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Determination of Phenolic Wood Smoke Components as Trimethylsilyl Ethers

Mary R. Kornreich[1] and Phillip Issenberg*[2]

A rapid and simple method, developed for qualitative and quantitative analysis of phenol mixtures, was applied to investigation of wood smoke condensates and model food components exposed to smoke vapor. Phenolic fractions were isolated by conventional methods based on acidity, followed by formation of trimethylsilyl (TMS) ethers by treatment with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at room temperature. An internal standard, 3,5-dimethylphenol, was added to eliminate volumetric errors in gas chromatographic (gc) injection. Mass spectra of TMS derivatives provide as much structural information as spectra of free phenols and the derivatives are more readily separated by gc. Phenol, guaiacol, 4-methylguaiacol, and syringol were the major components found in the wood smoke vapor phase. Their concentrations were determined in smoke vapor and in oil and water models exposed to the vapor for 4 hr.

Several previous studies of smoke or smoked foods included estimates of total phenolic compounds (Bratzler *et al.*, 1969; Foster and Simpson, 1961; Husaini and Cooper, 1957; Porter *et al.*, 1965; Simon *et al.*, 1966). No information was available, however, on quantities of individual phenolic compounds present because of the lack of an appropriate analytical method. Individual phenolic components of wood smoke condensate (Fiddler *et al.*, 1966; Lustre and Issenberg, 1969) and smoked pork (Lustre and Issenberg, 1970) have been investigated but without quantitative methods.

Phenols were isolated from aqueous smoke condensate by Lustre and Issenberg (1969) with a series of extractions to separate components by acidity. Recoveries ranging between 80 and 100% were achieved with a similar system for preparation of a weakly acidic or "phenolic" fraction from water and triolein solutions (Issenberg *et al.*, 1971).

Gas chromatographic (gc) separation of free phenols is complicated by their polar nature and low volatility. Chromatographic peaks tend to be asymmetric; long times and high temperatures are required to elute some of the higher boiling phenols. Despite these difficulties, gc was the most promising method for separation and quantitative analysis of individual phenols in complex mixture.

Peak symmetry in gc of phenolic compounds can be markedly improved by formation of derivatives of higher volatility and lower polarity. Silylating reagents react with hydroxyl, carboxylic acid, amine, and mercaptan groups by replacing the active hydrogens with trimethylsilyl (TMS) groups. Blocking the hydroxyl groups of phenolic compounds inhibits the formation of intermolecular hydrogen bonds, thus reducing interaction with the solid support and apparatus surfaces which improves peak symmetry. Decreased interaction with the stationary phase provides shorter elution times (Bhattacharyya *et al.*, 1968).

Early procedures for silylation of phenolic compounds utilized a mixture of hexamethyldisilazane (HMDS), catalyst, solvent, and a drying agent (Langer *et al.*, 1958). Silylation by N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) is faster and more complete than by HMDS or N,O-bis(trimethylsilyl)acetamide (BSA) (Stalling *et al.*, 1968). BSTFA

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

[1] Present address: 1326 Buttermilk Lane, Reston, Virginia 22070.

[2] Present address: Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska 68105.

and its by-product in the derivatization reaction, mono(trimethylsilyl)trifluoroacetamide, are more volatile than BSA and its by-product. BSTFA and its by-product are eluted from the column with or near the solvent peak and are less likely to interfere with peaks of analytical interest.

Methods are required for quantitative determination of individual phenolic components of smoke condensates, liquid smoke preparations, foods, and food models. Extraction and isolation procedures were reported previously (Issenberg et al., 1971). This paper reports development of quantitative methods for gc determination of TMS derivatives of wood smoke phenols.

EXPERIMENTAL SECTION

Isolation of Phenolic Fractions and Preparation of TMS Derivatives. The procedure for recovery of phenolic compounds from smoke vapor condensate, distilled water exposed to smoke vapor, and triolein solutions was described previously (Issenberg et al., 1971).

A known volume of a 1% (w/v) solution of the internal standard, 3,5-dimethylphenol in acetonitrile, was added to the phenol fraction (in ether), which was then dried with sodium sulfate, concentrated on a rotary evaporator to approximately 10 ml, and stored at 4° under nitrogen in a glass-stoppered centrifuge tube. This solution was concentrated further in a stream of prepurified nitrogen and a 50-μl portion was transferred to a Microflex tube (Kontes Glass Co., Vineland, N.J.). The exact degree of concentration was not critical, since the internal standard compensated for volumetric errors. Final volumes were approximately 3 ml for phenolic fractions from smoke vapor condensates and 0.5 ml for concentrates from water or triolein solutions. Fifty microliters of BSTFA (Regisil, Regis Chemical Co., Chicago, Ill.) was added, and the tube was sealed with a silicone rubber septum and screw cap. The reaction mixture was shaken for 30 sec and allowed to stand for 20 min.

