

IDENTIFICATION OF CARBONYL COMPOUNDS PRODUCED IN PRE-FERMENTS¹

BYRON S. MILLER, JOHN A. JOHNSON, AND ROBERT J. ROBINSON

ABSTRACT

Extraction of a pre-ferment with diethyl ether or of the condensed gases with carbon tetrachloride was ineffective for obtaining sufficient minor flavor constituents for analysis by gas chromatography using thermistors as detectors. Passing the natural gases from a pre-ferment through 2,4-dinitrophenylhydrazine reagent was a convenient way of isolating carbonyl compounds in reasonable quantity. Nitrogen also was effective for sweeping the carbonyl compounds from a pre-ferment.

Formaldehyde, acetaldehyde, acetone, isobutyraldehyde, n-butyraldehyde, methylethylketone, isovaleraldehyde, and n-valeraldehyde were detected by gas chromatography of the precipitated carbonyl derivatives by heating the mixed hydrazones in the presence of alpha-ketoglutaric acid. All of these but n-valeraldehyde were isolated from the precipitated mixture by passing it through a Celite column. In addition, 2-methyl-1-butanol was isolated by column chromatography. Individual components were identified by comparing them with authentic compounds using paper chromatography in two different solvents and by ultraviolet and infrared spectral analyses.

There is much interest in pre-ferments and their use in continuous processes for breadmaking. Some, however, consider the flavor of bread made by the continuous processes inferior to the flavor produced by the customary sponge process. Efforts are needed, therefore, to improve flavor characteristics of pre-ferments used in continuous processes. Since much of bread flavor originates during fermentation and since alterations in processing provide opportunities to modify or intensify flavor, knowledge concerning flavors produced in pre-ferments is essential to advance continuous breadmaking methods and to improve the organoleptic qualities of bread.

The small concentration of flavor compounds in pre-ferments makes analyses difficult. Even with the most sensitive methods, such as gas chromatography, it is necessary to concentrate the compounds by extraction, distillation, formation of derivatives, or by column or paper chromatography. Extreme care must be exercised while pre-ferments or pre-ferment extracts are being concentrated because of the volatile nature of flavor components and because of possible

¹Manuscript received February 27, 1961. Presented at the 46th annual meeting, Dallas, Texas, April 1961. Co-operative investigations between the Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, and the Department of Flour and Feed Milling Industries, Kansas Agricultural Experiment Station, Manhattan. Contribution No. 354, Department of Flour and Feed Milling Industries, Kansas Agricultural Experiment Station, Manhattan.

interactions of certain components.

Johnson, Miller, and Curnutte (5) and Smith and Coffman (13) have investigated chemical compounds produced in pre-ferments. Using paper and column chromatography, Johnson, Miller, and Curnutte (5) analyzed for organic acids and esters and obtained some quantitative data. Using gas chromatography, Smith and Coffman (13) separated and identified several of the neutral components of pre-ferment. Neish and Blackwood (6) determined the organic acids and certain other compounds produced during the dissimilation of glucose by yeast. One of the carbonyl compounds measured quantitatively was acetoin (6). Visser't Hooft and de Leeuw (14) demonstrated that acetoin is slowly oxidized to diacetyl, but because of its high volatility, little diacetyl was found in the finished bread.

Ng, Reed and Pence (7) and Wiseblatt and Kohn (15) examined baked bread for compounds that may be responsible for bread flavor. It is likely that several of the flavor compounds in bread arise from the baking, whereas others originate during fermentation (9). This paper describes the isolation and qualitative identification of several volatile compounds, mainly carbonyls, which are present in pre-ferments.

Materials and Methods

The pre-ferment used throughout this work was the salt-buffered type employed by Robinson *et al.* (11). The analyses of pre-ferments for constituents involved extraction with ether after 4 hours of fermentation, collection of gases evolved during fermentation, and formation of derivatives from extracted or collected carbonyl compounds. Further isolation and identification of flavor components were based on paper, column, and gas chromatography supplemented with ultraviolet and infrared spectroscopy.

