

# ISOLATION, ORIGIN, AND SYNTHESIS OF A BREAD FLAVOR CONSTITUENT<sup>1</sup>

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## ABSTRACT

A substance with a strong, pleasant cracker aroma was isolated from fermented liquid brews after boiling and was shown to be identical with that isolated from dried bread. The reacting compounds were determined by synthesis of the aroma substance and demonstration of its identity with those isolated from bread and from boiled brews. Aromas produced when dihydroxyacetone is heated in solution with various amino acids are described; this triose appears to react in a manner entirely typical of the Maillard browning system.

Numerous organic compounds have been identified in bread which, by their volatilities and pronounced odors, must play some part in the over-all aroma. Some of these have been compiled in a recent review (5). In very few instances, however, has a distinctively breadlike aroma characteristic been ascribable to a particular substance, rather than a mixture of substances. In the studies reported here, such a substance has been found in bread, and also as a constituent of a simple liquid ferment after the ferment had been boiled. After it was determined what substance in the ferment reacted to produce the aroma, it was possible to synthesize the aroma from the same substances in a variety of solvents, with heat the only necessary catalyst.

It has often been noted in the literature (1,3,4), and was observed by us, that the conditions under which model systems of amino acids and reducing sugars react to produce aroma substances seem severe by comparison with the conditions in such processes as bread-baking. A question was raised whether some of the volatile aldehydes arising during dough fermentation (2) might not themselves react with amino acids to engender yet other aromatic substances. During distillation of the volatiles from a liquid ferment to make a test of this possibility, a strong and easily recognized breadlike aroma developed in the boiling brew. Prolonged boiling yielded a weak, yeasty aroma in the distillate, while the undistilled liquid acquired an odor exactly like that of crackers. When the distillate and residue were recombined, the aroma again suggested fresh bread, and was seemingly undiminished.

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This observation led to the following investigations, all interest in the volatile aldehydes having become quite secondary at this point.

### Materials and Methods

*Liquid Ferments.* It was established immediately that the cracker aroma could be produced from a very simple brew, containing only glucose, yeast, water, and an inorganic buffering system like dipotassium phosphate-monopotassium phosphate. Brews were prepared in which the proportions of yeast and glucose were varied, and were buffered at several pH values. Fermentations were carried out under several conditions of temperature and time; the fermented brews were heat-processed both by boiling on a hot plate and by autoclaving at 20 p.s.i.g. Before the boiling or autoclaving, the brews were always adjusted to pH 7, although it was found that the aroma intensity developed fairly independently of pH in the range pH 6-8.

*Recovery of Aroma Material from Brews.* The cracker aroma is only feebly volatile with steam, and distillation of the brews was therefore not attempted as a rule. Ether and chloroform are moderately good solvents for the aroma substance, while such nonpolar solvents as hydrocarbons and carbon tetrachloride are virtually useless. Stubborn emulsions are common, and batch extraction of the salt-saturated, boiled brew with ether was most generally used to recover the crude aroma material, contaminated chiefly with brown pigment. A partial purification, at the expense of significant material losses, could be effected by repeatedly dissolving the extract in a small volume of absolute ether, precipitating with an excess of petroleum ether, and decanting the supernatant liquid. On one occasion the crude extract was vacuum-distilled in a short-path micro still, giving a very small yield of faintly yellow oil with enormous odor intensity.

*Recovery of Aroma Material from Bread.* When fresh bread was vacuum-dried at low temperature until totally odorless and ground to powder, and the powder was extracted by percolation of solvent, both methanol and water were highly effective in extracting the aroma material with an odor identical to that of the heated brews. Ether extracted only a small amount of odorous substance, the odor in this case being acrid and unpleasant, rather reminiscent of extremely stale bread. Methanol was used routinely, since water caused troublesome swelling of the bread powder. The crude methanol extracts were heavily contaminated with fat and sugars; the former could be largely removed by repeated washing with n-heptane, while the residual extract was taken up in water, saturated with salt, and extracted with

ether to segregate the aroma substance in a higher state of purity.

### Results

*Optimum Conditions for Aroma Development in Brews.* If a fermented brew was carefully filtered free of yeast cells before boiling, there was never any appreciable odor development. If some fresh yeast was added to a cell-free brew filtrate just before boiling, the aroma appeared. Whenever the aroma was appreciable, it was accompanied by rapid and intense browning. Fermentation is a necessary prerequisite for aroma production, since freshly prepared yeast-glucose-water mixtures failed to develop the cracker aroma on boiling. If an enzymatically active water extract of plasmolyzed yeast was substituted for whole yeast cells, both in the fermentation and at the boiling stage, aroma production was equally good. It was concluded that two constituents (at least) are necessary for the aroma to be formed: 1) an endocellular, water-soluble yeast component, and 2) an intermediate in the conversion of glucose to alcohol.

