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The Influence of Liquid Smoke on
N-Nitrosamine Formation

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THE INFLUENCE OF LIQUID SMOKE ON N-NITROSAMINE FORMATION

By

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ABSTRACT

THE INFLUENCE OF LIQUID SMOKE ON N-NITROSAMINE FORMATION

By

William George Ikins

The influence of liquid smoke on N-nitrosamine formation in bacon and on *in vivo* formation of N-nitroso compounds was investigated. Liquid smoke contains formaldehyde, a precursor of N-nitrosothiazolidine (NTHZ) and N-nitrosothiazolidine carboxylic acid (NTCA). The incorporation of liquid smoke into the curing-brine of bacon could greatly increase NTHZ concentrations in fried bacon. Phenols are also present in liquid smoke and have been reported to be catalysts of N-nitrosamine formation in aqueous systems. The catalysis of N-nitrosamine formation by phenols in bacon and endogenous formation of N-nitroso compounds in experimental animals was investigated.

Incorporation of liquid smokes into bacon curing-brines resulted in lower concentrations of N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR) and NTHZ upon frying than bacon processed by the traditional woodsmoke process. Levels of NDMA and NPYR in bacon treated with an atomized liquid smoke were not significantly lower than those in woodsmoked control bacon, but did contain less NTHZ. NTHZ was not found

in any samples of cookout-fat, indicating that the precursors of NTHZ are unlikely to be present in the adipose tissue of bacon.

Results of an aqueous model system study in which the smoke condensates were reacted with cysteamine and nitrite accurately reflected the trend in NTHZ concentrations in the corresponding fried bacon. NTCA concentrations in raw bacon correlated with NTHZ concentrations in the corresponding fried bacon, indicating that NTCA may be decarboxylated during frying. Residual nitrite levels in raw bacon treated with liquid smoke designed for inclusion in bacon curing-brines decreased as the pH of the smoke condensate decreased, while the concencentration of phenols in the smoke condensates did not appear to influence residual nitrite levels or N-nitrosamine concentrations.

The nitrosation of thiazolidine in an aqueous system generally increased as the pH was reduced from 7.0 to 1.0. The formation of NTHZ from cysteamine, formaldehyde and nitrite was optimal at pH 4.8 and decreased as the pH was raised or lowered.

Rats gavaged with one of two liquid smokes in combination with cysteine and nitrite excreted urine containing concentrations of NTCA that were parallel to the respective concentrations of NTHZ obtained in the aqueous model system. Ascorbate inhibited *in vivo* formation of NTCA by 90% in rats gavaged with cysteine, formaldehyde and nitrite. α-Tocopherol and a

phenol fraction of liquid smoke had little or no effect on NTCA formation. Thiocyanate did not catalyze NTCA formation in rats gavaged with cysteine, formaldehyde and nitrite, nor did it catalyze NTCA formation in rats given a nitrite solution ad libitum in their drinking water prior to gavaging the animals with cysteine, formaldehyde and thiocyanate. Formaldehyde was also ineffective in catalyzing the nitrosation of thiazolidine.

Dedicated to my wife, Nancy and my family

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INTRODUCTION

N-Nitrosothiazolidine (NTHZ) and N-nitrosothiazolidine carboxylic acid (NTCA) have been reported in smoked cured meats (Pensabene and Fiddler, 1983b, Sen et al., 1985). The formation of these compounds appears to result from the reaction of cysteamine and cysteine, respectively, with formaldehyde in the smoke, followed by nitrosation (Mandagere et al., 1984). Using scanning calorimetry, Mandagere et al. (1984) determined that the decarboxylation of NTCA to NTHZ occurs at a lower temperature than that used in frying. Sen et al. (1985) reported that at least a part of the NTHZ detected in fried bacon is formed as a result of the decarboxylation of NTCA present in raw bacon.

Little is known about the toxicity of NTHZ, although it has been reported to be a direct acting mutagen as determined by the Ames Test (Sekizawa and Shibamoto, 1980). NTHZ has also been implicated as a diabetogenic agent in human populations (Helgason et al., 1984).

The majority of the studies pertaining to the influence of liquid smoke on N-nitrosamine formation have been concerned with the inhibition of N-nitrosopyrrolidine (NPYR) formation (Sleeth et al., 1982, Theiler et al., 1984). Relatively little attention has been directed toward the influence of liquid smoke on NTHZ and NTCA formation in bacon, although Pensabene and Fiddler (1985) did demonstrate that liquid

smokes atomized onto the surface of pork bellies inhibited NTHZ formation during traditional smoking. The incorporation of liquid smokes in the curing brine, however, did not significantly reduce NTHZ formation.

Liquid smokes specifically designed for inclusion in the brines used to cure meats are now available. Because these smoke condensates all contain variable concentrations of formaldehyde (Potthast and Eigner, 1985), the pumping of liquid smoke throughout the meat system could maximize the exposure of free cysteamine or cysteine in the meat to formaldehyde and nitrite in the brine. Therefore, a primary objective of this study was to ascertain the effect of cure-solubilized liquid smokes on NTHZ concentrations in the resulting fried bacon. This study also investigated the relationship between the NTCA concentrations in raw bacon and the concentrations of NTCA and NTHZ in the corresponding fried bacon.

The phenols of smoke are the most important class of compounds to the flavor and aroma of smoked foods (Wasserman, 1966). Phenols also have been reported to be both inhibitors and catalysts of nitrosation reactions, (Challis and Bartlett, 1975; Davies and McWeeny, 1977). Another major objective of the present study was to ascertain if there was a relationship between the phenol content of bellies pumped with liquid smoke, the residual nitrite content of the raw bellies, and N-nitrosamine concentration of the fried product.

Ohshima et al. (1983) reported that rats gavaged with

cysteine, nitrite and formaldehyde will form NTCA *in vivo*. Liquid smoke treated meats and other food products flavored with liquid smoke will likely contain all of these precursors. In the present study, the effect of gavaging rats with liquid smoke, cysteine and nitrite on *in vivo* NTCA formation was investigated. In addition, the influence of potential inhibitors and catalysts of nitrosation reactions on *in vivo* NTCA formation was examined.

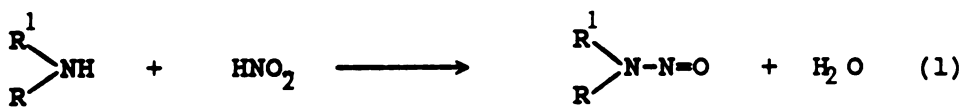
LITERATURE REVIEW

I N-Nitrosamines

N-Nitroso compounds are highly toxic, and of the more than 120 N-nitroso compounds tested, 80% have been shown to be carcinogenic (Magee et al., 1976). The carcinogenic activity of N-nitrosamines depends upon their structure (Wishnok et al., 1978). It appears necessary for N-nitrosamines to possess an α hydrogen that can be enzymatically hydroxylated to begin the metabolism of the compound. After several steps, either a diazonium ion or a diazoalkane is produced which can alkylate the nucleophilic sites on deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or proteins, thus producing the carcinogenic effect (Magee and Barnes, 1967).

Chemistry of Formation

N-Nitrosamines are stable compounds which are formed principally from the reaction of secondary amines with a nitrosating species.

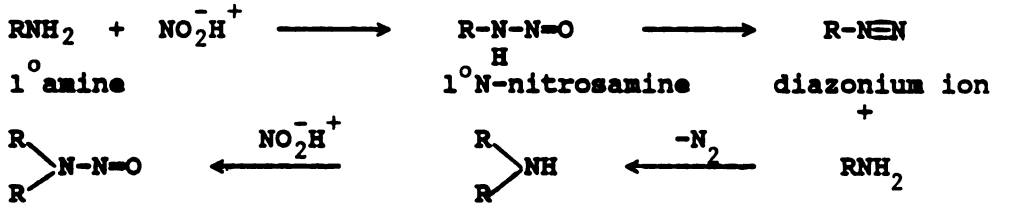


(1)

R^1 must be an alkyl group, while R may be any one of a large

number of functional groups. N-Nitrosamines can also be derived from primary amines, tertiary amines, and quaternary ammonium compounds (Fiddler et al., 1972).

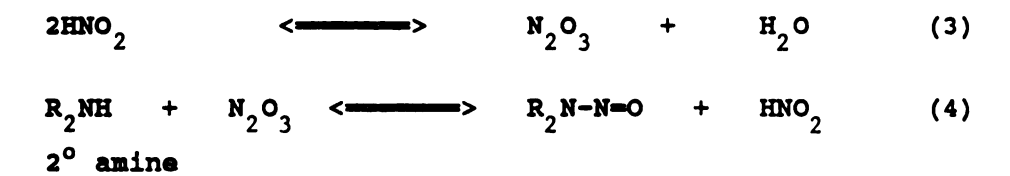
Primary amines can function as N-nitrosamine precursors, but they must first undergo conversion to a secondary amine (Ridd, 1961). The reaction proceeds through an unstable primary N-nitrosamine to a diazonium intermediate which can then react with the original primary amine to form a secondary amine.



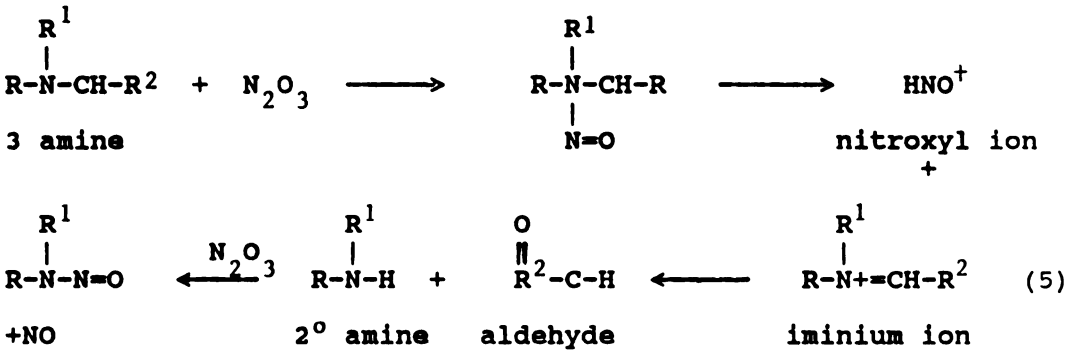
(2)

This reaction proceeds slowly because the conversion of the primary amine to a secondary amine must occur in a very acidic environment. Since only secondary amines will be nitrosated, and only a low proportion will be in this state at a low pH, the yield of N-nitrosamines will also be low.

Secondary amines undergo maximum nitrosation in an aqueous environment at a pH of 3.4. Under these mildly acidic conditions, nitrous anhydride (N_2O_3) becomes the principal nitrosating species.



Tertiary amines have generally been regarded as almost inert to nitrosation because of the high temperature required for dealkylation to the secondary amine (Smith and Loeppky, 1967). The tertiary amine is nitrosated to a N-nitrosammonium ion, which then undergoes cis elimination of the nitroxyl ion to form an iminium ion.



This ion is then hydroxylated to give an aldehyde and a secondary amine, which can then be nitrosated to the corresponding N-nitrosamine. Quaternary ammonium compounds react even more slowly than tertiary amines (Fiddler et al., 1972). An initial dealkylation must occur before any nitrosating agent can become involved, as in reaction (5).

In addition to the nitrosating species already mentioned, nitrogen oxides such as nitrogen dioxide (NO_2), dinitrogen tetraoxide (N_2O_4), and nitric oxide (NO) have been implicated in the formation of N-nitrosamines. In order for NO to act as a nitrosating agent, it must either react with an amine under anaerobic conditions in the presence of certain metal salts, or be oxidized to NO_2 (Douglass et al., 1978). Gaseous N_2O_4

exists in equilibrium with NO_2, and nitrosation by N_2O_4 yields a mixture of N-nitroso and N-nitro amino compounds (Challis and Kyrtopoulos, 1978). These reactions will occur in a gas phase, an organic or lipophilic environment, and in neutral or alkaline aqueous solutions.

Kinetics of Nitrosation

The nitrosation of a secondary amine under mildly acidic conditions proceeds via the mechanism depicted in reactions 6, 7, and 8 (Mirvish, 1975). The reaction kinetics are shown in equation 9.

$$2HNO_2 \longleftrightarrow N_2O_3 + H_2O \quad (6)$$

$$R_2NH_2 + H_2O \longleftrightarrow R_2NH + H_3O^+ \quad (7)$$

$$N_2O_3 + R_2NH \longrightarrow R_2NNO + HNO_2 \quad (8)$$

$$\text{Rate} = k\,[R_2NH]\,[HNO_2]^2 \quad (9)$$

In equation 9, the rate of nitrosation is proportional to the concentration of nonionized amine since only the unprotonated secondary amine can be nitrosated. The unprotonated amine exists in equilibrium with its conjugate acid (equation 7). The rate is also proportional to the concentration of N_2O_3, and thus to the square of the HNO_2 concentration. Although k will be independent of pH, the concentration of HNO_2 and R_2NH will vary with pH. At high pH, the concentration of N_2O_3 will diminish while at low pH, the concentration of R_2NH will be low.

The reaction rate of N-nitrosamine formation will depend on the basicity and concentration of the amino substrate, pH, the nitrite ion concentration, the presence of catalytic or inhibitory compounds, and temperature. The basicity of the amine influences the rate of N-nitrosamine formation because only unprotonated amines can be nitrosated. Thus, as the basicity or pKa of an amine decreases, the ease of nitrosation will increase.

The catalysis of a nitrosation reaction by an anion requires the initial conversion of nitrite to nitrous acid in an acid-catalyzed reaction. The NO group is then passed on to a catalytic anion, represented by the symbol Y^- in the following reactions:

$$HNO_2 + H_3O^+ \rightleftharpoons H_2O^+-N=O \quad (10)$$

$$H_2O^+-N=O + Y^- \rightleftharpoons Y-N=O + H_2O \quad (11)$$

$$R_2NH + YNO \longrightarrow R_2NN=O + HY \quad (12)$$

The nitrite ion (NO_2^-) will act as the nucleophile (and thus N_2O_3 as the nitrosating agent) under the appropriate conditions or in the absence of other stronger nucleophiles such as thiocyanate (NCS^-), bromide (Br^-), iodide (I^-), or chloride (Cl^-). In an environment of low pH (< 2.5) or when the concentration of thiocyanate or halide is high and that of nitrite is low, the thiocyanate- and halide-catalyzed mechanisms can dominate (Fan and Tannenbaum, 1973a). This could have important implications for cigarette smokers as the thiocyanate levels in

their saliva has been demonstrated to be three to four times higher than nonsmokers (Ladd et al., 1984). These authors speculated that higher salivary concentrations of thiocyanate in smokers may be responsible for the increased rate of gastric nitrosation of proline for this group in comparison to non-smokers. However, Lane and Bailey (1973) were not able to demonstrate in vivo catalysis of N-nitrosamine formation by thiocyanate in rats.

Temperature has a profound effect on the nitrosation reaction rate. Foreman and Goodhead (1975) reported that for every 10 °C rise in temperature, the reaction rate doubled. Freezing does not prevent amine nitrosation. Fan and Tannenbaum (1973b) demonstrated an enhancement of the nitrosation of morpholine in frozen buffers and milk which was attributed to the concentration of the reactants under frozen conditions.

There are, of course, many exceptions and deviations from these basic kinetic equations. The effect of other catalysts and inhibitors will be discussed in subsequent sections.

Occurrence of N-Nitrosamines in Foods and the Environment

Trace amounts of N-nitrosamines have been detected in a variety of commercial products. Cooked bacon, nitrite or nitrate-treated smoked fish, and salted or dried ocean fish consistently contain the highest concentrations of

N-nitrosamines (Sen, 1980). Of the nonfood items, high levels of N-nitrosamines have been detected in tobacco smoke, snuff, and chewing tobacco (Hoffman et al., 1984). Rubber products, and in particular, baby bottle nipples, have been reported to contain various N-nitrosamines (Preussmann et al., 1981).

The presence of N-nitrosamines in cured meats has been widely investigated. N-Nitrosodimethylamine (NDMA) and NPYR have been consistently detected in cooked bacon (Gough and Walters, 1976), while NTHZ has been identified recently as being associated with cured meats which are smoked (Mandagere et al., 1984). Free proline is the most probable precursor of NPYR and is present in substantial amounts (20 - 80 mg/kg) in connective tissue of pork bellies (Gray and Collins, 1977). Although there is some debate over the pathway of NPYR formation, the most probable mechanism appears to involve the nitrosation of proline to form the N-nitrosoproline (NPRO) intermediate (Lee et al., 1983). During the high temperature frying of bacon, NPRO is decarboxylated to NPYR. Bharucha et al. (1979) have suggested that the formation of NPYR in bacon occurs almost entirely in the fat phase after the bulk of the water is removed, and therefore by a free radical rather than an ionic mechanism.

The possible precursors of NDMA in bacon were investigated by Gray et al. (1978). They concluded that during the frying of bacon, sarcosine and phosphatidylcholine can contribute to the formation of NDMA. Dimethylamine and trimethyl-

amine are also likely precursors of NDMA, but these amines are more predominant in fish (Sen, 1980). Cured smoked fish and salted dried marine fish are a dietary staple for people in certain parts of the world, and in many countries the amounts of nitrate or nitrite added is not regulated. Concentrations of NDMA as high as 100 mg/kg have been reported in nitrite treated herring meal (Ender et al., 1964).

Beer was at one time a major source of N-nitrosamines for the populations of many Western nations (Spiegelhalder et al., 1980). The practice of direct drying malt in gas heated kilns resulted in significant N-nitrosamine formation. The switch to indirect drying eliminated the exposure of the free amines in malt to nitrogen oxides in the gas (Preussmann et al., 1981). Consequently, concentrations of NDMA in beer were greatly reduced. Other foods in which N-nitrosamines have been detected include various cheeses, mushrooms, and a solanaceous fruit (Ender and Ceh, 1967, DuPlessis et al., 1969, Crosby et al., 1972). In general, it can be said that these foods present little threat since N-nitrosamines were found in low concentrations and in a small percentage of the samples (Sen, 1980).

The presence of high concentrations of N-nitrosamines in chewing tobacco and snuff has sparked a great deal of controversy as to the safety of these products in recent years. In addition to NDMA and NPYR, tobacco products contain tobacco-specific N-nitrosamines (Hoffmann et al., 1982). The most

potent carcinogens of this class of N-nitrosamines are N-nitroso-nornicotine (NNN), N-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and N-nitrosoanatabine (NAT). A typical brand of snuff contains approximately 50 mg/kg of tobacco-specific N-nitrosamines, or roughly 10,000 times the N-nitrosamine concentration of fried bacon (Hoffmann et al., 1983). The use of popular sports figures in smokeless tobacco advertisments has encouraged young Americans to take up the habit of chewing tobacco, and the dramatic rise in chewing tobacco consumption reflects this trend (Economic Research Service, 1983). The evidence linking the use of smuff and chewing tobacco to cancer of the oral cavity is convincing and the use of these products by young people should actively be discouraged (Winn, 1984).

The fact that cigarette smoke contains tobacco specific N-nitrosamines has been known for some time (Hoffman et al., 1974). It is only relatively recently that research was conducted to determine the concentrations of N-nitrosamines in sidestream smoke and the health implications of passive smoking. Hoffmann et al. (1982) demonstrated that the concentrations of volatile N-nitrosamines in the sidestream smoke of cigarettes were consistently higher than in mainstream smoke. Conflicting epidemiological studies have been published on the risk of lung cancer for non-smoking spouses of heavy smokers (Hirayama, 1981; Garfinkel, 1981).

Other sources of N-nitrosamines include cosmetics, drugs,

pesticides, dishwashing liquids, and surface cleaners (Preussmann, 1984). Once these sources are identified, these products can be modified, banned altogether, or manufactured with N-nitrosamine inhibitors included in the formulation. This was the case when rubber products, in particular baby bottle nipples, were found to contain high concentrations of volatile N-nitrosamines (Ireland et al., 1980). A reduction in the amount of amine accelerators and stabilizers used in the formulation of rubber products greatly decreased the resulting N-nitrosamine levels (Preussmann, 1984).

Factors Influencing N-Nitrosamine Formation in Foods

Factors influencing the formation of N-nitrosamines in bacon are well documented (Gray, 1981), and include the method of cooking, nitrite concentration, cooking temperature and time, preprocess handling, smoke treatment, level of unsaturation of the lipids, and the presence of catalysts or inhibitors.

Several methods of cooking bacon were examined by Pensabene et al. (1974) who concluded that the frying temperature was more influential than time in NPYR formation. Baking produced the highest single sample yield of NPYR (35 ug/kg), while microwave cooking yielded essentially no NPYR. Bharucha et al. (1979) obtained a reduced yield of

NPYR in bacon lean while grilling the meat as opposed to pan frying. This observation was attributed to lower frying temperatures that result when the bacon fat is allowed to run out of the heated area. It should be noted that about 50% of the NPYR and 70% of the NDMA are released as vapor during the frying of bacon. Of the remaining N-nitrosamines, approximately two thirds are retained in the cookout fat and one third in the lean (Sen, 1980).

From the kinetics of nitrosation, it can be seen that the reaction rate is directly proportional to the square of the nitrite concentration. In light of this relationship, the levels of nitrite permitted in the processing of meat have undergone a great deal of scrutiny. Dudley (1979) reported that it is the lowest initial nitrite concentration that determines the probability of N-nitrosamine formation.

The length of storage of a pork belly prior to processing has an important influence on the final N-nitrosamine content of the fried bacon. Gray and Collins (1977) reported that the proline concentrations increased approximately 50% in the lean tissue and 90% in the adipose tissue of green pork bellies over a one week storage period at 20°C. Pensabene et al. (1980) concluded that bacon made from fresh bellies produced significantly less NPYR than that made from bellies that had either been stored for one week in a refrigerator or frozen for three months and then thawed before using. The effect of smoke treatments on N-nitrosamine formation will be discussed in a subse-

quent section of this literature review.

The level of unsaturation of pork belly adipose tissue can influence the N-nitrosamine concentration of the resulting fried bacon. Skrypec et al. (1985) supplemented the diet of pigs with various oils to modify the fatty acid composition of pork bellies. The addition of corn oil to the feed of pigs increased the degree of unsaturation of the adipose tissue and yielded bacon with higher N-nitrosamine concentrations than the control bacon. Conversely, the supplementation of pigs feed with coconut fat decreased the degree of unsaturation in the pork bellies and resulted in bacon with significantly lower NPYR concentrations than controls. A possible explanation for this was provided by the work of Walters et al. (1979) who reported that pseudonitosites (α-nitrosonitrite esters) of unsaturated triglycerides can transnitrosate to secondary amines.

Catalysts and Inhibitors of N-Nitrosamine Formation

The presence of catalysts of N-nitrosamine formation in foods or an environmental system is of concern because of the potential for increasing N-nitrosamine concentrations in that system many fold. As stated earlier, nucleophilic anions will accelerate the nitrosation of amines in direct proportion to their nucleophilic strength (Fan and Tannenbaum, 1973a).

Thus, thiocyanate is a much more effective catalyst than bromide, which is more effective than chloride ions. The catalysis exerted by these nucleophilic anions is more profound during the nitrosation of weakly basic rather than strongly basic amines (Fan and Tannenbaum, 1973a).

Phenols are deposited on the outside of cured meat as the result of the smoking process, or can be distributed throughout the meat system if liquid smoke condensates are included in the curing brine. Phenols are somewhat unique in that they can function as both inhibitors and catalysts of N-nitrosamine formation, depending upon the structure of the phenol and the relative concentrations of phenols and nitrite.

