The Production of Steam-Volatile Phenols during the Cooking and Alcoholic Fermentation of Grain

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The major steam-volatile phenols present in distillates from grain alcohol ferments are *p*-vinylphenol, *p*-ethylphenol, 4-vinylguaiacol, 4-methylguaiacol, and 4-ethylguaiacol. *p*-Vinylphenol and 4-vinylguaiacol are formed when corn is cooked, apparently by the thermal decarboxylation of *p*-coumaric and ferulic acids, respectively. Microbiological decarboxylation of these acids by yeast and bacteria also occurs during fermentation. *p*-Ethylphenol, 4-ethylguaiacol, and 4-methylguaiacol are produced during alcoholic fermentation by bacteria from *p*-coumaric and ferulic acids and vanillin, respectively. The vinyl phenols appear to be intermediates in the production of the ethylphenols. Ferulic acid, *p*-coumaric acid, and vanillin were found in corn and barley malt. The amounts in corn are increased by the cooking process.

Although "phenolic bodies" were reported as present in alcoholic distillates by Schidrowitz and Kaye in 1905 (*29*), no further work on phenols from grain was reported until 1958, when Braus and Miller (*5*) described the isolation and identification of steam-volatile phenols (SVPs) in fusel oil. Guaiacol, 4-methylguaiacol, 4-ethylguaiacol, phenol, *o*-cresol, and vanillin were identified. Vanillin was found primarily in the nonvolatile fraction of the fusel oil.

Schidrowitz and Kaye presumed that the phenols they detected in a pot-still whiskey originated from the peat and coke used in malting or were produced in the malt by the heat applied in kilning. Braus and Miller speculated that the phenols may originate from the degradation of lignin by bacteria or fungi during the fermentation.

This investigation was undertaken to determine the origin and mode of formation of the major phenolic components of grain alcohol distillates. Preliminary studies revealed that distillates from cooked grain appeared to contain several μg. per ml. of phenols when tested with Folin-Denis reagent (*11*). Some strains of yeast appeared to produce small amounts of SVPs during the fermentation of grain mash, i.e., 1 to 3 μg. per ml. increase to give 3 to 5 μg. per ml. in fermented grain. Larger amounts of SVPs are produced during fermentation by certain contaminating bacteria. (Fermented beer can contain as much as 15 μg. per ml. SVPs, depending on various factors such as cooking conditions, grain type, yeast strain, and severity of bacterial contamination.) Increased cooking temperatures and/or time increases the maximum amount of SVPs produced by bacteria. (Cooking conditions have only a slight effect on the smaller amount of SVPs produced by certain yeasts.) 4-Methylguaiacol and 4-ethylguaiacol represent the greater part of the SVPs produced by bacteria. In varying amounts, SVPs were found in all whiskies and brandies, foreign and domestic, which were examined.

Materials and Methods

Isolation of Steam-Volatile Phenols from Grain after Cooking and Fermentation. Cooked Grain. Steam released during the "blowdown" or pressure-release phase of the cooking cycle for corn was condensed and collected (Figure 1). Phenols were removed by continuous countercurrent extraction with ether (1.75 × 48 inch column), as generally described by Braus and Miller (*5*). About 10 gallons of condensate was extracted in each run, using a column input of 50 ml. of condensate per minute and 5 ml. of ether per minute. The ether from the column was evaporated to approximately 300 ml., and extracted by separatory funnel first with 5% aqueous $NaHCO_3$ and then with 5% aqueous NaOH. The NaOH extract was acidified and extracted with ether. This ether extract was dried over anhydrous K_2SO_4 and evaporated to give 1 to 2% phenols.

Fermented Grain. SVPs were isolated from grain ferments ("spirits" comprised of corn and barley malt, and "bourbon" comprised of corn, rye, and barley malt) by countercurrent extraction of condensed steam from stillage leaving the plant stills, and by the extraction of laboratory alcoholic distillates. The ether phase was treated as above.

Sufficient phenols for chromatographic analysis were obtained from 300 ml. of grain ferments. Grain ferments plus an equal volume of water were distilled and a volume collected equal to the volume of ferment used. This distillate was extracted with three portions of ether, and the phenols were isolated from the ether as described above for ether extracts.

Alcoholic Distillates. SVPs in alcoholic distillates (110 to 120 proof) were isolated by adsorption on 20- × 60-mesh Norit (American Norit Co., Inc., Jacksonville, Fla.) using 2% w./v. of the active charcoal with stirring for 2 hours. The charcoal was removed by filtration and extracted with ether by Soxhlet extraction for 60 hours. The ether was then extracted with aqueous $NaHCO_3$ and NaOH as previously described.

Active carbon was also used to isolate the SVPs in aged whiskey. The acidified NaOH extract was steam-distilled to separate the SVPs from wood extractives also adsorbed by the carbon. The isolation of SVPs from alcoholic distillates with active carbon was limited to distillates containing about 20 μg. per ml. or more SVPs, i.e., primarily of bacterial origin.