Qualitative Analysis by Gas Chromatographic Retention Data and by Combined Gas Chromatography–Mass Spectrometry (GCMS). For the samples investigated, the best chromatographic resolution was obtained on a 0.5 mm i.d. × 150 m stainless steel open tubular column coated with OV-17 (phenylmethylsilicone). Helium carrier gas flow rate was 8 cm^3 per minute. Temperature was programmed from 100 to 250° at 2° per minute. An F&M model 810 gas chromatograph with flame ionization detector was used. Special minimum volume injector and detector inserts were constructed and installed in the chromatograph.

Tentative identifications, based on gc retention times, were confirmed by GCMS. Mass spectra of components of the silylated phenol fractions and of reference TMS ethers were obtained using a Hitachi Perkin-Elmer double-focusing mass spectrometer (model RMU-7) with the OV-17 open tubular column installed in the mass spectrometer inlet chromatograph (Aerograph model 204-C). The Watson–Biemann (1965) separator was maintained at 200°. Ion source temperature was 250°; electron energy was 70 eV; and target current was 100 μA. The range, m/e 4–400, was scanned in 5 sec. Source and collector slits were set at 0.5 mm for a resolving power of approximately 400. Chromatograms were recorded from the total ion current monitor located between the electrostatic and magnetic sectors.

Quantitative Analysis. A 2.5 mm i.d. × 5 m stainless steel column, packed with 2% OV-17 and 0.1% Igepal CO-880 on Chromosorb G (100/120 mesh), gave adequate resolution of the TMS derivatives of major components of the phenol fractions from smoke vapor, solutions, and model systems. Peaks were relatively symmetrical and the column was stable over many cycles in the temperature range employed. Quantitative data are based on peak heights relative to the internal standard. It is desirable that the internal standard selected has chemical properties resembling those of the compounds under investigation. If the internal standard participates in the silylation reaction in a manner similar to the phenols being studied, the internal standard, added before silylation, can serve as a check on derivative formation, as well as on the injection procedure. The internal standard would, of course, have to be a compound not present in the fraction being analyzed and should also be well separated from those compounds. For the phenol fraction of smoke vapor condensate, 3,5-dimethylphenol met these criteria and was therefore selected for use in quantitative experiments.

Calibration curves were made by plotting the observed peak–height ratio of phenolic compound to internal standard as ordinate against actual weight ratio as abscissa. For this purpose, a stock solution of phenolic compounds was made containing 1% each of phenol, o-methoxyphenol (guaiacol), 2-methoxy-4-methylphenol (methyl guaiacol), and 2,6-dimethoxyphenol (syringol) in acetonitrile. A 1% stock solution of the internal standard, 3,5-dimethylphenol, in acetonitrile was also prepared. Seven mixtures were made from these two solutions and the silylating reagent. These mixtures contained phenolic solution and internal standard solution in the ratios 1:1, 2:1, 3:1, 4:1, 1:2, 1:3, and 1:4. Amounts of phenols (unsilylated weight) were between 0.67 and 2.7 μg. Duplicate 1-μl samples of each mixture were analyzed by gc.

Appearance of silyl derivatives and disappearance of phenolic compounds were observed simultaneously on a 2.5 mm i.d. × 1.8 m stainless steel column, packed with 4.5% FFAP on Ultrapak 100/120 mesh (Western Analytical Services, Orinda, Calif.). Experiments were carried out with one phenolic compound at a time, using naphthalene as an internal standard. Injections before the addition of BSTFA revealed the retention time of the free phenolic compounds. Reaction mixtures were injected at intervals of 1 min, 20 min, 1 hr, 2 hr, 3 hr, and 4 hr after the addition of BSTFA to measure the rate of derivative formation.

RESULTS AND DISCUSSION

Mass Spectrometry. Silylation prior to GCMS would provide an ideal method for separation and identification of phenols if mass spectra of TMS derivatives provided sufficient information for identification purposes. Abbreviated mass spectra (Hites and Biemann, 1968) of some silylated phenolic compounds are presented in Table I.

