Formation of the Mutagen IFP in Model Systems and Detection in Restaurant Meats

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Mixtures of the free amino acids, creatine and glucose, were dry-heated to model the potential formation of heterocyclic amines in meats. The formation of the mutagenic amine IFP (determined to be 2-amino-(1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*])pyridine) was investigated by varying heating time, heating temperature, and precursors. With an optimized mixture of glutamine, creatine, and glucose, heated at 200 °C for 60 min, 2 mg of IFP was purified for studies to define its structure. Trideuteriomethyl-IFP was made from trideuteriomethylcreatinine in the model system for use in LC-MS detection of IFP in foods. Analysis of well-done meats purchased from restaurants showed about half to contain IFP at levels from 1.4 to 46 ng/g of cooked meat, demonstrating human exposure to this mutagen.

Keywords: *Heterocyclic amines; cooked meats; IFP; model systems; food mutagen*

INTRODUCTION

Concern about the role of diet in human cancer has prompted the search for compounds in common foods that may act as tumor initiators by producing somatic cell mutations. Analyses of proteinaceous cooked foods, as well as pyrolyzed amino acids and proteins, led to the discovery of highly mutagenic heterocyclic amines (Sugimura et al., 1977).

Structural determination of mutagens and their quantification in cooked foods are essential to evaluate the role of the heterocyclic amines in human carcinogenesis. Although >20 heterocyclic amines have been isolated as mutagens from various kinds of heated materials, additional unidentified mutagens are present in cooked meats. These may play a role in risk assessment. Ten of the mutagenic amines already identified in foods have been proved to be carcinogenic in rodents (Ohgaki et al., 1991; Ito et al., 1991). There is a weak, yet quantitative, correlation between the mutagenicity of aromatic amines in *Salmonella typhimurium* TA98 and their carcinogenicities in rodents, suggesting that aromatic amine mutagens to which humans are exposed may be important in human carcinogenesis (Hatch et al., 1992).

Complete avoidance of exposure to heterocyclic amines is almost impossible for humans, and the link between heterocyclic amine exposure (determined by meat doneness preference) and human cancer is currently inconsistent (Probst-Hensch et al., 1997; Ward et al., 1997; Augustsson et al., 1999; Zheng et al., 1998). Yet variations in cancer incidence in studies of migrant populations suggest external causes are responsible (Moradi et al., 1998; Muir, 1996; Stanford et al., 1995), and the hope is that, once identified and understood, the exposures or their effects could be minimized.

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Research in our laboratory is concerned with the mechanisms of formation, the chemical identity, and the spectrum of genotoxicity of these mutagenic agents. We are interested in the mechanisms by which the heterocyclic amines arise in foods and the reaction conditions for their formation. Understanding the reaction conditions for the formation of the heterocyclic amines is necessary to predict which foods and process flavors have these compounds and to devise methods for reducing their concentrations and, thus, the human exposure.

To identify the precursors and to elucidate the reaction conditions that yield heterocyclic amines in cooked meat products and fish, we used a dried model system containing the heterocyclic amine precursors to simulate the dry reactions that seem to occur at the meat surface (Pais et al., 1998). This work involves modeling the formation of a mutagenic amine with unresolved structure, an aminodimethylimidazofuropyridine (IFP). This compound, having a molecular weight of 202, was reported to be present in cooked pork (Vahl et al., 1987), a creatine-added Norwegian sausage (Becher et al., 1988), a heated extract of beef (Taylor et al., 1986), and a milk- and creatine-added fried beef (Knize et al., 1990). We recently published the presence of IFP in six different kinds of meats (beef, chicken breast, chicken thigh, turkey, pork, and fish) in concentrations ranging between 2.6 and 16.0 ng/g (Pais et al., 1998). The mutagenic activity of IFP was reported to be 10000 TA1538 revertants/μg of pure compound (Knize et al., 1990). The observed mutagenic activity and the fact that IFP has been found in six different kinds of laboratory-cooked meats suggest that IFP contributes to the observed mutagenic activity in meats. Thus, we believe it is necessary to determine the occurrence of IFP in meats and its exact structure to facilitate its synthesis in high yield. We report here the formation, isolation, and structural determination of IFP in model systems and its occurrence in meats purchased in restaurants.