Inhibition by phenols can occur either by the reduction of nitrite to a non-nitrosating species (Figure 1, Reaction 1) or by reacting with nitrous acid to form a C-nitrosophenol (Figure 1, Reaction 2). The para-nitrosophenol isomer has been demonstrated to catalyze N-nitrosamine formation when the ratio of nitrite to phenol is high (Davies and McWeeny, 1977). The catalytic species was thought to be the quinone monoxime tautomer of the nitrosophenol whch is further nitrosated to form the O-nitroso derivative (Reaction 3, Walker et al., 1979). In a slower reaction, attack of the catalytic species by the amine results in N-nitrosamine formation and the regeneration of the nitrosophenol. These researchers concluded that only those nitrosophenols that could form a quinonemonoxime or a quinonemonoxime imine were able to

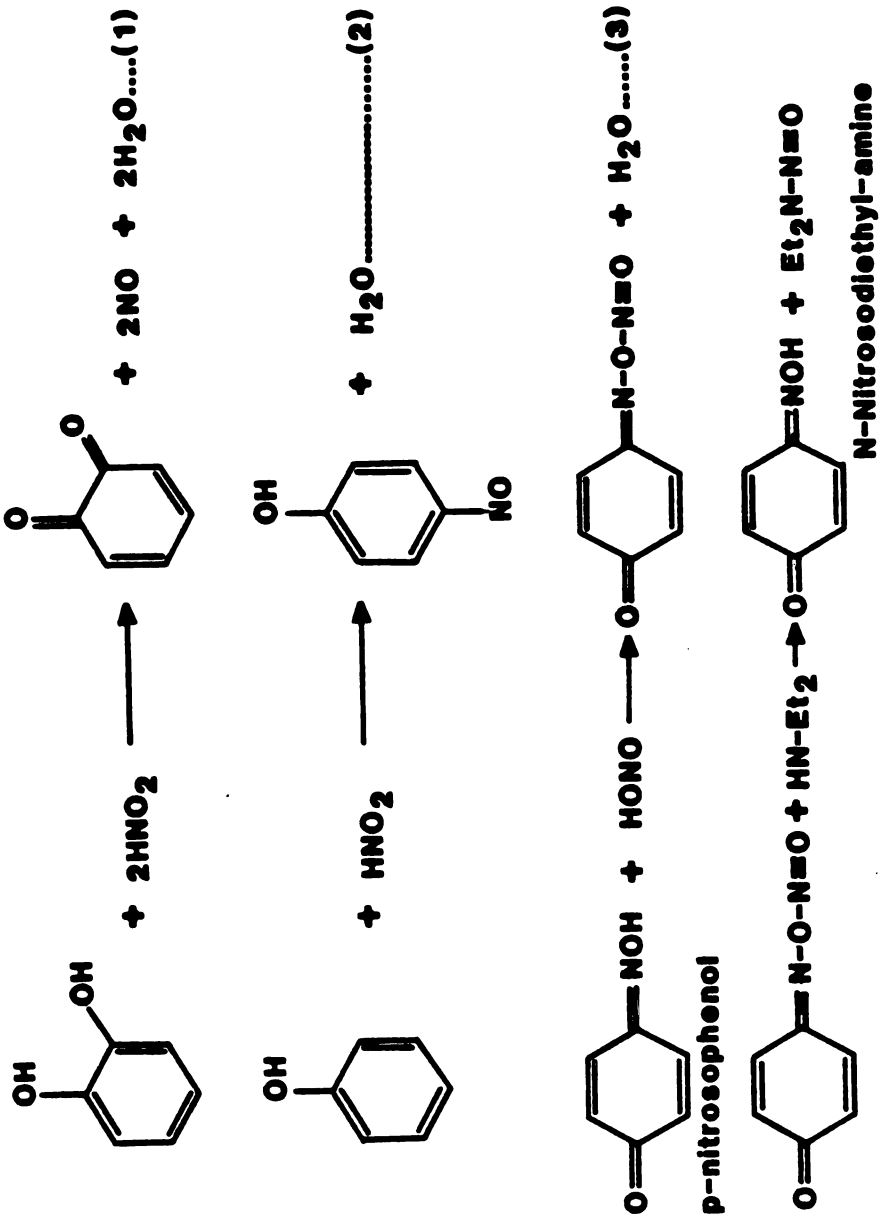


Figure 1. Reaction of phenols with nitrite

accelerate N-nitrosamine formation. Meta nitrosophenol, which cannot form a quinone tautomer, was shown to have no effect on the nitrosation of diethylamine. Catalysis by para nitrosophenol was found to modify the reaction mechanism with a resulting shift from second to first order kinetics with respect to nitrite.

Pignatelli et al. (1980) have shown that 1,2- and 1,4-dihydroxyphenols will inhibit N-nitrosamine formation by reducing nitrous anhydride (N_2O_3) to a non-nitrosating species (NO), while the dihydroxyphenols are oxidized to diquinones. The 1,3-dihydroxyphenol cannot oxidize to the quinone structure, and at a high nitrite to phenol ratio will catalyze N-nitrosamine formation (Figure 2). In this mechanism, an O-nitroso derivative of dinitrosoresorcinol is formed, and this species is capable of catalyzing the nitrosation of secondary amines (Walker et al., 1982).

Catalysis of the nitrosation of long chain dialkylamines can occur in the presence of surfactants that have the ability to form micelles (Okun and Archer, 1977). The catalytic effect increases as the chain length of the dialkylamine increases, i.e., as its solubility in the hydrophobic micellar phase increases. These authors have theorized that the electrostatic interactions at the surface of the micelle are responsible for destablizing the protonated amine, resulting in an acceleration of the nitrosation reaction. These findings could have important implications for <u>in vivo</u> N-nitrosamine formation because

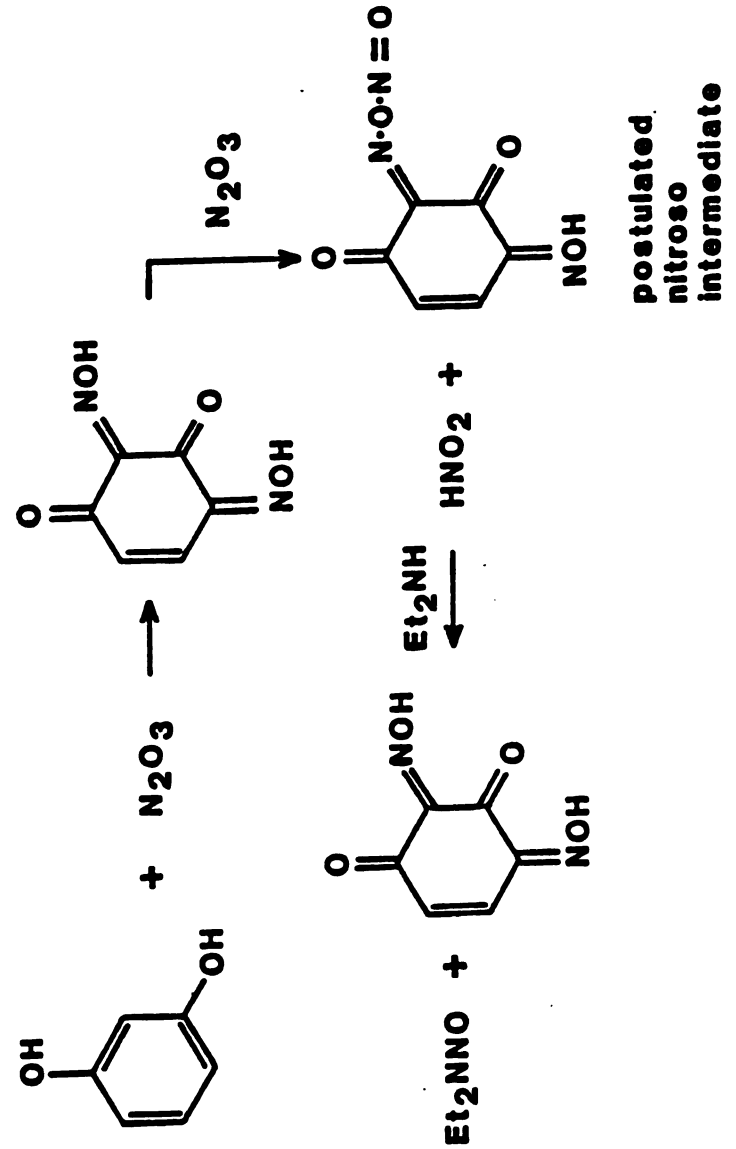


Figure 2. Proposed mechanism of catalysis of N-nitrosamine formation by 1,3-dihydroxyphenols (Walker et al., 1982)

of the omnipresence of emulsified lipids in the digestive tract.

N-Nitrosamine formation declines drastically as the pH is increased from 3 to 6. However, Keefer and Roller (1973) demonstrated that in the presence of some carbonyls like formaldehyde, N-nitrosamine formation can take place in the neutral and basic pH ranges. The reaction proposed involves the formation of an iminium ion from the reaction of a secondary amine and the aldehyde (Figure 3). Nitrite ion attack results in an unstable dialkylamino nitrite ester which can react further to regenerate the aldehyde and form the N-nitrosamine. Figure 3 also outlines the mechanism by which an N-nitrosamine is formed from a secondary amine and solid sodium nitrite in dichloromethane. In this reaction, a formaldiminium ion is produced as the result of a nucleophilic displacement reaction between the amine and a molecule of dichloromethane.

Any compound that can successfully compete with a secondary amine for a nitrosating species would reduce the possibility of N-nitrosamine formation (Gray and Dugan, 1975). These compounds are called blocking agents, and generally function by reducing the nitrosating species to a non-nitrosating oxide of lower oxidation state (Mergens and Newmark, 1980).

Ascorbic acid was first discussed as a N-nitrosamine inhibitor by Mirvish et al. (1972). The mechanism of inhibition involves the oxidation of the vitamin to dehydroascorbic acid as two moles of nitrous acid are reduced to nitric oxide

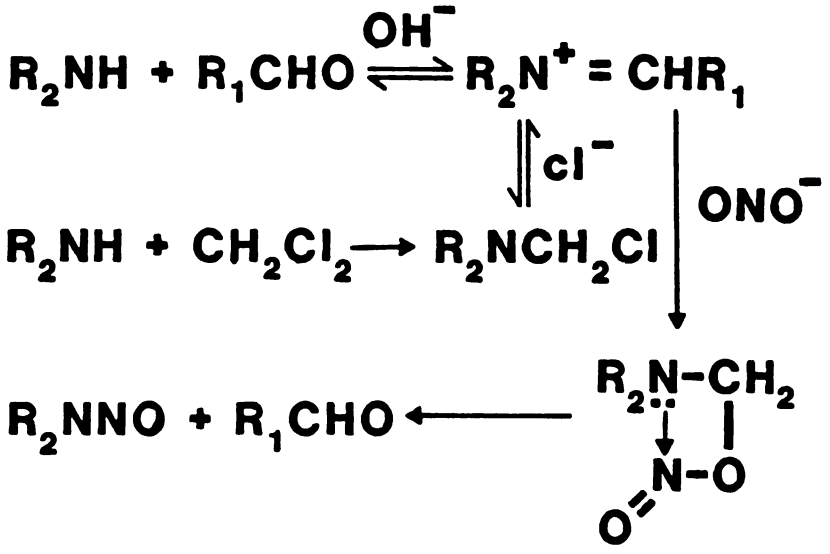


Figure 3. Catalysis of N-nitrosamine formation by aldehydes and dichloromethane

(Figure 4). The effectiveness of ascorbate is primarily dependent on the amine with which it is competing, as well as the pH of the environment. The pH determines which nitrosating species and ascorbic acid form will predominate. As a result of its greater nucleophilic activity, the ascorbate anion is 230 times more reactive than ascorbic acid, and is predominant in the pH range of 3 to 5 (Dahn et al., 1960).

Archer et al. (1975) reported that complete inhibition of N-nitrosomorpholine (NMOR) formation was obtained using an ascorbate/nitrite ratio of 2:1 at pH 4.0. However, when air was bubbled through the system, much more ascorbic acid was required to obtain this level of inhibition. This effect was thought to be due to the oxidation of ascorbate, and to the formation of additional oxidizing equivalents in the form of nitrogen dioxide.

Mirvish et al. (1972) demonstrated that ascorbic acid was an effective blocking agent in the pH range of 1 to 4. At pH 6.0, at least a 2:1 ratio of ascorbate to nitrite was required before significant inhibition of NDMA was recorded (Kawabata et al., 1974). Ascorbic acid concentrations equivalent to one tenth the nitrite concentration or less caused acceleration of N-nitrosamine formation at pH 6.0, regardless of temperature. Maximum inhibition occurred at pH 3.6.

To more closely represent a food system like bacon, Mottram and Patterson (1977) employed a two phase model system of buffer and corn oil to study the inhibitory effect of

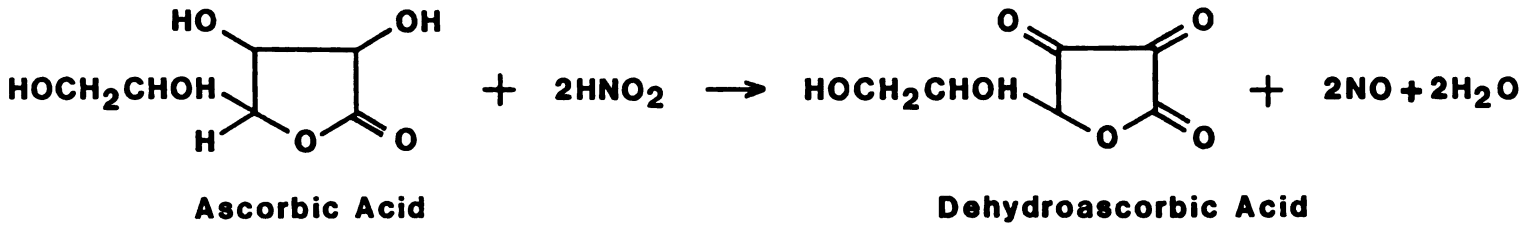


Figure 4. Reaction of ascorbic acid with nitrite

ascorbic acid. Sodium ascorbate greatly increased the yield of NPYR and N-nitrosodipropylamine (NDPA) and it was theorized that when nitrite is reduced in a purely aqueous system, the nitrogen oxides volatilize from solution or are kept in a non-nitrosating form (NO) by the reducing agent. In a two phase system, nitrogen oxides are free to migrate into the nonpolar phase away from the polar ascorbic acid and nitrosate amines there. Mottram et al. (1975) used pork slices spiked with dimethylamine to investigate the blocking potential of ascorbic acid. Ascorbate did inhibit N-nitrosamine formation in the lean, but did not influence its formation in the lipid phase.

Bharucha et al. (1979) observed that the formation of N-nitrosamines during the frying of bacon occurs almost entirely in the fat phase by a free radical mechanism rather than an ionic mechanism. They recommended that blocking agents in bacon should be able to serve as a NO radical trap, be fat soluble, be non-steam volatile, and be stable up to a maximum frying temperature of about 174°C.

Mottram and Patterson (1977) investigated the effect of ascorbyl palmitate on the nitrosation of pyrrolidine and dipropylamine in a two phase model system resembling adipose tissue. Ascorbyl palmitate inhibited the formation of NPYR at the 90% level, while N-nitrosodipropylamine (NDPA) formation was blocked by 20%. It was theorized that the solubility of ascorbyl palmitate in the nonpolar phase decreased

the reduction of nitrite in the aqueous phase, and thus lowered the production of nitrogen oxides. In addition, ascorbyl palmitate would be present to compete with amines for any nitrosating species migrating to the nonpolar phase. Bharucha et al. (1980) achieved 70% inhibition of N-nitrosamine formation in fried bacon by the use of ascorbyl palmitate applied as a slurry in soybean oil prior to frying. However, its effectiveness was reduced drastically after being stored for three weeks. These authors used ascorbyl acetal in bacon and obtained 80-90% inhibition of N-nitrosamine formation in the cook-out fat. This compound was found to remain stable for 35 days under refrigerated conditions, but it is not an approved food additive.

Alpha(α)-tocopherol is another compound which fulfills the requirements for a good blocking agent. In a mechanism analogous to the nitrite-ascorbate reaction, α-tocopherol reduces the nitrosating agent to a non-nitrosating species as outlined in Figure 5. Unlike many phenolic compounds, α-tocopherol cannot be C-nitrosated because of its fully substituted ring and thus cannot form a catalytic species (Walker et al., 1979).

Fiddler et al. (1978) investigated the effect of α-tocopherol on the formation of N-nitrosamines in fried bacon. α-Tocopherol is not water soluble and must be combined with an emulsifying agent to be dispersed in a brine. These authors reported that the α-tocopherol/polysorbate ratio must be

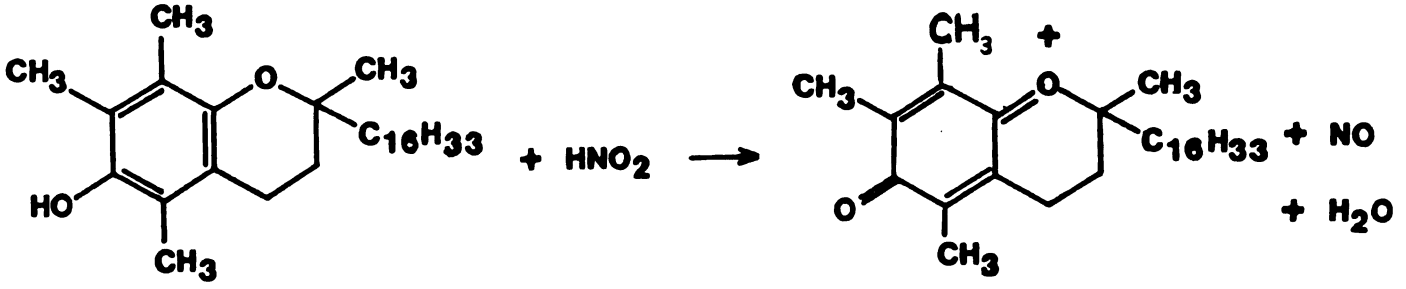


Figure 5. Reaction of α-tocopherol with nitrite

lower than 0.4 in order to obtain optimum distribution of the blocking agent. α-Tocopherol by itself significantly inhibited the formation of NPYR in the bacon and cook-out fat. In addition, an α-tocopherol-ascorbate combination treatment affected greater inhibition of the formation of NPYR and NDMA than did ascorbate alone. Reddy et al. (1982) investigated the feasibility of using α-tocopherol-coated salts as part of the dry cure for bacon. Approximately 96% inhibition of NPYR formation in the fried bacon was reported when α-tocopherol was used at the 500 mg/kg level in the cookout fat. The NPYR concentration was reduced 92%. α-Tocopherol was not as effective in blocking the formation of NDMA whose levels generally increased in the fried bacon treated with α-tocopherol-coated salt. Gray et al. (1982) reported that the inclusion of α-tocopherol-coated salt in the curing brine of bacon inhibited the formation of NTHZ by approximately 65%.

Mergens et al. (1978) determined the average loss of α-tocopherol under refrigerated conditions to be 3% per week, regardless of whether the packages were open or closed. Skrypec et al. (1985) reported the results of a study on the effect of storage on the ability of α-tocopherol-coated salts to inhibit N-nitrosamine formation in fried bacon. During the three month study, the percent inhibition of NDMA and NPYR formation did not change appreciably, although a 15% loss of α-tocopherol on the salts was detected. Lecithin is used in these salt systems to increase the dispersibility of

α-tocopherol in the brine. This compound has been identified as a possible precursor of NDMA (Gray et al., 1978). However, studies by Bernthal et al. (1986) revealed that lecithin in the α-tocopherol-salt system did not contribute to NDMA formation in fried bacon.

Pensabene and Fiddler (1985) investigated the effect of α-tocopherol on NTHZ formation in raw bacon during processing. α-Tocopherol sprayed on the surface of pork bellies or combined with an emulsifying agent and included in the brine did not significantly reduce NTHZ formation in raw bacon. Ascorbate by itself, or in combination with α-tocopherol, significantly decreased NTHZ formation in bacon. The difference in the efficacy of inhibition between ascorbate and α-tocopherol was attributed to the hydrophilicity of the former blocking agent and its ability to reduce residual nitrite in the raw product. It may also suggest that the precursors of NTHZ are water soluble (Gray et al., 1982).

Certain sulfur compounds have been reported to be effective N-nitrosamine inhibitors. Bisulfite reduces nitrite to nitrous oxide in a two step mechanism (reaction 13 and 14,

$$SO_2 + 2HNO_2 \longrightarrow 2NO + H_2SO_4 \qquad (13)$$

$$SO_2 + 2NO + H_2O \longrightarrow N_2O + H_2SO_4 \qquad (14)$$

$$NaNO_2 + H_2NSO_3H \longrightarrow NaHSO_4 + N_2 + H_2O \qquad (15)$$

Hisatsune, 1961), while sulfamate reduces nitrite to molecular

nitrogen (reaction 15, Jones, 1973). Gray and Dugan (1975) examined the effect of several sulfur compounds on the nitrosation of secondary amines. While greater than 99% inhibition of NDMA formation was achieved with sodium bisulfite and ammonium sulfamate at pH 3.5, the effectiveness of ammonium sulfamate dropped radically when a pH closer to that of meat was employed. Glutathione and cysteine were as effective as ammonium sulfamate in blocking the formation of NDMA. In contrast to the action of ammonium sulfamate, the effectiveness of the thiols dropped only slightly as the pH was increased to 5.5. However, Davies et al. (1978) concluded that thiols, like phenols, can form nitroso compounds which can themselves act as nitrosating agents. These researchers discovered that cysteine will catalyze an eleven fold increase in NPYR formation after 24 hours in an aqueous environment at pH 5.0. At pH 3.0, however, cysteine affected a 94% inhibition of NPYR formation with all other conditions being equal. The authors attributed this result in part to the decomposition of excess nitrite at this low pH value.

II The Smoking of Foods

The practice of smoking meats may be the oldest preservation technique known to man. Although the antioxidant and antimicrobial properties that smoke imparts to foods are

not as important today, the contributions of smoke to the characteristic flavor and color of these products are irreplaceable. It is therefore important to understand the chemistry of smoke so that compounds that contribute to the beneficial effects of smoking can be maximized, while toxic compounds in smoke can be eliminated or at least be reduced.

The Chemistry of Smoke

Smoke is strictly defined as the destructive anaerobic distillation of wood followed by partial oxidation (Wistreich, 1977). Wood is composed of approximately 50% cellulose, 25% hemicellulose, and 25% lignin, and it is the fracturing of these components that gives rise to the characteristic smoke aroma and flavor. Different types of wood vary somewhat in the relative proportions of these components, and thus the resulting smoke will impart unique flavor and aroma notes to the product. For example, hardwoods contain a higher hemicellulose content and as a result will deposit a higher concentration of acids on the food product being smoked (Mann, 1960). Hardwoods are more commonly used for the smoking of foods, but some are said to yield excessively dark colors and bitter taste (Mann, 1960). Softwoods contain more lignin with fewer methoxy groups and are thought to produce excessive soot and impart a resinous flavor (Clifford et al., 1980). In general, tradition

is the best indicator of what wood is best suited for the smoking of a certain product.

Smoke is composed of three principal phases; the particulate phase, the noncondensible phase, and the condensible phase. (Wistreich, 1979). The particulate phase consists of charcoal, fly ash, and long chain tars. Electrostatic precipitation of these compounds can be employed to prevent their deposition on food products. The noncondensible phase is composed of gases such as air, carbon dioxide, methane, and nitrogen oxides. The oxides of nitrogen can play an important role in N-nitrosamine formation and will be discussed later in this review of the literature. The condensible phase is the fraction of smoke that imparts all ofthe desirable and some of the undesirable qualities to smoked foods. The main subclasses of the condensible phase are the acids, carbonyls, phenols, furans, lactones, alcohols, esters, and the polycyclic hydrocarbons (PAH) (Hamm, 1977).

Smoke constituents

(a) Acids

The carboxylic acids of smoke have a profound effect on the overall quality of smoked food products. Aliphatic acids have been reported to contribute to the bacteriostatic effect of smoke (Clifford et al., 1980) and contribute to the

all smoke flavor (Toth and Potthast, 1984). In addition, the pH changes associated with the surface of smoked foods seem to denature and precipitate protein, adding to the stability of skinless franks (Wistreich, 1977). The lower pH also accelerates cure color formation. The main components of the acid fraction of smoke are acetic acid (3.7 g/100 g wood) and formic acid (0.8 g/100 g wood) (Toth and Potthast, 1984).

(b) Carbonyls

The carbonyl fraction of smoke contributes to the typical smoked food color via Maillard-like reactions (Ruiter, 1979). Both glycolaldehyde and methylglyoxal are reported to be the most active browners when reacted with amino groups and are present in high concentrations in smoke (Ruiter, 1979). Formaldehyde is also predominant in smoke and will readily react with amino groups. It will not form brown compounds, however, and actually inhibits brown color formation by reacting with amino groups (Chen and Issenberg, 1972). Color formation in smoked foods is directly related to the temperature in the smokehouse, concentration of the carbonyl and amine reactants, and the amount of moisture on the surface of the smoked product (Daun, 1979). Ideal color is obtained at a moisture level of between 6 and 10% in the smoked product exterior (Daun, 1979). At this level, the surface is

sufficiently moist to allow adsorption of the smoke constituents, but is dry enough to allow penetration of the smoke constituents into the product.