The spectra of TMS derivatives of smoke phenols were characterized by a series of intense peaks representing losses of methyl units from the molecular ion (M − 15, M − 30, etc.). The molecular ion was present in all TMS derivatives examined and varied in relative intensity from about 30 to 85% of the most intense (base) peak. Because of facile cleavage of a methyl group attached to silicon, the molecular ion was never the base peak. An intense M − 15 peak was always present and helped verify location of the molecular ion. The base peak in the spectra of silylated phenol and cresols was at M − 15. Silylated monomethyoxyphenolic compounds exhibited base peaks at M − 30 due to rapid loss of a second methyl group from the methoxy group by cleavage of the bond β to the aromatic ring. Spectra of dimethoxyphenols exhibit intense peaks at M − 15, M − 30, and M − 45. The relative intensities of these peaks and the molecular

Table I. Abbreviated Mass Spectra of TMS Ethers of Some Phenolic Components of Wood Smoke

Parent phenol	m/e (relative intensity)						
Phenol (M = 166)	167 (4)	166 (28)	153 (4)	152 (14)	151 (100)	135 (3)	95 (4)
	91 (7)	77 (10)	75.5 (6)	75 (7)	73 (6)	51 (6)	45 (6)
	43 (3)						
Guaiacol (M = 196)	197 (8)	196 (44)	182 (8)	181 (42)	169 (6)	168 (8)	167 (24)
	166 (100)	153 (2)	152 (5)	151 (26)	136 (15)	89 (4)	73 (14)
	59 (7)						
4-Methylguaiacol (M = 210)	211 (4)	210 (25)	195 (13)	182 (5)	181 (15)	180 (100)	179 (8)
	165 (7)	150 (5)	149 (6)	89 (4)	73 (15)	59 (6)	45 (5)
Salicylaldehyde (M = 194)	194 (4)	180 (16	179 (100)	161 (33)	149 (11)	135 (9)	91 (9)
	82 (11)	77 (11)	75 (8)	73 (17)	65 (6)	59 (9)	51 (9)
	45 (19)	43 (9)	39 (6)				
4-Ethylguaiacol (M = 226)	227 (8)	226 (42)	212 (6)	211 (35)	196 (5)	196 (19)	195 (100)
	180 (12)	179 (60)	149 (14)	97 (6)	91 (8)	89 (10)	77 (8)
	75 (9)	73 (53)	59 (19)	45 (6)	43 (7)		
Syringol (M = 226)	227 (14)	226 (85)	212 (17)	211 (100)	198 (17)	197 (52)	196 (91)
	181 (39)	166 (15)	153 (44)	151 (18)	95 (13)	89 (17)	79 (11)
	75 (17)	74 (10)	73 (94)	59 (47)	51 (13)	45 (56)	43 (21)
	39 (22)						
Eugenol (M = 236)	237 (9)	236 (43)	221 (15)	207 (19)	206 (100)	205 (20)	179 (12)
	103 (11)	73 (33)	59 (16)	45 (22)			
4-Ethylsyringol (M = 254)	255 (10)	254 (65)	239 (26)	225 (18)	224 (100)	209 (40)	77 (7)
	75 (10)	74 (5)	73 (53)	59 (20)	45 (22)		
trans-Isoeugenol (M = 236)	237 (10)	236 (46)	221 (7)	208 (6)	207 (20)	206 (100)	205 (16)
	179 (7)	103 (11)	91 (7)	89 (9)	77 (6)	75 (8)	74 (6)
	73 (31)	59 (13)	45 (14)				
Vanillin (M = 224)	225 (6)	224 (31)	209 (44)	195 (17)	194 (100)	193 (39)	73 (43)
	59 (19)	45 (23)	43 (11)				
4-Allylsyringol (M = 266)	267 (14)	266 (68)	252 (5)	251 (25)	238 (21)	237 (100)	205 (19)
	91 (10)	89 (11)	75 (21)	73 (63)	59 (26)	45 (30)	
Syringaldehyde (M = 254)	255 (11)	254 (63)	240 (15)	239 (92)	226 (12)	225 (36)	224 (67)
	223 (37)	209 (14)	153 (17)	89 (10)	79 (10)	77 (10)	75 (16)
	73 (100)	59 (41)	45 (45)	43 (17)	39 (11)		
Acetovanillone (M = 238)	238 (50)	224 (16)	223 (94)	209 (12)	208 (61)	194 (16)	193 (100)
	165 (12)	137 (12)	104 (10)	89 (16)	75 (10)	73 (74)	59 (27)
	45 (32)	43 (42)	41 (12)				
Acetosyringone (M = 268)	269 (13)	268 (59)	254 (14)	253 (74)	239 (24)	238 (100)	224 (18)
	223 (91)	137 (17)	119 (10)	104 (10)	89 (10)	77 (9)	75 (22)
	74 (10)	73 (95)	59 (41)	45 (39)	43 (56)		

ion peak varied, forming patterns characteristic of individual compounds.

