determination of residues in blood and tissues is being studied.

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Registry No. Penicillin G, 61-33-6; penicillin V, 87-08-1; cloxacillin, 61-72-3.

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High-Pressure Liquid Chromatographic Method for the Analysis of 2-Amino-3-methylimidazo[4,5-*f*]quinoline, a Mutagen Formed during the Cooking of Food

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A quantitative method for the analysis of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a mutagen formed during the cooking of beef and fish, has been developed. A crude extract containing basic materials is purified by preparative TLC and reverse-phase HPLC. Quantitation is done on a cyanopropyl bonded-phase column run in the reverse-phase mode. Recovery is about 45% and the limit of detection is less than 25 pmol/50 g of beef. Control experiments with spiked extracts show a high degree of precision. The method has been used to show that there are relatively large differences in the formation of IQ between meat samples with a high or low fat content. The presence of other additives such as BHA, Celite, and casein decreases the formation of mutagen during cooking.

There is a great deal of current interest in the formation of mutagenic activity during the cooking of food (Weisburger and Horn, 1982). A number of mutagens have been isolated and their structures characterized (Sugimura, 1982). At least six of these mutagens have been reported to occur in fried beef (Barnes et al., 1983).

Although there is one report of an attempt at quantitation in the literature, analytical methods have not yet been developed to measure routinely the amount of mutagen in a complex foodstuff. Partially, this may be due to the fact that the compounds are primary heterocyclic amines, and hence do not always chromatograph well, and partly because food is such a complex material that extensive preliminary cleanup is required.

It is important that a quantitative analytical method be available, because any assessment of human risk from these mutagens and potential carcinogens necessarily requires an accurate measure of exposure. Also, studies of the chemistry involved in the formation of the mutagens will demand a technique for quantifying the product. Up to now, the only method available to serve this purpose has been a test for bacterial mutagenicity. Although reasonably precise, such a bioassay may be subject to inaccuracy because of its response to enhancers or inhibitors of mutagenesis (Pariza et al., 1982; Sugimura et al., 1980).

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In this paper, we describe a quantitative chemical method for 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), an extremely potent mutagen, which is formed during the frying of ground beef. This compound is interesting because of its structural similarity to the known colon, breast, and prostate carcinogen 3,2′-dimethyl-4-aminobiphenyl, as shown in Figure 1.

MATERIALS AND METHODS

Chemicals. All solvents used in extraction and chromatography were HPLC grade. Butylated hydroxyanisole (BHA) and casein were from Sigma (St. Louis, MO) and Celite 503 was from J. T. Baker (Phillipsburg, NJ). 2-Methylbenzimidazole and 5-aminoquinoline were from Aldrich (Miwaukee, WI). IQ standard was synthesized according to published procedures (Kasai et al., 1980) and cochromatographed with a reference sample kindly provided by Dr. T. Sugimura (Tokyo). The structure of the synthesized compound was confirmed by mass spectroscopy and ^1H NMR and found to be identical with that reported in the literature.

Meat. Ground beef with high or low fat content was purchased from a local supermarket. Water content was determined by weight difference before and after lyophilization. Fat content was determined by hexane extraction in a Soxhlet apparatus for 24 h. The low-fat meat contained 56.2% water and 10.6% fat on a wet weight basis or 24% fat on a dry weight basis. High-fat meat contained 52% water and 27.5 or 57.3% fat on a wet weight and dry weight basis, respectively.

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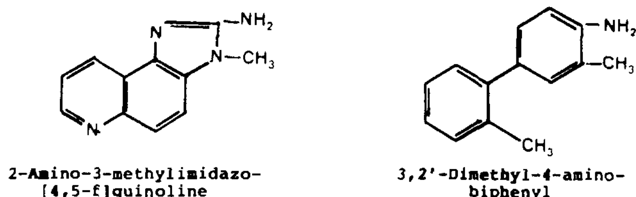

Figure 1. Structure of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ).