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MATERIALS AND METHODS

Materials. The heterocyclic amines used as analytical standards were purchased from Toronto Research Chemicals (Downsview, ON) and included 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo-[4,5-*f*]quinoxaline (DiMeIQx), 2-amino-1,6-dimethylimidazo-[4,5-*b*]pyridine (DMIP), and 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP). 2-Amino-1,5,6-trimethylimidazo[4,5-*b*]-pyridine (TMIP) was synthesized by Dr. Mary Tanga, (Tanga et al., 1994) and is available from Dr. Harold Seifried (National Cancer Institute, Chemical and Physical Carcinogenesis Branch, 6130 Executive Boulevard, EPN/700, Bethesda, MD 20892), and IFP was a natural product isolated from a creatine-added meat mixture (Knize et al., 1990). Amino acids, creatine, creatinine, and sugars were purchased from laboratory chemical suppliers. Meals containing meats were purchased from restaurants in the Livermore, CA, area.

Model System. A model system containing free amino acids, creatinine, and glucose in the proportions found in beef steak and shown to produce IFP (Taylor et al., 1986; Pais et al., 1998) was investigated. Mixtures of these precursors, simulating 50 g of meat, were dry-heated at different temperatures in a glass beaker (50 mL) in a muffle furnace (Blue M, Blue Island, IL). Additionally, model systems containing single amino acids (1 mmol), creatine or creatinine (1 mmol), and sugars (0.5 mmol) were dry-heated at 200 °C during 30 min.

Analysis of IFP in Model Systems. The analysis of IFP was performed using a modification of the solid-phase extraction method developed by Gross (1990) for the determination of other heterocyclic amines as we recently reported (Pais et al., 1998). Briefly, the samples were homogenized in sodium hydroxide (1 N) and mixed with diatomaceous earth (Hydromatrix, Varian Sample Preparation Products, Harbor City, CA). The amines were eluted from extraction columns, containing the diatomaceous earth mixture, directly to a propanesulfonic acid (PRS) cartridge (Varian Sample Preparation Products) using ethyl acetate. The PRS cartridge was washed, and IFP was eluted onto a C_{18} cartridge (Varian Sample Preparation Products) to concentrate the sample. The final elution was performed with methanol/ammonia (9:1). The extract was evaporated to dryness and redissolved in 50 μL of mobile phase. The duplicate samples of a single heated mixture were analyzed and averaged. Extraction recoveries were determined by spiking the samples with IFP. Amounts reported are corrected for incomplete recoveries. IFP quantification was performed using the extinction coefficient corresponding to PhIP (also an imidazopyridine) and the IFP absorbance maximum at 323 nm because a synthetic standard is still not available.

HPLC Determination. The IFP analysis was carried out on a Millennium 2010 HPLC system with a WISP autosampler, a model 996 diode array detector (Waters Corp., Milford, MA), and a Shimadzu model RF535 fluorescence detector set at excitation and emission wavelengths of 306 and 370 nm, respectively. A TSK-Gel ODS 80T_M column (5 μm, 22 cm × 4.6 mm i.d.) (Toso Haas, Montgomeryville, PA) was used. Aliquots of 20 μL were injected with a mobile phase of 0.01 M triethylamine phosphate at pH 3.2 and acetonitrile, operating in a linear gradient at a flow rate of 1 mL/min as we reported previously for other heterocyclic amines (Pais and Knize, 1998).

Peak confirmation of heterocyclic amines from foods was further done by LC-MS. Chromatography was done on a Microtech Ultra-Plus HPLC system (Sunnyvale, CA) equipped with an ODS-A column (25 cm × 3 mm) (YMC, Inc., Wilmington NC). Analytes were eluted at a flow rate of 200 μL/min using a mobile phase of A (water/methanol/acetic acid, 97:2:1) and 5% B (methanol/water/acetic acid, 95:4:1) for 2 min to 20% B at 5 min and a linear gradient to 60% B at 30 min followed by a hold for 5 min.

