# Carvacrol Facilitates Heat-Induced Inactivation of *Escherichia coli* O157:H7 and Inhibits Formation of Heterocyclic Amines in Grilled Ground Beef Patties

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Heating meat at high temperature and/or for a long time to kill foodborne pathogens increases the formation of potentially carcinogenic heterocyclic amines. To overcome this problem, 1% carvacrol, the main ingredient of oregano oil widely used in salad dressings, was added to ground beef, which was mixed well and then inoculated with *Escherichia coli* O157:H7. Beef patties were then prepared and heat-treated on a preheated electrical skillet to reach an internal temperature of 65, 70, or 80 °C at the cold spot. Samples were enumerated for surviving *E. coli* O157:H7 population by plating on appropriate media. Heterocyclic amines (MeIQ, MeIQx, and PhIP) were extracted from ground beef using solid phase extraction and analyzed by mass spectrometry. Multiple reaction monitoring (MRM) scan type in positive mode was used to monitor the amines of interest. Compared to controls, the population of *E. coli* O157:H7 was reduced by 2.5−5 logs. The corresponding highest reductions in the three major amines were MeIQ, 58%; MeIQx, 72%; and PhIP, 78%. The results show that carvacrol concurrently reduced *E. coli* O157:H7 and amines in a widely consumed meat product. Possible mechanisms of the beneficial effects and dietary significance of the results are discussed.

KEYWORDS: *Escherichia. coli* O157:H7; beef patties; heterocyclic amines; carvacrol; inhibition

## INTRODUCTION

Insufficient thermal treatment to inactivate pathogens such as *Escherichia coli* O157:H7 has been considered to be one of the major factors contributing to foodborne illness outbreaks following consumption of ready-to-eat meats. Hence, meat products need to be sufficiently heated to inactivate foodborne pathogens. However, high-temperature heat treatment of ready-to-eat meats could cause a serious health concern: formation of heterocyclic amines (HA) (*1−4*). An increased risk of developing colorectal, breast, and other cancers has been associated with the consumption of well-done, fried, or barbecued meats (*5−7*). There is therefore a need to develop methods to cook the meats in such a way as to inactivate the foodborne bacteria while concurrently reducing or eliminating the formation of potentially carcinogenic amines.

A variety of natural and synthetic compounds, primarily antioxidants, are reported to inhibit the formation of heterocyclic amines, reviewed in refs *8* and *9*. These include the following plant compounds, beverages, and foods: beer (*10*) and black, green, and white tea flavonoids (*11−15*); chrysin, a flavonoid compound present in honey and some fruits and vegetables (*16*); citrus flavonoids such as diosmin, naringenin, naringin, and rutin (*17*); chlorophylls (*8, 18*); fruit and vegetable extracts (*19*); garlic compounds (*20*); indole-3-carbinol from cruciferous vegetables (*21*); lycopene from tomatoes (*22−24*); soy isoflavones daidzein and genistein and soy sauce (*24*); phenolic compounds (*25*); olive phenolics (*26, 27*); sulforaphane, a constituent of broccoli (*28*); vitamin E (*29*); fruit extracts (*25*); and oil marinades with garlic, onion, and lemon juice (*30*).

Because some of these may also exhibit antimicrobial effects in meats, there is a need to investigate whether safe plant antimicrobials will allow the preparation of meat products at lower temperatures (*31*). In earlier investigations we found that carvacrol facilitated inactivation of *E. coli* O157:H7 both in liquid media (*32, 33*) and in heated ground beef (*34*). The objective of the present study was to investigate whether, and to what extent, the reduction in bacterial heat resistance by carvacrol may also be accompanied by significant reductions in levels of three major heterocyclic amines in ground beef patties heated at an internal temperature of 65, 70, or 80 °C.