Determination of Steam - Volatile Phenols. COLORIMETRIC ASSAY. SVPs were separated from grain mashes and ferments by a simple distillation. A 15-ml. sample was diluted with 15 ml. of water, and 15 ml. of distillate was collected. To an aliquot of the distillate containing not over 200 to 300 μg. of phenols, 1 ml. of Folin-Denis reagent (*11*, *22*) was added, and the mixture diluted to 50 ml. with distilled water and mixed with 5 ml. of 25% Na_2CO_3. The aliquot used contained no more than about 1.5 ml. of ethanol to avoid formation of cloudiness. Absorbance was determined 20 to 60 minutes after the addition of Na_2CO_3 against a standard curve prepared with 4-ethylguaiacol (Bios Laboratories, Inc., New York, N. Y.) (Lumetron Colorimeter, Model 402E, 700-mμ filter, 13-mm. cells, and distilled water set at 0 absorbance). Values obtained represent the μg. per ml. of 4-ethylguaiacol needed to produce the same amount of color with Folin-Denis reagent.

Unaged whiskeys and high wines were analyzed without redistillation. Aged whiskeys required distillation to separate SVPs from tannins.

PAPER CHROMATOGRAPHY. SVPs were paper chromatographed by the method of Hossfeld (*16*) and Chang *et al.* (*6*). Phenylazo dyes of the phenols were prepared by coupling the phenols with diazotized sulfanilic acid by a modification of the quantitative procedure given by Snell and Snell (*32*). One or two drops of the concentrated, final ether extract were made alkaline with 4% aqueous Na_2CO_3, and 1% diazotized sulfanilic acid was added dropwise until no increase in color occurred. If the dyes precipitated, ethanol was added to dissolve them.

An amount of the phenylazo dyes sufficient for paper chromatographic analysis was applied to the treated paper by repeated spotting, using a stream of air for drying each application.

GAS CHROMATOGRAPHY. Isolated SVPs were analyzed by gas chromatography using a Perkin-Elmer vapor fractometer Model 154B with a thermistor detector, and a Leeds and Northrup Speedomax recorder Model G.

Two $^1/_4$-inch × 2-meter, stainless steel, packed columns were used. One column (Perkin-Elmer column "C"; stationary phase silicon DC200) eluted phenols in order of their boiling points (*25*), while the other (Perkin-Elmer column "K"; stationary phase of Carbowax 1500) gave a significantly longer retention time for an alkylphenol than for an alkylguaiacol of comparable boiling point (*1*). This difference in the performance of the two columns was an aid in predicting the structure of unknown phenols.

Operating parameters used for the gas chromotograph were as follows: Carrier gas, helium; column temperature, 160° C.; attenuation, full sensitivity for unknowns and less for knowns; chart speed, $^1/_2$ inch per minute; sample size, 10 μl.; carrier gas pressure to column, 10 p.s.i.g. for column "C," and 20 to 17.3 p.s.i.g. for column "K"; carrier gas flow at detector outlet, 25 ml. per minute for column "C," and 52 to 43 ml. per minute for column "K."

To duplicate previous retention times for known phenols, the helium pressure to column "K" had to be reduced slightly each time the column was put in operation, presumably because of loss of column substrate.

Retention times for chromatogram peaks obtained with column "C" were measured from the air peak. Retention times obtained with column "K," however, were measured from the point of sample injection since air was not separated from ether by this column.

Peak areas were determined (*21*) and converted to per cent of the total peak areas of the chromatogram, excluding peaks appearing before phenol with column "C" or guaiacol with column "K."

Catalytic Hydrogenation of Vinylphenols. The vinyl group of *p*-vinylphenol and 4-vinylguaiacol was hydrogenated in diethyl ether to the ethyl group using platinum oxide as a catalyst (*8*). Hydrogen was bubbled for 20 minutes into 10 ml. of ether at 35° C. containing 5 to 10 mg. of the vinylphenol and 10 mg. of catalyst.

After hydrogenation, the catalyst was removed by filtration, and the ether was evaporated to concentrate the phenols for chromatographic analysis.

Conversion of Ferulic Acid, *p*-Coumaric Acid, and Vanillin to Steam-Volatile Phenols. BY BACTERIA. Ferulic acid, *p*-coumaric acid, or vanillin was added to 0.5 to 1 liter of cooked, malted, and yeast-inoculated grain mash to give 50 μg. per ml. (Such mashes contain viable lactic acid bacteria introduced with the malt.) SVPs were determined after 3 to 4 days incubation time at 32° C. Control determinations were made of ferment without added precursor and of ferment with the test precursor added just before the distillation. High levels of SVP formation were taken as presumptive evidence of the presence of active strains of bacteria. Confirmation was obtained by isolation and reinoculation of pure cultures into sterile mashes.

Bacteria also were isolated from barley malt by adding whole kernels to screened stillage supplemented with 1% glucose and 1% yeast extract, incubating 48 hours, and then adding appropriate dilutions to tomato juice agar tubes (*12*). Selected colonies were screened for SVP production in stillage medium to which 50 μg. per ml. of ferulic acid had been added.

