND	3.1	270
Chicken breast B, grilled	L	0.63	0.53	21
Hamburger A, charcoal grilled	R	0.89	ND	11
Hamburger B, pan fried	L	16.4	4.5	67.5
Hamburger C, charcoal grilled (for human studies)	L	2.2	ND	50
Beef, blackened, Cajun style	R	0.48	ND	1.0
Top sirloin steak	R	0.87	ND	13
Lamb, grilled	L	1.6	ND	11

[a] R=restaurant, L=laboratory cooked. [b] Average of duplicate analyses. [c] ND=not detected (<0.1 ng/g).

will be tested for recovery with the new and seemingly more universal method.

4. Conclusions

The heterocyclic amines are mutagens and animal carcinogens and are thus plausible human carcinogens. Relating the human consumption for exposed populations requires analysis to understand the amounts and the conditions for formation, and strategies to reduce formation and consumption if warranted.

If any of the additional heterocyclic amine mutagens are present in foods in masses comparable to the heterocyclic amines listed in Table 1, their mutagenic and carcinogenic potency should be evaluated to determine their contribution to the risk of human consumption of heterocyclic amines in foods.

Importantly, PhIP has been shown to have biological properties that appear to be relevant to risk assessment. PhIP has important tissue specificity with respect to carcinogenesis: the colon of male rats and the mammary gland and colon of female rats are targets. The same cancer targets are seen in humans

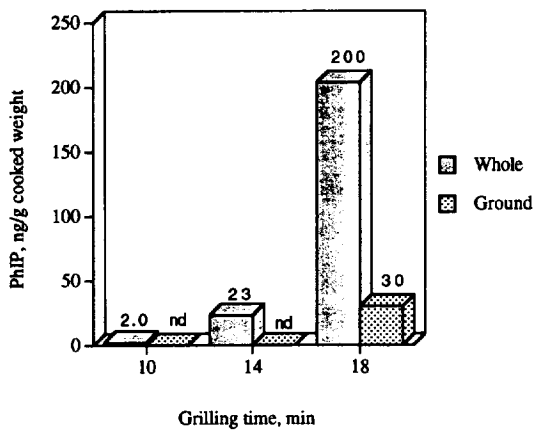

Fig. 4. Graph showing the PhIP in chicken breast meat samples ground and formed into patties or left intact and grilled for 10, 14 or 18 min. Analysis was by the PRS extraction method.

Table 2
Recovery of heterocyclic amines in the PRS method compared to the SCX method

Compound	% Recovered in PRS method	% Recovered in SCX method
IQx	95	58
DMIP	6	31
1,5,6-TMIP	0	81
3,5,6-TMIP	70	61
IQ	45	48
MeIQ	38	24
MeIQx	67	46
DiMeIQx	72	25
PhIP	30	28

on Western diets high in meat [24–26]. DMIP, TMIP and the IFP isomers are structurally related to PhIP, being pyridine imidazoamines, and their metabolic and carcinogenic properties are therefore important to assess. This further suggests a role for these compounds in human cancer etiology and shows a strong need for understanding the levels of consumption of PhIP and related compounds in our diet.

Analysis of foods requires reliable methods capable of determining the amounts of heterocyclic amine carcinogens present. The existing solid-phase extraction method and the new method under development appear capable of providing the analysis needed to support studies in the causes of human disease.

Acknowledgments

This work was performed under the auspices of the U.S. Department of Energy by LLNL under contract W-7405-Eng-48 and supported by NCI agreement YO1CP2-0523-01 and National Cancer Institute grant CA55861.