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Inhibition of *E. coli* O157:H7 and Heterocyclic Amines in Meat

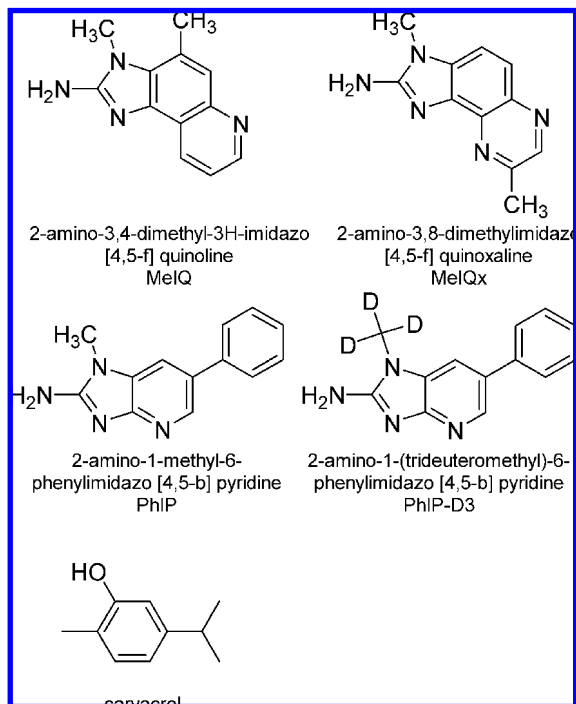*J. Agric. Food Chem.*, Vol. 57, No. 5, 2009 **1849**

**Figure 1.** Structures of three heterocyclic amines, of the trideuterated amine internal standard, and of the antimicrobial carvacrol evaluated in the present study.

To our knowledge, no published studies have as yet addressed this problem.

## MATERIALS AND METHODS

**Materials.** The following heterocyclic amine standards were purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada): 2-amino-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and 2-amino-1-(trideuteromethyl)-6-phenylimidazo[4,5-*b*]pyridine (PhIP-D3). Carvacrol (5-

isopropyl-2-methylphenol; 98% pure) was obtained from Sigma (St. Louis, MO). Methanol (Optima*) and 98% formic acid used in mass spectrometry were purchased from Fisher Scientific (Fair Lawn, NJ).

**Bacterial Cultures.** The test organism used was *E. coli* O157:H7 (ATCC strain 35150). Stock cultures of the organism were maintained in cryovials (Microbank, Austin, TX) at −80 °C and activated by transferring 100 $\mu$L into tryptic soy broth with 0.6% yeast extract (TSBYE; Difco Laboratories, Sparks, MD). The bacterial culture was maintained in TSBYE at 4 °C, and biweekly transfers were done. For experimental use, an overnight culture of the organism was grown in TSBYE at 37 °C for 18−24 h with shaking. All dilutions were done in buffered peptone water (BPW; Difco). Enumeration of *E. coli* O157:H7 was done by plating on Sorbitol MacConkey (SMAC) agar.

**Sample Preparation and Thermal Treatment of Ground Beef Patties.** Ground beef samples (93% lean) were purchased from a local grocery store and stored in a −20 °C freezer. Samples were removed from the freezer and transferred to 4 °C (refrigerator) for 1 day before the experiments. Samples were left at room temperature for about 30 min (to equilibrate to room temperature) before the experiments.

Carvacrol (0.35 mL) was added to 35 g of ground beef to obtain a 1% concentration. The sample was then mixed manually. Then, 0.35 mL of *E. coli* O157:H7 overnight culture was inoculated into the sample and again mixed manually for 1 min. The sample was made into a patty of 6 cm diameter. Another inoculated patty without added carvacrol was prepared and treated as a control. Ground beef patties were cooked on an electric skillet (West Bend Housewares LLC, West Bend, WI), which was preheated for 10 min. The surface temperature of the heating element is estimated to be ~200 °C. The internal temperature of each patty was monitored using a type K thermocouple (Fluke, Everett, WA), which was inserted into the geometric center of each ground beef patty. The patties were turned over when the internal temperature reached 45 °C. When the target internal temperature was achieved (65, 70, or 80 °C), patties were removed instantly from the skillet and then cooled in ice-cold water for 10 min. Samples were taken and used for both microbial analysis and for solid phase extraction and subsequent heterocyclic amine analysis.