The reaction of smoke carbonyls with the amines of food has been implicated in the loss of nutritional value of smoked products (Clifford et al., 1980). Lysine has been reported to be the most reactive of the basic amino acids by a factor of four (Tang, 1978) and losses as high as 33% have been cited (Hoffman et al., 1977). Losses of serine, threonine, and sulfur-containing amino acids have also been reported to result from the smoking of foods (Mauron, 1970).

Toth and Potthast (1984) reported that thirteen aldehydes, seventeen ketones, glycolaldehyde, and methylglyoxal have been identified in smoke. The major carbonyls of woodsmoke are formaldehyde and acetaldehyde and have been detected at levels up to 200 mg and 1150 mg, respectively, per 100 g of combusted wood. Ketones as well as glyoxal are very reactive with food components and may possess flavoring properties (Toth and Potthast, 1984).

(c) Phenols

The phenolic fraction of smoke contains the most important flavor and aroma compounds (Wasserman, 1966). The major phenols of smoke are guaiacol and syringol and their derivatives. The use of softwood to generate smoke results in a phenol

fraction dominated by guaiacol and its derivatives, while the pyrolysis of hardwoods results in smoke with syringol and its derivatives as the predominant phenols (Stahl et al., 1973). Conflicting data have been reported as to the generation temperature required for maximum phenol synthesis. Fenner and Lephardt (1981) indicated that maximum phenol formation occurred at 380°C, while Toth (1980) reported maximum phenol formation at 650°C. Other phenols consistently reported in smoke are phenol and its alkyl derivatives, the various isomers of cresol and pyrocatechol (Toth and Potthast, 1984).

The characteristic flavor and aroma of smoked foods are primarily due to phenolic constituents of smoke (Daun, 1979). The compounds most responsible for these sensory qualities are guaiacol, 4-methylguaiacol, and syringol (Wasserman, 1966). However, a more complex mixture of compounds must be present for full smoked food aroma. High molecular weight phenols contribute to smoked color formation by cross linking collagen via hydrogen bonding (Caurie et al., 1974).

The antioxidative and antimicrobial properties imparted to foods by smoking are present on the surface of the product, and therefore are effective only for an intact product (Kersken 1973). Syringol, 4-methylsyringol, and 4-ethylsyringol have been demonstrated to possess the greatest antioxidative properties (Kurko, 1959). Phenols with lower boiling points, such as phenol, cresol, and guaiacol, were shown to be less

antioxidative. Smoldering sawdust which produces higher concentrations of dihydroxyphenols possess greater antioxidative properties than smoke produced by friction burning of wood (Tilgner and Daun, 1970).

The antimicrobial effects of smoke have been attributed to phenols as well as other smoke constituents. While Radecki et al. (1975) concluded that phenols with higher boiling points possessed the most bactericidal activity in smoke, Incze (1965) reported that formaldehyde was responsible for this effect. The fungicidal effect attributed to phenols and formaldehyde are important in preventing mycotoxic mold growth (Kersken, 1973). However, the lowering of water activity and surface pH, and the heat treatment associated with the smoking process can all influence microbial growth (Lerche et al., 1957).

The deposition of phenols on the surface of smoked food products may produce some undesirable effects as well. Phenols can be nitrosated to form nitrosophenols which are potential catalysts of N-nitrosamine formation (Davies and McWeeny, 1977). In addition, nitrosophenols have been demonstrated to have mutagenic potential (Gilbert et al., 1980).

(d) Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are produced from thermally generated methylene radicals resulting from the

incomplete combustion of fuels (Barnett, 1976). Of the estimated 200 PAHs present in smoke, approximately 25 have been shown to be carcinogenic or mutagenic (Kaden et al., 1979). The concentration of one PAH, benzo[a]pyrene (B[a]P), is well documented in a variety of foods and in the environment. The total carcinogenicity of PAH exposure is estimated to be ten times that due to B[a]P alone (Toth and Blaas, 1972). While there is no direct evidence that the consumption of smoked or barbecued meat products containing PAHs cause cancer in humans, it has been speculated that these compounds are responsible for the high incidence of stomach and colon cancer in Iceland where heavily smoked meats containing high PAH concentrations are consumed (Fritz and Soos, 1980).

The concentrations of PAHs deposited on smoked products is affected by the method of smoke generation, the form of the smoke application, and the temperature of the smoke curing process (Potthast, 1978). A linear increase in the production of PAHs was shown to occur as the smoke generation temperature was increased from 400 to 1000°C (Toth and Blaas, 1972). Therefore, even heavily smoked foods need not contain over 1 ug/kg of B[a]P if smoke generation temperatures are kept down (Toth and Potthast, 1984).

Liquid Smoke

The smoked food industry has developed a variety of needs

for the modern day manufacture of their products (Hollenbeck 1977) These needs are: (1) better control of the flavor and color of smoked foods to reduce variability of the final product; (2) to eliminate the deposition of carcinogenic compounds on foods by smoking; (3) to reduce the air pollutants produced during smoked food manufacture; and (4) and to increase the efficiency of the smoking process (Hollenbeck, 1977). The use of liquid smoke can reduce all of these problems associated with traditional smoking.

The production of liquid smoke and its applications

In the United States, the most popular method of producing liquid smoke is by smoldering sawdust under controlled oxidation conditions and absorbing the smoke constituents in water (Hollenbeck, 1977). Maximum exposure of the smoke to water is achieved by forcing the smoke countercurrent to water flowing through an absorption tower. The solution is recycled until a desired concentration of smoke constituents is reached. The smoke condensate is then stored or aged to allow polymerization and precipitation of the tar to occur. The liquid smoke is then filtered through cellulose pulp to remove any particulates. Other processes for producing liquid smoke involve condensing smoke in a condensor, or treating finely cut wood chips with superheated steam and condensing the steam

distillate (Fessman, 1976).

Liquid smoke can be added directly to a food product such as cheese spreads, meat emulsions, and barbeque sauces. The product can also be dipped in liquid smoke as is done with some types of cheese and meat products (Waselewski and Kozlowski, 1977). Meat product manufacturers who use a continuous meat processing design generally shower their products with liquid smoke (Hollenbeck, 1977). A variation of this showering technique is to atomize the liquid smoke to form a fog in the smoke house. The latest method of applying liquid smoke involves the regeneration of smoke from a smoke condensate (Wistreich, 1979). Liquid smoke is atomized into a stream of air which is passed over a series of heating coils and is forced into the smokehouse. This regenerated smoke is recirculated through the coils and the smokehouse until a dense smoke is achieved. The reaction of the smoke constituents with the food product occurs at an accelerated rate, to the point that a 30 min static step is equivalent to an eight hour continous traditional smoke (Wistreich, 1979).

Benefits of using liquid smokes

Hollenbeck (1977) estimated that 65% of the smoked meats produced in the U.S. and Canada are treated with liquid smoke. The use of liquid smoke in the cured meat industry is becoming

more prevalent because it eliminates many of the problems of traditional smoking. Greater control of the smoked flavor can be exercised because measured concentrations of liquid smoke phenols can be added to the products (Gorbatov et al., 1971). Obtaining a consistent color with liquid smoke is a more difficult problem. It has been demonstrated that better color formation is achieved when products are showered with liquid smoke for two periods, with a rest period in between (Hollenbeck, 1977). The rest period allows penetration of the smoke constituents into the product, as well as providing a drying period so that the Maillard-like reactions can occur.

The use of liquid smokes virtually eliminates the presence of PAHs in smoke treated foods (Gorbatov et al., 1971). The aging of liquid smoke allows the PAHs to precipitate out of solution with the tars and can be further removed by filtering the liquid smoke through cellulose filters (Hollenbeck, 1977).

Pressure applied to the smoked food industry by a more environmentally concerned public and governmental agencies has led to a scramble for alternate processes. The use of liquid smoke greatly reduces the effluent released into the air. West (1976) described a closed smokehouse system that eliminates smoke effluent. Smoke from the smokehouse is forced through a condensor tower like those used in liquid smoke production. The washed smoke is then recirculated into the smokehouse so that heat is conserved. The use of liquid smoke

has also made possible the development of continuous processing tunnels for smoking foods (Hollenbeck, 1977). One of the largest problems with a continuous smoking process is applying a sufficiently uniform smoke to the moving product. The spraying of the product with liquid smoke while exposing the food to traditional smoke has reduced this problem.

Problems associated with the use of liquid smoke

The addition of sodium nitrite to cured meats is essential because it inhibits the outgrowth of Clostridium botulinum spores (Tompkin et al., 1978). These authors proposed that nitric oxides react with an essential iron-containing compound within the germinated botulinal cell and prevents outgrowth. Thus, the presence of sodium nitrite insures that these products will be free of the deadly toxin produced by C. botulinum. Unbuffered liquid smokes are very acidic because they contain substantial amounts of acids and phenols. The inclusion of an unbuffered liquid smoke in a curing brine can result in dangerously low levels of residual nitrite in the cured product (Sleeth et al., 1982). Nitrite can be reduced to nitric oxides which may then volatilize . The liquid smoke can be buffered, but solubilizing agents must be included to keep the phenolic compounds in solution (Sleeth et al., 1982).

Liquid smokes may also play a role in contributing to N-nitrosamine formation. This topic will be explored in the

next section of this review.

III The Smoking of Cured Meats

Results of an epidemiological study performed in Iceland revealed a high incidence of diabetes in males born in the month of October (Helgason and Jonasson, 1981). These authors noted that circumstantial evidence implicated the N-nitroso compounds present in a smoked mutton product as the causative agents. This product is heavily smoked and is traditionally eaten in large quantities at Christmas time in Iceland. Because some N-nitroso compounds have been determined to be diabetogenic (Rossini et al., 1977), it was suggested by Helgason and Jonasson (1981) that the ingestion of smoked mutton at the time of conception was the cause of the high incidence of juvenile diabetics born in October.

The Role of Smoke in N-Nitrosamine Formation

The analysis of cured smoked mutton products from Iceland revealed the presence of mg/kg concentrations of N-nitroso-thiazolidine carboxylic acid (NTCA) and ug/kg concentrations of NTHZ (Helgason et al., 1984). NTHZ had been previously identified by Gray et al. (1982) in samples of brine-cured

smoked bacon.

Mandagere et al. (1984) first proposed a mechanism for the formation of NTHZ and NTCA in smoked meat products. These researchers hypothesized that free cysteamine and cysteine in a food system can react with formaldehyde in smoke to produce thiazolidine and thiazolidine carboxylic acid, respectively (Figure 6). The nitrosation of these compounds by either nitrogen oxides present in the smoke or nitrite added to cured meat results in the formation of NTHZ and NTCA. Toth and Potthast (1984) have reported that 200 mg of formaldehyde are present in the smoke produced by the burning of 100 g of wood, and up to 50 mg/kg of formaldehyde has been detected in smoked meat products. Formaldehyde may also arise from the fragmentation of glucose in the meat system during the frying of bacon as suggested by Mandagere et al. (1984). Another major component of smoke is acetaldehyde (1150 mg/100 g of combusted wood) (Toth and Potthast, 1984). The reaction of acetaldehyde with cysteamine and cysteine, followed by nitrosation, results in the synthesis of the 2-methyl derivatives of NTHZ and NTCA (Ohshima et al., 1983).

While NTHZ may be formed by two pathways (Figure 6), Mandagere et al. (1984) determined by differential scanning calorimetry studies that the decarboxylation of NTCA occurs more readily than the decarboxylation of thiazolidine carboxylic acid. The observation by these authors that NTHZ concentrations of raw bacon were higher than that of fried

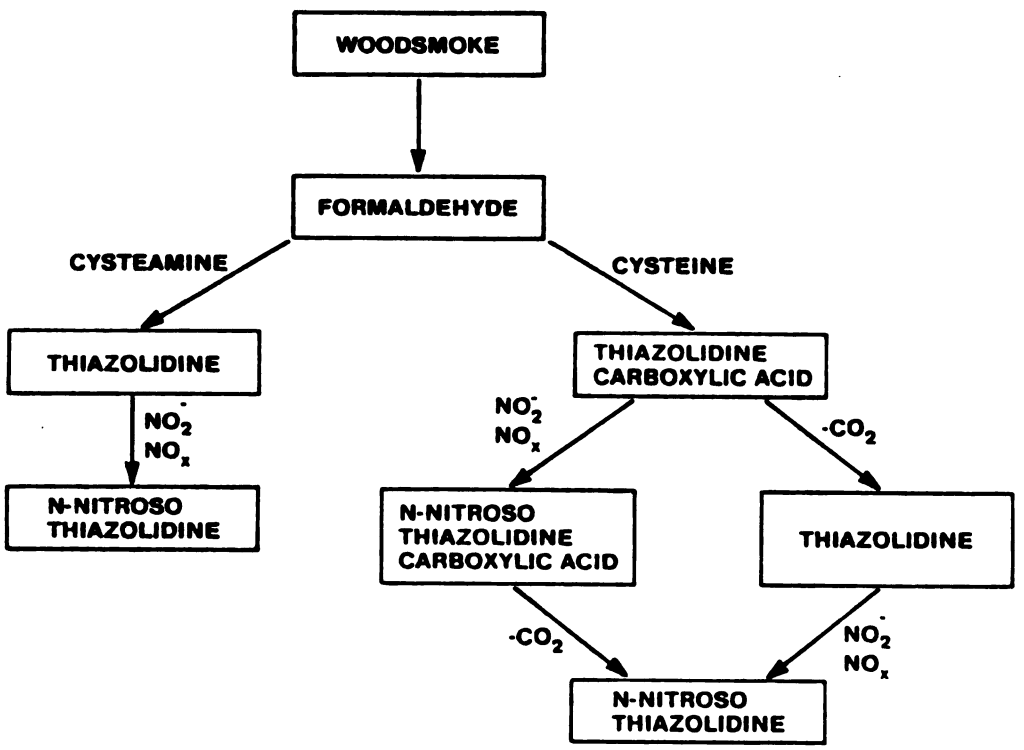


Figure 6. Possible pathways of N-nitrosothiazolidine formation

bacon suggests that cysteamine or thiazolidine is present in raw bacon, as the heat treatment applied during smoking would not be high enough to bring about the decarboxylation reactions.

Pensabene and Fiddler (1983a) also reported higher concentrations of NTHZ in raw bacon than in fried bacon. NTHZ levels were consistently higher in bacon processed in a gas fired wood burning smokehouse as opposed to an electrically heated house. This finding confirms the hypothesis of Mandagere et al. (1984) that NTHZ formation is associated with the smoking step. NTHZ was also detected in bacon that was sprayed with liquid smoke and cooked in an electrically heated smokehouse. The detection of an average NTHZ concentration of 22.5 ug/kg in ten samples of liquid smoke was given as a possible explanation for the presence of NTHZ in this bacon. The concentrations of NTHZ formed appeared to be independent of the residual nitrite levels in the raw bacon. The fate of NTHZ during frying was also investigated in this study. When nitrite-free bacon was spiked with known amounts of NTHZ and fried in a closed system, no destruction of the N-nitrosamine was observed. In an open frying system, no significant decrease in the concentration of NTHZ occurred, indicating also that NTHZ did not volatilize during frying.

These results contrast to those of Hotchkiss and Vecchio (1985) who fried uncured bacon in bacon drippings containing known amounts of NDMA, NPYR, and NTHZ. Significant amounts of NTHZ (2.0 ug/kg) were detected in the vapor and there was

a net synthesis of NTHZ (2.7 ug/kg) in the edible portion of the bacon. The frying of commercial bacon revealed that NTHZ concentrations are usually higher in the rasher than the cookout fat, and that the concentration of NTHZ did not always increase with increasing frying time.

Sen et al. (1985) demonstrated that at least a part of the NTCA present in raw bacon is decarboxylated to form NTHZ during frying. Raw bacon spiked with known amounts of NTCA were fried and analyzed for NTHZ. Various concentrations of NTHZ were obtained depending upon the frying conditions used. It was concluded that the NTCA concentrations in the raw bacon directly correlate with the NTHZ concentration in the fried bacon. In a limited bacon study, the concentrations of NTHZ in fried bacon were observed to be higher than those in raw bacon. In agreement with the results of Mandagere et al. (1984), these authors found less than 1 ug/kg NTHZ in the cookout fat of fried bacon.

Occurrence of NTHZ in Foods

NTHZ and NTCA have been isolated in a variety of smoked products. Helgason et al. (1984) detected mg/kg concentrations of NTCA in raw and cooked samples of Icelandic smoked cured mutton as well as ug/kg concentrations of NTHZ in the cooked product. The mutton analyzed in these experiments was a commercially processed meat which is typically cured with

regulated levels of nitrate and smoked for 36 hours. Traditional home smoked mutton can contain much higher concentrations of N-nitroso compounds. These meats are typically smoked intermittently for eight weeks, and the levels of added nitrate has been reported to be as high as 500 mg/kg (Ogmundsson and Adalsteinsson, 1979). Helgason et al. (1983) also reported high concentrations of NTHZ and NTCA in a variety of other Icelandic smoked products, including meat flavored drinks, sausages, ox tongue, salmon, and oysters.

Pensabene and Fiddler (1983b) surveyed a variety of commercially smoked products for the presence of NTHZ. Bologna, ham, pepperoni, hot dogs, and bacon all averaged less than 10 ug/kg. One product, a beef or pork breakfast stick, was treated with liquid smoke rather than woodsmoke. The presence of NTHZ in this product was attributed to preformed NTHZ in the liquid smoke.

Possible Toxic Effects of NTHZ and NTCA

The high incidence of juvenile diabetes in children born in October led researchers in Iceland to investigate the presence of N-nitroso compounds in the national diet. The diabetogenic effect of N-nitrosamides and N-nitrosomethylurea has been established (Rossini et al., 1977; Wilander and Tjaive, 1975), but it was not known whether N-nitosamines or their

acids could produce this effect. Helgason et al. (1982) fed Icelandic cured, smoked mutton to mice for ten days before fertilization. The parent mice were unaffected, but good evidence of a diabetic effect was produced in the progeny as measured by plasma glucose levels. The male mice seemed to be more succeptible to the diabetogenic agent than the females.

In a subsequent study, Helgason et al. (1984) identified the N-nitroso compounds present in the cured, smoked mutton product. While only trace amounts of NDMA were detected, high concentrations of NTCA (mg/kg) were found in both raw and cooked mutton. Significant concentrations of NTHZ (ug/kg) were detected in the cooked cured, smoked mutton product. Direct injection of mice with NTHZ and NTCA gave rise to highly significant increases in plasma glucose levels for many of the treated animals. The metabolites of NTHZ were speculated to be the sulfur oxides of the parent molecules that were detected in a variety of organs.

It is not known whether NTHZ is carcinogenic, but some research has been conducted to ascertain its mutagenic properties. When NTHZ was synthesized from cysteamine, formaldehyde, and nitrite, the product was found to be a direct acting mutagen on Salmonella typhimurium TA 100 (Mihara and Shibamoto, 1980). The response to this compound was nine times that of the spontaneous response level and was suppressed by a 9000 x g supernatant of rat liver homogenate (Sekizawa and Shibamoto, 1980).

Fiddler et al. (1984) demonstrated that NTHZ was not mutagenic when synthesized by direct nitrosation of thiazolidine and tested by the same Ames assay procedure employed by Mihara and Shibamoto (1980). These results implied that the mutagenic species in the cysteamine/formaldehyde/nitrite reaction was either a trace contaminant, a product of a side reaction, or a residual reaction precursor. HPLC separation of these products revealed that a fraction eluting after NTHZ demonstrated strong mutagenic potential. These researchers suggested the mutagenic compound may be formed in the processing or cooking of cured meats.

The Influence of Liquid Smokes on N-Nitrosamine Formation

The addition of liquid smoke to the curing brines of meat or the atomizing of liquid smoke on the surface of cured meat products has the potential to influence N-nitrosamine formation in several ways. The phenols in liquid smoke can act as either catalysts or inhibitors of N-nitrosamine formation. In addition, the presence of formaldehyde in liquid smokes could lead to the formation of high levels of NTHZ, particularly if liquid smokes are included in the curing brine. The distribution of formaldehyde throughout the meat system would most likely maximize the exposure of cysteine and cysteamine to formaldehyde.

Sleeth et al. (1982) employed a fraction of liquid smoke

in the curing-brine of bacon in an attempt to lower the N-nitrosamine concentration of fried bacon. Liquid smokes generally have a low pH, and if included in a curing-brine will reduce nitrite to volatile nitrogen oxides. Increasing the pH of the liquid smoke before it is added to the curing-brine results in a decrease in the solubility of the phenols and the subsequent clogging of the needles of the stitch pump injector. Sleeth et al. (1982) manipulated the pH of liquid smoke and extracted the smoke condensate with ethyl ether to obtain a fraction containing a carbonyl to phenol ratio of 0.5 to 5 - 1. A food grade emulsifier was used to keep the phenols in solution so that the extract could be buffered at a more neutral pH. An approximate 60% inhibition of NPYR formation was achieved in fried bacon when this modified liquid smoke was included in the curing-brines.

Theiler et al. (1984) used a ground pork model system to investigate the influence of liquid smokes on NPYR formation. The incorporation of liquid smoke in the model system resulted in significant reductions of NPYR in the fried meat without lowering residual nitrite levels. The surface application of liquid smokes to whole pork bellies, however, was found to have little effect on NPYR formation in fried bacon. The incorporation of liquid smoke in the curing-brine of bacon resulted in fried bacon with 60% lower NPYR concentrations than bacon prepared from a standard cure. The use of liquid smoke in combination with α-tocopherol and/or glucose inhibited

NPYR formation even further in the fried bacon model system.

Pensabene and Fiddler (1983a) examined the factors affecting NTHZ formation in bacon and reported that the atomizing of liquid smoke on the surface of bellies and cooking them in an electrically heated smokehouse produced bacon with much lower levels of NTHZ than woodsmoked bacon. All 10 samples of liquid smoke investigated contained NTHZ (average concentration of 22.5 ug/kg). These authors speculated that preformed NTHZ in the liquid smoke may contribute to the final NTHZ concentration in the treated bacon. The greater level of NTHZ in woodsmoked bacon appeared to be associated with the smoke itself, and the nitrosating species may be either nitrite in the meat or nitrogen oxides present in the smoke.

The influence of liquid smokes on NTHZ formation in bacon was further investigated by Pensabene and Fiddler (1985). Spraying liquid smoke on pork bellies before and during woodsmoking significantly inhibited the formation of NTHZ. This inhibition was thought to be due to the acidic nature of the liquid smoke which could destroy nitrite in the product or decompose NTHZ. In addition, the phenols of liquid smoke may compete with thiazolidine for the nitrosating species. The addition of liquid smoke to the curing brine of bacon did not significantly decrease NTHZ formation in woodsmoked bacon. Pensabene and Fiddler (1985) speculated that the ineffectiveness of liquid smoke in the cure was due to the distribution of the smoke constituents throughout the meat system, rather

than being concentrated at the surface.

IV In Vivo Formation of N-Nitrosamines

Epidemiological studies have demonstrated that a high risk of gastric cancer is associated with elevated exposure to nitrate in the water supplies of Columbia (Correa et al., 1975), Chile (Armijo and Coulson, 1975), and England (Hill et al., 1973). This relationship has directed attention to the posibility that in vivo formation of N-nitroso compounds may be occurring (Hart and Walters, 1983). Ingested nitrate can be reduced by the microflora of the mouth to nitrite which may react with amino compounds in the acidic conditions of the stomach to form N-nitroso compounds.

Sources of In Vivo Nitrosating Species

Nitrite is a normal constituent of human saliva and its concentration is largely dependent on nitrate intake from food and water (Spiegelhalder et al., 1976). The ingested nitrate is absorbed from the gastrointestinal tract into the blood. Serum nitrate is then excreted into the oral cavity, where about 5% of the dietary nitrate is reduced to nitrite in 24 hours by the oral microflora (Stephany and Schuller,

1980).