Gas Chromatography. The gas chromatography unit used was a Perkin-Elmer Vapor Fractometer Model 154-C² equipped with thermistors. The columns were 1/4-in. × 6-ft. tubes packed with the materials noted in Tables I and II. The experimental conditions for gas chromatography are noted in Tables I and II.

Extraction of Pre-ferments with Diethyl Ether. A 200-ml. portion of freshly centrifuged pre-ferment was extracted in a separatory funnel with 40 ml. of diethyl ether. This ether layer was removed and added to a fresh 200-ml. aliquot of pre-ferment along with 20 ml. of additional ether. Extraction was completed as for the first portion

²The mention in this publication of a trade product, equipment, or a commercial company does not imply its endorsement by the U.S. Department of Agriculture over similar products, companies, etc., not named.

and the process repeated until 1,200 ml. of pre-ferment were extracted. The ether layer from the last 200-ml. aliquot was centrifuged, and the ether was decanted into a glass-stoppered vial and dried over anhydrous sodium sulfate before being analyzed in the gas chromatograph, using 30- μ l. quantities.

Extraction of Condensate from Evolved Gases with Carbon Tetrachloride. Sixteen liters of pre-ferment (11) were allowed to ferment in a 5-gal. polyethylene bottle connected in series by tubing to two 1-liter wash bottles in Dewar flasks. The first bottle was surrounded by an ice-salt mixture and the second by a dry ice-acetone mixture. The liquid (approximately 12 ml.) in the first trap was added to the dry-ice trap and the resulting solution extracted in a separatory funnel with 5 ml. of carbon tetrachloride. The separated solvent was stored in a glass-stoppered vial over anhydrous sodium sulfate and placed in a deep freeze until analyzed by gas chromatography using 30 μ l. quantities. Carbon tetrachloride was chosen as a solvent because of its high density and stability and because all impurities could be removed by repeated distillations.

Confirming the Presence of Carbonyl Compounds in Extracts. To demonstrate the presence of carbonyl compounds in the extracts, a 1-ml. portion was shaken intermittently for 30 minutes with 1 ml. of water containing 100 mg. of sodium bisulfite (1). This treatment resulted in the formation of a complex between the bisulfite and the carbonyl compounds and the elimination of the peaks due to carbonyl compounds in the gas chromatogram. Most of the water was removed by freezing in a deep freeze for 15 minutes and the remainder by drying over anhydrous sodium sulfate. Thirty-microliter quantities were analyzed by gas chromatography.

Formation of Hydrazones from Carbonyl Compounds in Evolved Gases. Derivatives of carbonyl compounds formed during the first 6 hours of fermentation of a 16-l. pre-ferment were prepared by passing the gases first through a wash bottle surrounded by an ice-salt bath and then through 250 ml. of a filtered 2,4-dinitrophenylhydrazine solution prepared by stirring 3 g. of the solid reagent in 500 ml. of 2N hydrochloric acid. The solution containing the precipitated 2,4-dinitrophenylhydrazones (hereafter abbreviated 2,4-DNPH's) was cooled at 5°C. for 3 hours and filtered. When the filtrate was extracted twice with two-fifths the volume of ethyl acetate, a second batch of hydrazones was obtained. When the filtrate was stored at 5°C. several days, a third group of light-yellow crystals formed.

It was also possible to remove the carbonyl compounds which remained in the pre-ferment after active fermentation. This was

achieved by adding mercuric chloride to prevent further fermentation and sweeping the pre-ferment with nitrogen gas which was then passed through the 2,4-dinitrophenylhydrazine reagent. The carbonyl compounds isolated were the same as those isolated during active fermentation. Quantitative analyses of the flavor compounds isolated in this manner should be indicative of the quantities present in pre-ferments used commercially.