An aerobic organism which produced the corresponding vinylphenol from ferulic or *p*-coumaric acid was isolated from a commercial, dried, amylase product. Stillage medium with added ferulic acid and inoculated with the enzyme material gave an increase in SVPs after incubation for 1 to 3 days at 37° C. Since no increases were obtained when the same mixture was incubated under toluene, organisms from the enzyme preparation were plated on tomato juice agar and transferred to Fernbach flasks containing a syuthetic medium based on that of Skeggs *et al.* (*31*) to which precursors were added.

BY YEAST. The medium of Olson and Johnson (*23*), modified to contain 12% glucose, 0.25% $CaCl_2$, 2% KH_2PO_4, and 100 μg. per ml. of the phenolic cinnamic acid, was prepared in 150-ml. amounts in 250-ml. Erlenmeyer flasks. SVPs were determined after 72 hours of incubation at 32° C. with slow, rotary agitation.

Analysis of Grain for Ferulic Acid, *p*-Coumaric Acid, and Vanillin. Grains were analyzed by the method of McCalla and Neish (*19*, *20*) with some slight modifications. The initial extract of grain (80% ethanol) was not evaporated to dryness because the material extracted from corn formed a firm, rubbery cake. Ethanol was removed by continued evaporation on a steam bath with water added periodically.

Whatman No. 1 paper was used to chromatograph the extracted phenolic acids. The separated phenolic cinnamic acids were extracted from the paper by Soxhlet extraction, and Folin-Denis reagent (*11*) was used instead of Folin-Ciocalteu reagent (*10*).

Two "cooks" of corn were prepared in a 5-gallon pressure cooker. Each corn cook consisted of 1900 grams of corn meal and 10 grams of barley malt meal (premalt) with 5500 grams of water. An additional 50 grams of steam condensed in the cook. One batch was cooked by using a heating cycle with a maximum temperature of 280° F., with 30 minutes to reach 280° F., 30 minutes at 280° F., and 10 minutes to return to atmospheric pressure. For the other cook, a maximum of 250° F. was used with 27 minutes to reach 212° F., 28 minutes to reach 250° F., 5 minutes at 250° F., and 10 minutes to return to atmospheric pressure. These time cycles were patterned after cycles used in plant operations.

Grain meals were further ground in a micro-Wiley mill (1-mm. screen). For analysis, 100 grams of meal or 200 grams of cook were extracted with 300 ml. of boiling 80% ethanol.

For free, or unbound, cinnamic acids, one half of the initial ether extract was extracted with saturated aqueous $NaHCO_3$, and after acidification with dilute HCl and concentration by boiling

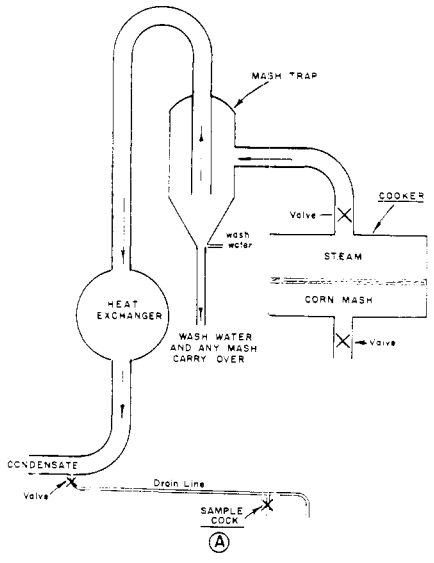

Figure 1. Diagram of cooker with sampling point for steam condensate

to a volume of 25 to 30 ml., the aqueous solution was continuously extracted with ether overnight. Total acids (free acids plus esterified acids) were determined on the remainder of the initial ether extract by using the saponification step.

Since free phenolic cinnamic acids were found in grain when the above method was used, the analyses were repeated with minimum heating of the sample. The ethanol was removed under vacuum, and several liquid-liquid extractors were used for each sample to eliminate concentrating the acidified aqueous extracts by boiling. The heating periods remaining were the two 5-minute periods the grain was boiled in 80% ethanol, the boiling water used for aqueous extraction, the vacuum evaporation of ethanol, and the continuous extraction with ether.

The corn cooks were preserved by freezing at 0 to 5° F. The grain meals used for the first analysis (same meals used for the cooks) were not preserved. Different lots of meals were used for the second analyses.

For identification, compounds present in the final ether were compared with known compounds as to R_F (toluene–acetic acid–water solvent, 5:1:4 v./v.), fluorescence in ultraviolet light after chromatographing, and colors developed by four spraying reagents [diazotized sulfanilic acid, ferric chloride, diazotized *o*-dianisidine (*27*), and diazotized *p*-nitroaniline (*2*)]. The diazotized reagents were prepared and mixed with two volumes of 4% aqueous Na_2CO_3 (or sufficient volume to make the reagent alkaline) and used immediately.

Vanillin was recovered from the ether extract with three portions of 5% aqueous NaOH and isolated by the procedure used for the phenolic cinnamic acids. The spraying reagent used was 2,4-dinitrophenylhydrazine (*27*). Because vanillin was lost when methanol (final extraction) was evaporated on the steam bath in preparation for colorimetric analysis, methanol was removed by evaporation at room temperature. Vanillin in the methanol extract was also determined by ultraviolet absorption by the method of Way and Gailey (*37*).