Analytes were detected with a Finnigan LCQ mass spectrometer (San Jose, CA) in the MS/MS mode, isolating M^+ ions in alternating scans at mass 163, 177, 203, 206, 214, 225, and 227 to detect DMIP, TMIP, IFP, IFP-d_3, MeIQx, PhIP, and 4,8-DiMeIQx, respectively.

Isolation of 2 mg of IFP from a Dry-Heated Mixture of Glutamine, Creatine, and Sucrose. Approximately 2 mg of IFP was purified from the mixture of glutamine (0.37 mol), creatine (0.37 mol), and sucrose (0.18 mol) dry-heated at 200 °C for 60 min. The total amount was heated in 16.69 g batches separately. Each heated mixture was dissolved in 200 mL of sodium hydroxide, mixed with diatomaceous earth (130 g), and extracted with ethyl acetate (700 mL). The solvent was evaporated to dryness, and the residues corresponding to 10 experiments (a total of 2.6 g of residue after the extraction) were dissolved in 10 mL of methylene chloride/methanol (95:5, v/v). Flash chromatography was carried out with a silica column (3 × 15 cm), using methylene chloride/methanol (95:5) for the elution. A total of 40 fractions of 20 mL each were collected, but none contained IFP as detected by HPLC analysis of fractions. Further elution was performed with methylene chloride/methanol (92.5:7.5), and 20 fractions of 20 mL each were collected. These fractions, which contained IFP, were evaporated to dryness, and the residue (0.2 g) was dissolved in 10 mL of methylene chloride/methanol (97:3). Additionally, a second flash chromatography was performed with these residues. In this case the elution was performed with methylene chloride/methanol (95:5) (24 fractions collected of 20 mL each), methylene chloride/methanol (94:6) (20 fractions collected of 20 mL each), and methylene chloride/methanol (93:7) (45 fractions collected of 20 mL each). Fractions 59−88 contained IFP and were evaporated to dryness. The residue (80 mg) was dissolved in 9 mL of mobile phase. The final purification was performed by HPLC fractionation, and 1 mL aliquots of the final extract were injected. The HPLC conditions described for the analysis of IFP in the model systems were used with an isocratic mobile phase of triethylamine phosphate (0.01 M, pH 7)/acetonitrile (80:20).

Structure Analysis. Proton and carbon-13 NMR spectra of purified IFP were recorded on a Gemini 300 spectrometer (Varian Analytical Instruments, Palo Alto, CA) in DMSO-d_6.

Synthesis of IFP-d_3. A mixture of glutamine (Sigma, St. Louis, MO) (1.1 mmol), creatinine-d_3 (98% purity, Isotec Inc., Miamisburg, OH) (1.1 mmol), and sucrose (EM Science, Gibbstown, NJ) (0.55 mmol) was dry-heated at 200 °C for 30 min in a 13 × 100 mm disposable test tube. The residue was dissolved in NaOH (10 mL, 1 N), mixed with diatomaceous earth, extracted, and purified by reversed-phase HPLC using the scheme described above. Recovered was 16 μg (0.078 μmol) of IFP-d_3.

RESULTS AND DISCUSSION

Factors Affecting the Formation of IFP. *Effect of Heating Temperature and Time.* Beginning with our dry-heated model system that formed heterocyclic amines including IFP (Pais et al., 1998), we experimented with simpler systems to determine the heating conditions and precursors to form IFP in optimum yields. Figure 1 shows the formation of IFP in the dry-heated model system containing the 20 amino acids, creatinine, and glucose, representing the composition of beef muscle. The dry-heated mixture formed IFP with increasing time at 200 or 250 °C to maximum concentrations of ~17 μg/g creatinine, and each showed that IFP formation is temperature dependent and that overheating decreased IFP yields, possibly due to decomposition. Other authors also observed the decrease in the concentration of heterocyclic amines at high temperatures and long heating times. For instance, Jackson and Hargraves (1995) found the decrease of MeIQx and DiMeIQx in a pressurized water model system of threonine, creatine, and glucose at temperatures of 225 and 250 °C. The same results were obtained for MeIQx and 7,8-DiMeIQx at 225 °C more recently by Arvidsson