Cooking and Extraction. Meat was made into 50-g patties by using a Petri dish as a form. Cooking and extraction procedures are fully described in Wang et al. (1982). Briefly, the cooking surface was preheated to 240 °C and patties were then cooked for 5 min/side. The temperature at the interface between the meat and cooking surface was initially 90 °C, increasing to about 130 °C at the end of 10 min. No difference in cooking temperatures was noted for any treatments. After being cooked, patties were extracted with methanol, reduced to the residual water in vacuo, adjusted to pH 1.5, and extracted with methylene chloride 3 times. The aqueous phase was adjusted to pH 10 and extracted again 3 times with methylene chloride. The methylene chloride phase, after back-extraction with 0.05 M sodium bicarbonate buffer (pH 10.0), is referred to as the crude basic extract. A total of 200 g of ground beef was cooked and extracted for each determination.

In experiments involving inhibitors, casein (10% w/w), Celite (10% w/w), and BHA (50 mmol/100 g of meat) were mixed into the meat by grinding twice through a meat grinder. Patties were then formed and cooked as above.

TLC. Preliminary cleanup of the crude basic extract was performed on Whatman LK6F analytical TLC plates. Development was in methanol–chloroform (20:80). An IQ standard was run beside each extract. A band, extending from 1 cm above the leading edge of the IQ standard spot to 1 cm below the trailing edge, was removed from the track of each extract, eluted in $MeOH-CHCl_3$ (1:1), and concentrated for HPLC.

HPLC. A Du Pont 8820 gradient liquid chromatograph, equipped with a Rheodyne Model 7125 injector and either a Beckman Model 153 fixed-wavelength UV detector or a Beckman Model 155 variable-wavelength UV detector, was used. Detection was at 254 nm. The TLC fraction was further purified by using an Altex LiChrosorb C_{18} column and a Whatman Partisil PXS 10/25 ODS 3 column, connected in tandem, and eluted with 15% methanol in 0.02 M triethylamine buffer, pH 3.0, at 1 mL/min. The fraction containing IQ was concentrated and rechromatographed on a Du Pont Zorbax CN column and was eluted with a linear gradient from 20% to 40% methanol in 0.015 M phosphate buffer, pH 7.0, at 1 mL/min. Quantitation was based on peak heights from the CN chromatogram.

Mass Spectrometry. Mass spectra were obtained with a Hewlett-Packard 5982A mass spectrometer equipped with a Model 5933A data system at 70 eV with a source temperature of 190–195 °C.

Statistics. Linear regression analysis was performed on a Hewlett-Packard 9815A calculator. For comparison of the amount of IQ in control extracts and those containing additives, a *t* test for the difference of two means with different standard deviations was used (Crow et al., 1960). *P* values for the *t* test were calculated on the HP9815A calculator.

RESULTS

The first cleanup step is TLC of the crude basic extract. Figure 2 shows a representative TLC chromatogram of a low-fat fried beef sample. A large number of bands are visible, and substantial purification can be accomplished at this stage by selective elution of the material with an R_f from about 0.15 to 0.30. Figures 3a shows an HPLC chromatogram of the broad IQ fraction obtained by TLC when it is chromtographed on a C_{18} column. Peak 1 is 2-methylbenzimidazole injected as an internal standard; peak 2 is IQ which is collected, concentrated, and rechromatographed on a cyanopropyl column for quantitation as shown in Figure 3b. Peak 1 is the internal standard, 5-aminoquinoline, and peak 2 is IQ.

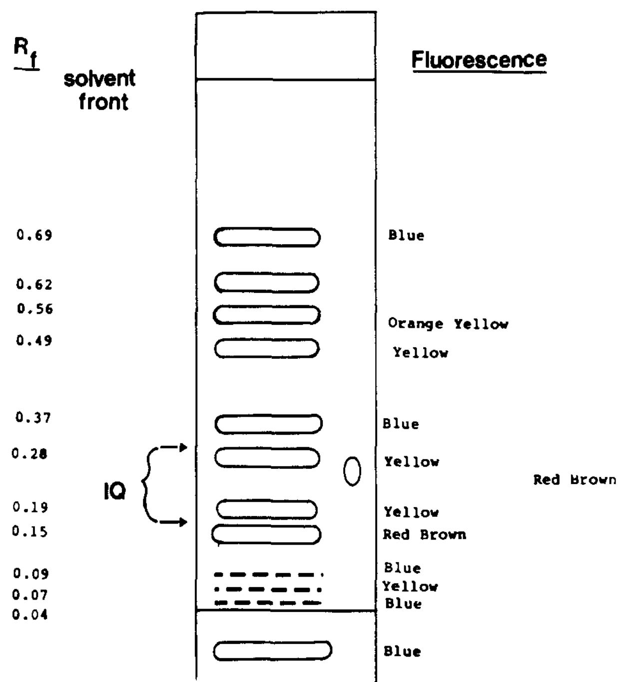

Figure 2. Thin-layer chromatogram of the extract from low-fat beef containing basic materials. The spot in the right-hand track is the IQ standard.