Results

Steam-Volatile Phenols from Grain. Phenols were found in condensed steam from corn cooked in the plant, and amounts sufficient for chromatographing were isolated. Paper chromatographic analysis revealed that the isolated phenols coupled with diazotized sulfanilic acid gave phenylazo dyes of the same color and R_F values as phenol and guaiacol. Gas chromatographic analysis, however, showed that the two phenols were not phenol and guaiacol but were probably a substituted phenol and guaiacol (Table I). The two phenols were found to be *p*-vinylphenol and 4-vinylguaiacol. Thus, the vinyl group was apparently expelled during coupling with diazotized sulfanilic acid.

The data in Table II and Figure 2 show that the two vinylphenols were found also in fermented grain and were the major SVPs present in grain fermented by a distillers' yeast (No. 54), which was known to produce several p.p.m. of SVPs during fermentation.

Table I. Retention Times for Phenol, Guaiacol, and Two Phenols Found in Condensed Steam from Corn Cooks

Compound	Retention Time, Minutes: Column "C"	Column "K"
Phenol	5.9	11.2
Guaiacol	11.3	7.7
Peak *A* (*p*-vinylphenol)	18.3	44.0
Peak *B* (4-vinylguaiacol)	30.3	24.1

Table II. Amounts of *p*-vinylphenol and 4-Vinylguaiacol Found in Condensed Steam from Cooked Corn and Stillage from Fermented Grain

Source of Phenols	Amount of Phenols Extracted, μg. per ml.[a]	Peak A, *p*-Vinylphenol, Area %[b]	Peak B, 4-Vinylguaiacol, Area %[b]	Peak A + B, Area %[b]
Steam from cooked corn	0.5	35	61	96
Stillage vapors from beer:				
Bourbon mash fermented by Yeast No. 54	2.2	30	55	85
Spirits mash fermented by Yeast No. 54	3.6	31	46	77
Bourbon mash fermented by Yeast No. 6	1.0	10	24	34
Spirits mash fermented by Yeast No. 6	0.6	16	19	35

[a] Expressed as μg. per ml. of 4-ethylguaiacol required to produce the same amount of color with Folin-Denis reagent.

[b] Values given are peak areas for the phenol expressed as % of the total peaks occurring in gas chromatograms of isolated phenols.

Table III. Phenols Found in Condensed Steam from Stillage, High Wines, and Aged Bourbon Whiskey of Above-Normal Steam-Volatile Phenol Content

Source	Total Phenols Extracted, μg. per ml.[a]	(1) 4-Methylguaiacol, Area %[b]	(2) 4-Ethylguaiacol, Area %	(3) *p*-Ethylphenol, Area %	Sum of (1), (2), & (3), Area %
Stillage from spirits mash, Yeast No. 54	13	36	23	39	98
High wines from beer above normal in volatile phenols	20	20	18	43	81
Aged bourbon whiskey of above-normal volatile phenol content	30	42	26	20	88

[a] Expressed as 4-ethylguaiacol to produce equivalent color with Folin-Denis reagent.

[b] Values given are peak areas for the phenol expressed as % of the total peaks occurring in gas chromatograms of isolated phenols.

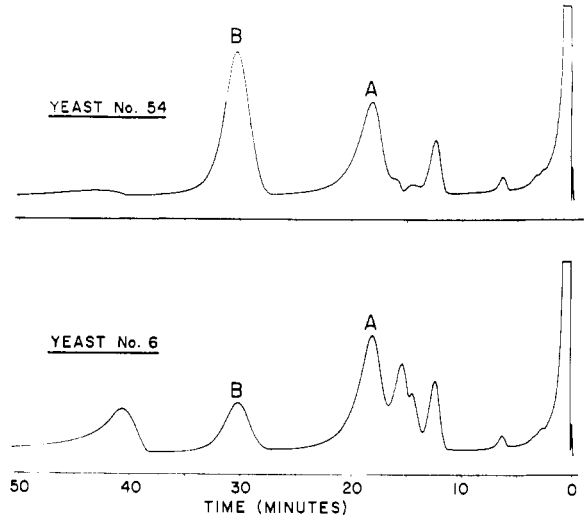

Figure 2. Steam-volatile phenols from stillage of bourbon mash fermented with Yeast No. 54 and Yeast No. 6 (column "C")

Peak A, *p*-vinylphenol; peak B, 4-vinylguaiacol

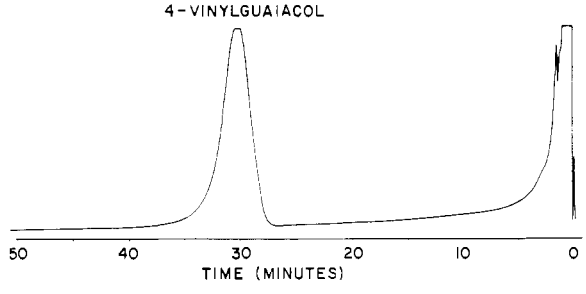

Figure 4. 4-Vinylguaiacol produced from ferulic acid by an aerobic organism (column "C")