**Microbial Sampling after Treatment.** After thermal treatment and cooling, each patty (10 g) was taken and added into chilled buffered peptone water (BPW; 90 mL) in a stomacher bag. Samples were stomached (Stomacher Laboratory Blender, model 400, Seward Medical, London, U.K.) at normal speed for 1 min. Serial dilutions in BPW were done from this sample as needed. Plating of an aliquot was done
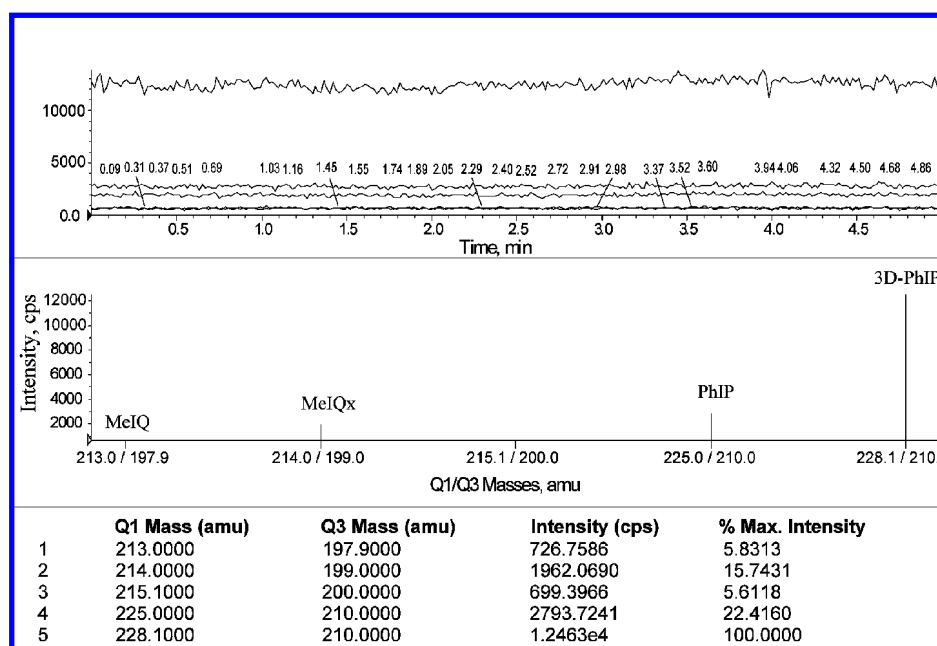


| | Q1 Mass (amu) | Q3 Mass (amu) | Intensity (cps) | % Max. Intensity |
|---|---|---|---|---|
| 1 | 213.0000 | 197.9000 | 726.7586 | 5.8313 |
| 2 | 214.0000 | 199.0000 | 1962.0690 | 15.7431 |
| 3 | 215.1000 | 200.0000 | 699.3966 | 5.6118 |
| 4 | 225.0000 | 210.0000 | 2793.7241 | 22.4160 |
| 5 | 228.1000 | 210.0000 | 1.2463e4 | 100.0000 |

**Figure 2.** Mass spectral trace of a beef extract (upper pane) and integrated intensity of MS signals of MeIQ, MeIQx, PHIP, and trideuterated PhIP, respectively (middle and lower panes).

on SMAC agar. The plates were incubated at 37 °C for 24−48 h and then enumerated for bacteria. Appropriate controls (patties inoculated and treated without antimicrobials) were also done for each sample.