From the numerous experiments performed with variations in yeast/glucose proportions, fermentation temperature, time, pH during fermentation, and heat-treatment conditions, the salient observations were these:

1. Low proportions of yeast to glucose gave the best aromas for equivalent fermentation time.
2. Short-time fermentations yielded more aroma for equivalent yeast levels.
3. Fermentation temperature is not at all critical.
4. The best aromas were generated from brews fermented in the range pH 5.0-5.6.
5. The pH during boiling is unimportant, at least over the range pH 6-8.
6. Boiling on a hot plate seems to give a slightly stronger aroma than autoclaving the same brew.
7. When the brew is fermented under vigorous aeration, the odor production is unaffected.

A further observation was that addition of some fresh yeast to a brew immediately prior to boiling always depressed odor production. Traces of cysteine or glutathione produced the same effect, suggesting that the inhibition is caused by sulfhydryl compounds in the yeast cells.

A series of experiments was performed in which various enzyme inhibitors were added to the brew ingredients before incubation. Unin-

hibited brews were incubated as controls. Aroma intensities after autoclaving were compared with the controls and between the variously treated brews. The inhibitors used were mercuric chloride, sodium pyrophosphate, sodium iodoacetate, and sodium fluoride, at several times the levels necessary to block the production of alcohol and carbon dioxide completely. Pyrophosphate and mercuric chloride prevented aroma development, but fluoride and especially iodoacetate dramatically enhanced the aroma intensities.

Iodoacetate is a powerful inhibitor of glyceraldehyde-3-phosphate dehydrogenase, while pyrophosphate effectively blocks the cleavage of fructose-1,6-diphosphate by aldolase. The above observations are best accounted for if it is assumed that one or both of the triose phosphates is a reactant in the formation of the cracker aroma. Blocking aldolase activity with pyrophosphate would prevent accumulation of the triose phosphates, while iodoacetate by blocking oxidation of the aldehyde isomer, would cause the triose phosphates to "pile up" in abnormal amounts.

*Synthesis of Cracker Aroma.* As the triose phosphates have reactive carbonyl groups, they should react readily with amino acids. Rather than the highly unstable phosphate esters, dihydroxyacetone and glyceraldehyde were selected. Seventeen amino acids were added to portions of dihydroxyacetone (DHA) in aqueous solution, using one mol. each of amino acid and DHA in all cases. The systems were all brought to pH 7.0 by addition of dipotassium phosphate, made up to 50 ml., and boiled on a hot plate. Table I records the aromas noted after about 15 minutes of boiling.

TABLE I

AROMAS PRODUCED ON BOILING AQUEOUS AMINO ACID-DIHYDROXYACETONE MIXTURES

AMINO ACID	DESCRIPTION OF AROMA
Proline	Very strong; crackers; crust; toast
Lysine	Strong; like dark corn syrup
Valine	Strong; yeasty; protein hydrolysate
Alanine	Weak; caramel
Glutamic acid	Moderate; chicken broth
Aspartic acid	Very weak
Arginine	Very weak
Cysteine	Mercaptan; hydrogen sulfide
Hydroxyproline	Weak; vaguely like proline
Phenylalanine	Very strong; hyacinth
Leucine	Strong; cheesy; baked potato
Isoleucine	Moderate; crust
Serine	Weak; vaguely breadlike
Threonine	Very weak
Methionine	Baked potato
Glycine	Baked potato
Histidine	Very weak

Thus proline, valine, and isoleucine gave the most significant aromas of the amino acids tested, with proline matching the cracker aroma recovered from the boiled brews and from bread perfectly. When D,L-glyceraldehyde was substituted for DHA in the reaction with proline, the aroma developed much more slowly, but was identical in nature. Other alpha-hydroxy carbonyl compounds were also tested, among which acetol (1-hydroxyacetone) and its acetate ester gave the same aroma. Acetylmethylcarbinol gave a totally different aroma with proline, exactly like that of scorching fruit preserves; while pyruvaldehyde, the aldehyde corresponding to acetol, gave only an overpowering burnt-sugar smell and almost instantaneous browning.

Most remarkable was the result when pyrrolidine was reacted with DHA in water. Without any external application of heat, the mixture grew warm, and within 5 minutes had browned deeply, the cracker aroma being easily recognizable through the foul odor of pyrrolidine. Further, when the vapors from a boiling proline-DHA-water mixture were passed into dilute aqueous barium hydroxide, barium carbonate precipitation was noted immediately. It could be inferred that proline decarboxylates to pyrrolidine during the reaction with DHA and that the decarboxylation is a necessary slow step in production of the cracker aroma.

The isolated, crude synthetic aroma material was dissolved in 2N sulfuric acid and boiled for 1 hour; it was observed here that below about pH 3, the aroma disappeared. On neutralization the aroma reappeared, seemingly undiminished by boiling in acid. A second sample was boiled for 45 minutes, under reflux, in 2N sodium hydroxide, again without discernible change in the aroma. Because of the apparent salt formation in acid medium, a sample of the synthesized material was steam-distilled exhaustively, and the distillate pH was measured at 9.0. This degree of alkalinity implies that the nitrogen from the proline or pyrrolidine is present in the aroma compound. Tests for primary and secondary amines were, however, negative, although an alkali fusion test confirmed the presence of nitrogen in the compound.