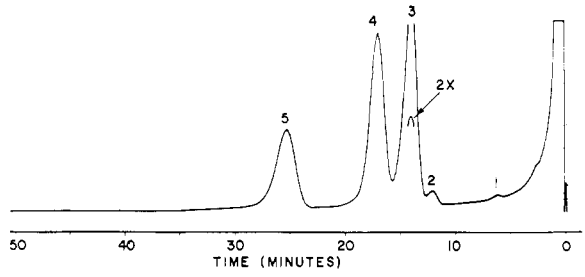

Figure 3. Steam-volatile phenols from grain spirits stillage above normal in phenol content (column "C")

Peak designations: 1 and 2, unknown; 3, *p*-ethylphenol; 4, 4-methylguaiacol; 5, 4-ethylguaiacol

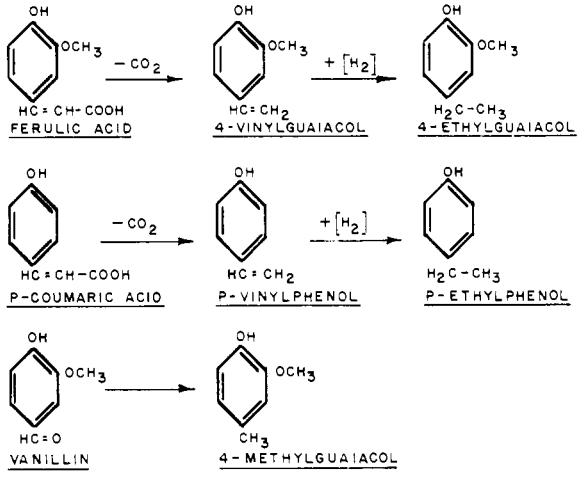

Figure 5. Transformation of precursors to steam-volatile phenols

Lower levels of these two vinylphenols were found in grain mash fermented with Yeast No. 6, which produces no detectable increase in SVPs during fermentation.

When high levels of SVPs were produced during fermentation, 4-methylguaiacol, 4-ethylguaiacol, and a phenol later identified as *p*-ethylphenol (see below) accounted for as much as 98% of the peaks appearing in the chromatograms, with a marked decrease in the vinylphenols (Table III and Figure 3). None of these three phenols was found in steam from cooked corn; apparently they were formed during fermentation. No 4-ethylguaiacol and only small amounts of 4-methylguaiacol and *p*-ethylphenol were found in grain ferments of normal SVP level.

Identification of *p*-Ethylphenol. By comparing peak sizes in the gas chromatograms of SVPs from fermented grains and alcoholic distillates, the compound appearing at approximately 14 minutes with the silicone column (column "C") was found to be the compound appearing at 20 minutes with the Carbowax column (column "K"). The greater retention time with column "K" suggested a substituted phenol. A salmon-pink phenylazo dye with an R_F of 0.90 to 0.95 appeared on paper chromatograms when this compound was present, and the relative amount of dye appeared to be proportional to the relative peak size in gas chromatograms. Chang *et al.* (*6*) found *p*-ethylphenol produced a pink dye with an R_F of 0.95 when coupled with diazotized sulfanilic acid. A sample of authentic *p*-ethylphenol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) had the same retention times with the two gas chromatograph columns and formed a phenylazo dye with diazotized sulfanilic acid having the same color and R_F as the unknown phenol. This was taken as positive identification of the phenol appearing at 14 minutes with column "C" and at 20 minutes with "K" as *p*-ethylphenol.

Identification of *p*-Vinylphenol and 4-Vinylguaiacol. The two phenols found in steam from cooked corn and produced by Yeast No. 54 were usually absent in grain ferments containing 4-methylguaiacol, 4-ethylguaiacol, and *p*-ethylphenol. This fact, coupled with estimated boiling points from data with column "C," suggested that the two unknown phenols might be *p*-vinylphenol and 4-vinylguaiacol.

Thermal decarboxylation of ferulic acid produces 4-vinylguaiacol (*26*). Synthesized ferulic and *p*-coumaric acids (*28*), upon heating, yielded products with the same gas chromatographic behavior as the two unknown phenols.

When, as discussed below, the two phenols thought to be *p*-vinylphenol and 4-vinylguaiacol were obtained by microbial action from *p*-coumaric acid and ferulic acid, respectively, sufficient quantities were obtained to prove their identity.

The presence of a vinyl group para to the hydroxyl group of the two phenols produced microbiologically from ferulic and *p*-coumaric acids was proved by catalytically hydrogenating the vinyl group to produce the corresponding ethylphenol. Hydrogenation decreased the vinylphenol and resulted in the appearance of ethylphenol. Production of the ethylphenols was confirmed by paper chromatographing the phenylazo dyes as well as by gas chromatography.

The vinylphenols produced microbiologically from *p*-coumaric and ferulic acids behaved chromatographically the same as the two phenols found in steam from cooked corn and produced by Yeast No. 54. Thus, *p*-vinylphenol and

4-vinylguaiacol occur in steam from cooked corn and also in grain ferments (along with *p*-ethylphenol, 4-methylguaiacol, and 4-ethylguaiacol). The relative retention times for the five SVPs found in grain ferments are given in Table IV, with *p*-ethylphenol as the reference compound.

