REDUCTION OF HETEROCYCLIC AROMATIC AMINE MUTAGENS /CARCINOGENS IN FRIED BEEF PATTIES BY MICROWAVE PRETREATMENT

J.S. Felton, E. Fultz, F.A. Dolbeare, and M.G. Knize

Biology and Biotechnology Research Program, L-452, Lawrence Livermore National Laboratory, P.O. Box 808, Livermore, CA 94551-9900

RUNNING TITLE: Microwave pretreatment of beef.

Abstract: To investigate a method to reduce the amount of mutagenic/carcinogenic heterocyclic aromatic amines formed during frying of ground beef, we measured both the mutagenic activity in *Salmonella* strain TA98 and the amount of known heterocyclic amines by solid-phase extraction and HPLC. The beef patties received microwave treatment for various times before frying. Microwave pretreatment for 0, 1, 1.5, 2, or 3 min before frying at either 200°C or 250°C for 6 minutes per side reduced heterocyclic aromatic amine precursors (creatine, creatinine, amino acids, glucose), water, and fat up to 30%, in the patties and resulted in a decrease in mutagenic activity up to 95%. The sum of the four heterocyclic aromatic amines shown to be present, MeIQx, IQ, DiMeIQx, and PhIP, decreased 3- to 9-fold compared to control, non-microwaved beef patties fried under identical conditions.

Introduction

Human exposure to dietary chemicals may play a significant role in the initiation of cancer (Doll and Peto, 1981). The great variation in human diets and in the dietary content of food mutagens may explain the observed variation in human cancer rates worldwide and may offer strategies for intervention to prevent cancer in humans (IARC, 1993).

Among these dietary chemicals are the heterocyclic aromatic amines produced during the cooking of meats, particularly at higher temperatures (Sugimura et al., 1988; Felton and Knize, 1991). The heterocyclic amines are mutagenic in *Salmonella* bacteria, cause chromosomal damage and mutations in cells in culture, and are carcinogenic in mice and rats (reviewed in Aeschbacher, 1991), and in one instance, in monkeys (Adamson et al., 1990).

Efforts in several laboratories have been directed at reducing the amount of mutagens produced during food preparation. Reduction of mutagenic activity in bacon was investigated using low temperature cooking methods or reduced heating time (Miller and Buchanan, 1983).

The addition of various substances to the meat before or during cooking to reduce the formation of mutagenic activity has also been investigated. Reduced mutagenic activity has been achieved using antioxidants (Wang et al., 1982; Pearson, et al., 1992), soy or cottonseed flour (Rhee et al., 1987, Wang et al., 1982), tryptophan (Jones and Weisburger, 1988), other food additives (Chen et al., 1992) or sugars, either alone or with starch (Skog et al., 1992; Nakamura and Tsuji, 1986).

Muscle meats contain creatine and creatinine which can react with free amino acids and sugars during cooking to form a series of heterocyclic amines depending on time and temperature (Knize et al., 1994). Modeling experiments in aqueous or dry-heating conditions have produced many of the same heterocyclic amines found in cooked meat products as reviewed by Jägerstad et al. (1991). Removing the known precursors of heterocyclic amines from beef patties by microwave pretreatment before frying, was shown by Taylor et al. (1986) to reduce greatly the formation of mutagenic activity as measured in the Ames/*Salmonella* test.

In this study, we examined the effect of varying microwave pretreatment times used to remove creatine, creatinine, amino acids and fat in meat, and we determined the mutagenic activity formed during the subsequent frying of the meat at 200 or 250°C. We used solid-phase extraction and HPLC analysis to measure the levels of MeIQx, DiMeIQx, IQ, Trp-P-1, Trp-P-2, AaC, and PhIP formed as a result of frying after varying microwave pretreatment time from 0 to 3 min. We compared the mutagenic activity measured in the extracts of the beef patties to the mutagenic activity accounted for by the measured known heterocyclic amines.