**Solid Phase Extraction of Meat for Mass Spectrometry of Heterocyclic Amines.** For solid phase extraction, cooked ground beef (25 g) was mixed with 1 M sodium hydroxide (75 mL) and then homogenized in a blender (Oster, Boca Raton, FL). The homogenate (16 g) was spiked with the internal standard trideuterated PhIP (PhIP-D3; 75 μL; 0.5 μg/mL) and then mixed with 18 g of Extrelut NT 20 (Merck, Darmstadt, Germany) sorbent packing material (Merck). This mixture was transferred into an Extrelut extraction column and then eluted with ethyl acetate. A Bond-Elut PRS cartridge (500 mg; Varian, Palo Alto, CA) was coupled with the Extrelut column as soon as the liquid level reached the bottom of the column. The extraction was stopped following collection of 40−50 mL of eluted solution. The PRS cartridge was removed and washed with 0.1 N HCl (6 mL) and then with methanol/0.1 M HCl (40:60; 15 mL) and distilled water (2 mL). The PRS cartridge was then coupled with a Bond-Elut C18 cartridge (100 mg, Varian), preconditioned with methanol (2 mL) and water (2 mL), and then eluted with 0.5 M ammonium acetate (pH 8.0; 20 mL). The C18 cartridge was rinsed with water (1 mL), dried on a vacuum manifold for 20−30 min, and then eluted with methanol/ammonium hydroxide (9:1; 0.8 mL). The eluted solution was stored in 1.5 mL centrifuge tubes at −20 °C prior to MS analysis.

**Sample Preparation for Mass Spectrometry of Heterocyclic Amines.** Trideuterated PhIP was used as an internal standard. Before extraction, the internal standard (75 μL; 0.5 μg/mL) was added to each meat sample. The volume of the extracted sample was reduced to 60−80 μL via an SC110A SpeedVac Plus concentrator (Savant Instruments, Inc., New York). The dried sample was reconstituted in 500 μL of 0.1% (w/w) formic acid in methanol. The concentration of the internal standard in the reconstituted sample was 75 pg/μL based on 100% recovery. Preliminary experiments demonstrated that this concentration provided an MS signal intensity comparable to the signal intensities for the compounds of interest.

**Mass Spectrometric Analysis.** MS analysis of the samples was performed on an ABI/Sciex 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) with a nanospray source and Analyst 1.4.1 software. The analytes were introduced into the instrument via infusion at 2 μL/min by syringe infusion pump model 22 (Harvard Apparatus, Holliston, MA). The changes in HA levels were estimated using multiple reaction monitoring (MRM) scan type in positive mode. As the compounds of interest are being fragmented, the instrument detects *m/z* values of the analytes (precursors) paired with the *m/z* values of the predetermined fragment ions derived from the corresponding precursor ions (see **Figure 2**). Both precursor ion and fragment ion must be observed for detection. The parent mass chosen was only due to the analyte of interest. Fourier transform ion cyclotron resonance mass analysis (FT-ICR MS) showed the absence of isobaric interferences (results not shown).

MRM transitions in this experiment are as follows: MeIQ *m/z* 213.00 → 197.90; MeIQx *m/z* 214.00 → 199.00; PhIP *m/z* 225.00 → 210.00; trideuterated PhIP *m/z* 228.10 → 210.00. The following instrument parameters were used: source temperature, 150 °C; source voltage, 2100 V; collision energy (CE), 30 V; declustering potential (DP), 80 V; dwell time for all transitions, 200 ms; sample acquisition duration, 5 min.