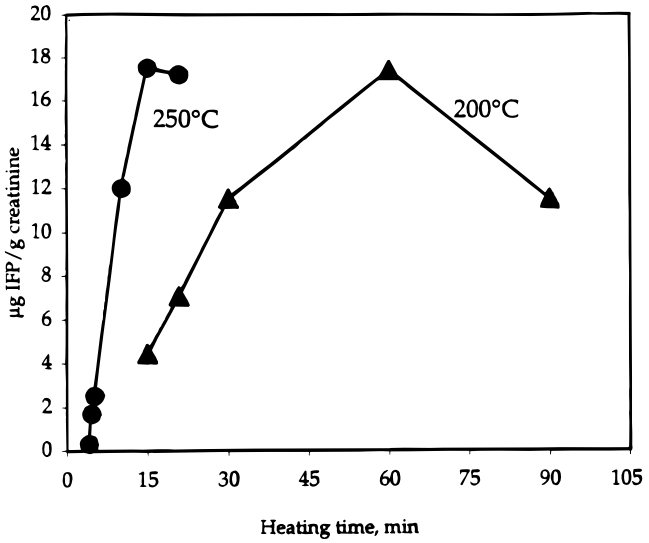

Figure 1. Formation of IFP in a mixture of free amino acids, creatinine, and glucose simulating the composition of beef, after heating for various times at 200 or 250 °C. Shown are averages of duplicate analyses.

et al. (1997) in a water model system simulating the composition in meat. This decrease has also been reported in cooked meat and fish. For example, MeIQx and PhIP levels decreased with time when salmon was pan-broiled at 200 °C (Gross and Grüter, 1992).

Effect of Precursors. Mutagenic activity was first reported in protein-rich foods. However, when proteins instead of amino acids were reflux-boiled in a model system, no mutagenic activity was detected (Jägerstad et al., 1983). The free amino acids, the creatin(in)e, and the sugars present in foods are responsible for the formation of heterocyclic amines. We studied the amino acids and sugars and compared creatinine and creatine for their importance as precursors for the formation of IFP in a dry-heated model system.

The 20 amino acid model system simulating the composition of beef (Figure 2, left) did not yield higher amounts of IFP than the simpler system with only the 10 (lysine, arginine, threonine, aspargine, serine, glutamic acid, glycine, alanine, leucine, and glutamine) or 7 (lysine, arginine, aspargine, glutamine, glutamic acid, alanine, and leucine) most abundant amino acids. We then tested each of the seven amino acids alone with creatinine and glucose (molar ratios of 1:1:0.5), and all of the reaction mixtures formed IFP. We compared creatinine to creatine and found little difference between them in the formation of IFP when combined with glutamine and glucose and then heated.

We compared glucose to sucrose and found greater IFP formation with sucrose. Sucrose increased the formation of IFP by almost 100%, and after glutamine and creatinine were heated without sugar, IFP was not detected. These results concur with those of Skog et al. (1995), who reported the increase of mutagenic activity in a model system when substituting glucose by sucrose. Simpler mixtures of glutamine and creatine or glutamine and glucose failed to form IFP, as did individually heating glutamine, creatinine, or glucose. IFP is formed from creatinine and glucose alone, with no amino acid. Glutamic acid or glutamine are the amino acids that do increase the formation of IFP.

IFP is the only heterocyclic amine found in cooked foods containing an oxygen atom in its structure. A mutagenic heterocyclic amine containing oxygen atoms was also isolated from a creatine pyrolysate and identified as 4-amino-1,6-dimethyl-2-methylamino-1*H*,6*H*-pyrrolo[3,4-*f*]benzimidazole-5,7-dione (Cre-P-1) (Nukaya et al., 1991). However, the presence of Cre-P-1 in cooked foods or derived food products has not yet been reported. To determine if atmospheric oxygen was involved in IFP formation, we reacted creatine and glucose in a nitrogen atmosphere instead of air, but no difference in the IFP amount was observed (Figure 2). From these results we conclude that the presence of oxygen in the IFP structure does not come from the air, but from the glucose or the creatine.