For the TMS phenols investigated, the locations and relative intensities of these high mass peaks at 15 mass unit intervals are the most informative components of the spectra. In the lower mass portion of the spectra, relatively strong peaks were always found at m/e 45, 59, and 73. The peak at m/e 73 was always the most intense in this portion of the spectrum. Less intense peaks were found at m/e 43 and 75. The pattern formed by these five peaks was indicative of a silylated phenol, but was of no use in differentiating among these compounds. Cleavage of the TMS group at the bond β to the aromatic ring is responsible for the intense m/e 73 peak [$(CH_3)_3Si^+$]. A rearrangement (Budzikiewicz et al., 1967) produces the m/e 75 peak. The presence of most substituent groups is recognized by the molecular weight of the molecule, rather than from characteristic fragments.

Spectra of free phenols and of their TMS derivatives have many similar features and give equivalent amounts of information. In spectra of both, methoxy substituents fragment easily at the bond β to the ring. In both, the spectral pattern of a dimethoxy compound is similar to that of its corresponding monomethoxy analog, except that the high mass peaks for the former are m/e 30 units higher. In both, substituents with β bonds are cleaved from the aromatic ring prior to cleavage of the aromatic ring or of resonance-stabilized bonds α to the ring.

Identification of Major Components of Phenolic Fraction of Whole Smoke Condensate. The chromatogram in Figure 1 illustrates open tubular column separation of TMS ethers of components of a phenol fraction from whole smoke condensate. Resolution of components was superior to that achieved by packed column chromatography of free phenols from similar condensates (Lustre and Issenberg, 1969). The principal goal of the present studies was investigation of quantitative methods for phenol determination. Therefore, identification of all components was not attempted. Some major components were identified by GCMS so that their location in the chromatograms could be established for comparison with previous work. In all cases, gc retention times and mass spectra were identical to those recorded for pure reference compounds.

It is clear, from the discussion of mass spectra and from the chromatogram shown, that GCMS utilizing open tubular columns and TMS derivatives will be a valuable aid in further investigations of wood smoke, liquid smoke preparations, and smoked food composition. Open tubular column separation of free phenols was impossible because of excessive peak-tailing on a variety of liquid phases examined. A number of quantitatively minor components, not detected in separations of free phenols, were observed in open tubular column chromatograms of TMS ethers.