Separation and Identification of Carbonyl Hydrazones. The carbonyl hydrazones were separated by two different techniques, both of which employed the recrystallized solids separated from the 2,4-dinitrophenylhydrazine reagent. One method involved separating individual hydrazones by column partition chromatography (4), using Hyflo Super Cel in place of Celite Analytical Filter-aid because satisfactory separation was obtained in one-fifth the time. The second method involved gas chromatography of the volatile carbonyl compounds which were liberated from these hydrazones by heating in the presence of alpha-ketoglutaric acid (10).

Identification of the carbonyl compounds separated by column chromatography (4) was also accomplished by descending chromatography using Whatman No. 1 paper. The solvents used were 2-phenoxyethanol-heptane and N,N-dimethylformamide-decalin (7).

The ultraviolet spectrum analyses of the separated 2,4-DNPH's were made on a Beckman (Model DU) spectrophotometer by two methods. Absorbance was measured by using the chromatographed paper spot of the respective carbonyl compound (8) and by using chloroform solution of the eluted purified carbonyl compounds. The latter method was more reproducible and reliable.

The infrared spectrum analyses of the separated and purified 2,4-DNPH's and known carbonyl compounds were obtained by the potassium bromide technique (13-mm. pellets) and a Perkin-Elmer Model 137 Infracord spectrophotometer. In cases where only micro quantities of 2,4-DNPH's were available a 1.5-mm. pellet was employed.

Preparation of Known Carbonyl Derivatives. The 2,4-DNPH's of known carbonyl compounds were prepared and purified by the techniques of Shriner, Fuson, and Curtin (12).

Results and Discussion

Flavor Components in Ethyl Ether Extracts of Pre-ferments. The ether extracts of pre-ferments were analyzed by gas chromatography with the results shown in Table I. Ethyl alcohol was the main component, but lesser amounts of ethyl acetate, isobutyl alcohol, and

TABLE I
 FLAVOR COMPOUNDS DETECTED BY GAS CHROMATOGRAPHY IN AN ETHYL ETHER EXTRACT
 OF PRE-FERMENTS AND IN A CARBON TETRACHLORIDE EXTRACT OF
 EVOLVED GAS CONDENSATE FROM PRE-FERMENTS

IDENTIFIED COMPOUNDS IN PRE-FERMENTS	RETENTION TIME			
	Column A ^a		Column X ^b	
	Compound in Extract	Authentic Compound	Compound in Extract	Authentic Compound
	minutes	minutes	minutes	minutes
Compounds extracted from pre-ferment with ethyl ether				
Ethyl alcohol	3.0	3.0
Ethyl acetate	5.6	5.6	12.6	12.6
Isobutyl alcohol	9.3	9.3	21.7	21.3
Isoamyl alcohol	20.5	21.2	47.8	48.0
Compounds present in carbon tetrachloride extract of dry ice-acetone condensed gases				
Acetaldehyde	2.0	1.9	4.5	4.4
Ethyl alcohol	3.0	3.0	7.0	6.8
Ethyl acetate	5.7	5.6	12.4	12.6
Isoamyl alcohol	21.2	21.2	48.2	46.7
Solvents				
Diethyl ether		1.9		4.2
Carbon tetrachloride		7.5		16.4

^a Celite 545 (60-80 mesh) coated with 20% by weight of diisodecyl phthalate; column at 98°C.; helium flow 32 ml. per minute.

^b Celite 545 (80-100 mesh) coated with 30% by weight of di(2-ethyl-hexyl)phthalate; column at 98°C.; helium flow 11.3 ml. per minute.

^c Same retention time as ether.

isoamyl alcohol were present. Columns A and X both detected the same compounds, although ethyl alcohol and ether emerged together in column X. Using column F, isoamyl alcohol was not detected, probably because of the extremely long retention time (95 minutes) of this compound. Micro amounts of compounds retained a long time generally are released slowly and exhibit only a very small millivolt response over several minutes.

These data confirm the presence of four of the twenty-seven compounds isolated from a pre-ferment by Smith and Coffman (13), using an elaborate distillation and concentration procedure. Undoubtedly, if a more sensitive detector, such as a hydrogen flame device, were used many more components would have been detected. Because of the inadequacy of simple ether extraction of the pre-ferment and the inability of the thermistors to detect more than a few compounds, other procedures were employed to study compounds which were extremely volatile or present in micro amounts.