The most important source of dietary nitrate is reported to be vegetables, followed by drinking water (Selenka and Brand-Grimm, 1978). Nitrate concentrations of vegetables can be particularly high when the plants have been treated with nitrogen-containing fertilizers. The Netherlands and Switzerland have recently imposed regulations on nitrate levels in vegetables (Preussmann, 1984). The exposure of humans to nitrogen oxides in the air may also contribute to *in vivo* formation of N-nitroso compounds (Mirvish, 1982). The exhaust of gas fired appliances, tobacco smoke, and urban air are all sources of elevated levels of air-borne nitrogen oxides.

Amino Compounds Involved in In Vivo Nitrosation

It is not known which specific amino compounds present the greatest threat of being nitrosated *in vivo* to carcinogenic species, and therefore any evaluation of risk from an amine precursor is difficult. However, the same rules apply to *in vivo* formation of N-nitroso compounds as to *in vitro* formation. That is, secondary anines and alkyl amides react more readily that primary, tertiary, or quaternary amine compounds. In addition, weakly basic amines will be nitrosated faster than strongly basic amines.

A method of estimating *in vivo* nitrosation in humans was

developed by Oshima and Bartsch (1981). These investigators measured increases in urinary excretion of NPRO in subjects who had ingested nitrate and proline. The consumption or *in vivo* synthesis of NPRO was deemed safe for humans by Chu and Magee (1981). These researchers determined that when NPRO was administered orally to rats, greater than 80% of this compound was excreted unchanged within 24 hours. Rats gavaged with radioactively labelled NPRO showed negligible alkylation of DNA by the labelled carbon of NPRO and negligible production of labelled carbon dioxide. NPRO has also been reported to be noncarcinogenic and nonmutagenic by Mirvish et al. (1980).

Oshima and Bartsch (1981) gave vegetable juice and proline to a male volunteer and observed a significant increase in the excretion of urinary NPRO. Simultaneous ingestion of ascorbic acid or α-tocopherol suppressed NPRO excretion. The formation of NPRO *in vivo* appeared to be directly proportional to the dose of proline and increased exponentially with increasing concentrations of nitrate.

Ellen and Schuller (1983) administered high concentrations of nitrate to volunteers to investigate its effect on urinary NPRO excretion. NPRO levels in the urine remained constant until 6 hours after nitrate ingestion, at which time they increased rapidly until peaking at 10 to 15 hours. The diet of the experimental subjects was not controlled in this study. Analysis of the urine of patients given ammonium

nitrate for the treatment of renal stones was performed. Over half of these individuals had urinary NPRO levels equal to the baseline levels of healthy subjects, with the remainder having only slightly elevated concentrations of urinary NPRO.

Another amine that has been investigated for its potential to be nitrosated *in vivo* is piperazine, an anthelmintic drug used for treating humans. Piperazine nitrosates *in vitro* to two compounds; the mutagenic and carcinogenic N,N'-dinitrosopiperazine (DNPIP) (Druckery et al., 1967), and the less toxic N-nitrosopiperazine (NPIP) (Love et al., 1977). Bellander et al. (1984) orally administered piperazine to four healthy, male volunteers and examined samples of their gastric contents. From a dose of 480 mg of piperazine, a synthesis of 30-66 ug of NPIP was estimated to occur in the stomach. No NPIP was detected in the blood and almost all of the NPIP appeared to be excreted in the urine in the first 8 hours after administration of the drug. The toxic DNPIP was not detected in the urine, gastric juice, or blood of the subjects. The co-administration of ascorbic acid significantly inhibited the nitrosation of piperazine in the stomach.

Another drug that can act as a precursor for *in vivo* N-nitrosoamine formation is amidopyrine (AP). This drug is still prescribed as an analgesic in some parts of the world, despite the fact that it can be easily nitrosated to form NDMA (Lijinsky et al., 1972). In animal experiments, simultaneous ingestion of AP and nitrite resulted in high levels of liver

tumors due to the in vivo formation of NDMA (Lijinsky et al., 1973). Spiegelhalder and Preussman (1984) administered AP to healthy volunteers and monitored urinary NDMA excretion. Normally, NDMA excretion cannot be monitored by urinary excretion because of the high metabolic conversion of this compound. However, the simultaneous ingestion of ethanol with the drug significantly increases NDMA excretion. This effect is most likely due to a competitive inhibition of NDMA metabolizing enzymes (Peng et al., 1982). A single oral dose of AP accompanied by 30 g of ethanol resulted in the excretion of significant amounts of NDMA in the urine, estimated to be 1-2% of the total NDMA synthesized in vivo. Salivary nitrite concentrations were found to be directly correlated with the levels of NDMA formed.

Ladd et al. (1984) investigated the possible differences in the rates of in vivo nitrosation between smokers and nonsmokers. The ingestion of proline by itself resulted in no significant differences in urinary excretion of NPRO between smokers and nonsmokers. However, the simultaneous consumption of beet juice and proline resulted in the synthesis of 2.5 times more NPRO by smokers than nonsmokers. While salivary nitrite levels were not significantly different between the two groups, salivary thiocyanate concentrations were approximately three times higher in smokers. These authors suggested that the catalytic ability of thiocyanate was responsible for the increased rate of gastric nitrosation of proline for

smokers. The direct nitrosation of amines in the respiratory tract by oxides of nitrogen in cigarette smoke was not thought to contribute greatly to the nitrosation of proline.

The analysis of human urine for NPRO by Ohshima et al. (1983) consistently yielded several unidentified N-nitroso compounds. In a subsequent paper, Ohshima et al. (1984) identified these compounds as NTCA and 2-methyl N-nitrosothiazolikine carboxylic acid (MNTCA). At an optimum pH *in vitro*, thiazolidine carboxylic acid (TCA) and its methyl derivative (MTCA) were nitrosated 250-500 and 60-300 times faster than NPRO, respectively. Up to 95% of an oral dose of NTCA and MNTCA was recovered in urine of rats in the first 24 hours, while less than 2% was recovered in the feces. The gavaging of rats with TCA or MTCA and nitrite resulted in a significant increase in the excretion of NTCA and MNTCA. In addition, NTCA and MeNTCA were readily formed in rats after gavaging them with cysteine, nitrite and the respective aldehyde. The analysis of human urine revealed that smokers normally excrete greater quantities of NTCA and MNTCA, although the difference was not statistically significant. The administration of ascorbic acid three times daily significantly decreased the excretion of NTCA, MNTCA, and NPRO. These authors suggested that NTCA and MNTCA may appear in human urine as the result of (i) the intake of preformed N-nitroso compounds; (ii) nitrosation of the amines *in vivo*; (iii) or the *in vivo* synthesis of these compounds from the reaction of cysteine with the respective aldehyde, followed by nitrosation.

EXPERIMENTAL

Important safety note: Caution should be exercised in the handling of N-nitrosamines since they are potential carcinogens. Direct contact with these chemicals should be avoided. Safety gloves should be worn whenever N-nitrosamines are being handled. All experimental work should be done in a hood or a well-ventilated area.

Materials

All chemicals employed were of analytical grade and used without further purification. The solvents were purchased from Mallinckrodt Inc. (Paris, KY) and were redistilled prior to use. Sodium nitrite, sodium ascobate, sodium nitrate, sodium hydroxide, and sodium tripolyphosphate were also obtained from Mallinckrodt Inc.

NPYR, NDMA, N-nitrosopipecolic acid (NPIC), cysteine, guaiacol, 4-methylsyringol, phenol, isoeugenol, 4-allylsyringol, eugenol, and syringol were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Fisher Scientific Company (Fair Lawn, NJ) provided the 2,4-dimethylphenol, formaldehyde, potassium thiocyanate, mercuric chloride, and acetaldehyde. 4-Methylguaiacol was purchased from Pfaltz and Bauer Inc. (Stamford, CN), while sulfanilic acid, o-cresol, p-cresol, m-cresol, N-1-naphthylethylenediamine, cysteamine, thiazolidine, thiazolidine carboxylic acid, and N-methyl-N-nitroso

p-toluene sulfonamide (for diazomethane synthesis) was obtained from Sigma Chemical Co (St Louis, MO). N-Nitrosothiomorpholine (NTMOR), NTHZ, NTCA, and MeNTHZ were synthesized from their corresponding amines and sodium nitrite as described by Pensabene et al. (1972). MeNTCA was prepared according to the method of Riemschneider and Hoyer (1962).

The liquid smokes were donated by Red Arrow Production Co. (Mantowoc, WI), Griffith Laboratories (Alsip, IL), and Hickory Specialties Inc. (Crossville, TN). The α-tocopherol-coated salt was donated by Diamond Crystal Salt Co. (St Clair, MI). The pork bellies used for bacon manufacture were purchased from local suppliers.

Methods

I Effect of Smoking on N-Nitrosamine Formation in Bacon

Bacon Processing

Pork bellies were processed into bacon within 48 hours of slaughter. The bellies were stitch pumped to 110% of their original weight to obtain target concentrations of 120 mg/kg sodium nitrite, 1.5% sodium chloride, 0.5% sucrose, 550 mg/kg sodium ascorbate, and 0.35% sodium tripolyphosphate in the finished bacon. Four solubilized liquid smokes were added to the brines at levels recommended by the manufacturers.

Another liquid smoke preparation was atomized in the smokehouse for two 30 minute intervals during the 8 hour cooking process. The control bacon was smoked with hickory woodsmoke.

The bellies were placed in plastic bags after stitch pumping and allowed to equilibrate overnight in a cooler at 2°C. The bellies were transferred to an Elec-Trol laboratory smokehouse (Drying Systems Inc., Chicago, IL) and cooked at a temperature of 58°C (dry bulb) for 4 hours followed by three further hours of cooking at 52°C (dry bulb) and ambient relative humidity. Smoke was applied throughout cooking for the control bacon using a midget size Mepaco smoke generator (Meat Packers Equipment Co., Oakland, CA.). The cooked bellies were transferred to a tempering cooler (-2° C) where they were held overnight prior to slicing and packaging.

The bellies were sliced to 20-25 slices /lb or 1/8 " thickness and packaged in a manner similar to that described by Robach et al. (1980). After each belly was sliced, it was packaged in 15 packages with the first slice going into the first package, the second slice into the second package, the third slice into the third package and so on, until the bacon was completely packaged. Thus, each of the 15 packages could be considered representative of an entire belly. Two packages from each belly, or ten packages from each smoke treatment group were analyzed for N-nitrosamines, while remaining packages were analyzed for nitrite and N-nitrosamino acids.

Model System Studies

The role of smoke in NTHZ and MeNTHZ formation in bacon was further evaluated using an aqueous model system. Cure-solubilized liquid smokes were included in the model system at the same concentrations as those employed in the curing brines of bacon. The liquid smoke and a condensate from woodsmoke were included in the model system at a concentration of 3.5%. The condensate from woodsmoke was obtained by drawing smoke through ice cooled traps for 6 hours with a vacuum pump. Solutions of nitrite, cysteamine, and liquid smoke were combined and the volume adjusted to 100 ml with distilled water. The reaction systems contained 120 mg and 25 mg of nitrite and cysteamine, respectively.

The samples were transferred to stoppered 250 ml Erlenmeyer flasks, and the pH of the samples was adjusted to 5.5 with concentrated sodium hydroxide. The samples were reacted for 1 hour at 30°C in a water bath, and extracted with three 75 ml aliquots of methylene chloride. The extract was dried over anhydrous sodium sulfate and concentrated in a Kuderna Danish apparatus to a final volume of 100 ml.

Analysis of Liquid Smokes for N-Nitrosamines

The liquid smokes were diluted with water to obtain

concentrations similar to those employed in the previous model system. The liquid smoke solutions (100 ml) were extracted with three 75 ml aliquots of methylene chloride. The combined extracts were dried over anhydrous sodium sulfate and concentrated in a Kuderna Danish apparatus to a volume of 7 ml. The extracts were transferred to centrifuge tubes and concentrated to 1 ml under a gentle stream of nitrogen.

Isolation of N-Nitrosamines from Fried Bacon

N-Nitrosamine concentrations in fried bacon were determined using the mineral oil distillation procedure of Fine et al. (1975), as modified by Robach et al. (1980). Bacon was fried for 3 minutes per side in a calibrated skillet set at 340°F. The cook-out fat was poured off and set aside for later analysis. The fried bacon was frozen with dry ice and ground to a powder with a Futura II blender (Waring Products Division, New Hartford ,CN). Ground bacon (25 g) and ammonium sulfamate (2 g) were weighed into a distillation flask along with 25 ml of mineral oil. NTMOR (200 ng) was also added to the sample as an internal standard. Water (1.0 ml) was placed in the vacuum trap and shaken vigorously before plunging it into liquid nitrogen. The distillation flasks were slowly heated in oil baths to 110°C in one hour under vacuum.

The vacuum was adjusted to prevent excessive foaming of the sample. After the samples reached 110°C, the oil baths were removed and the samples were allowed to sit for 10 minutes under vacuum.

The traps were then removed and set aside in a dark place to thaw. Methylene chloride (20 ml) was added to the trap and shaken vigorously. The solvent was passed through anhydrous sodium sulfate into a Kuderna Danish apparatus. The rinsing of the trap was repeated 5 more times. The combined extracts were concentrated to 7 ml and transferred to a graduated centrifuge tube. The extract was concentrated to a final volume of 1 ml under a gentle stream of nitrogen.

Isolation of N-Nitrosamines from Bacon Cookout-Fat

N-Nitrosamines in the fat drippings were isolated and quantitated essentially by the method of Owens and Kinast (1980), except that 2.0 g of ammonium sulfamate were added to the distillation flask prior to distillation. The bacon drippings were spiked with 200 ng of NTMOR so that recoveries of N-nitrosamines could be estimated.

Quantitation of N-Nitrosamines

N-Nitrosamines levels were quantitated using a GC-TEA system comprised of a Varian 3700 gas chromatograph coupled to a TEA model 502 LC (Thermo Electron Corp., Waltham, Ma.).

The N-nitrosamines were separated on a 3m x 2mm i.d. glass column packed with Carbowax 20 M on 80/100 Chromosorb W (Supelco Inc., Bellefonte, PA.). Gas chromatographic conditions included: temperature programming, 100-180 °C at 15 C/minute; carrier gas (nitrogen) flow rate, 30 ml/minute; TEA pyrolyzer temperature, 475 °C; and oxygen flow rate 10 ml/minute.

Quantitation of NTCA in Bacon

NTCA levels in raw and fried bacon were determined by the method of Mandagere (1986). Bacon was ground and thoroughly mixed in a Hobart grinder (Model 84181D) and a representative sample (25 g) was blended in 200 ml distilled water for 2 minutes. The mixture was centrifuged at 1800 rpm in a Model K centrifuge (International Centrifuge, Needham Hts, MA.) for 10 minutes and the supernatant filtered through glass wool. The filtrate was treated with 5 ml of 20% ammonium sulfamate in 3N sulfuric acid and 15 g of sodium chloride. After sitting for 10 minutes, the mixture was centrifuged for 10 minutes at 1800 rpm and the supernatant was filtered through Whatman No. 1 filter paper. The filtrate was transferred to a 500 ml separatory funnel and extracted with three 75 ml aliquots of ethyl acetate. The extract was dried over

anhydrous sodium sulfate and concentrated on a rotary evaporator to 2 ml. The concentrate was derivatized with diazomethane and further concentrated to 1 ml under a steady stream of nitrogen. NTCA levels were determined by the GC-TEA system using a 3m x 2mm i.d. glass column packed with a mixed phase, 1% OV 210 and 2% OV 17, on 80/120 mesh Chromobsorb W (Supelco Inc., Bellefonte, PA.). The gas chromatograph was temperature programmed from 100-180°C at 8°C/minute.

Nitrite Analysis

Residual nitrite in raw bacon samples was quantitated according to the standard AOAC procedure (1984).

II Analysis of Phenols in Liquid Smoke

Phenols in liquid smoke were analyzed essentially by the procedure of Lustre and Issenberg (1970). Liquid smoke (5 ml) was diluted to 50 ml in a volumetric flask with a 5% sodium hydroxide solution. Neutral compounds were extracted with two 100 ml and one 50 ml aliquot of diethyl ether. The aqueous fraction was neutralized by bubbling carbon dioxide through the ice cooled samples. When a pH of 6.8 was reached, the samples were extracted with two 200 ml and one 100 ml

aliquots of diethyl ether. The combined extracts were dried with anhydrous sodium sulfate and transferred to a 500 ml round bottom flask. The solvent was removed at 30°C using a rotary evaporator (Buchi Instruments Co., Switzerland). The phenol fraction was removed from the flask by rinsing it with four 2 ml volumes of acetone. The combined acetone rinses were made up to 10 ml in a volumetric flask.

The phenol fraction was analyzed with a Hewlett Packard 5840A gas chromatograph equipped with an FID detector and a 50m 20M Carbowax capillary column (Alltech Associates, Inc., Deerfield, Illinois). Gas chromatographic conditions included: temperature programming, 50-190°C at 10°C/minute; nitrogen flow rate 25 ml/minute; FID temperature, 350°C; injection temperature, 225°C. The identities of the phenols were confirmed using a Hewlett Packard 5983A gas chromatograph-mass spectrometer unit equipped with a 2m x 3mm i.d. glass column packed with 1% SP 1240-DA on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA.) Operation conditions included: electron energy 70 eV; actual source temperature, 200°C; helium carrier gas, 25 ml/minute.

III Effect of pH on NTHZ Formation

A buffer system prepared according to the specifications

of Gomori (1955) was employed. This system was composed of variable volumes of a 0.1 mole/L sodium citrate solution and a 0.1 mole/L hydrochloric acid solution to obtain samples ranging in pH from 1.0 to 7.0 in 0.5 pH unit increments. The pH of the buffer solutions was monitored using a digital pH meter (Model 601A, Orion Research Inc., Cambridge, MA). Buffer (5 ml) at the appropriate pH was pipetted into screwcap test tubes. To the buffer, standard solutions of thiazolidine (5.0 µmoles) and nitrite (25.0 µmoles) were added and the pH was again adjusted to the desired value using sodium hydroxide and hydrochloric acid when required. The samples were sealed and placed in a 37°C water bath. The samples at each pH were reacted in duplicate.

After one hour, the reaction was quenched by the addition of 1 g of ammonium sulfamate. The samples were extracted with three 5 ml aliquots of methylene chloride. The combined extracts were dried over anhydrous sodium sulfate and concentrated to 5 or 10 ml depending on the NTHZ content of the sample. The samples were analyzed on the GC-TEA system containing a glass column (2m x 2mm) packed with a mixed phase packing (1% OV 210, 2% OV 17 on Chromosorb WMP).

The effect of pH on NTHZ formation from cysteine, formaldehyde and nitrite was also investigated. Standard solutions of cysteamine (5 µmoles), formaldehyde (5 µmoles), and nitrite (25 µmoles) were added to the buffer. The rest of the procedure was similar to that described above.

IV In Vivo Formation of N-Nitroso Compounds

Male Sprague-Dawley rats (25) were obtained through Michigan State University Laboratory Animal Care Service and were weighed to insure they were all approximately the same weight (200 ± 25 g). The rats were housed in metabolic cages and weighed each week to monitor their growth and health.

The rats were fasted overnight before administration of NTCA precursors via gavage in the morning, but were given water ad libitum. Six rats composed each treatment group. Treatment groups were administered saline or individual NTCA precursors as described in Table 1. The desired volume of precursor solution was administered using a gavage needle attached to a 3.0 ml disposable syringe. Combinations of NTCA solutions were given by mixing the appropriate ratio of precursor volumes in a test tube with a vortex prior to treating the rat (Table 1). The lag time between the initial mixing of the solutions and the of the rat was held to under 10 seconds with the use of automatic pipet bottles. The order of mixing was cysteine, aldehyde, and nitrite. Liquid smoke D and E (as designated in the bacon study) were administered to rats in combination with cysteine and nitrite (Table 1).

Measured volumes of nitrate were included in the water bottles of rats deprived of water for 24 hours. Two hours later, the rats were administered cysteine and formaldehyde

Table 1 NTCA precursors, catalysts and inhibitors administered to rats

Treatment Number	Reactants	Volume (ml)	Concentration (moles/L)
1	saline	1.0	0.166
2	cysteine	0.5	0.050
3	formaldehyde	0.5	0.050
4	nitrite	1.0	0.025
5	cysteine	0.5	0.050
	+ formaldehyde	0.5	0.050
6	cysteine	0.5	0.050
	+ nitrite	1.0	0.025
7	formaldehyde	0.5	0.050
	+ nitrite	1.0	0.025
8	cysteine	0.5	0.050
	+ formaldehyde	0.5	0.050
	+ nitrite	1.0	0.025
9	cysteine	0.5	0.050
	+ acetaldehyde	0.5	0.050
	+ nitrite	1.0	0.025
10	treatment #8	2.0	-----
	+ liquid smoke D	0.5	-----
11	treatment #8	2.0	-----
	+ liquid smoke E	0.5	-----
12	treatment #8	2.0	-----
	ascorbic acid	0.5	0.100
13	treatment #8	2.0	-----
	α-tocopherol	0.5	0.00005
14	treatment #8	2.0	-----
	phenols of LS A	0.5	-----

Table 1 (continued) NTCA precursors, inhibitors and catalysts administered to rats

Treatment Number	Reactants	Volume (ml)	Concentration (moles/L)
15	treatment #8	2.0	-----
	+ thiocyanate	0.5	0.010
16	cysteine	0.5	0.050
	+ formaldehyde	0.5	0.050
	+ nitrate	variable	0.250
17	treatment #16	variable	-----
	+ thiocyanate	0.5	0.010
18	thiazolidine	0.5	0.050
	+ nitrite	1.0	0.025
19	treatment #18	1.5	-----
	+ formaldehyde	0.5	0.050

(Table 1). The water bottles were periodically checked to insure they were not leaking. After 24 hours, the urine was collected and the volume of nitrate solution remaining in the water bottle was recorded.

Potential inhibitors and catalysts of NTCA formation were given to rats in combination with cysteine, formaldehyde, and nitrite (Table 1). Potassium thiocyanate was combined with cysteine and formaldehyde and administered to rats whose water had been replaced with a nitrate solution. Six rats were also treated with formaldehyde, thiazolidine, and nitrite (Table 1).

The urine was collected in centrifuge tubes containing 1.0 mole/L sodium hydroxide and cooled in ice baths. Twenty four hours after treating the rats, the screens and funnels of the metabolic cages were rinsed with distilled water. The urine and rinse was combined and transferred to a 50 ml volumetric and made to mark. The urine was analyzed by the procedure of Oshima et al. (1984).

An aliquot of the diluted urine (15 ml) was poured into a separatory funnel containing 5 g of sodium chloride. One ml of a NPIC solution (5 ug/ml in ethyl acetate) was added as an internal standard. Two ml of a 20% ammonium sulfamate in 1.8 mole/L sulfuric acid solution were added to the funnels to prevent artifactual N-nitroso compound formation. The urine was extracted three times with 10% methanol in methylene chloride (25 ml). The combined extracts were dried over

anhydrous sodium sulfate and transferred to 250 ml roundbottom flasks. The samples were concentrated to 1 ml in a rotary evaporator at 35°C and pipetted to a glass centrifuge tube. A small volume of ethyl ether was used to rinse the flask and was combined with the urine extract. The sample volume was reduced to 1 ml under a gentle stream of nitrogen and derivatized with diazomethane. The volume was again reduced to 1 ml under nitrogen and analyzed by the GC-TEA system. A 2m x 3 mm glass column was used containing 5% FFAP on Chromobsorb W (80/100 mesh)(Supelco, Bellefonte, PA). The gas chromatograph was temperature programmed from 100 to 180°C at 10°C per min.

V Statistical Treatment of N-Nitroso Compound Data

Statistical analysis of N-nitrosamine data for the different bacon treatment groups was performed using Bonferroni t statistics (Gill, 1978). Analysis of NTCA excretion for groups of rats treated with different inhibitors or catalysts of NTCA formation was also performed using Bonferroni t statistics. Where a single contol group was compared to a single treatment group of rats, an F-test was employed (Gill, 1978).

RESULTS AND DISCUSSION

I Bacon Study

Analysis of Fried Bacon for N-Nitrosamines

Bacon was processed by various smoke treatments to investigate their effect on N-nitrosamine formation. Four groups of pork bellies were stitch pumped with different cure-solubilized liquid smokes, while another group was treated with an atomized liquid smoke. The concentrations of volatile N-nitrosamines in the fried bacon produced by these treatments were compared to those in fried bacon prepared from traditionally smoked bellies (Table 2).