Materials and Methods

Preparation and microwave pretreatment of fried beef

Ground beef containing 15% fat was obtained from a local market and formed into 100 g patties approximately 1.5 cm by 9 cm. Single beef patties were placed in a petri dish and pretreated in a Kenmore microwave oven (Sears, Chicago, IL; 1,450 W maximum output; 2450 MHz) at 80% power for 0, 1.0, 1.5, 2.0, or 3.0 min, the liquid was poured

Abbreviations: AaC=2-amino-9H-pyrido[2,3-b]indole (CAS # 261148-68-5); DiMeIQx=2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (95896-78-9); IQ=2-amino-3-methylimidazo[4,5-f]quinoline (76180-96-6); MeIQx=2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (77500-04-0); PhIP=2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (105650-23-5); Trp-P-1=3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (62450-06-0); Trp-P-2=3-amino-1-methyl-5H-pyrido[4,3-b]indole (62450-10-3).

off, and the beef patties were fried at 200 or 250°C for 6 minutes per side on a steel griddle (Cecilware Corp., Long Island, NY). A scheme of the microwave pretreatment process is shown in Fig. 1. Griddle surface temperatures were monitored with a Cole-Parmer digital probe thermometer. Cooked meat samples were homogenized in a blender (Waring, New Hartford, CT) to produce a uniform sample, and frozen at -4°C until extraction.

Untreated ground beef or the liquid from the pretreated meat patties was analyzed for creatine by the a-naphthol-diacetyl method (Wong 1971) and creatinine by the picrate method, (creatinine determination kit #555, Sigma Chemical Co., St Louis, MO). Glucose content was measured using HPLC by the National Food Agency, Dublin, CA. Amino acids and dipeptides were measured by HPLC using ortho-phthaldehyde precolumn derivatization. Fat content of the meat was determined by lypholyzation followed by extraction with dichloromethane/methanol (2:1) and weighing. For the liquid released during microwave pretreatment of the beef patties, the water immiscible phase was allowed to separate during refrigeration and then weighed to determine the amount of fat.

Sample extraction for mutagenicity testing

Food samples were extracted by the solid-phase extraction method of Gross (1990). Meat samples (4 g) were homogenized with 8 g of 1 M NaOH, mixed with 15 g Extrelut diatomaceous earth, poured into an empty Extrelut 20 column (EM Science, Gibbstown, NJ) and extracted by collecting 40 ml dichloromethane through the coupled Bond Elut PRS (propylsulfonyl) extraction column (Varian Sample Preparation Products, Harbor City, CA). The PRS column was eluted with 1.5 ml methanol/ammonium hydroxide solution (9:1), the solution was evaporated under a stream of nitrogen, and the residue was dissolved in 400 µl dimethylsulfoxide for use in the Ames/*Salmonella* test.

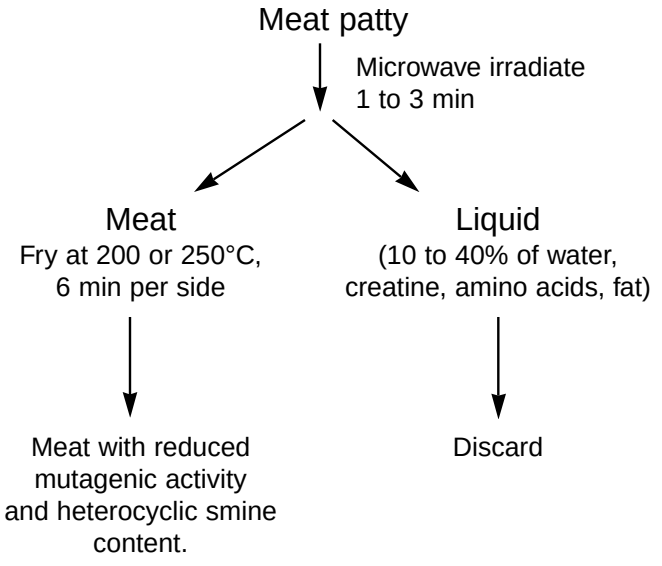

Figure 1. Scheme for microwave pretreatment of beef patties.

Heterocyclic amine extraction and HPLC analysis of samples

For the determination of heterocyclic amines, the sample retained by the PRS column was processed and analyzed by HPLC according to the procedures of Gross and Grüter (1992). Replicate samples were analyzed unspiked or spiked with 250 ng each of IQ, MeIQx, DiMeIQx, PhIP, Trp-P-1, Trp-P-2, and AaC to determine extraction recoveries.