Quantitative Determination of Phenolic Components. Gc analysis of samples of reaction mixtures, examined at times between 1 min and 4 hr after addition of BSTFA, showed that the reaction was complete in less than 1 min. No free phenol peaks were detected, even when the gc detector sensitivity was increased by a factor of 256. Conversion to TMS
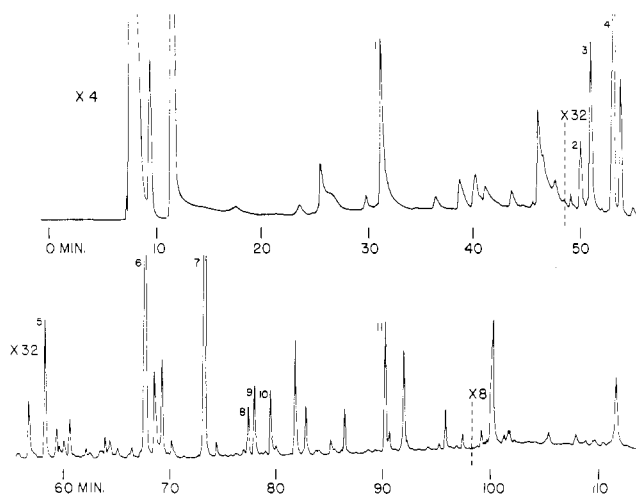
Figure 1. Gas chromatogram of silylated phenol fraction of wood smoke condensate: 0.5 mm i.d. × 150 m stainless steel open tubular column coated with OV-17. Peak identities (GCMS), TMS ethers of: (1) phenol; (2) 2-hydroxy-2-methylcyclopent-2-en-1-one (cyclotene); (3) o-methoxyphenol (guaiacol); (4) 3-hydroxy-2-methylpyrone (Maltol); (5) 2-methoxy-4-methylphenol (4-methylguaiacol); (6) 2,6-dimethoxyphenol (syringol); (7) 2,6-dimethoxy-4-methylphenol; (8) 2,6-dimethoxy-4-ethylphenol; (9) 2-methoxy-4-trans-propenylphenol (trans-isoeugenol); (10) 4-hydroxy-3-methoxybenzaldehyde (vanillin); (11) 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde)
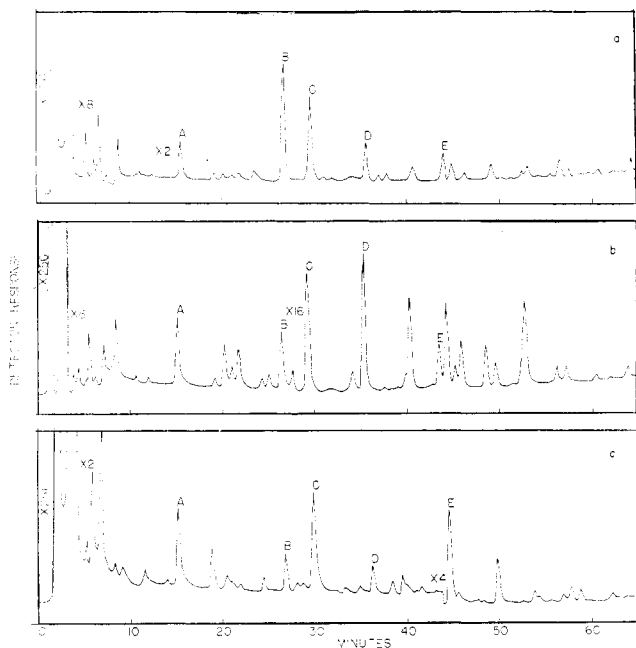

Figure 2. Gas chromatograms of silylated phenol fractions from: (a) smoke vapor condensate; (b) water model; (c) oil model (triolein). 2.5 mm i.d. × 5 m stainless steel column packed with 2% OV-17 + 0.1% Igepal CO-880 on Chromosorb G (100/120 mesh). Peak identities: A. phenol; B. 3,5-dimethylphenol (internal standard); C. guaiacol; D. 4-methylguaiacol; E. syringol. Amounts of internal standard solution (1%) added: to (a), 200 μl; to (b) and (c), 8 μl

derivatives appeared to exceed 99.6% for all phenols examined. There was no loss of TMS ethers after 4 hr at room temperature.

Linear relationships were found between ratios of observed peak heights and actual weight ratios in the range from 1:4 to 4:1. All calibration lines passed through the origin (±0.02). Slopes of calibration lines for phenol, guaiacol, 4-methyl-

Table II. Concentrations[a] of Major Phenols in Wood Smoke Condensate and Vapor Phase, and in Oil and Water Models[b]

	Whole smoke, μg/l.	Vapor phase, μg/l.	Oil model, μg/g	Water model, μg/g
Phenol	59.4	5.9	33.1	29.2
Guaiacol	417	32.1	176	77.7
4-Methylguaiacol	333	13.6	241	24.2
Syringol	392	6.5	102	68.5

[a] Mean coefficient of variation for replicate smoke generations was 24%. [b] Whole smoke and vapor phase were collected for 2 hr. Models were exposed to smoke vapor for 2 hr.

guaiacol, and syringol were 1.24, 0.751, 0.665, and 0.619, respectively. Concentration of each phenol in a mixture could be calculated from $x = c \cdot y/S$, where x is the concentration of phenol in the mixture, S is the slope of the calibration line, c is the concentration of internal standard, 3,5-dimethylphenol, and y is the observed ratio of peak height of compound of interest to that of the internal standard. Concentrations may be expressed in any consistent units. A major limitation on analytical precision in gc analysis is the inability to reproduce injected volumes. Use of an appropriate internal standard eliminates this problem. The maximum observed difference in relative peak heights in duplicate chromatograms of the TMS ethers studied was less than 0.5%, an error negligible in comparison with the standard deviations of recoveries of phenols from aqueous (4 to 13%) and triolein (1 to 5%) solutions (Issenberg et al., 1971). Quantitative determination of any component of the mixture could be made in this manner, but only the four major phenols were investigated in this work.

Typical chromatograms of silylated phenolic fractions from wood smoke vapor phase and from oil and water models exposed to the smoke vapor for 2 hr are shown in Figure 2. Concentrations of the four major phenols are listed in Table II.