The highest yields for the formation of IFP were obtained with the reaction of glutamine, creatine (or creatinine), and sucrose. Thus, we scaled up the reaction to isolate the milligram amounts needed for analytical studies and as a spiking standard for food analysis.

Structural Determination. We heated high amounts of glutamine, creatine, and sucrose in 10 different aliquots to produce IFP that was purified from the reactions. The purification started with an extraction with ethyl acetate of $^1/_{10}$ of the total reaction in a solid support of diatomaceous earth. The extraction efficiency in this step was ~76%. Two different flash chromatography steps were carried out with the residue combined from all 10 extractions. Dichloromethane/methanol at different percentages (from 95:5 to 92.5:7.5, v/v) was used for the elution. In these steps good extraction efficiencies were obtained, 96 and 98%, respectively. Finally, an HPLC fractionation with a mobile phase of triethylamine phosphate (0.01 M, pH 7.0)/acetonitrile (80:20) was performed to obtain pure IFP. The recovery in this last step was 70%. With this product we could perform NMR experiments, and we hoped to obtain crystals to carry out X-ray crystallography experiments, which would elucidate the structure of IFP.

The structure of IFP was previously determined to be one of four isomers (Knize et al., 1990). The fusion of the furan and pyridine rings and the position of the *N*-methyl group could not be determined due to limited sample mass at that time. The sample isolated from our model system had a proton NMR spectrum identical in peak signal position and relative signal strength to the spectrum published in 1990, but the increased overall signal strength from the 60-fold-increased mass allowed us to determine the isomeric structure.

Nuclear Overhauser effect (NOE) experiments showed a coupling of the proton at the 4-position of pyridine at 7.65 ppm to the *N*-methyl protons at 3.52 ppm, showing that the methyl is in the 1-position as shown in Figure 3. Irradiation of the peak at 7.65 ppm failed to affect the furan proton (6.53 ppm), indicating these protons are separated in space by a distance >5 Å and are on opposite sides of the fused rings, supporting the structure shown in Figure 3. Irradiating the C methyl protons at 2.44 ppm increased the area of the proton at 6.53 ppm, confirming both types of protons to be on the furan ring. Similarly, irradiation of the peak at 6.53 ppm increased the peak at 2.44 ppm without affecting any other peak.

The carbon-13 spectrum generally supports the structure shown (data not shown). Proton NMR predictions did not reveal any new angular or linear fused-ring structure that would fit the available data. The structure shown in Figure 3 is consistent with the NMR data and shows the observed proton interactions.