Briefly, 20 g samples of ground meat were homogenized with 60 g 1 M NaOH. Four aliquots of 16 g of homogenized mixture (equal to 4 g meat sample) were used, two were spiked with a mixture of heterocyclic amines in 50 µl methanol. Each sample was mixed thoroughly with 1 packet of Extrelut diatomaceous earth (EM Science, Gibbstown, NJ) and poured into an Extrelut 20 column. The extractions were made by collecting 40 ml dichloromethane through attached PRS columns with subsequent washing and extraction onto C_{18} columns, separating polar and apolar extracts as described by Gross and Grüter (1992). The eluted polar and apolar mixtures were evaporated to dryness at 50° C, redissolved in 50 µl of methanol containing 5 ng/ml caffeine as an internal standard.

HPLC separation was done as optimized by Gross and Grüter (1992), on a TSK gel ODS80TM column (TosoHaas, Montgomeryville, PA, 250 mm x 4.6 mm I.D.) with a mobile phase of triethylamine phosphate, 0.01M, pH 3.6 (solvent A) and acetonitrile (solvent B), but without the ternary buffer (pH 3.2), which is not necessary for the separation of the heterocyclic amines we used in this study. 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.

Samples were analyzed on a Millenium 2010 HPLC system (Millipore Corp., Milford, MA) with a 996 photodiode array detector and a Hewlett-Packard 1046A Programmable Fluorescence Detector. The identity of chromatographic peaks was confirmed by comparing the UV absorbance spectra to library spectra acquired from standard solutions.

Chemicals

Heterocyclic amines IQ, MeIQx, DiMeIQx, and PhIP were purchased from Toronto Research Chemicals (Downsview, Ont.). AaC was a kind gift from Dr. Gian Gross, Nestec, Ltd, Lausanne Switzerland. Trp-P-2 and Trp-P-1 were purchased from Wako Pure Chemicals (Dallas, TX). Concentrations of standard solutions were determined using published extinction coefficients and measured on a Shimadzu 2100 spectrophotometer (Shimadzu Scientific instruments, Inc., Columbia, MD).

Calculations were made by linear regression analysis of the individual results of the spiked and unspiked extracts using a microcomputer spread sheet macro “Trace_Calc”, a kind gift of Dr. Gian Gross, Nestec Ltd., Lausanne, Switzerland, running on Wingz (Informax Software Inc., Menlo Park, CA).

Salmonella mutagenicity assay

The mutagenic activity of the extracts was determined with the standard plate assay described by Ames et al.

(1975), using *Salmonella* 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. Dose-response curves were produced using the method of Moore and Felton (1983). A minimum of four dose points from duplicate platings were used, and the linear portion was used to calculate the number of revertants per gram of cooked meat. A positive control, 2-aminoanthracene, gave 800-1200 revertants per microgram. Dimethylsulfoxide gave a background of 30-55 revertants per plate. Calculated values of revertants per gram for each meat sample were based on values from Knize et al. (1987) and Felton and Knize (1990) for individual heterocyclic amines: MeIQx = 99 rev/ng; DiMeIQx=320 rev/ng; IQ = 200 rev/ng; PhIP = 2 rev/ng. The quantities of heterocyclic amines determined by the HPLC analysis were then multiplied by these values to determine the calculated mutagenic activity.

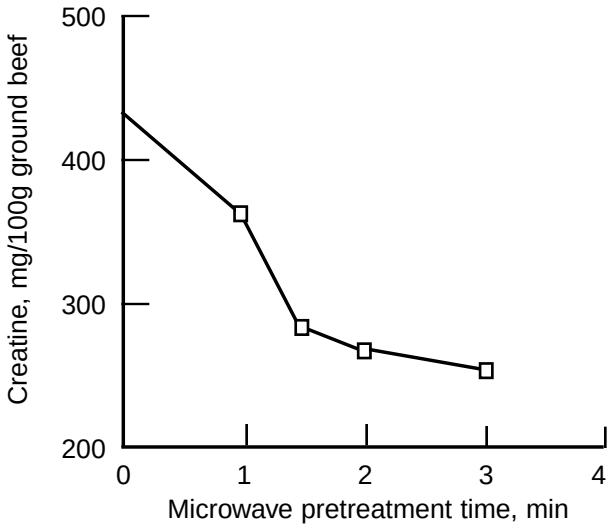

Figure 2. Graph of creatine remaining with varying microwave pretreatment times.