The method described for preparation of TMS ethers was rapid and reliable. It has been applied to quantitative studies of partition of phenolic components between smoke vapor and food models (Kornreich, 1970) and to identification of phenols in commercial smoked foods (King, 1970). The method is generally applicable to quantitative gc and qualitative GCMS determination of phenolic components of foods, beverages, and environmental samples. The principal problems remaining in quantitative studies of the smoking process are the difficulty of completely extracting phenols from smoked food products and poor reproducibility of smoke composition.

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Interactions of Some Wood Smoke Components with ε-Amino Groups in Proteins

Lan-Bo Chen and Phillip Issenberg*[1]

Interactions of some previously identified wood smoke components with meat and pure proteins were investigated. Uncured lean beef sirloin strips, exposed to wood smoke for 10 hr, lost 44% of the available lysine initially present. Heating in air (65°, 10 hr) caused a 15% loss of available lysine. Beef homogenate, treated with acidic, phenolic, or neutral fractions of smoke condensate, lost 14, 38, and 45% available lysine, respectively. A model system consisting of a soluble protein, bovine serum albumin (BSA), was treated with pure smoke components and the degree of interaction was determined spectrophotometrically by measuring the extent to which the protein reacted with dinitrobenzyl sulfonate. The reduction in available ε-amino groups observed when BSA was treated with sinapaldehyde and coniferaldehyde was comparable to that observed for some previously known active aldehydes (formaldehyde, glyoxal, pyruvaldehyde, and furfural). No significant interaction was detected when BSA was treated with phenol, cyclotene, eugenol, or syringol. A variety of colors, ranging from yellow to red, was produced by treating casein with aldehydes and phenolic aldehydes.

The preservative effects of wood smoke on foods result from partial surface dehydration and from deposition of compounds with antimicrobial and antioxidant activity. In current commercial practice in the United States, smoking is employed primarily for color and flavor, but significant extension of shelf-life may result from the smoke components added to the products.

In view of current concern about the nutritional value of processed foods and about chemicals added to foods, it is important not only to know the identities and amounts of smoke components consumed, but also the nature of reaction products formed during exposure of foods to smoke. Many wood smoke components have been identified (Fiddler *et al.*, 1966, 1967, 1970a,b; Hamid and Saffle, 1965; Hoff and Kapsalopoulou, 1964; Jahnsen, 1961; Love and Bratzler, 1966; Lustre and Issenberg, 1969; Porter *et al.*, 1964; Ruiter, 1970), some of these were identified in smoked food products (Bratzler *et al.*, 1969; Howard *et al.*, 1966; Lustre and Issenberg, 1970; Shewan, 1953; Tucker, 1942), but little attention has been given to chemical reactions in smoked foods or to the reaction products.

Knowledge of chemical reactions during the smoking process can provide background necessary for evaluation of the functional and nutritional properties of protein in smoked foods. Such studies may also reveal the presence of reactive

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.
[1] Present address: Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska 68105.

compounds with potential commercial value as coloring reagents and antimicrobial compounds.

Reactions of aldehydes with free amino groups are well known. Loss of available lysine in meats treated with wood smoke was attributed to reactions of formaldehyde with the ε-amino group of lysine (Dvorak and Vognarova, 1965; Inagami and Horii, 1966). Ruiter (1970) suggested that glycolic aldehyde and pyruvaldehyde can react with protein. The reaction between glycolic aldehyde and aminoethanol was examined in detail (Ruiter, 1971). The present investigation undertakes to determine the extent to which some specific wood smoke components react with ε-amino groups of lysine in protein and to establish the significance of these components in causing loss of available lysine during smoking. We wished to determine whether interaction of proteins with coniferaldehyde and sinapaldehyde, major phenolic components of wood smoke condensates, could account for inability to recover these compounds from smoked food products (King, 1970; Lustre and Issenberg, 1970).

EXPERIMENTAL SECTION

Smoking of Samples. The glass smoke generator and the smokehouse were described previously (Lustre and Issenberg, 1970). Fresh lean beef sirloin was purchased locally. Samples to be smoked were sliced as strips 2 to 3 mm thick, 1 to 2 cm wide, and 15 to 20 cm long. Individual strips were held at one end with straightened paper clips and suspended from a rack located at the top of the smokehouse. They were hung in four rows, 5 cm apart, and samples within a row were separated by 6 to 10 cm. This arrangement maximized the