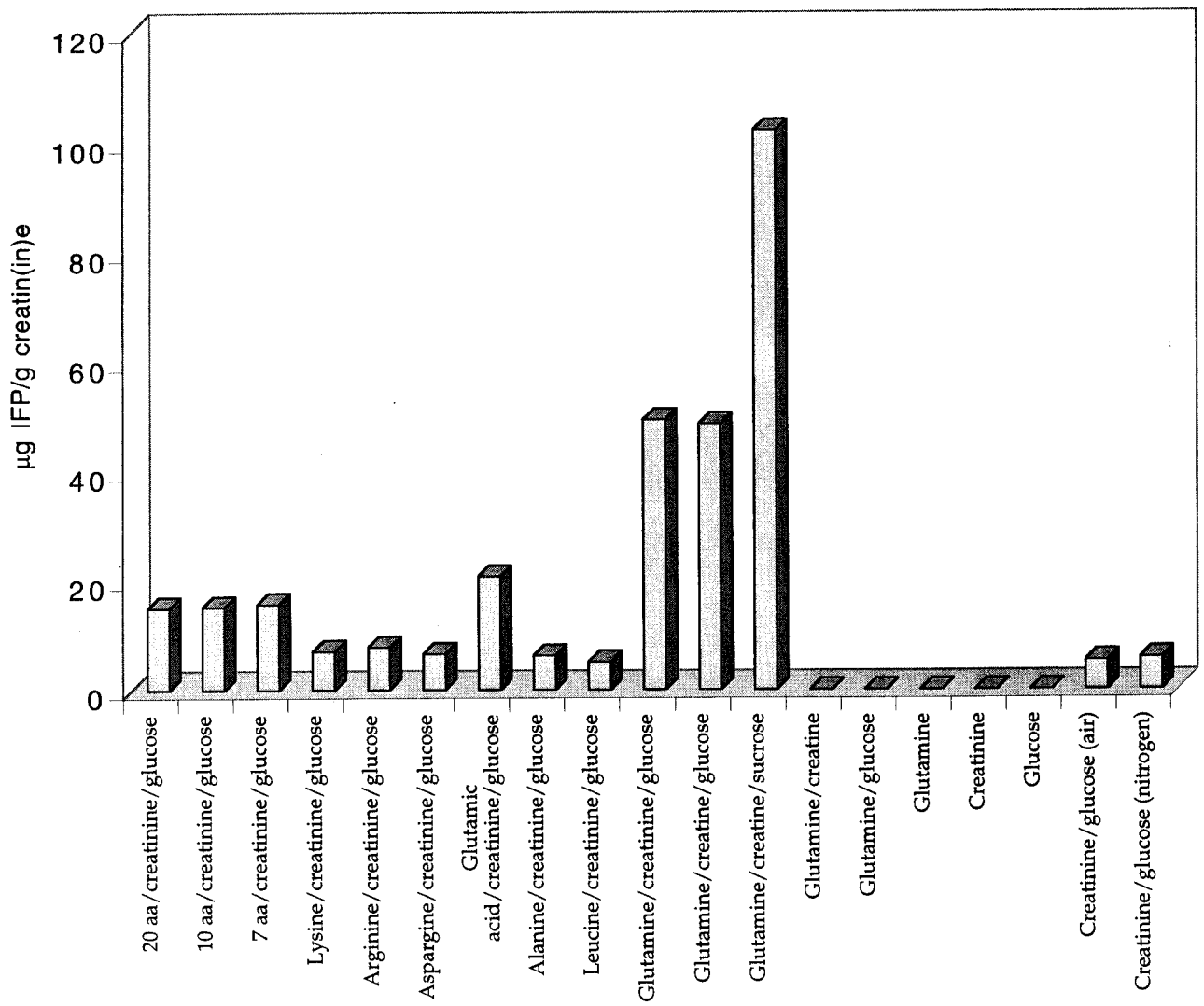

Figure 2. IFP formation in model systems containing different combinations of precursors in molar ratios of 1:1:0.5 of amino acid/creatinine/sugar. Values shown are averages of duplicate analyses.

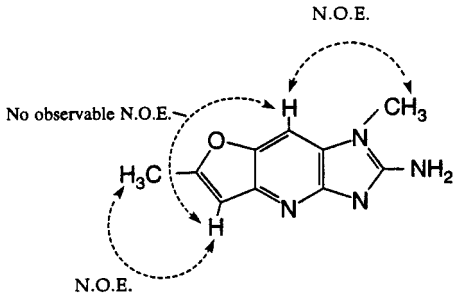

Figure 3. Structure of IFP determined from NMR NOE experiments: 2-amino-(1,6-dimethylfuro[3,2-*e*])imidazo[4,5-*b*]-pyridine.

Attempts to grow crystals suitable for structure determination by X-ray diffraction were unsuccessful.

Occurrence of IFP in Restaurant Foods. Optimization of the extraction conditions for IFP and other mutagenic heterocyclic amines showed that ethyl acetate gave better recoveries than the usual dichloromethane/toluene but more complex UV chromatograms (Pais et al., 1998); thus, IFP-d_3 was made to facilitate sample analysis by mass detection. LC-MS of IFP-d_3 (detected at mass 206) showed ~1.2% IFP at mass 203 as expected from the starting creatinine-d_3 that was only 98% pure.