Table IV. Relative Retention Times Found for Steam-Volatile Phenols Isolated from Grain Ferments

Compound	Column "C" (Silicone D.C. 200)	Column "K" (Carbowax 1500)
p-Ethylphenol	1.000	1.000
4-Methylguaiacol	1.220	0.524
p-Vinylphenol	1.253	2.200
4-Ethylguaiacol	1.803	0.658
4-Vinylguaiacol	2.082	1.217

Table V. Effect on Ferulic and p-Coumaric Acids of Exposure to Cooking Temperatures Used for Corn

Fraction No.	Phenol Content of Each 25-Ml. Fraction of Distillate Collected, μg. per ml.[a]: From aqueous solution of ferulic acid	From aqueous solution of *p*-coumaric acid
1	1.6	1.3
2	2.7	1.8
3	3.9	2.8
4	5.2	3.5
	Remaining aqueous solution (200 ml. with 0.1 gram of acid) autoclaved for 40 minutes at 15 p.s.i.g., then distillation continued	
5	46	10.0
6	30	10.1
7	19	10.2

[a] Expressed as 4-ethylguaiacol required to produce equivalent color with Folin-Denis reagent.

Ferulic and *p*-Coumaric Acids: Precursors of Vinylphenols. The synthesis of *p*-vinylphenol and 4-vinylguaiacol by the thermal decarboxylation of *p*-coumaric and ferulic acids suggested that this might be the mode of formation of these two vinylphenols during the cooking of corn. Both ferulic and *p*-coumaric acids occur widely distributed in plants in ester form, and the *Gramineae* are especially rich in ferulic acid (*2*). The two acids have been reported in grain straw (*3*, *4*), testinic acid of barley (*33*), and recently in barley (*35*) and sake (*39*). Ferulic and *p*-coumaric acids, along with caffeic and sinapic acids, have been postulated as intermediates in the biosynthesis of lignin in *Salvia splendens* (*20*).

Also pertinent, *p*-vinylphenol has been isolated from poppy straw and reported as a new compound, although this may be an artifact produced by the isolation procedures (*17*, *30*). This compound has been reported also to be the aglycon of a glycoside of *Virburnum furcatum* (*13*).

The thermal stability of ferulic and *p*-coumaric acids at the cooking temperature of corn was tested by autoclaving a dilute aqueous solution of the two acids. Although this temperature was well below the decomposition temperatures of the two phenolic cinnamic acids, sufficient amounts of SVPs were produced to explain the small amounts produced when corn is cooked (Table V).

If ferulic and *p*-coumaric acids are the precursors of the vinylphenols produced during cooking, the two cinnamic acids also may be precursors of SVPs produced during fermentation.

A preliminary investigation was made to determine if ferulic and *p*-coumaric acids were converted to SVPs by various commercial, dried amylase products. Although no volatile phenols were produced directly by these enzymes, several of the products contained aerobic organisms which produced the vinylphenol when grown in a defined medium (*31*) to which ferulic acid or *p*-coumaric acid had been added. The lower-boiling homologs were not detected (Figure 4).

Recently, Finkle *et al.* (*9*) reported finding a constitutive, nonoxidative decarboxylase in strains of *Aerobacter* which decarboxylates 4-hydroxycinnamic acids to the corresponding 4-vinylphenol.

When the two yeasts used during this study were grown in a defined medium, greater increases in SVPs occurred when ferulic acid or *p*-coumaric acid was added to the medium. Yeast No. 54, which produces SVPs during fermentation, gave the largest increase in phenols (Table VI).

When cinnamic acid was added to the defined medium, Yeast No. 54 produced the typical odor of styrene. The conversion of cinnamic acid to styrene by yeast and molds has been previously reported (*7*, *36*).

Attempts to isolate bacteria from plant grain ferments which would consistently produce SVPs from ferulic acid were unsuccessful. Some of the bacterial isolates produced significant increases in SVPs when isolated colonies were transferred to media supplemented with ferulic acid, but this activity was not retained upon repeated transfers.

Since malted grains (barley and rye) are sources of contamination in fermenting grain mashes, barley malt was tested for bacteria capable of producing volatile phenols from ferulic acid and *p*-coumaric acid. Three isolates were obtained from barley malt which gave good conversion of ferulic acid to 4-vinylguaiacol when grown in a stillage medium with 40 μg. per ml. of ferulic acid added (Table VII). These bacteria, which were microaerophilic (probably lactobacilli), did not lose this ability when transferred to fresh medium every 2 to 3 days. "Stab" cultures in tomato juice–agar medium lost about half of their activity. No 4-ethylguaiacol was produced by the bacteria from barley malt, even when grown with yeast in grain mash.

Table VI. Levels of Steam-Volatile Phenols Produced (μg. per Gram)[a] by Distillers Yeasts in Defined Media (72 Hours at 32° C.)