Of the four groups of bellies that were treated with cure-solubilized liquid smokes, treatments B, D, and E produced fried bacon containing significantly lower ($p < 0.05$) NDMA concentrations relative to bacon produced by traditional smoking. NDMA concentrations in bacon treated with the atomized liquid smoke were not significantly different from those present in the woodsmoke controls ($p < 0.10$). These results agree with those of Theiler et al. (1984) who reported that surface application of liquid smoke had little or no effect on N-nitrosamine formation. These authors did not, however, specifically discuss the influence of liquid smokes on NDMA concentrations.

Table 2. N-Nitrosamine formation in fried bacon processed with various smoke treatments

Smoke Treatment	Mode of Application	N-Nitrosamine concentration (μg/kg) NDMA[a,e]	NPYR[a]	NTHZ[a]
Wood smoke	Traditional	4.7±1.5 (2.8-6.7)	13.6±7.3 (6.3-25.0)	5.1±1.2 (3.1-6.4)
Liquid smoke A	Atomized in smokehouse	4.2±1.7[b] (2.4-6.9)	9.3±7.0[b] (4.6-21.4)	0.9±0.8[d] (0.1-1.8)
B	In brine	2.9±0.9[c] (1.5-4.1)	2.5±1.5[d] (0.4-4.5)	tr[d]
C	In brine	3.9±1.0[b] (2.4-5.2)	4.0±2.6[d] (1.0-7.2)	tr[d]
D	In brine	2.3±1.4[d] (1.1-4.5)	4.8±4.2[d] (2.0-12.0)	2.1±2.1[d] (0.1-5.5)
E	In brine	1.2±0.4[d] (0.8-1.6)	2.5±0.8[d] (1.6-3.7)	tr[d]

[a] NDMA, N-nitrosodimethylamine; NPYR, N-nitrosopyrrolidine; NTHZ, N-nitrosothiazolidine

[b] Not significantly different from traditionally smoked bacon ($p < 0.10$)

[c] Significantly different from traditionally smoked bacon ($p < 0.05$)

[d] Significantly different from traditionally smoked bacon ($p < 0.005$)

[e] Five bellies were analyzed per treatment, and duplicate analyses of each belly were performed

A more pronounced reduction was observed in NPYR concentrations in bacon treated with the cure-solubilized liquid smokes (Table 2). Bacon processed in this manner had an average NPYR concentrations at least 60% lower than those in traditionally smoked bacon. The most effective liquid smoke treatments were B and E, which produced bacon samples with an average NPYR concentration of 2.5 ug/kg. This value represents an 82% reduction relative to the woodsmoked samples where the average NPYR concentration was 13.6 ug/kg. Theiler et al. (1984) reported that bacon produced with liquid smoke dispersed in the curing brine contained much less NPYR (57% reduction) than unsmoked control bacon samples. These investigators also observed no inhibition of NPYR formation when liquid smoke was sprayed onto the outside of the belly. In the present study, fried bacon processed with atomized liquid smoke contained an average NPYR concentration of 9.3 ug/kg. This value was 32% lower than that for the woodsmoked controls. The difference, however, was not statistically significant ($p < 0.10$).

NTHZ concentrations in fried bacon produced from bellies which had been stitch pumped with liquid smoke were significantly lower ($p < 0.005$) than those in bacon produced by traditional woodsmoking. Bacon prepared with liquid smoke D had an average NTHZ concentration of 2.1 ug/kg. This was the only cure-solubilized liquid smoke preparation that produced greater than trace amounts of NTHZ in the fried bacon. The most likely

explanation for the difference in these data is that liquid smoke D probably contains a higher concentration of formaldehyde than the other liquid smokes, as this aldehyde is believed to be involved in the formation of the thiazolidine compound (Mandagere et al., 1984). However, no carbonyl analyses were performed on the liquid smokes to substantiate this hypothesis.

Atomizing liquid smoke A and depositing it on the surface of pork bellies resulted in fried bacon samples averaging 0.9 ug/kg of NTHZ. These results are similar to those obtained by Pensabene and Fiddler (1983a) who concluded that liquid smoke sprayed on the surface of bellies makes only a minor contribution to NTHZ formation in fried bacon. However, a part of this contribution to NTHZ in fried bacon was thought to be due to the presence of preformed NTHZ in the liquid smoke. Pensabene and Fiddler (1983a) reported an average of 22.5 ug/kg NTHZ in ten samples of liquid smoke. While formaldehyde and a nitrosating species are likely to be in liquid smoke, the origin of a sulfur containing compound like cysteamine was not explained. Analysis of the five commercial liquid smokes used in the present study revealed no detectable levels of NTHZ. These results indicated that atomized liquid smoke spray did not deposit preformed NTHZ on the surface of the pork bellies, and that the small levels of NTHZ in the smoked bacon came from reaction of formaldehyde in the liquid smoke with meat components. Formaldehyde

has been detected in smoke condensates in concentrations ranging from trace amounts up to 1.68 g/l (Potthast and Eigner, 1985). These researchers speculated that liquid smoke could impart up to 10 g of formaldehyde to treated meat products.

Volatile N-nitrosamine concentrations in the cookout-fat of fried bacon are shown in Table 3. In general, the concentrations of NDMA and NPYR in the cookout-fat followed a similar trend to those observed in the fried bacon samples. Cookout-fat from traditionally smoked bacon contained higher average concentrations of NDMA (2.5 ug/kg) and NPYR (5.3 ug/kg) than the cookout-fat from other smoke-treated bacons. The differences were not statistically significant for the cookout-fat from bacon treated with atomized liquid smoke A or for the cookout-fat from bacon stitch pumped with liquid smoke B and C ($p < 0.10$). The other three cure solubilized liquid smoke treatments generally yielded cookout-fat with significantly lower NDMA and NPYR concentrations ($p < 0.05$). NTHZ was not detected in any of the cookout-fat samples analyzed in this study. These results agree with those of Gray et al. (1982) who suggested that the absence of NTHZ in the cookout-fat might indicate that the major precursors of NTHZ in cured meats were water soluble and would most likely reside in the lean tissue of bacon. In addition, NTHZ may be volatilized from cookout-fat during frying (Hotchkiss and Vecchio, 1985). Pensabene and Fiddler (1983a) demonstrated that NTHZ does not undergo appeciable thermal decomposition during frying, and

Table 3. N-Nitrosamines in the cookout-fat of fried bacon treated with various smokes

Smoke Treatment	Mode of Application	N-Nitrosamine concentration (µg/kg) NDMA	NPYR	NTHZ
Wood smoke	Traditional	2.5±0.7 (1.8-3.4)	5.3±2.9 (2.8-10.2)	ND[e]
Liquid smoke A	Atomized in smokehouse	2.1±0.7[a] (1.3-3.0)	3.1±1.6[a] (1.9-5.3)	ND
B	In brine	2.4±1.4[c] (0.5-4.1)	2.4±2.2[a] (0.5-5.5)	ND
C	In brine	1.7±0.6[a] (1.0-2.3)	2.0±2.4[d] (0.1-5.9)	ND
D	In brine	0.5±0.3[b] (0.2-0.8)	0.3±0.5[b] (0.0-1.2)	ND
E	In brine	1.1±0.3[c] (0.9-1.7)	1.1±0.9[c] (0.6-2.6)	ND

[a] Not significantly different from cookout-fat of woodsmoked bacon ($p < 0.10$)

[b] Significantly different from cookout-fat of woodsmoked bacon ($p < 0.005$)

[c] Significantly different from cookout-fat of woodsmoked bacon ($p < 0.025$)

[d] Significantly different from cookout fat of wooksmoked bacon ($p < 0.05$)

[e] Not detected, limit of detection 0.1 µg/kg

reported low concentrations of NTHZ in the cookout-fat of fried bacon (<2.5 ug/kg). Similarly, Hotchkiss and Vecchio (1985) reported a mean NTHZ value of 3.1 ug/kg in the cookout-fat of fried bacon.

Aqueous Model System Studies with Liquid Smoke

In order to confirm the relative contribution of various liquid smoke preparations to NTHZ formation in bacon, a model system study was carried out in which the liquid smokes were reacted with cysteamine and nitrite to promote N-nitrosamine formation (Table 4). Liquid smoke concentrations in the the model systems were similar to those employed in the brines used to produce the various bacon samples. A woodsmoke condensate was obtained by drawing smoke from the smoke house for six hours through traps which were cooled in ice water. The model system N-nitrosamine data parallel those of the bacon study. The reaction of woodsmoke condensate with cysteamine and nitrite in the model system resulted in the formation of NTHZ in concentrations at least six fold greater than the liquid smokes. N-Nitrosamine levels in bacon processed by woodsmoking were generally higher than those levels in bacon produced with liquid smokes (Table 2). Furthermore, liquid smoke D produced NTHZ concentrations that were at least six times higher than the other liquid smokes designed for stitch pumping (liquid smoke B, C, and E) (Table 4).

Table 4. N-Nitrosothiazolidine formation in an aqueous model system consisting of smoke condensate, cysteamine and nitrite[a]

Smoke condensates	Amount of N-nitrosamine (μg)	
	NTHZ[b]	MeNTHZ[b]
Woodsmoke[c]	2,044.9	81.6
Liquid smoke A	226.3	3.6
B	51.1	8.3
C	22.3	32.4
D	328.3	26.9
E	37.8	ND

[a] Model system consisted of cysteamine (25 mg), nitrite (120 mg), and smoke condensate at manufacturers recommended levels where applicable (Woodsmoke = 3.0%; A = 3.0%; B = 1.8%; C = 1.8%; D = 2.0%; E = 3.1%). Total volume of 100 ml was reacted in flasks for 1 hour at 30°C

[b] NTHZ, N-nitrosothiazolidine; MeNTHZ, 2-Methyl-N-nitrosothiazolidine; ND = not detected

[c] Smoke from the smokehouse was pulled through ice water-cooled aqueous traps for six hours using a vacuum pump

This trend correlates well to the fried bacon data as liquid smoke D was the only cure-solubilized liquid smoke that produced bacon which upon frying contained greater than trace amounts of NTHZ. Liquid smoke A produced relatively high concentrations of NTHZ in the model system, and the atomization of this smoke condensate and its deposition on the surface of bellies yielded fried bacon with detectable concentrations of NTHZ. MeNTHZ was detected when five of the six smoke condensates were reacted in the model system. This would indicate the presence of acetaldehyde in the liquid smoke and would support the hypothesis that formaldehyde in smoke is the precursor of NTHZ. Toth and Potthast (1984) reported that acetaldehyde and formaldehyde were present in woodsmoke at concentrations of 1150 mg and 200 mg, respectively, per 100 grams of wood burned. However, the concentrations of MeNTHZ in the model system were much lower than the NTHZ concentrations and consequently was not detected in any of the smoked bacon samples. This apparent anomaly would seem to indicate that formaldehyde is either much more reactive than acetaldehyde with cysteamine, or that it can also catalyze the nitrosation of the formed thiazolidine.

Analysis of Fried Bacon for N-Nitrosothiazolidine Carboxylic Acid (NTCA)

The results of the analysis of NTCA in raw and fried

bacon are summarized in Table 5. The concentrations of NTCA and NTHZ in fried bacon have been adjusted for fat and moisture loss to facilitate comparisons with the NTCA levels in the raw bacon. This adjustment was performed by frying several packages of randomized sliced bacon and calculating the loss in weight of the bacon as a consequence of frying.

The pattern of NTCA concentrations in the raw bacon was similar to that for NTHZ concentrations in the fried bacon. Woodsmoked bacon contained the highest concentration of NTCA (average 87.3 ug/kg), followed by bacon processed with liquid smoke D (average 76.5 ug/kg) and liquid smoke A (average 37.8 ug/kg). The other liquid smoke treatments resulted in raw bacon with relatively low concentrations of NTCA as compared to the woodsmoke controls. The average concentrations of NTCA in all fried bacon samples when adjusted to account for the loss of fat and moisture during frying were 15 to 45% lower than the average NTCA concentration in raw bacon. Sen et al. (1985) spiked raw bacon with known amounts of NTCA and ascertained that 1 to 3% of the added NTCA was converted to NTHZ under their frying conditions. However, this percent conversion does not account for the differences in NTCA levels between the raw and fried bacon samples in the present study.

The data in Table 5 support the observation of Sen et al. (1985) that NTCA levels in the raw bacon correlate with NTHZ levels in the corresponding samples of fried bacon. If the trace levels of NTHZ in the fried bacon are taken as

Table 5. N-Nitrosothiazolidine carboxylic acid and N-nitrosothiazolidine concentrations in raw and fried bacon produced by different smoke treatments

Smoke Treatment	Mode of Application	N-Nitrosamine concentration (µg/kg) NTCA[a] (raw)	NTCA[b] (fried)	NTHZ[a,b] (fried)
Wood smoke	Traditional	87.3±20.6 (67.6-113.1)	64.1±18.2 (43.5-89.6)	1.5 (0.4-3.0)
Liquid smoke A	Atomized in smokehouse	37.8±9.1[c] (30.4-52.5)	29.0±12.3[c] (14.1-44.5)	0.5 (0.0-0.1)
B	In brine	24.6±6.0[c] (18.8-32.6)	12.9±2.7[c] (9.6-17.0)	0.0 (0.0-0.1)
C	In brine	12.1±1.9[c] (10.5-15.1)	9.9±3.7[c] (7.3-13.4)	0.0 (0.0-0.1)
D	In brine	76.5±21.2[d] (51.2-107.5)	63.6±14.3[d] (49.4-83.0)	0.6 (0.0-1.6)
E	In brine	23.0±5.6[c] (18.5-29.9)	18.3±6.8[c] (6.3-22.8)	0.0 (0.0-0.1)

[a] NTCA, N-nitrosothiazolidine carboxylic acid; NTHZ, N-nitrosothiazolidine

[b] Fried NTCA and NTHZ values were adjusted for fat and moisture loss for comparison with raw bacon data

[c] Significantly different from woodsmoked bacon ($p < 0.005$)

[d] Not significantly different from woodsmoked bacon ($p < 0.05$)

0.1 ug/kg, the concentrations of NTHZ detected in the fried bacon consistently ranged between 0.5 and 2.0% of the NTCA concentrations detected in the raw bacon. This compares well with the 1 to 3% yield of NTHZ obtained by Sen et al. (1985) from raw bacon spiked with NTCA. However, the mechanism of NTHZ formation in bacon requires further elucidation as there are several conflicting reports in the literature. Pensabene and Fiddler (1983a) as well as Mandagere et al. (1984) reported higher NTHZ concentrations in raw bacon than in fried bacon. Hotchkiss and Vecchio (1985) also reported that NTHZ levels in bacon, unlike NPYR levels, did not always increase with frying time. Sen et al. (1985) on the other hand, implicated the intermediacy of NTCA in NTHZ formation based on a comparison to the involvement of N-nitrosoproline in the formation of NPYR (Sen et al., 1976; Lee et al., 1983). During frying, a large percent of the NTHZ formed during the smoking process is probably steam volatilized from the raw bacon. The steam volatility of NTHZ during frying has been recently established by Hotchkiss and Vecchio (1985). During the first few minutes of frying, the temperature of the rasher barely exceeds the boiling point of water (Bharucha et al., 1979). However, after the bulk of the water has been volatilized, N-nitrosamine formation increases as the frying temperature increases (Bharucha et al., 1979) The decarboxylation of NTCA to form NTHZ also probably occurs towards the end of the frying process after the bulk

of the water has been volatilized. Mandagere et al. (1984) has reported that the decarboxylation of NTCA will begin at 108°C. Therefore, it is likely that the decarboxylation of NTCA that will occur towards the end of the frying process is responsible for the parallel relationship between the NTCA concentration of raw bacon and the NTHZ concentration of the fried bacon. It is also possible that a part of the NTHZ present in raw bacon is carried over to the fried product.

Further studies are neccessary to establish the concentration of cysteamine in bacon as this compound could concievably be involved in NTHZ formation in raw bacon (Mandagere et al., 1984). The decarboxylation of NTCA cannot account for the small amounts of NTHZ in raw bacon as the normal smoking temperature (58°C) is not high enough to effect the decarboxylation reaction. Thus, NTHZ formation in raw and fried bacon may arise through two different mechanisms, one involving cysteamine for NTHZ in raw bacon, and the other involving cysteine for fried bacon NTHZ.

Residual Nitrite in Smoked Bacon

The effect of smoke treatments on residual nitrite in bacon is shown in Table 6. Bellies treated with liquid smoke in the curing brine (treatments B, C, D, and E) all had significantly lower ($p < 0.01$) residual nitrite concentrations than

Table 6. Residual nitrite levels in bacon exposed to various smoke treatments

Smoke Treatments	Mode of Application	pH of condensate	Residual nitrite[a] (mg/kg)
Wood smoke	Traditional	------	61.0±10.9 (44.2-72.9)
Liquid smoke A	Atomized in smokehouse	2.2	66.6±24.3[b] (50.4-107.8)
B	In brine	5.3	38.4±7.0[c] (33.2-50.6)
C	In brine	5.5	46.4±11.1[d] (38.9-65.6)
D	In brine	4.5	29.6±4.2[c] (24.6-35.3)
E	In brine	4.6	37.2±8.6[c] (31.1-51.1)

[a] Three determinations per belly were made, five bellies per treatment were used

[b] Not statistically different from woodsmoked bacon ($p < 0.10$)

[c] Statistically different from woodsmoked bacon ($p < 0.005$)

[d] Statistically different from woodsmoked bacon ($p < 0.01$)

bellies treated with either an atomized liquid smoke or woodsmoke. The acid nature of liquid smokes promote the reduction of nitrite to volatile nitric oxides, thus lowering the residual nitrite concentration (Sleeth et al., 1982). This was evident in the present study as the stitch pumping of bellies with liquid smoke D, the most acidic cure-solubilized liquid smoke (pH of 4.5), resulted in raw bacon with the the lowest average residual nitrite concentration (29.6 mg/kg). Treatment of bellies with liquid smoke E, the next most acidic liquid smoke (pH of 4.6), yielded the next lowest average residual nitrite concentration (37.2 mg/kg). The average residual nitrite concentration of the bellies stitch pumped with cure-solubilized liquid smoke appeared to increase as the pH of the smoke condensate increased. Although the pH of liquid smoke A was much lower than the other smoke condensates (pH of 2.2), the atomizing of this liquid smoke on the surface of bellies did not reduce the residual nitrite levels of the resulting bacon in comparison to the woodsmoke controls.

The lower residual nitrite levels observed in raw bacon treated with cure-solubilized liquid smoke (Table 6) correlate well with the lower NDMA and NPYR concentrations detected in the resulting fried bacon in comparison to the wooksmoke controls (Table 2). While three of the four cure-solubilized liquid smoke treated bacon samples contained less NDMA than the woodsmoked bacon, all four had lower concentrations of NPYR in the fried bacon. The bacon treated with atomized

liquid smoke A did not differ appreciably in residual nitrite levels from the woodsmoked raw bacon, and the resulting fried bacon contained only slightly less NDMA and NPYR than the woodsmoked control.

The NTHZ concentrations in fried bacon prepared with the various smoke treatments appeared to be independent of the residual nitrite levels in the raw bacon. For example, raw bacon stitched pumped with liquid smoke D contained the lowest average concentration of residual nitrite. The analysis of the resulting fried bacon, however, revealed the highest average NTHZ concentration among the bacon samples prepared with liquid smoke. Raw bacon processed with liquid smoke C contained 50% more residual nitrite than bacon prepared with liquid smoke D, yet only trace concentrations of NTHZ were detected in the resulting fried bacon. These results are in agreement with those of Pensabene and Fiddler (1983a), who observed that the nitrite content of raw bacon did not appear to influence the NTHZ concentration of fried bacon. This was particularly apparent in the case of a nitrite-free bacon which was found to contain 3.9 ug/kg NTHZ after smoke processing.

II Phenols in Liquid Smoke Condensates

In order to ascertain if there was a relationship between

the concentration of phenols in smoked bacon, residual nitrite, and N-nitrosamine concentrations of the fried product, the phenol contents of the various liquid smokes were analyzed by the method of Lustre and Issenberg (1970). It has been demonstrated that this method is somewhat selective and that the concentrations of certain phenols such as trans-isoeugenol and propenylsyringol will be underestimated (Knowles et al., 1975). These researchers suggested that the partial solubility of their sodium salts in diethyl ether or incomplete salt formation may result in the partitioning of these phenols in the neutral fraction.

Knowles et al. (1975) also analyzed the phenol content of bacon prepared by several smoking techniques, again using a method outlined by Lustre and Issenberg (1970). This method is very similar to the procedure employed for the analysis of smoke condensates and suffers from the same problem of selectively extracting certain phenols. However, Knowles et al. (1975) proposed that some of the differences between the condensates and the corresponding bacon were due to the selective uptake of the phenols by bacon. This selective uptake was found to be dependent on the method of applying the smoke condensate. The incorporation of liquid smoke in the curing brine favored an uptake of guaiacol, phenol, 4-methylguaiacol, and m- and p-cresol, with negligible uptake of syringol and its derivatives, or the eugenols. Traditionally smoked bacon contains a phenol profile that

more closely resembles that of smoke condensates, with higher levels of guaiacol and its derivitives.

In the present study, the phenol content of the smoke condensates was analyzed rather than that of the treated bacon because of the selective extraction of phenols inherent in either method, and the difficulty in isolating phenols from the meat constituents. In addition, differences in phenol concentrations of smoke-treated bacon will most likely reflect differences in the phenol content of the corresponding smoke condensate.

The major phenols in liquid smoke were seperated by capillary column-gas chromatography (Figure 7) and quantitated (Table 7). The identities of 13 phenols were confirmed by mass spectrometry. The mass spectra of these phenols are presented in Appendix 1. The total measured phenol concentration was calculated and multiplied by the liquid smoke manufacturer's recommended usage level to obtain the theoretical phenol concentration of the bellies. While the liquid smokes with the lower phenol concentrations had slightly higher recommended usage levels, large discrepancies between treatment groups in the theoretical phenol concentration of raw bacon still existed. For example, liquid smoke A contained a five fold higher concentration of measured phenol than did liquid smoke E. Although the recommended usage level of liquid smoke E in the curing brine was higher, the theoretical concentration of liquid smoke A in bacon was three fold greater than

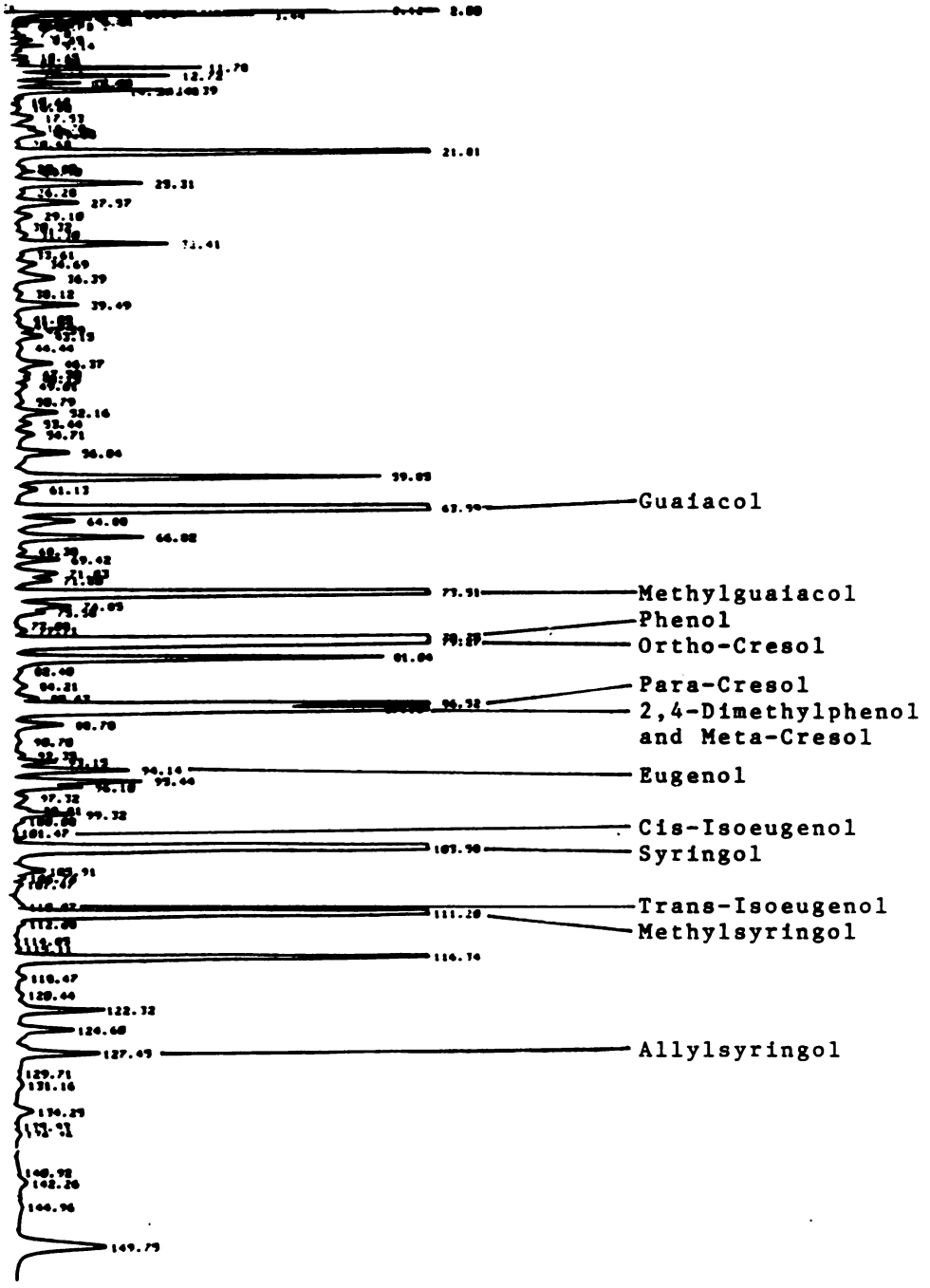


Figure 7. Analysis of phenols of liquid smoke by capillary column-gas chromatography

Table 7. Major phenol concentrations from ether extracts of liquid smokes used in the curing brines of bacon

	µg phenol/ml liquid smoke			
Liquid smoke	B	C	D	E
guaiacol	2,692	1,656	938	422
methylguaiacol	1,560	1,056	600	294
phenol	1,856	981	398	193
o-cresol	768	417	153	60
p-cresol	524	293	138	43
m-cresol and 2,4 dimethyl phenol	839	482	160	28
eugenol	105	69	53	16
cis isoeugenol	82	54	51	10
syringol	4,075	2,211	2,211	1,392
trans isoeugenol	32	125	61	16
4-methylsyringol	2,154	1,575	967	548
4-allylsyringol	381	298	135	94
Total measured phenol	15,174	9,732	5,870	3,120
Manufacturers recommended level in meat	0.19%	0.19%	0.20%	0.31%
Theoretical concentration in meat (µg/g)	28.8	18.5	11.7	9.7

in bacon treated with liquid smoke E.