The amount of IQ in the crude basic extract is quantitated by spiking aliquots of the extract with different amounts of IQ standard. Each aliquot is purified and quantified individually. When a regression analysis is applied to these peak heights, the *y* intercept estimates the amount of IQ in an unspiked extract and the precision of the assay is indicated by the standard error of the estimate and the correlation coefficient. Percentage recovery is obtained by dividing the slope of the extract regression line by the slope of the regression line for an IQ standard.

This method has been applied to extracts from a low-fat and a high-fat sample of fried beef. Data are shown in Figure 4 and 5, respectively. The horizontal axis represents the amount of IQ added to the crude basic extract; the vertical axis represents peak heights obtained by HPLC on a CN column. The dotted lines are the calibration curves for IQ standard. The solid curves are the regression lines for the spiked extracts. These data were standardized by using the appropriate internal standard after each HPLC step. However, we have found that the difference between correlation coefficients for standardized and unstandardized data is very small, so that this procedure is not obligatory. When quantitative extraction of IQ from the cooked meat into the basic extract is assumed, these determinations yield values of 20.1 μg of IQ/kg of meat for the high-fat sample and 0.53 μg/kg of meat for the low-fat sample, with the standard error of the estimate equal to 0.61 and 0.19 μg/kg, respectively. In actuality, this is a slightly conservative estimate because the actual extraction efficiency is approximately 85%. The recovery

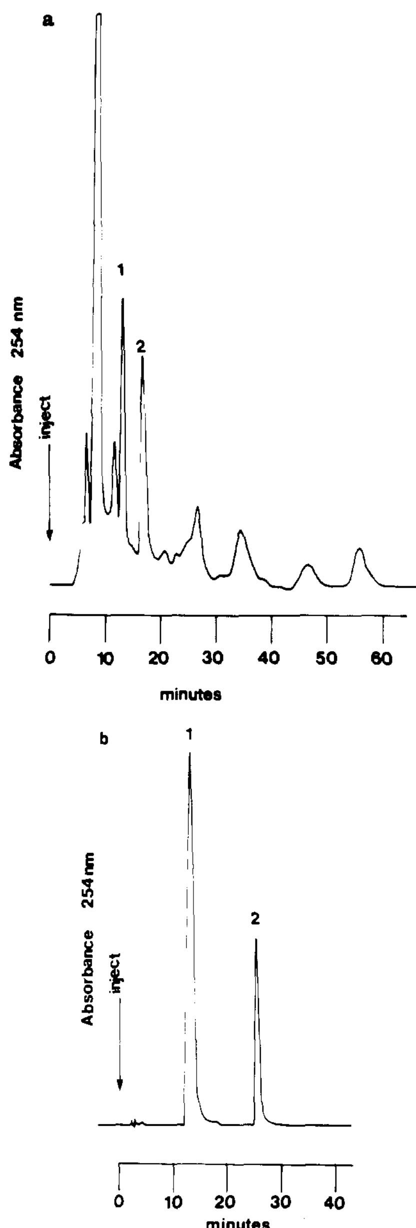

Figure 3. (a) HPLC chromatogram on a C_{18} column of compounds in the IQ band after cleanup by TLC. 1 = 2-methylbenzimidazole; 2 = IQ. (b) HPLC chromatogram on a CN column of IQ purified on the C_{18} column. 1 = 5-aminoquinoline; 2 = IQ.