Results

The amount of creatine remaining in the meat is shown in Fig. 2, with most of the loss into the microwave-released liquid occurring at 1.5 min or more of microwave pretreatment time (original content of 437 mg of creatine in 100 g of meat). Analysis of the creatine content of the liquid resulting from the microwave pretreatment accounted for the creatine lost in the meat. Creatinine was present in the meat at 15 mg/100g, and decreased to 14.1, 13.3, 12.6, and 11.1 mg/100g following microwave pretreatment for 1.0, 1.5, 2.0, and 3.0 min, respectively (data not shown).

Analysis of the liquid released upon microwave treatment showed that the fat in the beef patties was reduced from 12.7% by weight without microwaving, to 11.2%, 10.4%, 8.9% and 7.3% for microwave treating for 1, 1.5, 2, and 3 min, respectively. Glucose was reduced from 250 mg per 100 g of meat to 237 and 215 mg per 100 g for microwaving for 1 and 2 min, respectively (data not shown). Free amino acids were reduced by 5 to 10% for 1 min of pretreatment to 10 to 30% for 3 min treatment compared to untreated meat.

Weight loss during frying for 6 min per side from untreated patties was 34.2 % at 200°C and 37.8% at 250°C. For the microwave-treated patties, additional weight loss from the release of water, fat, and mutagen precursors further reduced the weight of the fried patties by 1.1% and 2.9% for 1 and 2.0 min pretreatment at 200°C, and 3.9% and 2.0% when pretreated for 1.0 and 2.0 min and then fried at 250°C, respectively.

Table 1 shows the amount of specific heterocyclic amines quantified using the solid-phase extraction and HPLC analysis method, calculated per gram of meat after cooking. For each of the four compounds detected, a decrease in the mass amount is measured with microwave pretreatment. The difference in the loss of PhIP with the different cooking temperatures is unclear, but may be due to a coeluting impurity present after frying at 200°C that fluoresces and has a UV absorbance spectrum similar to authentic PhIP.

Table 1. Heterocyclic amines in cooked beef patties fried for 6 min per side following various microwave pretreatment times.

Microwave time (min) Frying at 200°C	IQ*	MeIQx	DiMeIQx	PhIP
0	ND	3.0 (0.2)	0.3 (0.1)	2.7 (0.4)
1.0	ND	1.3 (0.3)	0.1 (0.0)	0.7 (0.2)
1.5	ND	0.5 (0.3)	0.2 (0.0)	1.6 (0.2)
2.0	ND	0.5 (0.1)	0.2 (0.1)	1.6 (0.2)
3.0	ND	ND	0.1 (0.0)	2.2 (0.3)
Microwave time (min) Frying at 250°C				
0	1.0 (0.2)	5.1 (0.7)	1.2 (0.3)	13.3 (1.8)
1.0	ND	1.7 (0.1)	0.3 (0.1)	9.4 (2.5)
1.5	ND	ND	0.7 (0.1)	3.3 (0.5)
2.0	ND	ND	0.5 (0.2)	1.9 (0.1)
3.0	ND	ND	0.1 (0.0)	2.2 (0.3)

*ng/g (SD)

ND-not detectable with these methods. Limit of detection 0.1 to 0.5 ng/g, depending on sample and compound. Trp-P-1, Trp-P-2, and AaC were not detected.

The total mass of heterocyclic amines formed decreased approximately 3- to 9-fold with microwave pretreatment. No Trp-P-1, Trp-P-2, or AaC were found in the fried-beef samples cooked under the described conditions with our detection limit of approximately 0.1 ng/g. Extraction recoveries ranged from 26 to 80% for MeIQx, DiMeIQx, and IQ; 30 to 57% for PhIP; and 7 to 37% for Trp-P-1, Trp-P-2, and AaC which are within the range of published values for this method. The values in Table 1 are corrected for the measured loss of heterocyclic amines during sample preparation.

Table 2 lists the mutagenic activity measured by *Salmonella* strain TA98 for beef patties fried at 200°C or 250°C and the mutagenic activity calculated from the heterocyclic amine content from our analysis of the meat patties. The level of mutagenic activity decreases approximately 90% with 3 minutes of microwave pretreatment. The measured mutagenic activity in each sample is similar to the mutagenic activity calculated from the measured amounts of heterocyclic amines. This comparison shows that the heterocyclic amines we are capable of measuring are responsible for most of the mutagenic activity. At 250°C, 0 microwave time, there appears to be a substantial portion of the mutagenic activity unaccounted for by the heterocyclic amines we measured. This is consistent with other samples of beef patties cooked at higher temperatures that we have analyzed (data not shown).