References

[1] IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 56, IARC, Lyon, 1993, p. 165.
[2] T. Sugimura, S. Sato and K. Wakabayashi, in Y.T. Woo, D.Y. Lai, J.C. Arcos and M.F. Argus (Editors), Chemical Induction of Cancer, Structural Bases and Biological Mechanisms, Academic Press, New York, 1988, p. 681.
[3] B. Stavric, Food Chem. Toxicol., 32 (1994) 977.
[4] S. Robbana-Baranat, M. Rabache, E. Rialland and J. Fradlin, Environ. Health Perspect., 104 (1996).
[5] F.T. Hatch, M.G. Knize, S.K. Healy, T. Slezak and J.S. Felton, Environ. Molec. Mutag., 12 (1988) 1.
[6] K. Skog, Food Chem. Toxicol., 31 (1993) 655.
[7] M. Jägerstad, K. Skog, S. Grivas and K. Olsson, Mutat. Res., 259 (1991) 219.
[8] G.A. Gross and A. Grüter, J. of Chromatogr., 592 (1992) 271.
[9] S. Murray, A.M. Lynch, M.G. Knize and N.J. Gooderham J. Chromatogr., 616 (1993) 211.
[10] K. Wakabayashi, H. Ushiyama, M. Takahashi, H. Nukaya, S.B. Kim, M. Hirose, M. Ochiai, T. Sugimura and M. Nagao Environ. Health Perspect., 99 (1993) 129.
[11] M.A.E. Johansson and M. Jägerstad, Carcinogenesis, 15 (1994) 1511.
[12] L.S. Jackson, W.A. Hargraves, W.H. Stroup and G.W. Diachenko, Mutat. Res., 320 (1994) 113.
[13] G.A. Gross, Carcinogenesis, 11 (1990) 1597.
[14] M.G. Knize, N.H. Shen, S.K. Healy, F.T. Hatch and J.S. Felton, Dev. Ind. Microbiol., 28 (1987) 171.
[15] G. Becher, M.G. Knize and J.S. Felton, Vår Föda, 42 (1989) 85.
[16] G. Becher, M.G. Knize, I.F. Nes and J.S. Felton, Carcinogenesis, 9 (1988) 247.
[17] M.G. Knize, M. Roper, N.H. Shen and J.S. Felton, Carcinogenesis, 11 (1990) 2259.
[18] M.G. Knize, R. Sinha, N. Rothman, E.D. Brown, C.P. Salmon, O.A. Levander, P.L. Cunningham and J.S. Felton, Food Chem. Toxicol., 33 (1995) 545.
[19] R. Sinha, N. Rothman, E. Brown, O. Levander, C.P. Salmon, M.G. Knize and J.S. Felton, Cancer Res., 55 (1995) 4516.
[20] M.J. Tanga, J.E. Bupp and T.J. Tochimoto, Heterocycl. Chem. 31 (1994) 1641.
[21] H. Nukaya, S. Koyota, F. Jinno, H. Ishida, K. Wakabayashi, R. Kurosaka, I-S. Kim, Z. Yamaizumi, H. Ushiyama, T. Sugimura, Carcinogenesis, 15 (1994) 1151.
[22] I-S. Kim, K. Wakabayashi, R. Kurosaka, Z. Yamaizumi, F. Jinno, S. Koyota, A. Tada, H. Nukaya, M. Takahashi, T. Sugimura and M. Nagao, Carcinogenesis, 15 (1994) 21.
[23] R. Kurosaka, K. Wakabayashi, H., Ushiyama, H. Nukaya, N. Arakawa, T. Sugimura and N. Nagao, Jpn. J. Cancer Res., 83 (1992) 919.
[24] N. Ito, R. Hasegawa, M. Sano, S. Tamano, S. Takayama and T. Sugimura, Carcinogenesis, 12 (1991) 1503.
[25] K. El-Bayoumy, Y-H. Chae, P. Upadhyaya, A. Rivenson, C. Kurtzke, B. Reddy and S.S. Hecht, Carcinogenesis, 16 (1994) 431.
[26] A. Ghoshal, K-H. Preisegger, S. Takayama, S.S. Thorgeirsson and E.G. Snyderwine, Carcinogenesis, 15 (1994) 2429.