Flavor Components in Carbon Tetrachloride Extracts of Condensed Gases from Pre-ferments. The compounds detected in the condensed gases (Table I) were somewhat different from those de-

tected in the ether extracts. Acetaldehyde was found because this highly volatile substance was retained at the cold temperature of the acetone-dry ice trap. Ethyl alcohol, ethyl acetate, and isoamyl alcohol were detectable but isobutyl alcohol was not. Acetaldehyde and isoamyl alcohol also were detected using column F (described in Table II), but ethyl alcohol and ethyl acetate emerged with carbon tetrachloride in this column. All these compounds were also detected by Smith and Coffman (13).

Carbonyl Compounds in Gases Evolved from Pre-ferments. The mixed 2,4-DNPH's obtained from the gases of the pre-ferment were analyzed by two methods. The simplest technique used was that of Ralls (10) employing the gas chromatograph to detect carbonyl compounds released from their 2,4-DNPH's by heating in the presence of alpha-ketoglutaric acid. The eight compounds (Table II) detected by

TABLE II
CARBONYL COMPOUNDS PRESENT IN GASES EVOLVED FROM PRE-FERMENTS, MADE INTO 2,4-DNPH DERIVATIVES, AND DETECTED BY GAS CHROMATOGRAPHY^a

IDENTIFIED COMPOUNDS IN PRE-FERMENTS	RETENTION TIME					
	Column A ^b		Column X ^c		Column F ^d	
	Compound in Gases	Authentic Compound	Compound in Gases	Authentic Compound	Compound in Gases	Authentic Compound
	minutes	minutes	minutes	minutes	minutes	minutes
Formaldehyde	1.7	1.6	3.2	3.2	1.0	1.1
Acetaldehyde	1.9	1.9	4.4	4.4	3.0	3.1
Acetone	3.5	3.6	7.7	7.7	6.9	6.8
Isobutyraldehyde	4.7	4.8	10.3	10.2	8.3	8.5
n-Butyraldehyde	5.7	5.9	13.4	13.4	12.0	12.1
Methylethylketone	6.5	6.5	14.1	14.1	13.5	13.7
Isovaleraldehyde	9.5	9.4	19.9	19.9	18.6	18.5
n-Valeraldehyde	11.6	11.9	26.8	26.6	26.9	26.5

^a See reference 10.

^b Celite 545 (60-80 mesh) coated with 20% by weight of diisodecyl phthalate; column at 98°C.; helium flow 32 ml. per minute.

^c Celite 545 (80-100 mesh) coated with 30% by weight of di(2-ethyl-hexyl)phthalate; column at 98°C.; helium flow 11.3 ml. per minute.

^d Firebrick (30-60 mesh) coated with 25% by weight of tetraethyleneglycol dimethyl ether; column at 74°C.; helium flow 80 ml. per minute.

this technique contrast with only three volatile carbonyl compounds (acetaldehyde, acetone, and diacetyl) found in pre-ferments by Smith and Coffman (13). Acetoin, found by Smith and Coffman (13) in ether extracts of pre-ferments, was not found using the present technique because of its limited volatility (b.p. 188°C.).

The analysis of the 2,4-DNPH mixture by Ralls' technique (10) revealed that the amount of acetaldehyde 2,4-DNPH in the mixture was many times the concentration of the next most abundant com-

ponent. The small quantities of most of the 2,4-DNPH's made it impossible to identify the free carbonyls emerging from the column using infrared spectroscopy.