An example of the HPLC chromatograms for the polar extracts of beef patties that were spiked with the solution of standard heterocyclic amines (a) or pretreated 0 (b), 1 (c) or 2 (d) min are shown in Fig. 3. The spiked sample shows the elution position of IQ (1), MeIQx (2) internal standard (3), DiMeIQx (4) Trp-P-2 (5) and PhIP (6). Trp-P-1 is extracted into the apolar extracts and is not seen in figure 3. Chromatograms based on fluorescence provided a more sensitive detection for PhIP, Trp-P-1, Trp-P-2, and AaC and the apolar fraction gave higher recoveries for Trp-P-1, Trp-P-2, and AaC; and so these chromatograms and extracts were used for calculating amounts and recoveries of these compounds (data not shown). A peak coeluting with IQ (peak 1 in Fig. 3) in all three unspiked samples does not match the UV spectrum expected for IQ and is thus considered an interference. For chromatogram "c" in Fig. 3, the peak coeluting with PhIP (peak 6) did not match the library UV spectrum of PhIP (showing PhIP plus the interference) and the fluorescence chromatograms were needed for quantitation of PhIP. A scheme for further sample clean-up to help resolve these important peak identification problems has been published (Gross et al., 1992)

Discussion

We clearly show in this report that the reduction of heterocyclic amines and the concomitant mutagenic activity is possible by eliminating the known precursors of heterocyclic amine formation. Although creatine, sugar, and amino acids were only reduced up to 30%, heterocyclic amines were reduced up to 90% after two minutes of microwave pretreatment. These differences are best explained by assuming second-order reaction kinetics. If 2 reactants are needed and they each are reduced by 30%, then the product formation would be reduced to 50%. If 3 reactants were required and all were reduced 30%, then the reaction product would be reduced 70-80%. An alternative explanation is that the remaining precursors may not be available as reactants, perhaps due to water loss during microwaving, and thus preventing the transport of small molecule precursors to the meat surface and preventing the reactions from occurring. Examination of creatinine and creatine levels and mutagenic activity formation in meats from 16 animal species suggested that a threshold level of creatine is necessary to produce a high mutagenic response (Vikse and Joner, 1993). The addition of creatine or creatinine to meat before cooking does increase the mutagenic activity as first shown by Nes (1986).

The effect of frying temperature shows that increasing the temperature from 200 to 250°C increases the mutagenic activity about three-fold. This is in agreement with previous results showing the effect of increased temperature on levels of heterocyclic amines in fried beef patties (Knize et al., in press).

Microwave cooking alone has been shown not to form mutagenic activity during cooking in three studies (Dolara et al., 1979; Nader et al., 1981; Baker et al., 1982), but Barrington et al. (1990) showed high mutagenic activity in one beef steak sample microwave cooked 5 to 7 min per side. Our results suggest no contribution to the mutagenic activity from the short duration of microwaving used in our study.

Microwave-assisted natural product extraction has been patented as an effective method for extraction of natural products from plants into appropriate microwave-transpar-

Table 2. Measured and calculated mutagenic activity in *Salmonella* strain TA98 from beef patties pretreated in a microwaveoven and then fried at 200 or 250°C.

Microwave time ,min	T98, rev/g 200°C	Calc*, rev/g 200°C	T98, rev/g 250°C	Calc*, rev/g250°C
0	450 ± 31.5 †	398	1400 ± 140	880
1.0	220 ± 23	301	369 ± 26	305
1.5	130 ± 17	135	216 ± 8.5	185
2.0	47 ± 8.5	65	67 ± 2.8	57
3.0	16 ± 3.5	3	41 ± 4.2	36

*Calculated from sum of the measured amounts of heterocyclic amines (from Table 1) multiplied by the mutagenic activity per nanogram of compound.

† Slope from the linear portion of dose response curve, standard error of the line.

ent solvents (Pare' et al., 1991). In our study, water soluble mutagen precursors were effectively extracted by endogenous water in the ground beef during the microwaving process.