Figure 4 shows the results from the HPLC analysis of a meat sample. The lower chromatogram shows a meat sample spiked with the four heterocyclic amines that fluoresce under these HPLC conditions: DMIP, TMIP, IFP, and PhIP. The unspiked chromatogram (Figure 4, middle) shows peaks at the appropriate retention times for three heterocyclic amines detected. The UV absorbance chromatogram at 323 nm (Figure 4, top) shows a complex chromatogram. Confirmation of IFP peaks by UV absorbance spectra was difficult, so further confirmation was obtained by LC-MS. Extracts were spiked with IFP-d_3, and the presence of IFP was confirmed with peaks at the retention time of 21.34 min (Figure 5), with 20% of the area of the d_3 IFP at time of 21.21 min, along with the other heterocyclic amines (data not shown). The presence of 1.2% IFP in the IFP-d_3 limits the usefulness of the internal standard for samples having low levels of IFP. Thus, samples with low levels were confirmed without spiking, relying only on retention times to confirm peaks. A sample needed to have a fluorescence peak at the appropriate retention time, an absorbance peak at 323 nm, and a peak at mass 203 at the appropriate retention time to be considered confirmed. Quantitation was made on the fluorescence chromatogram peak areas, as these chromatograms showed fewer interfering peaks.

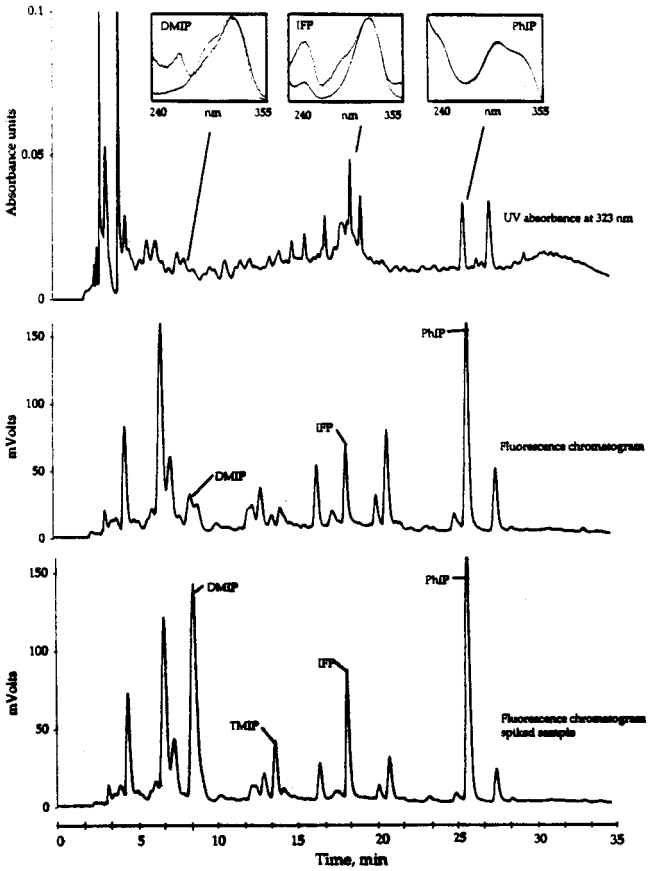

Figure 4. HPLC chromatograms of the analysis of a London broil steak purchased in a restaurant: (bottom) recovery of four heterocyclic amines spiked before extraction; (middle) presence of DMIP, IFP, and PhIP; (top) UV absorbance of unspiked sample. Inset boxes are a comparison of UV photodiode array spectra of the sample peak and a reference spectrum of the pure analyte.

Table 1 shows that IFP was detected in many well-done meat samples cooked in restaurants. The amount of IFP can exceed that of other known heterocyclic amines and ranges from undetectable levels to 46 ppb. The amount seems to be very cooking-dependent and appears to be formed under the same conditions that favor the formation of PhIP. Results shown for the more commonly found heterocyclic amines are in the same range as reported for other steaks cooked in restaurants (Knize et al., 1998) or for laboratory studies of cooked beef steaks (Sinha et al., 1998; Skog et al., 1997; Wakabayashi et al., 1992).