Techniques other than gas chromatography were necessary to separate sufficient quantities of most of the carbonyl compounds so that they might be identified by the use of ultraviolet and infrared spectroscopy. The technique which proved most useful for analyzing the mixed 2,4-DNPH's was a combination of their partial separation on a Celite column by the technique of Day, Bassette, and Keeney (3), followed by further purification by paper chromatography using N,N-dimethylformamide-decalin solvent. The ultraviolet spectra of the separated components were initially obtained directly on the air-dried paper chromatographs by the method of Nonaka, Pippen, and Bailey (8) and as used by Ng, Reed, and Pence (7). Results obtained by this method were highly erratic. Data obtained with the solvent extracts of the 2,4-DNPH spots were much more reliable. The purified carbonyl compounds were also identified by infrared spectra.

TABLE III
R_H AND R_F VALUES AND ULTRAVIOLET ABSORPTION MAXIMA FOR 2,4-DINITROPHENYL-HYDRAZONES OF CARBONYLS ISOLATED FROM PRE-FERMENTS AND FOR COMPARABLE AUTHENTIC CARBONYL DERIVATIVES

CARBONYL COMPOUND	R _H OR R _F VALUES OF 2,4-DNPH ^a		ABSORPTION MAXIMUM OF 2,4-DNPH	
	Isolated	Authentic	Isolated	Authentic
			<i>mμ</i>	<i>mμ</i>
2-Phenoxyethanol:heptane solvent				
Formaldehyde	0.17	0.16	363	364
Acetaldehyde	0.32	0.32	369	368
Acetone	0.32	0.31	375	374
Methylethylketone	0.49	0.49	374	374
2-Methyl-1-butanol	0.60	0.60	356.7	356.7
n-Butyraldehyde	0.37	0.37	368	368
iso-Butyraldehyde	0.39	0.39	367	368
iso-Valeraldehyde	0.45	0.45	368	369
N,N-dimethylformamide:decalin solvent				
Formaldehyde	0.12	0.12	362	362
Acetaldehyde	0.15	0.15	362	364
Acetone	0.25	0.26	368	368
Methylethylketone	0.38	0.38	377	376
2-Methyl-1-butanol	0.68	0.69	368	368
n-Butyraldehyde	0.38	0.39	370	370
iso-Butyraldehyde	0.36	0.36	368	368
iso-Valeraldehyde	0.39	0.37	364	364

^aR_H values (distance of travel compared with that for 3-heptanone) are reported for the first solvent.
R_F values are reported for the second solvent.

Table III lists the R_F and R_H values in two different solvents and the maximum ultraviolet absorbances for the 2,4-DNPH's of carbonyl compounds isolated from the volatile gases of the pre-ferment. Similar data for corresponding known 2,4-DNPH's are also recorded. All the 2,4-DNPH's except formaldehyde and methylethylketone were isolated from the mixture separated by the first filtration of the 2,4-dinitrophenylhydrazine reagent. Formaldehyde and methylethylketone 2,4-DNPH's were isolated from the reagent by extraction with ethyl acetate. From all the mixed 2,4-DNPH crystals, at least seven carbonyl compounds were isolated but unidentified, in addition to the nine positively identified. Some of the unidentified compounds appeared to be unstable. The melting points of isolated acetaldehyde and methylethylketone 2,4-DNPH's were 162° and 119°C ., respectively. These agree well with the values of 160° and 120°C . for similar derivatives of authentic acetaldehyde and methylethylketone. Other 2,4-DNPH's of carbonyl compounds found in the pre-ferment were not crystallized in sufficient quantities to permit the determination of melting points.

Should be n-Val. . .

The 2,4-DNPH's isolated by column and paper chromatography agreed with those identified by gas chromatography (Table II), except for 2-methyl-1-butanal and n-valeraldehyde. The 2-methyl-1-butanal was not identified in the 2,4-DNPH mixture by gas chromatography. N-valeraldehyde could not be distinguished from isovaleraldehyde by paper chromatography, even though two different solvents were employed. The maximum ultraviolet absorbances of the isolated 2,4-DNPH's agreed well with those for authentic samples. However, this technique failed to distinguish among the aldehydes and between the ketones.