The removal of fat from meat by a complex washing process has been reported by Small et al. (1991), but microwave extraction also appears to be a practical means of fat removal with 30% removed (from 12.7% to 8.9% for 2 min microwaving) before frying. The organoleptic properties of the fried beef patties prepared by microwave pretreatment needs to be evaluated, as the water and fat losses from the beef patties may be associated with reduced consumer acceptance.

The amount of beef consumed per day in many countries is relatively high, so fried beef appears to be a major source of dietary heterocyclic amines (Plumlee et al., 1981). Since it is estimated that 92% of homes in the U.S. have a microwave oven (Geise, 1992), microwave pretreatment is a practical way to reduce fat and heterocyclic amine content of even well-done ground beef for many consumers. Additional studies determining heterocyclic amine reduction in other meat products and possible changes in taste, texture, and nutritional content of the meat need to be explored.

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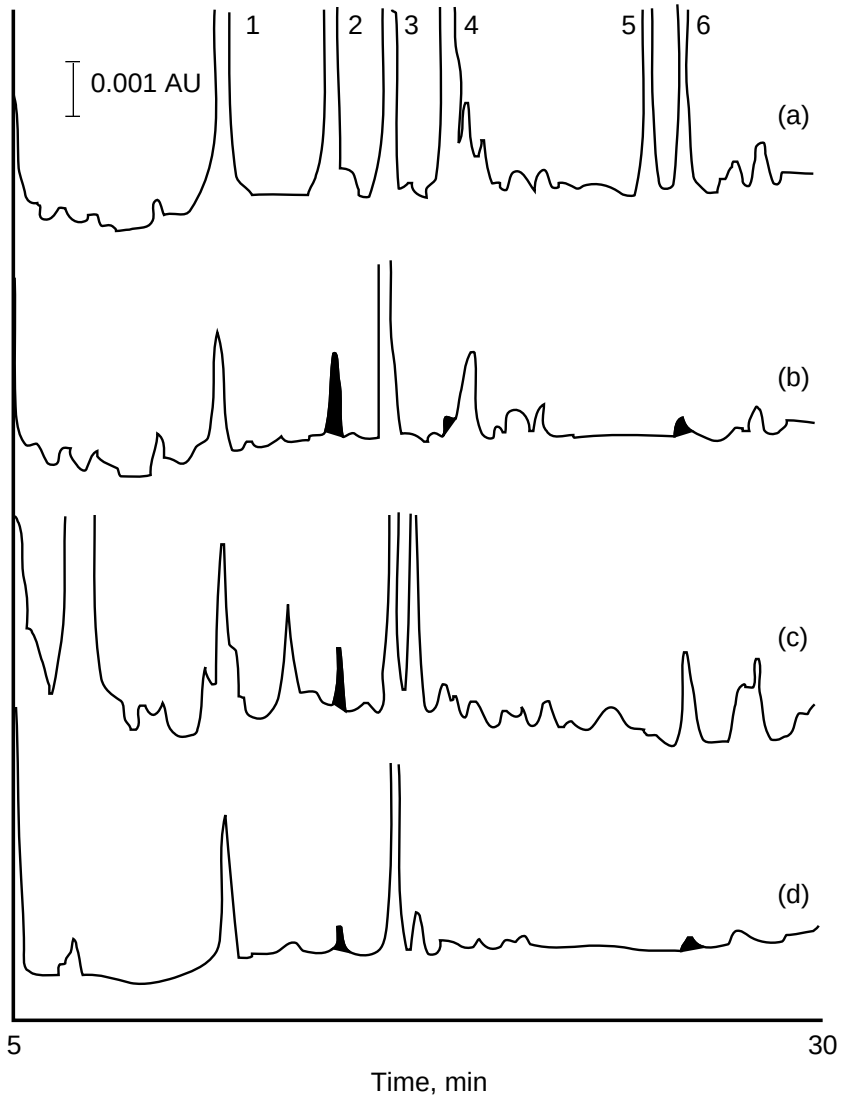

Figure 3. Chromatograms of polar extracts of beef patties fried at 250°C and spiked with standards(a) or microwave pretreated for 0 (b), 1 (c), or 2 min (d). Numbered peaks are IQ (1), MeIQx (2), internal standard (3), DiMeIQx (4), Trp-P-2 (5), and PhIP (6). Trp-P-1 is extracted into the apolar extracts and is not seen. Filled peaks represent heterocyclic amines in unspiked samples confirmed by absorbance spectral matching.

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