The ability of the aroma compound to form a salt with a mineral acid presented a simple and effective method of concentrating small amounts. The crude synthesis mixture is steam-distilled, and the distillate is carefully acidified, then evaporated at reduced pressure nearly to dryness. The extremely hygroscopic syrup has been kept in a stoppered bottle in the refrigerator for 3 weeks with no detectable alteration in aroma when neutralized.

Several syntheses of the cracker aroma were performed in various organic solvents, both with and without the use of acid or base catalysts. In general, no dramatic differences in yields could be obtained in any of these experiments; in all cases, a high yield of aroma was accompanied by intense browning, although the pure aroma substance has little color itself.

*Identity between Natural and Synthetic Aromas.* Attempts to prepare 2,4-dinitrophenylhydrazones and 3,5-dinitrobenzoates from the natural or synthetic cracker aroma principles have been unsuccessful. The vacuum-distilled material from the ether extract of a cooked brew was insufficient for chemical analysis, but was used to obtain an ultraviolet spectrum in the range 220–360  $m\mu$ , in methanol solution, using a Beckman DU instrument. A single maximum was recorded at 295  $m\mu$ , with a minimum at 243  $m\mu$ . Ultraviolet spectra of the concentrated steam distillates from the synthetic, brew-extracted, and bread-extracted aroma materials showed the same maxima and minima.

In an attempt to separate a silver salt from the proline-DHA reaction mixture, it was noted that metallic silver was quickly deposited as a mirror. This occurred also in the case of the purified synthetic product from DHA-pyrrolidine or acetol-pyrrolidine. When the partially purified extracts from boiled brews and from bread were treated with 5% silver nitrate, the identical result occurred. In every instance, the odor was changed to an unpleasantly acrid one, strongly resembling that of stale bread or the inside of a long-unwashed breadbox. This was reminiscent of the odor extracted from bread by ether. When a quantity of air-dried stale bread was extracted with acetone, the same odor was isolated in larger yield. Finally, silver nitrate solution oxidized the steam-distilled synthetic cracker aroma substance in identical fashion, with appearance of the stale odor and the silver mirror.

The stale-odor material proved much more volatile than the cracker aroma, and was easily distilled from the aqueous reaction mixtures. The distillates originating from the following sources were collected: (a) acetone extract of stale bread; (b) methanol extract of fresh bread, oxidized by silver nitrate; and (c) synthetic cracker aroma, oxidized by silver nitrate.

Each distillate was treated with 0.5% 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid, and a finely divided solid hydrazone was isolated in each case. The derivatives were spotted on a paper chromatogram, together with the corresponding hydrazones of acetone and acetol, and with the reagent. After ascending development with heptane:methanol, the oxidized synthetic derivative showed only a station-

ary spot, while the oxidized fresh bread extract derivative gave a strong stationary spot plus a diffuse streak in the  $R_f$  range of 0.06–0.30. The acetone-extracted stale bread derivative gave a strong stationary spot and a weaker spot of acetone-DNPH. The stationary spots were not due to reagent, as was shown by spraying with alcoholic potash; the reagent spot was unaffected, the others turning blue, as typical of the bis-hydrazones from dicarbonyl compounds.

The three crops of derivatives were recrystallized from chloroform/petroleum ether, and melting-point determinations were attempted on a heated metal block. In each instance, there was no melting, but rapid sublimation occurred at 283°C. Portions of all three sublimates were collected on a single microscope cover glass and rubbed together, and the mixed sample again sublimed sharply at 283°C.

From the ultraviolet and sublimation data, together with the odors observed, it seems very likely that the natural and synthetic cracker aromas are identical, and that both are readily oxidized by silver nitrate to a product identical with the stale odor formed in bread by autoxidation.

### Discussion

Although the experimental results offer some evidence for the origin of one important aroma component in bread, a proof of structure would make the evidence much more positive. It has not yet been possible to increase the reaction yield in the synthesis to a point where the product can be obtained in greater than milligram amounts, which makes the aroma intensity the more remarkable. On an equimolar basis, the proline-DHA reaction product dominates all the others listed in Table I, and it is this product that appears to develop the characteristic stale odor on oxidation. Furthermore, the proline-DHA product is considerably less volatile than the aromas formed by the other amino acids, which may account for the persistence of the cracker aroma in bread, and especially in crackers themselves.

What is less apparent is how the cracker aroma can be extracted from bread that has been vacuum-dried to an odorless state. The compound is hygroscopic and obviously highly polar, besides being ionizable; such a substance could readily be bound through ionic or hydrogen-bond forces to any of several possible sites in the bread. A study of this phenomenon should be of great value in explaining some hitherto mysterious aspects of flavor in baked foods, as for example the partial return of fresh flavor when dry rolls, etc., are "refreshed" in an oven.

If the reaction between dihydroxyacetone and amino acids follows

a common mechanism, it may become possible to control flavors by additive combinations of the appropriate amino acids. A few preliminary experiments in this laboratory suggest that such a common mechanism may not hold good, however, except for the simple alpha-monoamino acids. This area is being studied further.

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