## RESULTS AND DISCUSSION

**Analysis of Heterocyclic Amines.** We used nano-FT-ICR-MS to measure the mass of the amines in question because it provides a more accurate mass measurement than MS on the 400 QTRAP. The analyses were highly reproducible in terms of the mass measurement made and confirmed the elemental composition of the analytes as well as the absence of isobaric interferences by other meat ingredients. The results will be discussed with reference to **Figure 1**, which illustrates the structures of the three heterocyclic amines, the trideuterated PhIP used as internal standard, and the antimicrobial plant compound carvacrol evaluated in the present study. **Figure 2** illustrates
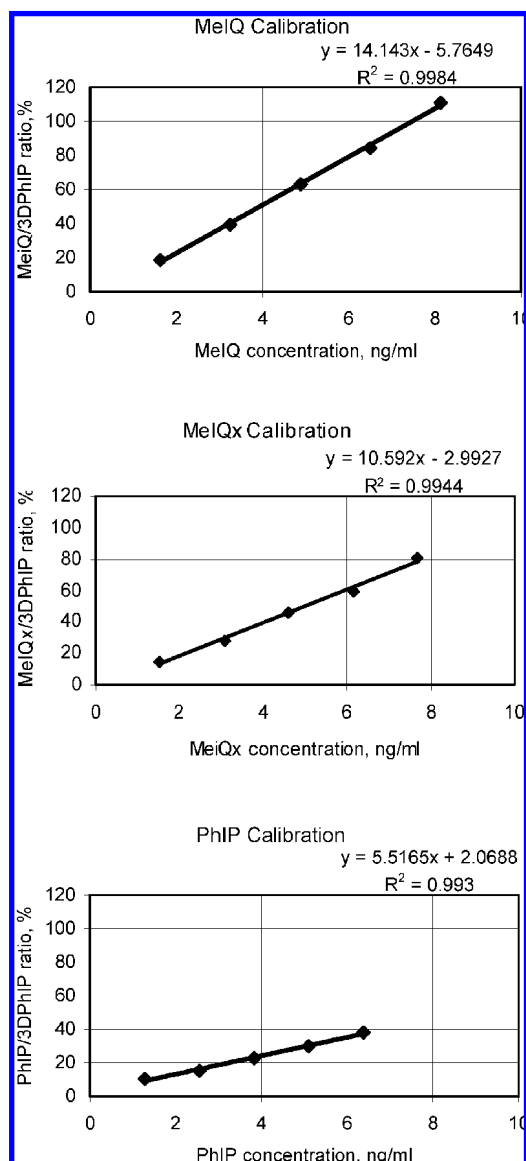


**Figure 3.** Standard calibration curves for three heterocyclic amines evaluated in the present study.

the mass spectra of an extract and **Figure 3**, the calibration curves in the following concentration ranges (in ng/mL): MeIQ, 1.63−8.13; MeIQx, 1.54−7.69; PhIP, 1.28−6.38.

**Effect of Carvacrol Added to Ground Beef.** Previously, we reported that HPLC analysis showed that the essential oil from oregano contained up to 85% of carvacrol (*32*). The objective of the present study was to apply carvacrol to meat, grill it, and then test the meat for HA and bacterial counts. We used the single-extract cleanup procedure followed by mass spectrometric analysis of heterocyclic amines adapted and modified from the literature (*16, 35*). The results of analysis for each sample are presented as the ratio of signal intensities of MeIQ, MeIQx, and PhIP to the intensity of the internal standard. Each sample was run in triplicate on the same day. The results of samples with added carvacrol were also compared to controls without carvacrol. The results are summarized in **Table 1**.

**Table 1** and **Figure 4** show the effect of adding 1% carvacrol to ground beef on both heterocyclic amine and *E. coli* O157: H7 levels in the resulting beef patties cooked at three internal temperatures. As expected, added carvacrol facilitated heat inactivation of *E. coli* O157:H7 during heating of the patties.

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**Table 1.** Concurrent Reduction by 1% Carvacrol Added to Ground Beef of Levels of Three Heterocyclic Amines (MeIQ, MeIQx, and PhIP) and of *E. coli* O157:H7 Populations in Ground Beef Patties Heated at Three Different Internal Temperatures