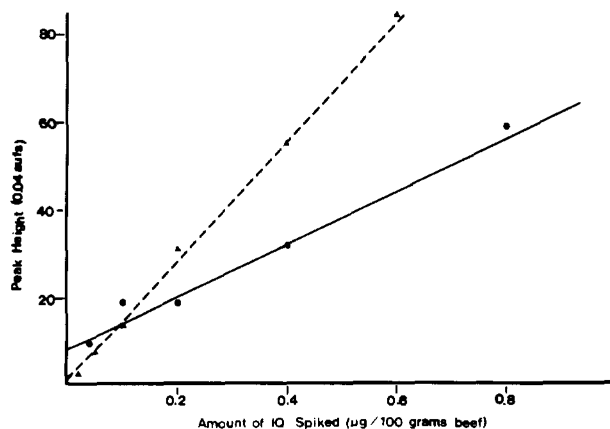

Figure 4. Quantitation of low-fat samples (100 g equiv) by spiking with an IQ standard. (- - -) IQ standard; (—) extracts from spiked meat samples.

of IQ from the basic extract itself is 44.3% and 44.4% for the high- and low-fat samples, respectively. The limit of

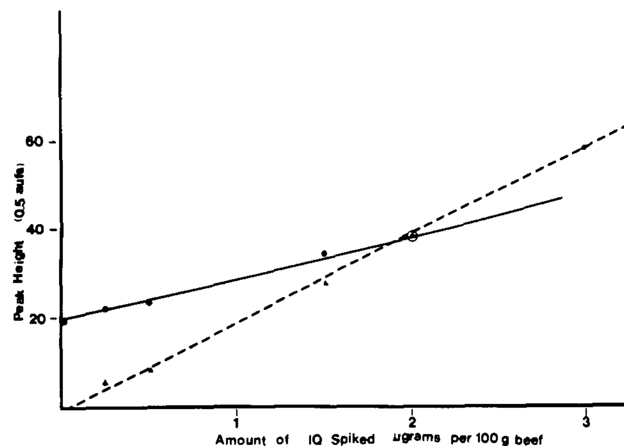

Figure 5. Quantitation of high-fat samples (100 g equiv) by spiking with an IQ standard. Circle indicates two identical points. (- - -) IQ standard; (—) extracts from spiked meat samples.

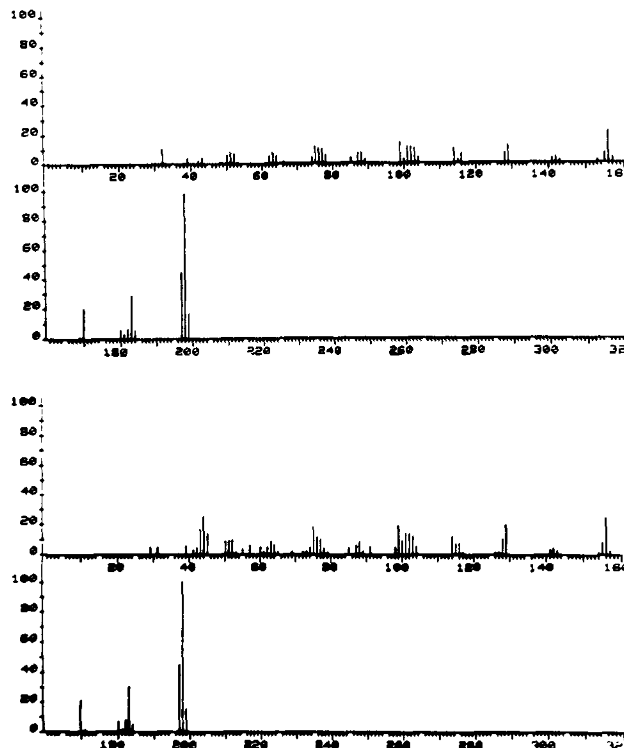

Figure 6. Upper: Mass spectrum of an IQ standard. Lower: Mass spectrum of product isolated from the beef extract as IQ.

detection for this method is approximately 5 ng of IQ (25 pmol)/50 g of beef. The correlation coefficients are 0.98 for the low-fat sample and 1.0 for the high-fat sample. Such high correlation coefficients indicate a low degree of error and extreme linearity of the data.

The identity of IQ was confirmed by mass spectrometry of peaks collected from unspiked extracts. Figure 6 shows the mass spectrum of an IQ standard and the IQ purified from a high-fat sample of fried beef. The extracted IQ and standard IQ also exhibited identical mobilities by TLC and coeluted from the C_{18} and CN columns by HPLC.