Medium	Precursor Added, 100 μg. per Ml.: None	Ferulic acid	*p*-Coumaric acid	Cinnamic acid
		Trial 1		
Uninoculated medium	0.1	1.3	0.9	...
Medium fermented with Yeast No. 54	2.9	10.0	9.1	...
		Trial 2		
Uninoculated medium	0.6	1.7	1.1	0.6
Medium fermented with Yeast No. 54	2.0	8.3	7.6	2.0
Medium fermented with Yeast No. 6	0.7	4.1	2.5	0.7

[a] Reported as 4-ethylguaiacol required to produce equivalent color with Folin-Denis reagent.

Table VII. Production of Steam-Volatile Phenols by Bacteria Isolated from Barley Malt

Medium: grain stillage, 1% glucose, 1% yeast extract plus 40 μg. per ml. of ferulic acid

Inoculation	SVPs, μg. per Ml.		
None	3.2		
Yeast No. 54	4.4		
	Bacterial Isolates: A	B	C
(1) 3rd Transfer in stillage medium	43	43	41
(2) Tomato juice stab cultures	17	18	16
(3) Inoculum (2) plus Yeast 54	20	22	21

Table VIII. Amounts of Phenol Precursors (μg. per Gram) Found in Corn, Barley Malt, and Cooked Corn

Sample	Ferulic Acid Trial 1	Ferulic Acid Trial 2[a]	p-Coumaric Acid Trial 1	p-Coumaric Acid Trial 2[a]	Vanillin By Folin-Denis reagent	Vanillin By ultraviolet absorption
Corn (free)	6.0	5.2	6.4	5.3	4.0	3.2
Corn (total)	8.2	...	9.2	...	...	...
Barley malt (free)	7.8	3.2	8.7	2.2	3.2	1.8
Barley malt (total)	11.8	...	11.0	...	...	...
Corn (free) cooked at:						
250° F.	26	39	33	36	19	15
280° F.	27	47	46	52	31	30

[a] Minimum heating during extraction.

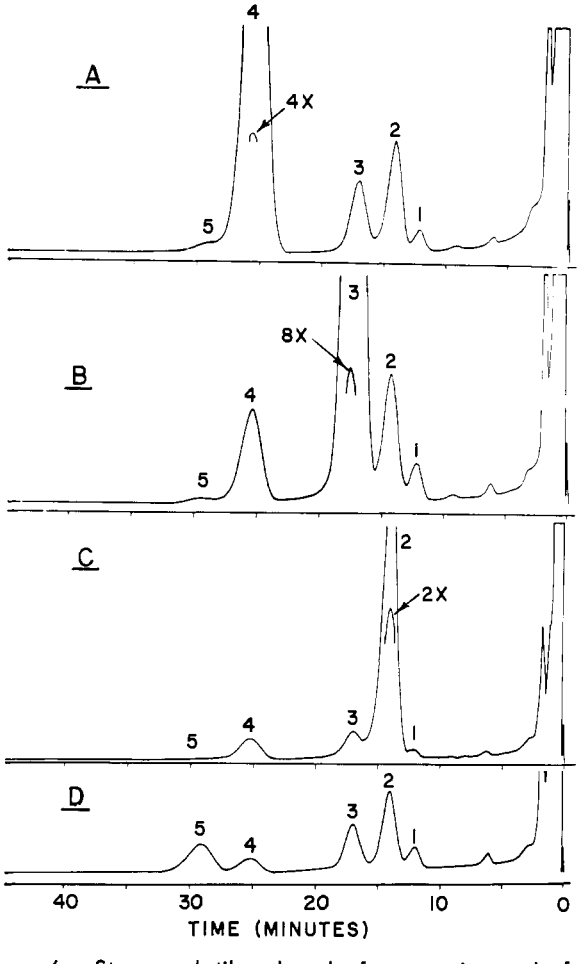

Figure 6. Steam-volatile phenols from grain mash fermented 72 hours at 32° C. after addition of 50 μg. per ml. of (A) ferulic acid, (B) vanillin, (C) p-coumaric acid, and (D) with no phenol precursor added (column "C")

Peak designations: 1, unknown; 2, p-ethylphenol; 3, 4-methylguaiacol; 4, 4-ethylguaiacol; 5, 4-vinylguaiacol

Ferulic Acid, *p*-Coumaric Acid, and Vanillin: Precursors of *p*-Ethylphenol, 4-Ethylguaiacol, and 4-Methylguaiacol. Addition of ferulic and *p*-coumaric acids to distillery mash which had been cooked, malted, and yeasted revealed that SVPs increased during fermentation but to different degrees, apparently due to the types and number of bacteria present. Vinylphenols were increased after incubation at 25° C. for 3 days, whereas ethylphenols were increased after incubation for 3 to 4 days at 32° C., suggesting that the vinylphenols were intermediates in the conversion of the phenolic cinnamic acids to ethylphenols.

Since the conversion of ferulic acid to 4-ethylguaiacol produced no increase in 4-methylguaiacol, the authors concluded that ferulic acid was not the precursor of 4-methylguaiacol. The absence of *p*-cresol, which would be expected from *p*-coumaric acid, supported this conclusion.