There appeared to be no relationship between the theoretical phenol concentration of the bacon treated with cure solubilized liquid smoke (Table 7) and the residual nitrite content of raw bacon (Table 6). For example, bellies stitch pumped with a brine containing liquid smoke E had a theoretical phenol concentration approximately one third that of bellies treated with liquid smoke B. The average residual nitrite content for bellies treated with these two smoke condensates were, however, nearly equal. The pH of the liquid smoke appeared to correlate much better with the residual nitrite concentrations of the raw bellies than did the theoretical phenol content of the corresponding bellies. The N-nitrosamine concentrations in fried bacon did not appear to be greatly influenced by the presence of phenols in the meat system. Bacon cured with a brine that included liquid smoke E (the smoke condensate with the lowest concentration of phenols) yielded fried bacon with the lowest concentration of NDMA and NPYR. Bacon treated with liquid smoke C theoretically contained twice the concentration of phenols than did bacon processed with liquid smoke E, yet the resulting fried bacon had a three fold greater average NDMA concentration, and a nearly two fold higher average concentration of NPYR.

The concentration of individual phenols, expressed as a percentage of the total phenol fraction, is shown in Table 8. In addition to the cure solubilized liquid smokes, liquid

Table 8. Major phenol concentrations of ether extracts from various smoke condensates.

Liquid smoke	Percent of total phenol fraction					
	A	B	C	D	E	TS[a]
guaiacol	8.5	13.5	12.4	8.5	7.7	13.6
methylguaiacol	5.9	8.4	8.5	5.9	5.7	11.1
phenol	6.1	11.8	9.3	4.6	4.5	9.5
o-cresol	1.5	4.4	3.5	1.6	1.3	3.7
p-cresol	1.3	3.4	2.8	1.6	1.0	4.0
m-cresol and 2,4-dimethylphenol	2.0	5.3	4.6	1.9	1.3	4.9
eugenol	0.2	0.6	0.6	0.5	0.3	0.6
cis-isoeugenol	0.3	0.3	0.3	0.3	0.1	0.3
syringol	21.3	17.4	17.4	18.0	21.6	9.3
trans-isoeugenol	0.1	0.5	0.7	0.4	0.2	0.4
4-methylsyringol	7.5	8.7	9.5	7.6	8.1	3.2
4-allylstringol	1.2	1.2	1.4	0.8	1.1	0.6

[a] TS is a smoke condensate prepared by drawing smoke from the smokehouse through ice cooled aqueous traps using a vacuum pump.

smoke A, the smoke condensate atomized in the smokehouse, was analyzed. The phenols of the smoke condensate prepared from smoke drawn from the smokehouse were also quantitated and are shown in Table 8. The results support the observations of Potthast (1976) who noted that liquid smoke preparations differ in total amounts of phenols as well as in individual phenol concentrations. Although all of the smoke condensates analyzed in this study were made from hardwood sawdust, levels of major phenols varied markedly. The concentration of guaiacol in the various smoke condensates ranged from 7.7 to 13.6% of the total phenol content, while the syringol concentrations varied between 9.3 to 21.6% Higher levels of syringol and its derivatives have been reported in smoke condensates prepared from hardwood sawdust (Toth, 1980).

The results in Table 8 compare very well to those of Toth (1982) who analyzed the phenolic compounds in ten liquid smoke preparations. Toth (1982) reported guaiacol concentrations in the smoke condensates ranging from 5.7 to 10.8% of the total phenol content, while syringol levels varied from 11.0 to 20.5%. The phenol levels varied between 4.5 amd 11.8% of the total phenol concentration in the present study, while the phenol concentrations ranged between 4.2 and 9.8% in the smoke condensates analyzed by Toth (1982). The corresponding levels of guaiacol, syringol, and phenol reported by Knowles et al. (1975) were lower than those reported by Toth (1982) or those shown in Table 8. This may

be due to a better recovery of trans-isoeugenol and allyl syringol achieved with the analytical procedure employed by Knowles et al. (1975). Indeed, these researchers reported trans-isoeugenol levels ranging from 7.6 to 11.9% of the total phenolic content of three liquid smokes. The results shown in Table 8 agree with those of Toth (1982), who found trans-isoeugenol composed less than one percent of the total phenol content of ten liquid smokes.

III The Effect of pH on N-Nitrosothiazolidine Formation

An aqueous model system was employed to investigate the effect of pH on NTHZ formation. The effect of pH on the formation of NTHZ from thiazolidine and nitrite is shown in Figure 8. In general, the yield of NTHZ increased with decreasing pH. However, there was no optimum pH for NTHZ formation. A similar trend was reported by Ohshima et al. (1984) for NTCA formation from thiazolidine carboxylic acid and nitrite. The yield of NTCA increased with decreasing pH, and no optimum pH was observed over the pH range of 1.0 to 6.0.

The formation of NTHZ from the reaction of formaldehyde, cysteamine, and nitrite over the pH range of 1.0 to 7.0 is shown in Figure 9. The yield of NTHZ formation increased as the pH decreased from 7.0 to the optimum pH for this

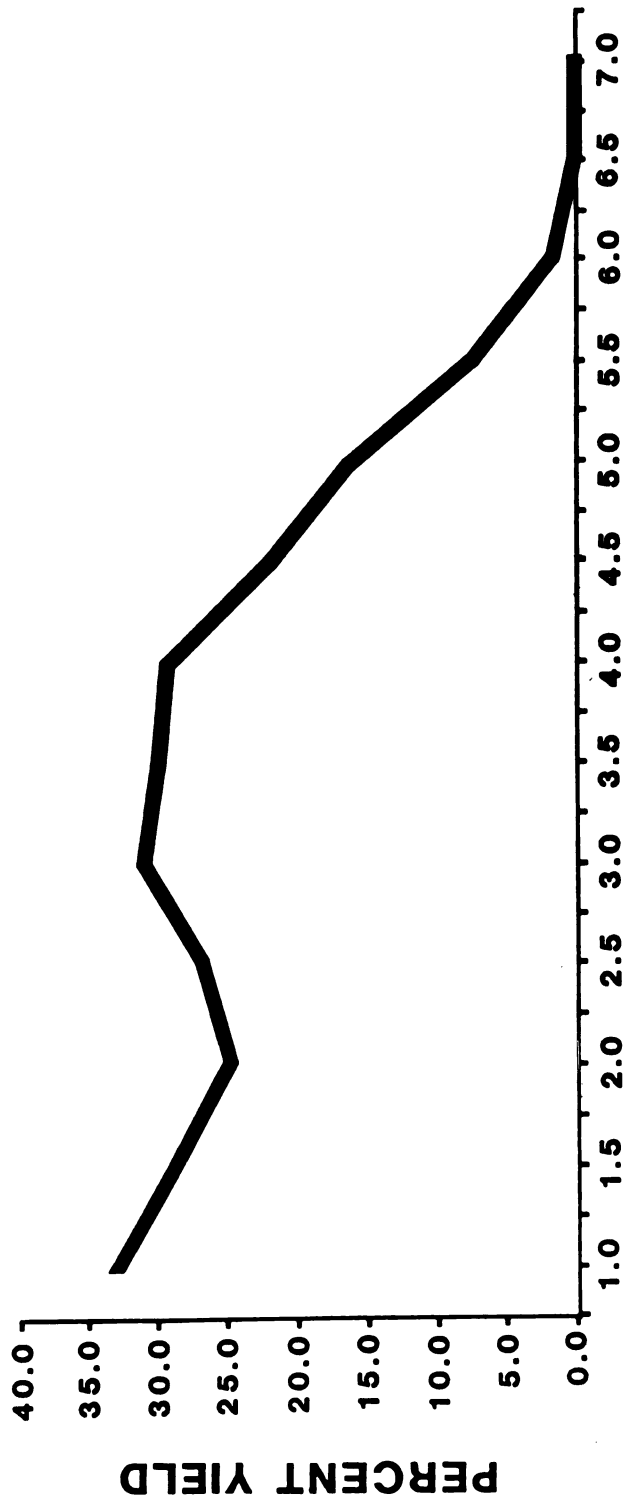


Figure 8. The influence of pH on the formation of N-nitrosothiazolidine from thiazolidine and nitrite

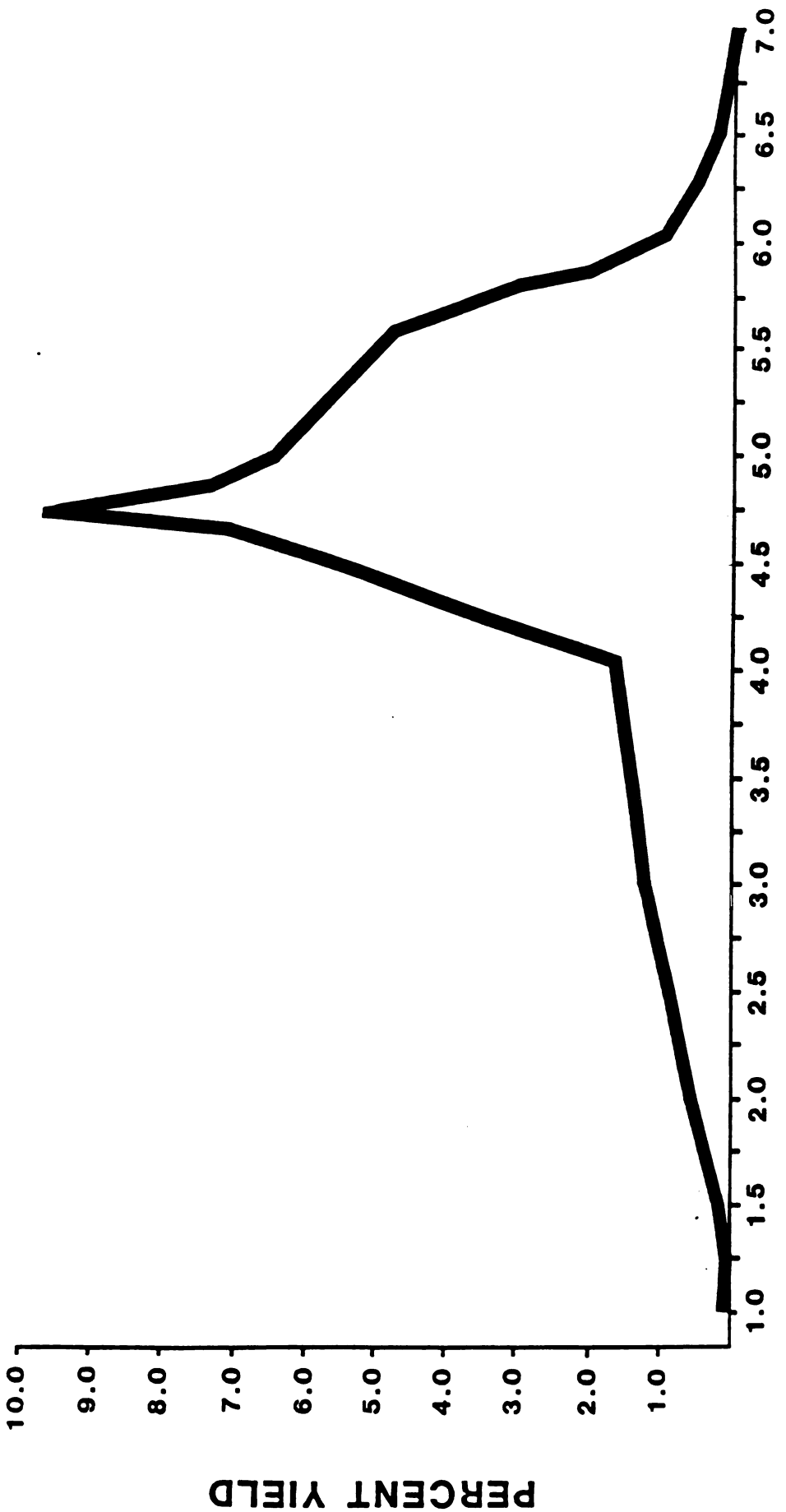


Figure 9. The influence of pH on the formation of N-nitrosothiazolidine from cysteamine, formaldehyde and nitrite

reaction, 4.75. As the pH was decreased from 4.75, a rapid decline in the yield of NTHZ occurred until a pH of 4.0 was reached. At this point, a more gradual decline in the yield of NTHZ was observed as the pH was decreased from 4.0 to 1.0.

The pattern of NTHZ formation shown in Figure 8 is in sharp contrast to that obtained from the reaction of thiazolidine and nitrite shown in Figure 9. The cysteamine/formaldehyde/nitrite reaction demonstrated a definite optimum pH, while the reaction of thiazolidine and nitrite yielded no clearcut pH of maximum formation. While the nitrosation of thiazolidine increased as the pH of the aqueous system was decreased to 1.0, the yield of NTHZ from cysteamine, formaldehyde, and nitrite decreased as the pH approached 1.0. The yield of NTHZ from cysteamine, formaldehyde, and nitrite at optimum pH was approximately 10% of the theoretical yield, while the nitrosation of thiazolidine resulted in NTHZ formation at approximately 35% of the theoretical yield.

The optimum pH of 4.75 for the reaction of cysteamine, formaldehyde, and nitrite has important implications in regard to the occurrence of NTHZ formation in a meat system. The more neutral pH of the cure solubilized liquid smokes (averaging 4.98 in this experiment), as well as the slightly acidic pH of a meat system would favor the formation of NTHZ. The highly acidic properties of the atomized liquid smoke (pH 2.2) or the predominance of acidic compounds on the surface of traditionally smoked meat reduces the formation of NTHZ in

smoked foods. However, the formation of NTHZ is generally greater in products exposed to a surface smoke treatment because of the greater concentration of NTHZ precursors at the surface of the meat.

IV In Vivo Formation of N-Nitroso Compounds

The endogenous formation of N-nitroso compounds has been proposed to be a major source of human exposure to these toxic compounds (Tannenbaum, 1980). In the present study, the possible role of liquid smoke in endogenous NTCA formation was investigated, as was the influence of known inhibitors and catalysts.

Individual precursors of NTCA as well as combinations of two precursors were administered to rats to monitor their influence on background excretion levels of NTCA. The results are shown in Table 9. The administration of saline to rats via gavage produced no measurable levels of NTCA in the urine. In addition, NTCA was not detected in the urine of rats administered either nitrite, formaldehyde, or cysteine. Rats treated with formaldehyde and nitrite excreted urine that contained trace concentrations of NTCA. This result suggested that small concentrations of free cysteine in the rat were available for NTCA formation. Other treatments involving the administration

Table 9. *In vivo* formation of N-nitrosamino acids in rats administered precursor compounds via gavage

Reactants[a,b]	Number of rats	% Average recovery[c]	Yield (nmole/rat per 24 hours) NPRO[d]	MeNTCA[d]	NTCA[d]
Saline	6	82.7	-	-	-
CYS	6	80.2	-	-	-
$NaNO_2$	6	78.3	-	-	-
FOR	6	80.9	-	-	-
CYS, $NaNO_2$	6	95.0	-	-	-
CYS, FOR	6	87.1	-	-	-
FOR, $NaNO_2$	6	94.0	-	-	tr
CYS, FOR, $NaNO_2$	11	85.5	-	-	1763.5±347.5 (1257.3-2229.3)
CYS, ACET, $NaNO_2$	6	91.7	-	1883.7±413.5 (1529.5-2432.7)	-

[a] CYS = cysteine, FOR = formaldehyde, NaNO = sodium nitrite, ACET = acetaldehyde

[b] Rats were treated with cysteine (0.05 moles/L), 0.5 ml; nitrite (0.025 moles/L), 1.0 ml; formaldehyde (0.5 moles/L), 0.5 ml; and saline (0.9%), 2.0 ml

[c] Average recovery of internal standard, N-nitrosopipecolic acid

[d] NPRO = N-nitrosoproline, MeNTCA = Methyl-N-nitrosothiazolidine carboxylic acid, NTCA = N-nitrosothiazolidine carboxylic acid, tr = trace.

of two NTCA precursors (cysteine/nitrite; cysteine/formaldehyde) produced no detectable levels of NTCA in the collected urine. However, Ohshima et al. (1984) generally reported somewhat higher NTCA levels in the urine of rats when fed similar concentrations of precursors. These investigators also reported that the treatment of rats with formaldehyde or cysteine essentially resulted in no NTCA formation, while an oral dose of nitrite yielded less than one nmole of NTCA in urine excreted over 24 hours. As in the present study, Ohshima et al. (1984) detected the highest concentration of NTCA (2.67 nmoles/24 hours) in the urine of rats treated with formaldehyde and nitrite as compared to the other two possible combinations of NTCA precursors.

The treatment of rats with either formaldehyde or acetaldehyde in combination with cysteine and nitrite resulted in approximately similar concentrations of NTCA (1763.5 nmoles/ 24 hours) and MeNTCA (1883.7 nmoles/24 hours), respectively, in the urine (Table 9). These results contrast to those of the aqueous model system studies (Table 4) in which greater concentrations of NTHZ as opposed to MeNTHZ were produced from the reaction of liquid smokes with cysteamine and nitrite. This occurred despite the fact that there are greater concentrations of acetaldehyde (the precursor of MeNTCA) than formaldehyde (the precursor of NTHZ) woodsmoke/liquid smokes. The more acidic environment of the rats stomach in comparison to the mildly acidic aqueous model system may

favor increased formation of MeNTCA relative to NTCA. Ohshima et al. (1984) treated rats with concentrations of NTCA precursors that were 20% less than those employed in the present study. These authors obtained urinary NTCA concentrations that were approximately half those detected in the present study and MeNTCA concentrations that were approximately a fifth of those shown in Table 9. Differences in the strain of rat employed in the present study and that used by Ohshima et al. (1984) may be responsible for the disparity in the yields of NTCA and MeNTCA in rat urine. In addition, different precursor administration procedures between the two studies could result in different initial reaction rates and different ultimate yields of N-nitroso compounds in urine.

The levels of NTCA detected in the urine of rats treated with liquid smoke, cysteine, and nitrite are shown in Table 10. Two of the liquid smokes investigated in the aqueous model system were utilized in the rat feeding trials. Liquid smoke D yielded higher concentrations of NTHZ in the aqueous model system than any other smoke condensate (Table 4), and also resulted in the formation of higher NTHZ concentrations in fried bacon (Table 2). Liquid smoke E, on the other hand, resulted in low NTHZ concentrations in both the model and bacon systems. A similar pattern was observed for the endogenous formation of NTCA in rats. Treating rats with liquid smoke D, cysteine and nitrite resulted in 24 hour urine samples which had a nine fold higher concentration of NTCA than

Table 10. *In vivo* N-nitrosamino acid formation in rats treated with liquid smoke, nitrite and cysteine.

Reactants[a]	Number of rats	% Average recovery[b]	Yield (nmoles/24 hours per rat)	
			MeNTCA	NTCA
Liquid smoke D, CYS + $NaNO_2$	6	89.5	11.3±3.0 (7.7-16.1)	796.0±288.0 (475.3-1313.0)
Liquid smoke E, CYS + $NaNO_2$	6	96.9	--	82.8±27.7 (44.2-114.0)

[a] Liquid smoke, 0.5 ml; sodium nitrite (0.025 moles/L), 1.0 ml; cysteine (0.05 moles/l), 0.5 ml

[b] Average recovery of internal standard, N-nitrosopipecolic acid

those of rats given liquid smoke E. A similar trend was observed in the aqueous model system study where liquid smoke D produced an approximate nine fold higher concentration of NTHZ than did liquid smoke E when reacted with cysteamine and nitrite (Table 4). MeNTCA was detected in the urine of rats treated with liquid smoke D, but not in the urine of rats treated with liquid smoke E. These results are similar to those obtained from the aqueous model system (Table 4). MeNTHZ was produced from the reaction of liquid smoke D with cysteamine and nitrite, but not when liquid smoke E was reacted. These results again suggest that the acetaldehyde content of liquid smoke D is greater than the acetaldehyde level in liquid smoke E.

The endogenous formation of NTCA in experimental animals confirms the presence of formaldehyde in liquid smoke and its reactivity towards cysteine and nitrite in vivo. The toxicological implications of these findings are less clear. Keefer and Roller (1973) have demonstrated that formaldehyde can act as a powerful catalyst of N-nitroso compound formation and extends the pH range of the reaction into the alkaline region. The use of liquid smoke as a flavoring agent in products which have not undergone heat treatment could lead to in vivo catalysis of N-nitroso compound formation. In addition, formaldehyde may contribute to endogenous formation of NTCA and possibly NTHZ in humans. Potthast and Eigner (1985) have estimated that heavily smoked meats contain a maximum of 5 mg/kg

of formaldehyde and its presence, therefore, is of little toxicological importance. The relationship of formaldehyde to NTCA formation *in vivo* or in a meat system was not considered, however.

The inclusion of potential inhibitors of N-nitrosamine formation in the oral dose administered to rats reduced *in vivo* NTCA formation to varying degrees (Table 11). Ascorbic acid was very effective in blocking NTCA formation. The amount of NTCA detected in urine from rats treated with ascorbic acid/cysteine/formaldehyde/nitrite was 88% less (204.3 nmoles/24 hours) than the control rats treated with the NTCA precursors alone (1763.5 nmoles/24 hours). Ascorbate has been reported to be effective in blocking the nitrosation of amino compounds in humans (Ohshima and Bartsch, 1981). These investigators reported that ascorbate inhibited the *in vivo* nitrosation of proline in humans by 80 to 85% when ingested simultaneous with beet juice and proline. The resulting levels of NPRO were nearly equal to the background levels excreted by the control subjects.

α-Tocopherol was not as effective in blocking *in vivo* NTCA formation as ascorbic acid. The urine of rats treated with α-tocopherol/cysteine/formaldehyde/nitrite contained 14% less NTCA (1499.8 nmoles/24 hours) than did the urine from rats administered the NTCA precursors alone (1763.5 nmoles/24 hours). The difference in NTCA levels between these two treatment groups was not statistically significant ($p < 0.10$). The

Table 11. The influence of potential inhibitors on *in vivo* NTCA formation in rats.