Conclusions. The work described here shows that the mutagenic heterocyclic amine IFP is formed from mixtures of creatine or creatinine and glucose or sucrose, and can be augmented by mixtures of amino acids, especially glutamine or glutamic acid. The model system developed permitted scale up for studies to define the structure of IFP, to have sufficient IFP to routinely spike food samples for quantitative analysis, and to produce trideuterio-IFP for use as a standard in LC-MS analyses. We also determined the amount of IFP in meat products purchased in local restaurants and found concentrations from 1.4 to 46 ng/g in 6 of 11 meat samples. When detected, levels are less than those of PhIP but consistently exceed the amount of MeIQx.

Because this compound is a potent mutagen and people are exposed to IFP in well-done meats, the role of this compound in cancer etiology needs to be determined.

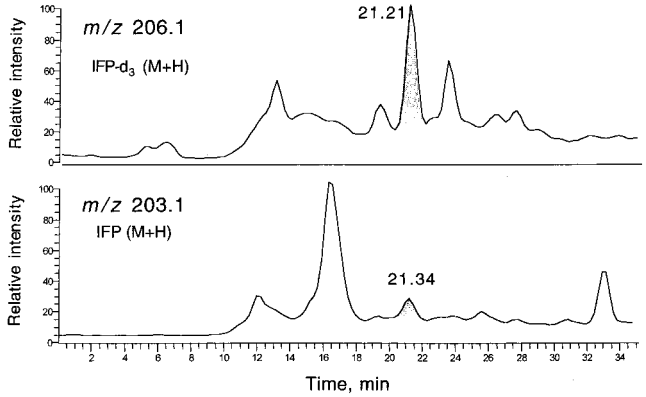

Figure 5. IFP peak confirmation by LC-MS. London broil steak sample was spiked with IFP-d_3. The upper chromatogram shows the IFP-d_3 peak at the expected retention time of 21.21 and natural IFP in the sample at 21.34, confirming the finding of IFP.

ABBREVIATIONS USED

DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline [CAS Registry No. 95896-78-5 (CAS Registry No. have been provided by the author)]; DMIP, 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine; HPLC, high-performance liquid chromatography; IFP 2-amino-(1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*])pyridine; LC-MS, liquid chromatography−mass spectrometry; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (CAS Registry No. 77500-04-0); PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (CAS Registry No. 105650-23-5); PRS, propanesulfonic acid silica; TMIP, 2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine; UV, ultraviolet.

Table 1. IFP and Other Heterocyclic Amines in Restaurant Foods (Nanograms of Heterocyclic Amine per Gram of Cooked Meat)

sample	restaurant, doneness	meat cut	IFP	MeIQx	PhIP	DMIP	TMIP	DiMeIQx
top sirloin	A, well done	loin	nd[a]	1.2[b]	1.8	nd	nd	nd
New York steak	A, well done	loin	nd	0.12	0.86	nd	nd	nd
pork chop	A, unspecified	loin	nd	0.4	2.4	nd	nd	nd
beef (French dip sandwich)	A, unspecified	loin	nd	nd	nd	nd	nd	nd
New York steak	B, well done	loin	7.0	1.3	7.7	7.2	1.5	0.77
tenderloin steak 1	C, well done	loin	7.6	1.9	16	nd	nd	nd
tenderloin steak 2	C, well done	loin	21	0.67	49	nd	nd	nd
top sirloin steak	D, well done	loin	3.3	2.0	7.8	nd	nd	nd
London broil steak	C, well done	round	46	3.0	182	3.4	nd	nd
prime rib	C, well done	rib	nd	nd	nd	nd	nd	nd
beef (fajitas)	D, unspecified	unknown	1.4	0.93	1.7	0.59	nd	0.06
au jus gravy	A, unspecified		nd	nd	nd	nd	nd	nd

[a] nd, not detected. [b] Average of duplicate analyses of a single sample.

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