The analysis of grain for ferulic and *p*-coumaric acids (discussed below) revealed that these two cinnamic acids are increased by the cooking process. Since vanillin is a well known degradation product of lignin (*24*) and has been found in fusel oil (*5*), vanillin was added to set mash. This resulted in large increases in 4-methylguaiacol. Thus, with proper bacterial flora present, the addition of ferulic acid, *p*-coumaric acid, and vanillin regularly resulted in increases in 4-ethylguaiacol, *p*-ethylphenol, and 4-methylguaiacol, respectively. These transformations are shown in Figure 5.

Figure 6 shows the effect of adding the three precursor compounds to yeast-inoculated grain mash upon the SVPs produced during fermentation.

Ferulic Acid, *p*-Coumaric Acid, and Vanillin in Corn and Barley Malt. Phenolic cinnamic acids are usually present in plants in ester form (*14, 20*), although ferulic and *p*-coumaric acids have been reported to occur unbound in barley (*35*) and tomato wall tissue (*34*). Ferulic acid also has been found in unbound form along with other phenolic acids in wheat germ (*18*).

Corn, barley malt, and corn cooked at two different temperatures were examined for ferulic and *p*-coumaric acids and vanillin. The results of two trials (Table VIII) show that these grains contain small amounts of free ferulic and *p*-coumaric acids and vanillin. By cooking corn, the value for each of these compounds was raised substantially.

Some increases in the phenolic cinnamic acids were produced by saponification but not in sufficient amounts to suggest that the phenolic cinnamic acids released by cooking were originally present in ester form. Nor do glycosidic linkages appear probable, since boiling the acidified grain extracts did not increase the precursors nearly as much as cooking.

The higher temperature cycle used for cooking corn had the greatest effect on the amount of vanillin released. This finding is supported by the ratio of SVPs in grain ferments having high phenol levels. 4-Methylguaiacol is the major SVP when corn is cooked at 280° F. (see Table III, aged whiskey) while *p*-ethylphenol is the major SVP when corn is cooked at 250° F. (Table III, high wines and stillage.)

Other phenolic acids were present in the grain extracts. Sinapic acid was found in barley malt and cooked corn, and caffeic acid appeared to be present in all extracts. Sinapic and caffeic

acids (Mann Research Laboratories, New York, N.Y.), however, gave no increase in SVPs when added to grain ferments, possibly because of the lower volatility of the phenols produced from these acids.

Discussion

Precursors of five steam-volatile phenols found in alcoholic grain ferments are present in small amounts in free form in raw corn and barley malt. Larger amounts are present in bound form and released when corn is cooked. The bound form of the precursors is not known. They do not appear to be present as glycosides nor do the phenolic cinnamic acids appear to be present as esters. The close relationship of the precursors to lignin suggests the thermal breakdown of lignin or an analog of lignin at cooking temperatures. Although cooking conditions are relatively mild, the amount of breakdown necessary to produce the amount of precursors found in cooked corn is small. By calculation, the precursors found in corn cooked at 280° F. represent about 5% of the lignin of the corn hull (*15*).

Vinylphenols are produced by the decarboxylation of ferulic and *p*-coumaric acids. Small amounts of *p*-vinylphenol and 4-vinylguaiacol can be produced by cooking and by some strains of yeast during fermentation. Larger amounts, apparently limited by the amount of cinnamic acids produced when grain is cooked, can be produced by bacteria. In view of these observations, it is quite possible that *p*-vinylphenol and 4-vinylguaiacol were present in the fusel oil extracted by Braus and Miller (*5*) and that guaiacol and phenol were produced by the breakdown of the vinylphenols during distillation of the SVPs. Also, the vinylphenols in the distillate fractions would not have been distinguished from guaiacol and phenol by chromatographing the phenylazo dyes.

Formation of the ethylphenols and 4-methylguaiacol is not completely clear. In most distillates containing above-normal levels of phenols, *p*-ethylphenol, 4-methylguaiacol, and 4-ethylguaiacol were the major phenols. However, above-normal amounts of the vinylphenols were found early in the fermentation when the cinnamic acids were added to the medium, and bacteria were isolated from barley malt which would produce the vinylphenols from the cinnamic acids. Thus, the ethylphenols appear to be produced by the reduction of the vinyl group of the vinylphenols. The production of the ethylphenols from ferulic and *p*-coumaric acids may result from the synergistic action of the microflora, although the conversion of *p*-coumaric acid to *p*-ethylphenol by a single organism (*L. pastorianus*) has been reported (*38*). In all cases, when distillates from plant grain ferments containing above-normal amounts of SVPs were analyzed, the two ethylphenols and 4-methylguaiacol were found. This suggests that the reduction of the carbonyl group of vanillin and the vinyl group of the vinylphenols requires the same conditions, or possibly the same organisms.

Acknowledgment

The authors are indebted to Hiram Walker & Sons, Inc., for permission to conduct the study and for making equipment and preliminary data available, and to J. M. Van Lanen for helpful advice during the course of the work and in preparation of this manuscript.

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Received for review August 12, 1963. Accepted December 16, 1963. Data in this paper have been taken from a thesis submitted by one of the authors (RDS) in partial fulfillment of requirements for the M.S. degree, Bradley University.