Reactants[a,b]	Number of rats	% Average[c] recovery	NTCA Yield (nmoles)	% Inhibition
CYS, FOR, $NaNO_2$	11	85.5	1763.5±347.5 (1257.3-2229.3)	--
CYS, FOR, $NaNO_2$, AA	6	88.0	204.3±67.6[d] (104.1-280.5)	88.2
CYS, FOR, $NaNO_2$, TOC	6	88.4	1499.8±358.5[e] (1100.5-1954.8)	13.7
CYS, FOR, $NaNO_2$, LS A Phenols (unsol)	6	84.3	1740.5±478.6[e] (955.4-2270.9)	--
CYS, FOR, $NaNO_2$, LS A Phenols (sol)	6	94.3	1444.3±425.2[e] (1035.3-2063.3)	16.9

[a] AA = ascorbic acid, α-TOC = α-tocopherol-coated salt, LS A = liquid smoke A (solubilized with polysorbate 80 or unsolubilized)

[b] Cysteine (0.05 moles/l), 0.5 ml; formaldehyde (0.05 moles/L), 0.5 ml; sodium nitrite (0.025 moles/L), 1.0 ml; liquid smoke phenol, 0.5 ml ascorbate (0.10 moles/L), 0.5 ml; α-tocopherol (0.05 millimoles/L) on salt, 0.5 ml.

[c] Average recovery of internal standard, N-nitrosopipecolic acid

[d] Significantly different from CYS/$NaNO_2$/FOR samples ($p < 0.005$)

[e] Not significantly different from CYS/$NaNO_2$/FOR samples ($p < 0.10$)

reduced efficacy associate with α-tocopherol in comparison to ascorbate is due to the fact that the dosage of α-tocopherol was 2000 times less concentrated than the ascorbate solution on a molar basis. α-Tocopherol-coated salt was employed in the present study because this product is a practical way of including α-tocopherol in food products. Further studies employing higher concentrations of solubilized α-tocopherol are neccessary to evaluate the efficacy of this blocking agent in vivo. Ohshima et al. (1981) had human volunteers consume a small wafer capsule of α-tocopherol (500 mg) while solutions of proline and beet juice were ingested. α-Tocopherol was not effective as ascorbate, but did reduce NPRO excretion by 50% in the 24 hour urine.

In order to ascertain the influence of the phenols of liquid smoke on in vivo NTCA formation, an ether extract of liquid smoke A was administered to rats with the NTCA precursors. After removal of the ether, the phenols were administered to the rats in acetone because of the high solubility of the phenols in this solvent. The urine excreted by the treated rats contained high concentrations of a compound that eluted at the same time as MeNTCA. Confirmation of the identity of this compound by mass spectrometry and further studies on the formation of NTCA related compounds from ketones was not performed. The acetone probably reacted with cysteine in the rats stomach to form MeNTCA or a closely related N-nitroso compound. It is also conceivable that acetone was metabolized

by the rats to form acetaldehyde or some similar compound. Acetone is one of the ketone bodies and is formed as a result of the slow, spontaneous decarboxylation of acetoacetate (Stryer, 1975). Acetone, acetoactate, and β-hydroxybutyrate are synthesized in the liver and are transported through the bloodstream to peripheral tissues for energy utilization (Lehninger, 1977). In the peripheral tissues, the ketone bodies are converted to two carbon units (acetyl-CoA), which may then enter the tricarboxylic acid cycle. It is possible that a high concentration of cysteine in the blood of treated rats may react with the metabolic products of acetone to form NTCA. The starving of the rats overnight prior to gavaging may have important implications to in vivo NTCA formation. In fasting individuals or those with the disease diabetes mellitus, ketones can accumulate in the blood resulting in the condition known as ketosis (Lehninger, 1977). Renal conservation of ketone bodies also increases as the central nervous system and kidneys increase ketone utilization so as to reduce the need for glucose (Hawkins et al., 1971). Therefore, the starvation of rats to the point of increasing blood ketone levels could increase in vivo formation of MeNTCA.

The phenol fraction from liquid smoke was subsequently contained in an aqueous medium. The phenols appeared to be a dark oil that had only slight solubility in the aqueous solution. When shaken vigorously, the oil would break up to form a dispersion of tiny droplets. The gavaging of rats with

the phenol fraction of liquid smoke A dispersed in water along with the precursors of NTCA resulted in NTCA concentrations almost identical to the controls (Table 11). Rats treated with the unsolubilized phenols of liquid smoke A/cysteine/formaldehyde/nitrite excreted urine containing 1740.5 nmoles per 24 hours, while the urine of those given cysteine/formaldehyde/nitrite averaged 1763.5 nmoles per 24 hours.

The phenol fraction of liquid smoke A was again isolated and solubilized in an aqueous environment using polysorbate 80. The solubilized phenol fraction inhibited the formation of NTCA by 16.9%. The difference in the amount of NTCA in the 24 hour urine of rats treated with solubilized phenols and NTCA precursors and those administered just NTCA precursors was not statistically significant ($p < 0.10$). The improved dispersion of the solubilized phenols in the aqueous solution probably increased the inhibitory efficiency of these compounds.

Thiocyanate is a powerful catalyst of N-nitrosamine formation in aqeous systems under moderately acidic conditions (Fan and Tannenbaum, 1973a). Ladd et al. (1984) reported that smokers have three to four times higher levels of salivary thiocyanate than nonsmokers. These authors suggested that the higher level of salivary thiocanate in smokers may be responsible for the increased rate of gastric nitrosation of proline observed for these individuals in comparison to nonsmokers.

The influence of potential catalysts on NTCA formation from the cysteine/formaldehyde/nitrite reaction is shown in Table 12. In addition to the NTCA precursors, rats were treated with 1.0 ml of a thiocyanate solution (0.01 moles/L) at a concentration similar to the thiocyanate content found in the saliva of smokers (Ladd et al., 1984). Thiocyanate did not catalyze the formation of NTCA in vivo. Rats administered thiocyanate and NTCA precursors excreted urine containing 34% less NTCA than those rats given NTCA precursors alone. The difference in urinary NTCA concentrations between the control and thiocyanate treated rats was not, however, significant ($p < 0.10$).

A more accurate representation of endogenous N-nitroso compound formation in humans was performed by administering nitrate to rats rather than nitrite. Dietary nitrate is absorbed from the gut and rapidly distributed throughout the body via the blood stream (Spiegelhalder et al., 1976). The nitrate is excreted into the oral cavity via the salivary glands where oral microflora can reduce nitrate to nitrite. In the present study, rats were deprived of water for 24 hours and then allowed to consume a nitrate solution ad libitum. Two hours after the rats were given the nitrate solutions, the control group was treated with cysteine and formaldehyde, while another group was administered cysteine, formaldehyde, and thiocyanate. Because the rats consumed different amounts of the nitrate solution, the concentrations of urinary NTCA

Table 12. The influence of potential catalysts on *in vivo* NTCA formation in rats

Reactants[a,b]	Number of Rats	% Average recovery[c]	NTCA yield (nmoles)	% Catalysis
CYS, FOR, $NaNO_2$	11	85.5	1,763.5±347.5 (1,257.3-2,229.3)	--
CYS, FOR, $NaNO_2$, SCN	6	91.6	1,139.8±636.7 (476.0-2,031.0)	-34.4[d,e]
TCA, $NaNO_2$	6	83.9	7,330.7±679.1 (6,291.6-8,116.5)	--
TCA, FOR, $NaNO_2$	5	83.7	7,777.8±1885.0 (4,487.3-9,671.2)	6.1[d]

[a] SCN = thiocyanate; TCA = thiazolidine carboxylic acid

[b] Cysteine (0.05 moles/L), 0.5 ml; formaldehyde (0.05 moles/L), 0.5 ml; sodium nitrite (0.025 moles/L), 1.0 ml; potassium thiocyanate (10 millimoles/L), 1.0 ml; thiazolidine carboxylic acid (0.05 moles/L), 0.5 ml

[c] Average recovery of internal standard, N-nitrosopipecolic acid

[d] Not significantly different from control ($p < 0.10$)

[e] Negative sign indicates inhibiton of reaction occurred

were expressed as nmoles per ml nitrate consumed (Table 13). Again, the results indicated that thiocyanate does not catalyze *in vivo* NTCA formation. Rats treated with thiocyanate had a 12% lower concentration of NTCA per ml of nitrate consumed in their 24 hour urine than did the contol rats. The difference was not statistically significant ($p < 0.10$) These results do not support the hypothesis of Ladd et al. (1984) who suggested that the greater excretion of NPRO for smokers is due to their higher salivary thiocyanate concentrations. It is possible that species differences between rats and humans, such as differences in stomach pH could affect the catalysis of *in vivo* NTCA formation. Further studies are neccessary to ascertain the effect of thiocyanate concentration on this reaction. Fan and Tannenbaum (1973a) reported that while thiocyanate catalyzed N-nitrosomorpholine formation in an aqueous system, increased thiocyanate concentrations decreased the rate of morpholine nitrosation.

Higher excretion rates of NTCA and MeNTCA for smokers have been reported by Ohshima et al. (1984). These authors suggested that the higher concentrations of these N-nitroso compounds are due to the presence of formaldehyde and acetaldehyde in cigarette smoke. Formaldehyde has been reported to catalyze N-nitrosamine formation by forming an iminium ion with the secondary amine (Keefer and Roller, 1973). After attack by a nitrite ion, the adduct collapses to form an N-nitrosamine. In the present study, rats were used to

Table 13. The influence of thiocyanate on NTCA formation from cysteine, formaldehyde and nitrate

Reactants[a]	Number of rats	% Average[b] recovery	NTCA (nmoles) per ml NaNO consumed	% Catalysis
CYS, FOR, $NaNO_3$	6	84.3	3.05=1.58 (1.17-5.73)	--
CYS, FOR, $NaNO_3$, SCN	6	93.1	2.70=0.60[c] (1.69-3.34)	-12.0[d]

[a] Cysteine (0.05 moles/L), 0.5 ml; formaldehyde (0.05 moles/L), 0.5 ml $NaNO_3$ (0.25 moles/L), ad libitum; thiocyanate (10 millimoles/L), 1.0 ml

[b] Average recovery of internal standard, N-nitrosopipecolic acid

[c] Not significantly different from control ($p < 0.10$)

[d] Negative sign indicates inhibition of NTCA formation

investigate the influence of formaldehyde on the nitrosation of thiazolidine carboxylic acid (TCA) (Table 12). The rats treated with formaldehyde excreted urine containing 6% higher concentrations of NTCA. The difference between this treatment group and the control rats was not significantly different ($p < 0.10$). Further studies are neccessary to determine if there is a certain ratio of reactants at which catalysis by formaldehyde becomes significant. If so, the presence of formaldehyde in products treated with liquid smoke could influence *in vivo* N-nitroso compound formation.

SUMMARY AND CONCLUSIONS

The influence of liquid smoke on N-nitrosamine formation in bacon was investigated, with particular reference to their role in NTHZ formation. Formaldehyde is a precursor of NTHZ and is probably present in all liquid smokes. The incorporation of liquid smokes into the curing brine of bacon could influence NTHZ concentrations in fried bacon. Phenols are also present in liquid smoke and have been reported to be catalysts of N-nitrosamine formation in aqueous systems. The possible contribution of selected compounds on NTHZ and NTCA formation *in vivo* was also investigated.

Fried bacon processed by cure-solubilized liquid smoke contained less NDMA than the woodsmoked control samples. A more pronounced reduction was observed in the concentrations of NPYR for fried bacon treated with cure-solubilized liquid smoke in comparison to the woodsmoked bacon. Bellies treated with an atomized liquid smoke produced bacon containing only slightly less NDMA and NPYR than the woodsmoked controls.

Woodsmoke-treated bacon contained a higher concentration of NTHZ than did the liquid smoke treated bacon samples. Bacon treated with one of the cure-solubilized liquid smokes contained greater concentrations of NTHZ than either the bacon treated with an atomized liquid smoke or the bacon treated with the other three cure-solubilized liquid smokes. This

pattern was repeated in an aqueous model system containing smoke condensates, cysteamine and nitrite. Results of these studies demonstrated that the model system can be employed to predict approximate NTHZ levels in cure-solubilized liquid smoke treated bacon. MeNTHZ was also detected in some of the model system samples indicating the presence of acetaldehyde in the liquid smokes. NTHZ was not found in any of the cookout-fat samples, indicating that the precursors of NTHZ are unlikely to be present in the adipose tissue of bacon.

The concentration of NTCA in raw bacon paralleled the NTHZ levels in the corresponding fried bacon. NTCA concentrations of the fried bacon were approximately 35% lower than those in raw bacon after correcting for fat and moisture loss. These results support the hypothesis that NTCA is decarboxylated during frying, probably after the bulk of the water has volatilized. The majority of the NTHZ thus formed is also volatilized, while a fraction remains in the rasher. The NTHZ content of the fried bacon thus reflects the NTCA content of the raw bacon.

Nitrite analysis of the raw bacon revealed an apparent relationship between the residual nitrite values and the pH of the corresponding cure-solubilized liquid smoke. Residual nitrite concentration of the raw bacon decreased as the pH of the liquid smoke decreased. The atomized liquid smoke-treated bacon and the woodsmoke controls contained higher levels of residual nitrite than did the cure-solubilized liquid smoke

treated bacon. The lower residual nitrite levels observed in the cure-solubilized liquid smoke treated bacon correlated well with the lower concentrations of NDMA and NPYR detected in the resulting fried bacon.

An analysis of the phenols in the liquid smokes revealed a wide variance in the total phenol content among the smoke condensates. There appeared to be no relationship between the theoretical phenol concentration in the bacon treated with cure-solubilized liquid smoke, the residual nitrite concentration of the corresponding raw bacon, and the N-nitrosamine content of the resulting fried bacon.

The nitrosation of thiazolidine in an aqueous system generally increased as the pH was reduced from 7.0 to 1.0. The formation of NTHZ from cysteamine, formaldehyde, and nitrite, however, was optimal at pH 4.8 and decreased as the pH was raised or lowered.

Rats treated by gavage with either cysteine, formaldehyde, or nitrite excreted urine containing no detectable levels of NTCA. Treating rats with nitrite and formaldehyde produced trace amounts of NTCA in their urine. When either of two liquid smokes were administered to rats in combination with cysteine and formaldehyde, the excreted urine contained NTCA concentrations that paralleled the respective concentrations of NTHZ obtained in the aqueous model system. Ascorbate inhibited the *in vivo* formation of NTCA by nearly 90% when given to rats simultaneously with cysteine, formaldehyde, and

nitrite. α-Tocopherol was much less effective than ascorbate in blocking NTCA formation. However, the concentration of α-tocopherol employed was much less because of the difficulty in solubilizing this compound. The phenol fraction of a liquid smoke also had a slight inhibitory effect on the *in vivo* formation of NTCA. These compounds also had to be solubilized to have any effect in an aqueous system. Thiocyanate slightly inhibited the formation of NTCA in rats administered cysteine, formaldehyde, nitrite and thiocyanate. Thiocyanate also did not catalyze NTCA formation in rats given a nitrate solution *ad libitum* in their drinking water prior to treating the animals with cysteine, formaldehyde and thiocyanate. Formaldehyde was also ineffective in catalyzing the nitrosation of thiazolidine.

As a result of these studies, it can be concluded that the use of cure-solubilized liquid smokes can result in fried bacon with less NDMA, NPYR, and NTHZ than traditionally wood-smoked bacon. A relatively high formaldehyde concentration in some liquid smokes, however, may result in detectable levels of NTHZ in fried bacon. This study also describes a simple model system that can be used to evaluate the potential contribution of liquid smoke to NTHZ formation in bacon. The acidic nature of liquid smoke appears to reduce residual nitrite levels resulting in lower N-nitrosamines concentrations in fried bacon. The phenols of liquid smoke appear to have a very minor effect on N-nitrosamine formation in bacon and

N-nitroso compound formation *in vivo*. Ascorbate, however, was very effective in blocking endogenous NTCA formation.

PROPOSALS FOR FUTURE RESEARCH

The investigation into the influence of liquid smoke on N-nitrosamine formation in meat systems and *in vivo* has raised questions which merit further study:

1) While the results of this study support the hypothesis that a part of the NTHZ detected in fried bacon is formed as the result of the decarboxylation of NTCA during frying, the identification of the dominant pathway of NTHZ formation in cured meats requires further study. An experiment employing radioactively labeled compounds to gain further insight into the mechanism of NTHZ formation is one approach .

2) The present study reacted five commercial liquid smokes in the aqueous model system. The remaining liquid smokes on the market should be tested in the model system to estimate the potential of these smoke condensates to form NTHZ in fried bacon.

3) The absence of the formation of the characteristic brown color on the surface of cured meats treated with an atomized liquid smoke is a problem associated with the use of this product. Research directed at increasing the formation of brown pigments on the surface of atomized liquid smoke treated meat should be conducted. A possible approach is to add long chain aldehydes as browning pigment precursors. However, their possible involvement in thiazolidine formation should also be

evaluated.

4) Further study is needed to identify any NTCA-related compounds produced from the reaction of ketones or glycolytic aldehydes with cysteine and nitrite. Specifically, the identity of the product formed when acetone was administered to rats simultaneously with cysteine and nitrite should be determined. Ultimately, it should be established whether acetone or a metabolite of acetone reacts with cysteine. If it is established that cysteine and nitrite are reacting with a metabolite of acetone, it would be interesting to investigate the endogenous formation of NTCA in rats in a ketotic state. Because ethanol is metabolized to acetaldehyde, experiments investigating the formation of MeNTCA in rats administered alcoholic beverages and gavaged with cysteine and nitrite should be conducted. These studies could be extended to humans where alcohol consumption and cigarette smoking form part of the life style.

5) Further studies need to be conducted to investigate whether higher concentrations of α-tocopherol would increase the inhibitory effect of this compound on NTCA formation *in vivo*. In addition, lower concentrations of the potential catalysts thiocyanate and formaldehyde should be administered to rats to determine if this increases the catalytic effect of these compounds.

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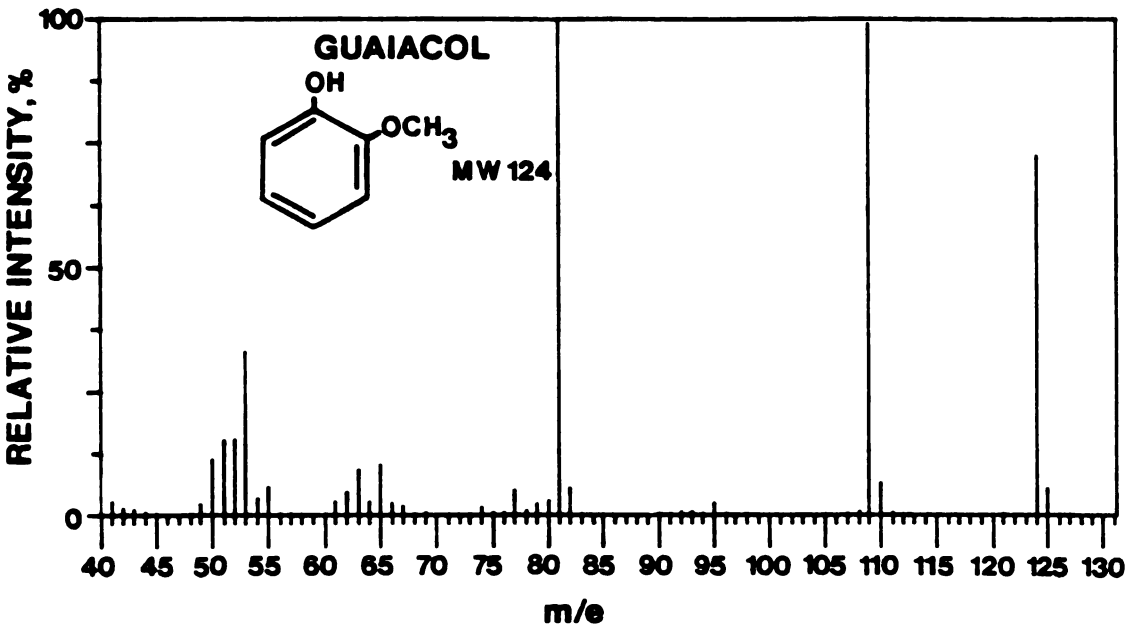
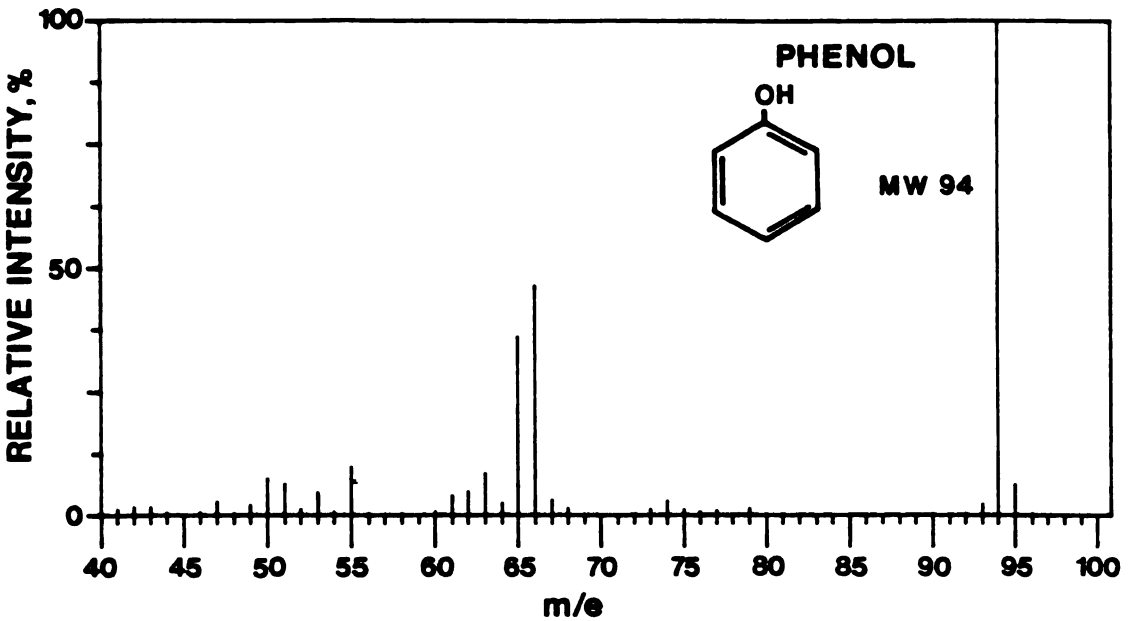
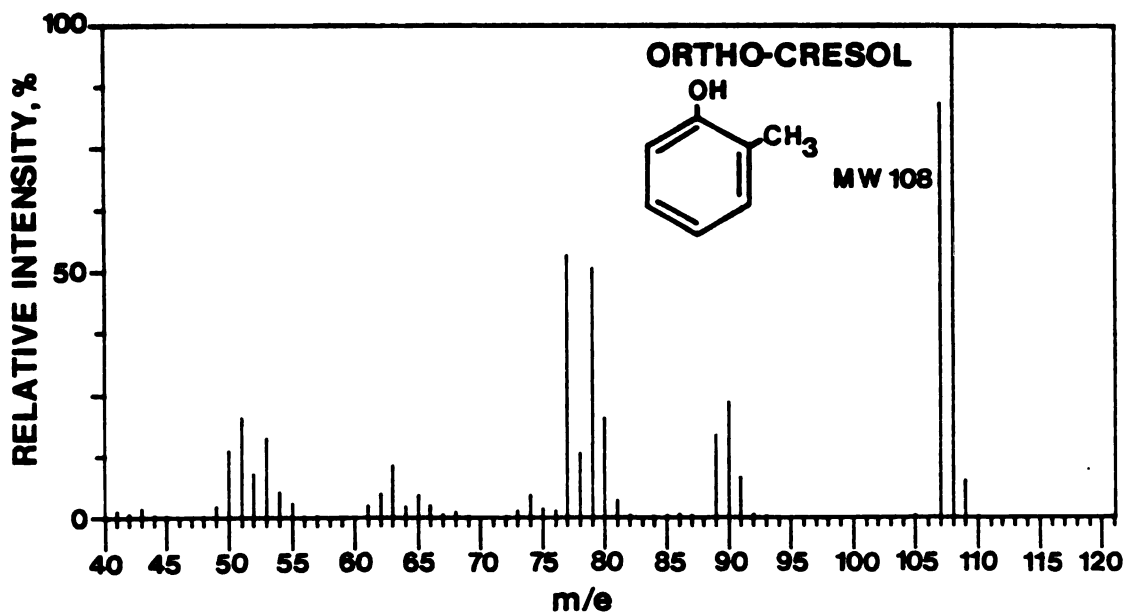
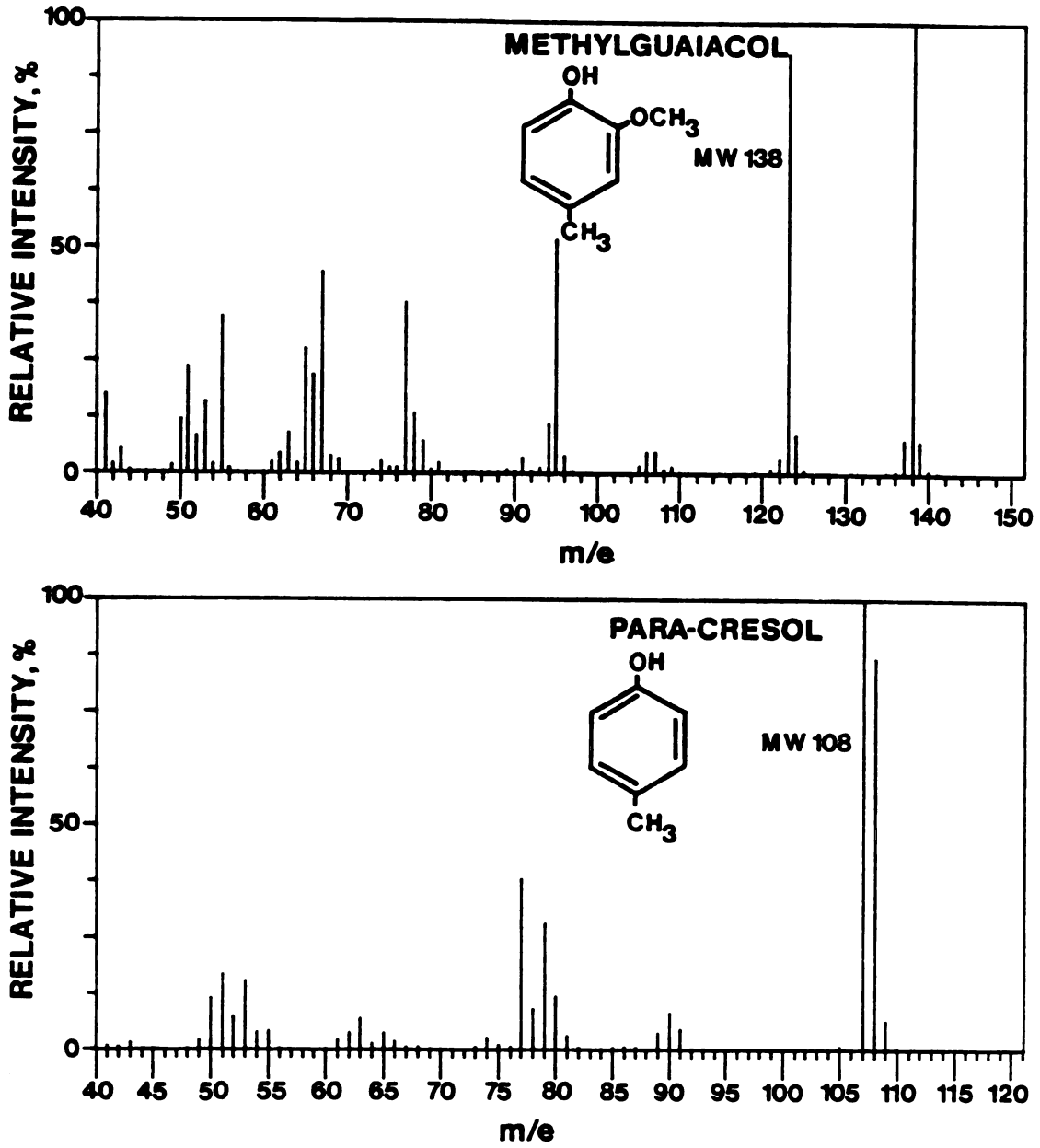
Appendix I