| meat internal temp (°C) | amine | heterocyclic amine | | | *E. coli*[a] (log CFU/g) | |
|---|---|---|---|---|---|---|
| | | heat (control) ratio[b] | heat plus carvacrol ratio[c] | reduction (%) | heat (control) | heat plus carvacrol[c] |
| 65 | MeIQ | 9.01 | 6.57 ± 0.56 | 27 | 6.36 | 3.9 ± 0.15 |
| | MeIQx | 29.51 | 17.65 ± 1.55 | 40 | | |
| | PhIP | 38.74 | 23.22 ± 1.42 | 40 | | |
| 70 | MeIQ | 21.07 | 8.76 ± 0.82 | 58 | 5.63 | 2.38 ± 0.36 |
| | MeIQx | 84.34 | 23.72 ± 2.98 | 72 | | |
| | PhIP | 131.63 | 28.76 ± 1.94 | 78 | | |
| 80 | MeIQ | 27.02 | 23.38 ± 4.51 | 13 | 5.00 | 0.00 |
| | MeIQx | 126.83 | 84.06 ± 7.73 | 34 | | |
| | PhIP | 197.02 | 95.61 ± 6.30 | 51 | | |

[a] Initial amount in ground beef, ~7 log CFU. [b] Ratio of signal intensity of each heterocyclic amine to the signal intensity of the internal standard. [c] Mean ± SD (*n* = 3).
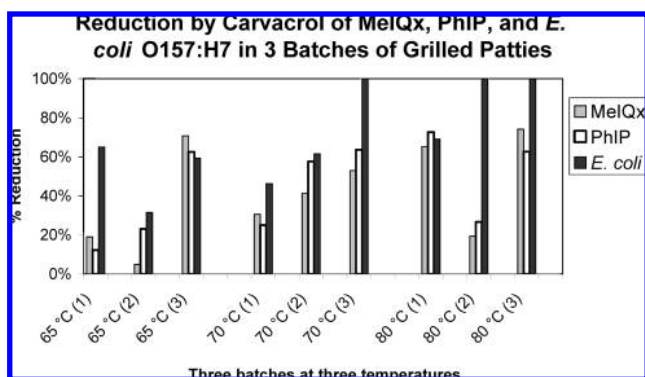


**Figure 4.** Reduction in MeIQx, PhIP, and *E. coli* O157:H7 in grilled beef patties by added carvacrol. Each point represents the percent reduction when the carvacrol-containing patty is compared to the non-carvacrol-containing patty grilled at the same temperature.

The surviving populations of pathogens in beef were significantly lower in beef heated in the presence of carvacrol than in the absence of this plant antimicrobial. At 65 °C, the added carvacrol induced about a 2.5 log decrease in *E. coli* O157:H7 surviving population. The corresponding decreases at 70 and 80 °C were about 3 and 5 log, respectively. **Table 1** also shows that, as expected, heterocyclic amine levels in terms of ratios to the internal standard decreased with decreasing internal patty temperature. Comparison of the treated patty at 65 °C to the untreated patty at 80 °C shows that both the bacterial count and the level of HA are lower in the treated sample. The patties heated at 70 °C had the greatest relative reductions among the three temperatures tested: MeIQ decreased by up to 58%, MeIQx by up to 72%, and PhIP by up to 78% versus control patty without carvacrol heated at the same temperature (**Table 1**).

**Figure 4** shows batch to batch reproducibility. There seems to be variation between batches at a given temperature. This could be due to the difficulties of sampling real foods and in amine extraction and/or to the nonhomogeneity of the complex meat samples (e.g., perhaps one patty had more fat in contact with the grill, whereas another patty had more protein in contact). The trend in *E. coli* O157:H7 reductions is more regular than that in the reductions of the amines. This is not surprising because the highest levels of surviving *E. coli* O157:H7 would be expected to be present in the middle of the heat-treated patty, where we know by measurement what temperature was attained. By contrast, the outside of the patty is where we expect the highest levels of HA to be formed. Here we expect the most variation in local temperature at the grill interface due to small

variations in composition of the ground meat (fat/protein particles), in the structure or the surface of the patties (valleys and ridges), and in high-intensity heat from the grill. Additionally, small differences in meat composition throughout the meat matrix could affect the rate at which heat radiates through the patty. Such variations are difficult to quantify but need to be taken into account in the determination of optimal conditions for food safety.