This method has been used to measure differences in the formation of IQ found in high-fat beef extracts as a function of added inhibitors (Figure 7). Data are the means and standard error of the mean for three independent determinations of each type of extract. The significance of this reduction was analyzed by the *t* test, as explained under Materials and Methods, and results are tabulated in Table I. The difference between control and

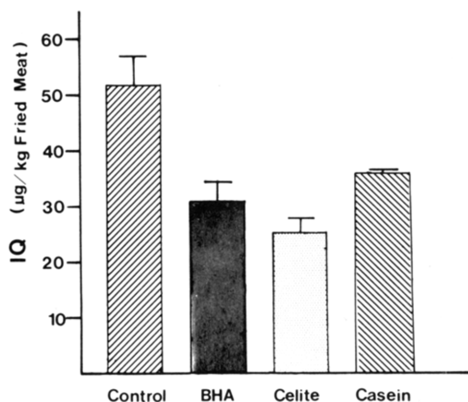

Figure 7. IQ content of extracts from beef fried after adding different inhibitors.

Table I. *t* Test of Significance for the Difference between Means of IQ Content in High-Fat Ground Beef Samples after Different Treatments

	$\overline{X}^a$	SEM	*P*
control	51.8	5.0	
BHA	31.0	3.3	0.03
Celite	25.2	2.5	0.01
casein	36.0	0.58	0.14

[a] Mean IQ (μg/kg) in samples of meat treated with BHA, Celite 503, or casein (see Materials and Methods for details).

BHA or control and Celite are significant at the 3% and 1% levels, respectively. The null hypothesis that control and casein extracts contain the same amount of IQ can only be rejected at the 14% level. However, this is probably due to the fact the one sample was lost, reducing the degrees of freedom to only 1.

DISCUSSION

IQ was originally isolated by using open column silica gel and LH-20 chromatography followed by HPLC on a reverse-phase C_{18} column (Spingarn et al., 1980). Subsequently, IQ and a number of other such products in food were quantitated by mass spectroscopy using these procedures and a deuterated internal standard (Nishimura and Yamaizumi, 1981).

The method reported here represents a time saving over the previous procedures. It has been possible to eliminate open-column chromatography altogether by substituting analytical TLC. This has the additional advantage of increasing resolution, and hence purification, at the initial step. Moreover, a reasonable analysis can be performed by using only the material from 50–100 g rather than 1 kg of fried meat.

In the past, IQ has proved to be a difficult compound to chromatograph. In this work, a new method, employing HPLC on a cyanopropyl bonded phase column, produces peak shapes clearly superior to any in the literature.

Although this is a precise, quantitative, sensitive, and relatively simple method, it is not yet routine because of the lack of a labeled internal standard. Since the levels of IQ found in fried meat are rather low, a compound of very high specific activity will be required; with this, we estimate that analysis time will be 0.5 day/sample.

The concentration of IQ which we find in low-fat samples of fried beef is very similar to the value of 600 ng/kg reported previously (Nishimura and Yamaizumi, 1981). However, we find that fried beef which initially contained higher proportions of fat also generated considerably more IQ during cooking. This is consistent with earlier work where it was found that mutagenicity of basic extracts was dependent on the fat content of the meat (Spingarn et al., 1981). The effect of several additives on the formation of IQ in beef generally parallels earlier findings on the mutagenicity of such extracts (Wang et al., 1982).

The reasons for modulation of IQ formation as a result of lipid content, or the presence of various additives, are somewhat speculative at this time. Several trivial explanations are possible. Lipids might act to conduct heat more efficiently into the meat, but we do not see the changes in heating kinetics which would be expected if this were the case. Alternatively, a higher lipid content might inhibit thermal degradation of IQ, once it had been formed. Of more interest is the fact that a large number of alcohols, aldehydes, ketones, organic acids, and even N-heterocycles can be formed during lipid peroxidation and thermal degradation (Aust and Svingen, 1982; Watanabe and Sato, 1971, 1972). It is possible that intermediates for mutagen formation and Maillard reactions originate from this source. Moreover, free radicals are generated during lipid peroxidation and this may enhance certain Maillard reactions (Namiki and Hayashi, 1981). Such a mechanism is attractive because it could explain the inhibition mechanism of BHA and other antioxidants. The action of Celite 503 and perhaps casein as well seems to suggest that the physical properties and texture of the meat may also influence mutagen formation. This is a new aspect which requires further study.

Registry No. IQ, 76180-96-6; BHA, 25013-16-5.

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