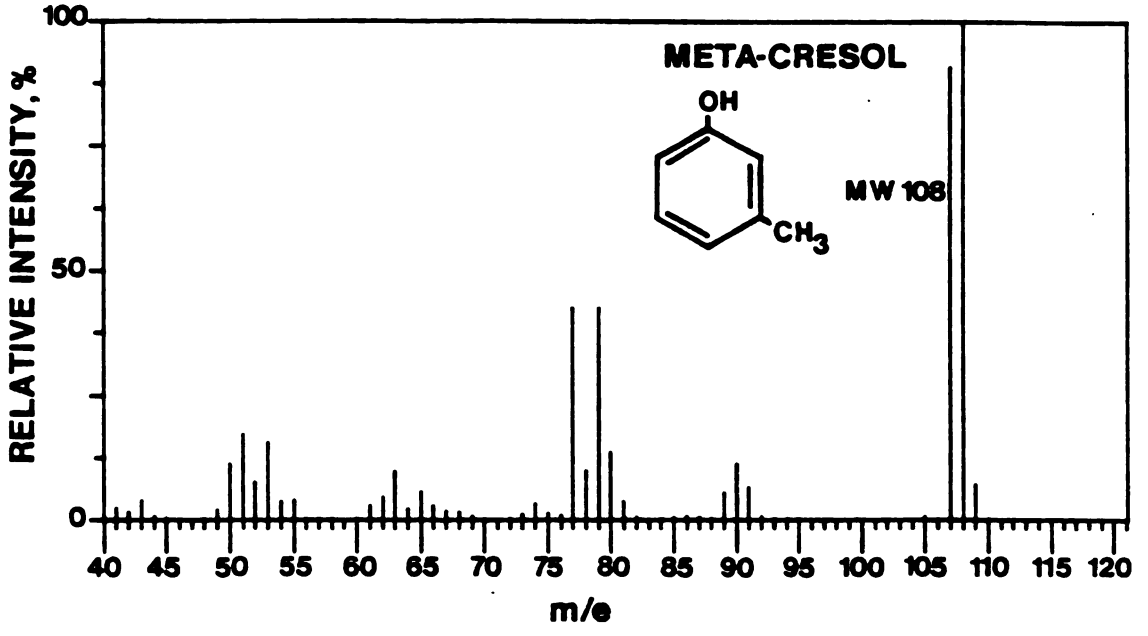
Mass Spectra of Phenols from Liquid Smoke

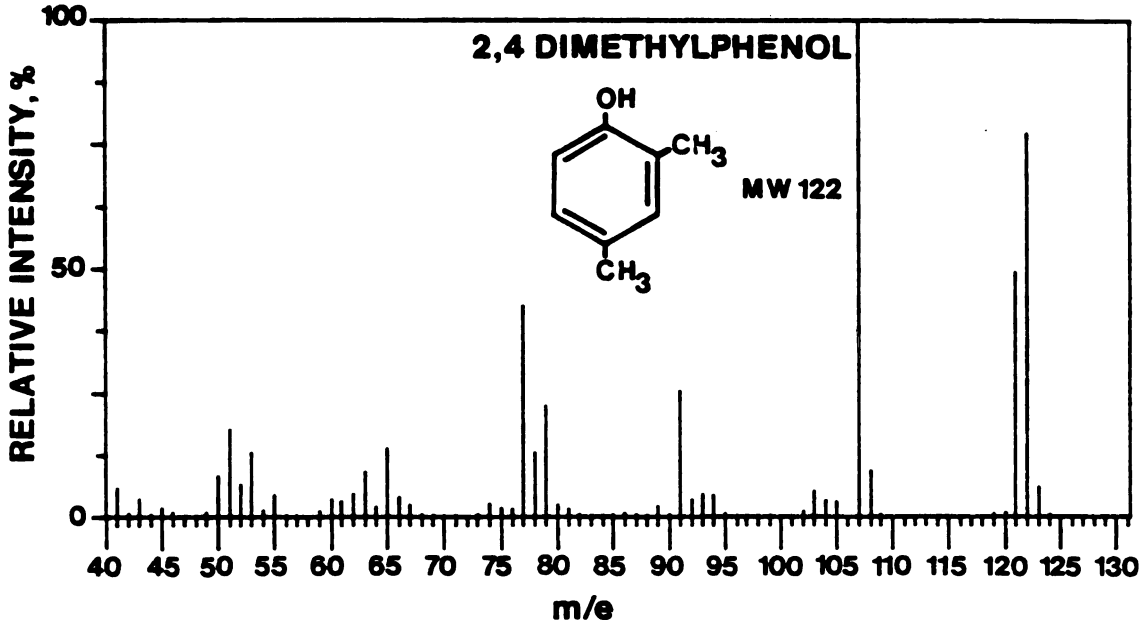

GUAIACOL
OH
OCH3
MW 124
RELATIVE INTENSITY, %
100
50
0
40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130
m/e

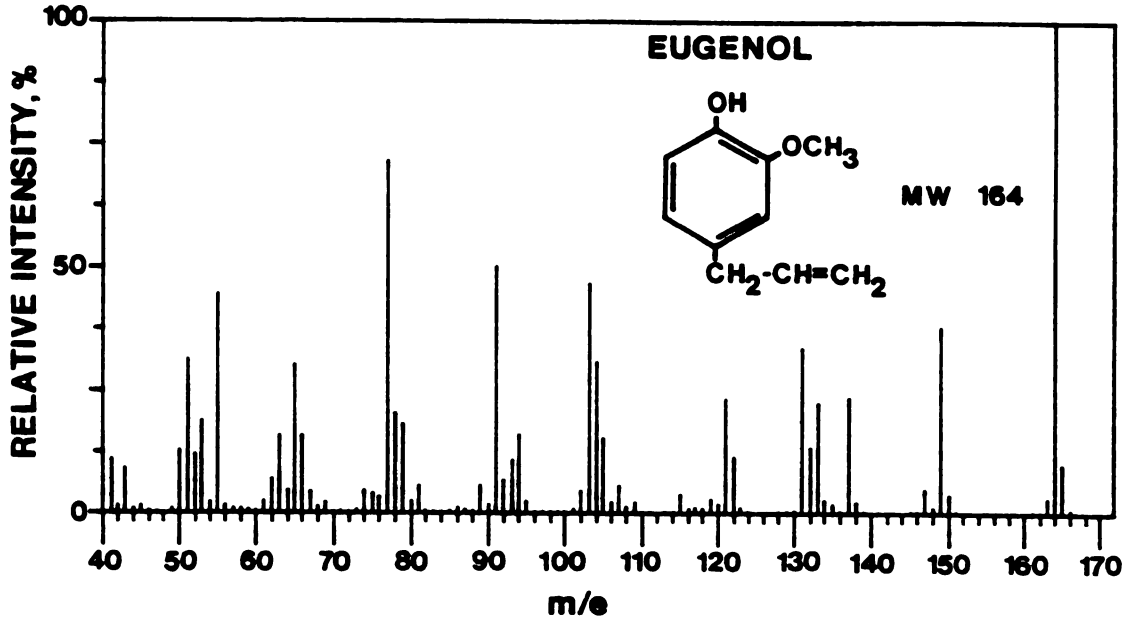

PHENOL
OH
MW 94
RELATIVE INTENSITY, %
100
50
0
40 45 50 55 60 65 70 75 80 85 90 95 100
m/e

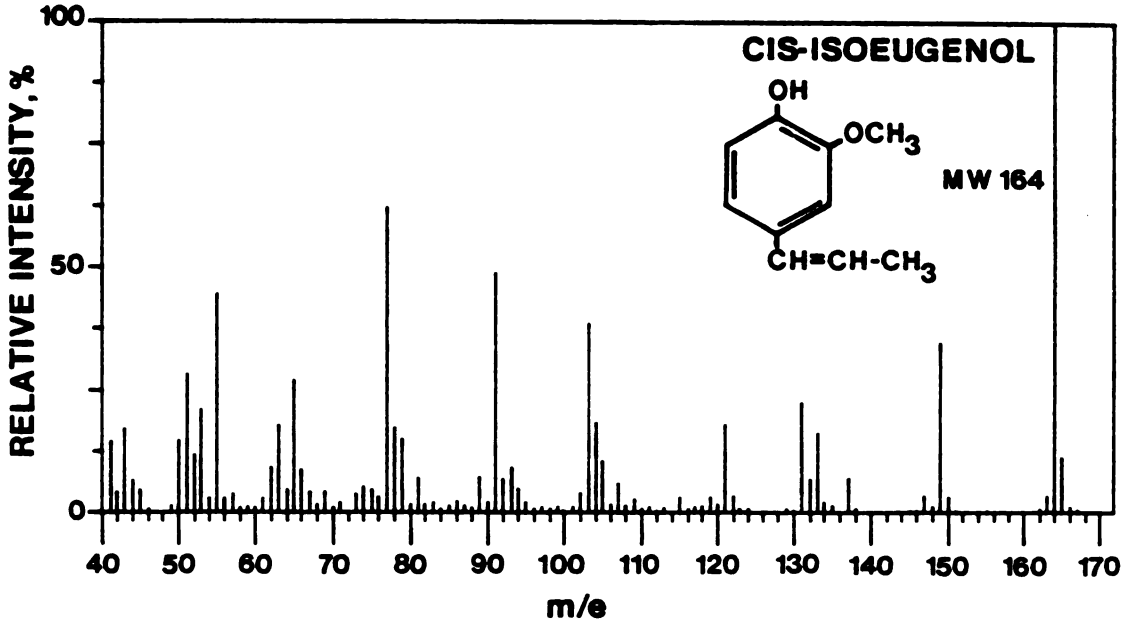

ORTHO-CRESOL
OH
CH3
MW 108
RELATIVE INTENSITY, %
100
50
0
40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
m/e

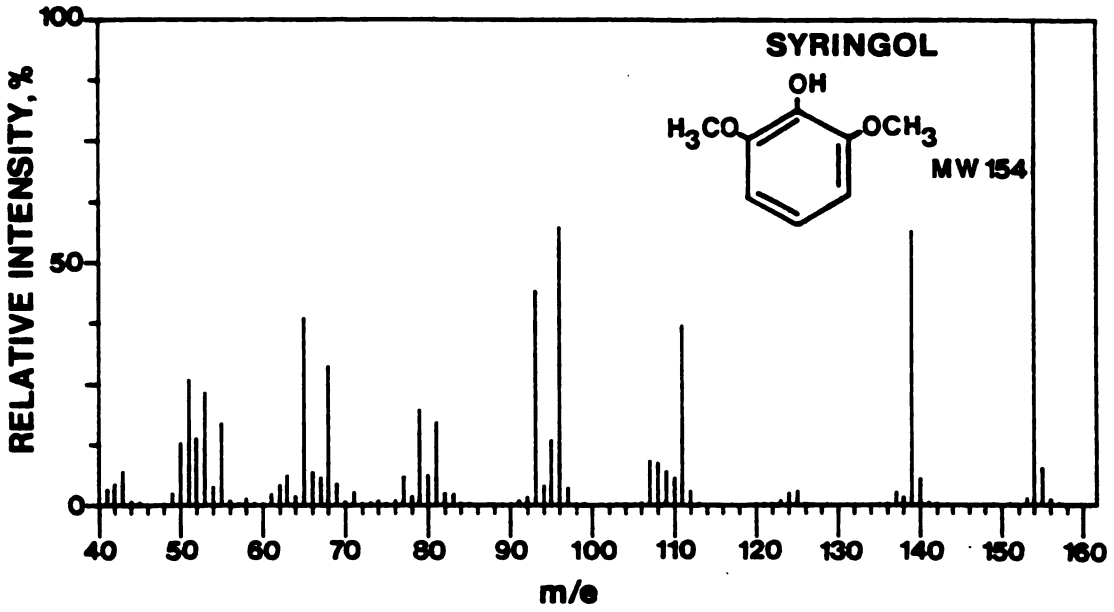

METHYLGUAIACOL
OH
OCH3
MW 138
CH3
RELATIVE INTENSITY, %
100
50
0
40 50 60 70 80 90 100 110 120 130 140 150
m/e
PARA-CRESOL
OH
MW 108
CH3
RELATIVE INTENSITY, %
100
50
0
40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
m/e

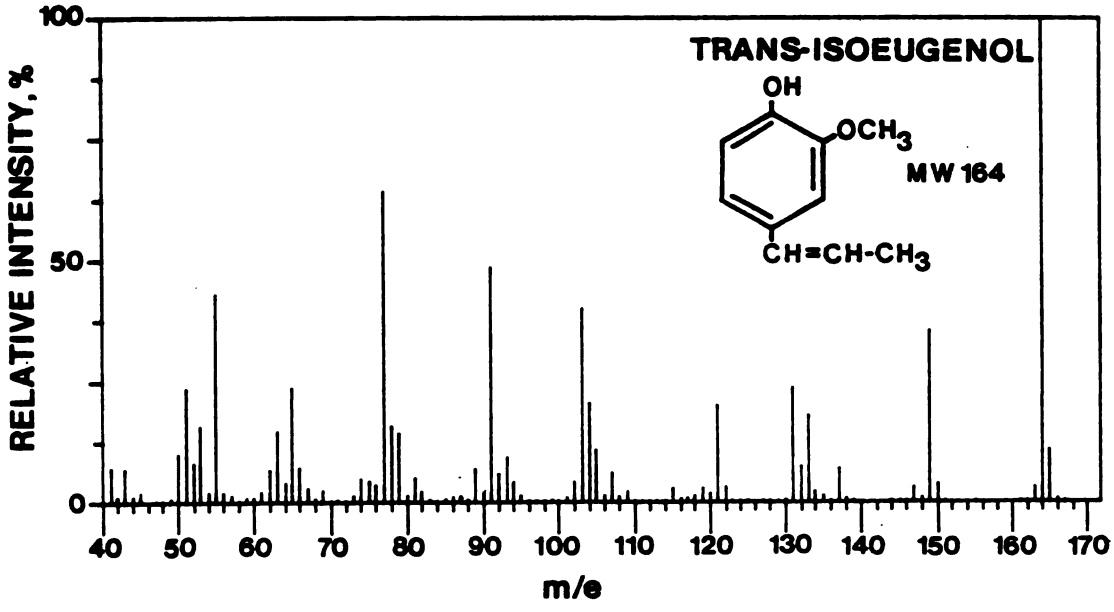

META-CRESOL
OH
CH3
MW 108
RELATIVE INTENSITY, %
100
50
0
40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
m/e

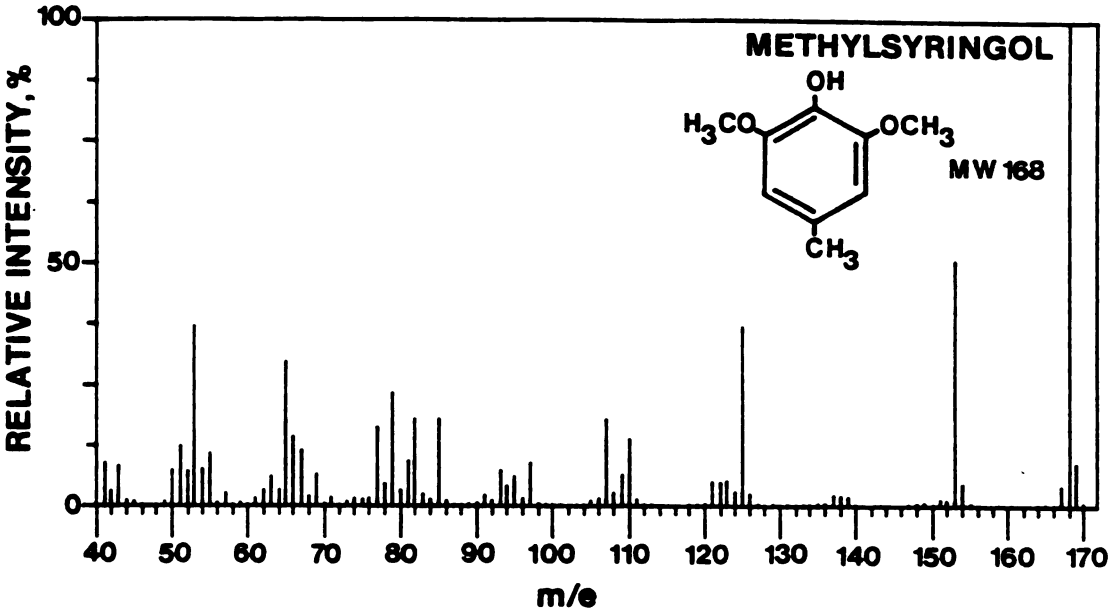

2,4 DIMETHYLPHENOL
OH
CH3
CH3
MW 122
RELATIVE INTENSITY, %
100
50
0
40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130
m/e

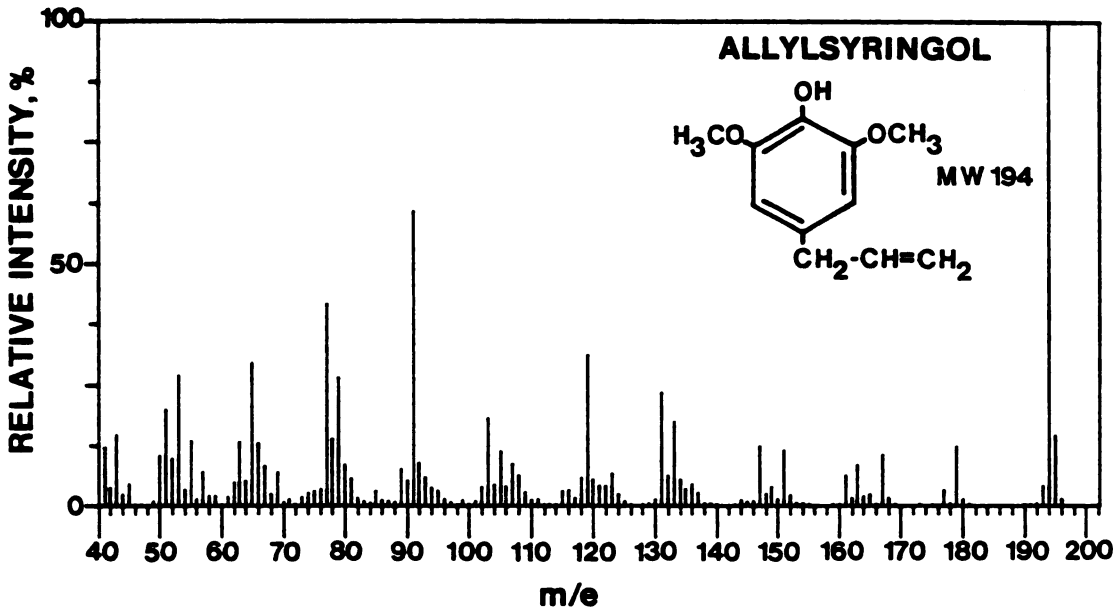

EUGENOL
OH
OCH3
MW 164
CH2-CH=CH2
RELATIVE INTENSITY, %
100
50
0
40 50 60 70 80 90 100 110 120 130 140 150 160 170
m/e


CIS-ISOEUGENOL
OH
OCH3
MW 164
CH=CH-CH3
RELATIVE INTENSITY, %
100
50
0
40 50 60 70 80 90 100 110 120 130 140 150 160 170
m/e


SYRINGOL
OH
H3CO
OCH3
MW 154
RELATIVE INTENSITY, %
100
50
0
40 50 60 70 80 90 100 110 120 130 140 150 160
m/e


TRANS-ISOEUGENOL
OH
OCH3
MW 164
CH=CH-CH3
RELATIVE INTENSITY, %
100
50
0
40 50 60 70 80 90 100 110 120 130 140 150 160 170
m/e


METHYLSYRINGOL
OH
H3CO
OCH3
MW 168
CH3
RELATIVE INTENSITY, %
100
50
0
40 50 60 70 80 90 100 110 120 130 140 150 160 170
m/e


ALLYLSYRINGOL
OH
H3CO
OCH3
MW 194
CH2-CH=CH2
RELATIVE INTENSITY, %
100
50
0
40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
m/e