The cited data indicate that (a) carvacrol in beef simultaneously reduces both the bacterial count and the amount of heat-induced heterocyclic amine levels, (b) the reduction varied with the temperature of heating and with the nature of amine, and (c) at the three temperatures evaluated, the reduction ranged in approximately the following order: PhIP > MeIQx > MeIQ.

**Mechanisms of Antimicrobial and Antiheterocyclic Amine Effects.** The antimicrobial consequences of exposing foodborne pathogens to carvacrol include depletion of the intracellular ATP pool, change in membrane potential, and increase in permeability of the cytoplasmic membrane for protons and potassium ions (*36−38*). The loss of the ion gradient is responsible for the loss of essential metabolic processes of the cell and consequently cell death.

Although the cited mechanism of antimicrobial activity of carvacrol appears to be plausible, we can only hypothesize about possible mechanisms of inhibition of HA by carvacrol. As noted elsewhere (*39*), the structure of carvacrol [2-methyl-5-(1-methylethyl)phenol] shown in **Figure 1** suggests that it is an antioxidative hydrophilic−hydrophobic phenolic compound. However, the calculated hydrophilic−lipophilic balance (HLB) number for carvacrol of 4.15 by the semiempirical method suggests that the compound is predominantly a hydrophobic molecule that would dissolve preferentially in oil, stabilize oil-in-water emulsions, and form micelles in water.

A postulated reaction route for the formation of HA involves reaction of free amino acids with glucose to form pyridine or pyrazine and aldehyde intermediates, which then combine with creatinine in a single third-order reaction step to form the HA (*1, 2, 40*). Different amino acids produce different amines. For example, alanine forms MeIQ and phenylalanine forms PhIP. Detailed discussion of the role of amino acids in heat-induced formation of the HA are described in refs *41−44*.

These considerations suggest two possibilities for the carvacrol inhibition of HA. In the first, the phenolic OH group of carvacrol inhibits free radical intermediates involved in the formation of HA by a radical-trapping mechanism described elsewhere (*45*). In the second, carvacrol forms insoluble complexes with the amines, analogous to those postulated for chlorophyll (*46*).

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Whether some of the naturally occurring and safe food additives reported to inhibit the formation of carcinogenic acrylamide in heated plant-based foods (*47*) will also inhibit HA during grilling of muscle foods (beef, poultry, and fish) merits study.

**Conclusions.** The results of the present study demonstrate that carvacrol added to ground beef contaminated with *E. coli* O157:H7 exhibits two beneficial effects. The plant compound facilitates heat inactivation of the bacteria during grilling of the patties (hamburgers) and concurrently induces reduction in the formation of heat-induced potentially carcinogenic HA. These results imply that by using safe natural compounds as additives, it may be possible to reduce the need to consume well-done meat. Because well-done meat contains >10 times the concentration of mutagens/carcinogens than rare-cooked meat, keeping the temperature as low as possible and sufficient to inactivate microbial pathogens should minimize mutagen/carcinogen formation. The approach recommended in the present study with carvacrol and possibly other safe and food-compatible natural antioxidative/antimicrobial food additives may make it possible to achieve this objective. The recommended change in culinary practices can benefit microbial food safety and human health. The use of less heat to cook meat will also save energy.

Finally, because carvacrol and other plant-derived antimicrobials also inactivated other foodborne pathogens, including *Campylobacter jejuni*, *Clostridium perfringens*, *Listeria monocytogenes*, *Mycobacterium avium paratuberculosis*, and *Salmonella enterica* in vitro and in meats (*39, 48−53*), we expect that these natural compounds will also inactivate these bacteria and concurrently reduce heterocyclic amine formation in cooked meat and poultry products. This aspect as well as the palatability of the antimicrobial-containing foods merit further study.

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