The identification of all but two of the compounds in Table III was confirmed by infrared spectra. Insufficient 2,4-DNPH's of formaldehyde and 2-methyl-1-butanal were obtained for identification by this technique. The identity of formaldehyde 2,4-DNPH, however, was confirmed using chromotropic acid (2).

Wisblatt and Kohn (15) isolated acetaldehyde, acetone, 2-ethyl hexanal, 2-hexanone, 3-heptanone, crotonaldehyde, diacetyl, methyl glyoxal, and furfuraldehyde from fresh bread. The only carbonyls of this group found in pre-ferments during the present investigation were acetaldehyde and acetone. Diacetyl and pyruvaldehyde (methyl glyoxal) are probably present among the unidentified carbonyls produced during fermentation. The other carbonyls found by Wisblatt and Kohn (15) may result from the browning reactions during baking and processing.

Ng, Reed, and Pence (7) found formaldehyde, acetaldehyde, acetone, methylethylketone, ethyl pyruvate, 2-methyl-1-butanol, hexanal, and furfural in the ethanol extract of bread. They also found isobutyraldehyde and n-valeraldehyde in vapors of baking bread, but not in the ethanol extract of the bread crumb. In this work, most of these compounds were found in pre-ferments. Exceptions were furfural, which probably results from the browning reaction during baking, hexanal, and ethyl pyruvate which may be among the unidentified carbonyl compounds.

Acknowledgments

The authors gratefully acknowledge the assistance of John W. Sullivan in preparing infrared absorption curves used in the identification of the carbonyl compounds, and the kindness of J. W. Pence, Western Utilization Research and Development Division, for supplying a sample of 2-methyl-1-butanol 2,4-DNPH. The pioneering work of James F. Lawrence with gas chromatography of pre-ferment extracts and the financial assistance of Corn Products Sales Company, New York, N. Y., are also gratefully acknowledged.

Literature Cited

1. BASSETTE, R., and WHITNAH, C. H. Removal and identification of organic compounds by chemical reaction in chromatographic analysis. *Anal. Chem.* **32**: 1098-1100 (1960).
2. BRICKER, C. E., and JOHNSON, H. R. Spectrophotometric method for determining formaldehyde. *Ind. Eng. Chem. Anal. Ed.* **17**: 400-402 (1945).
3. DAY, E. A., BASSETTE, R., and KEENEY, M. Identification of volatile carbonyl compounds from cheddar cheese. *J. Dairy Sci.* **43**: 463-474 (1960).
4. JOHNSON, G. D. Correlation of color and constitution. I. 2,4-dinitrophenylhydrazones. *J. Am. Chem. Soc.* **75**: 2720-2723 (1953).
5. JOHNSON, J. A., MILLER, B. S., and CURNUTTE, B. Pre-ferments in breadmaking. Organic acid and esters produced in pre-ferments. *J. Agr. Food Chem.* **6**: 384-387 (1958).
6. NEISH, A. C., and BLACKWOOD, A. C. Dissimilation of glucose by yeast at poised hydrogen ion concentrations. *Can. J. Technol.* **29**: 123-129 (1951).
7. NG, H., REED, D. J., and PENCE, J. W. Identification of carbonyl compounds in an ethanol extract of fresh white bread. *Cereal Chem.* **37**: 638-645 (1960).
8. NONAKA, M., PIPPEN, E. L., and BAILEY, G. F. Separation and identification of n-alkanals, 2-alkanones, 2-alkenals, and 2,4-alkadienals by paper chromatography and direct spectrophotometric examination. *Anal. Chem.* **31**: 875-877 (1959).
9. OTTERBACHER, T. J. A review of some technical aspects of bread flavor. *Baker's Digest* **33**(3): 36-38, 40, 42 (1959).
10. RALLS, J. W. Rapid method for semiquantitative determination of volatile aldehydes, ketones, and acids. *Anal. Chem.* **32**: 332-336 (1960).
11. ROBINSON, R. J., LORD, T. H., JOHNSON, J. A., and MILLER, B. S. The aerobic microbiological population of pre-ferments and the use of selected bacteria for flavor production. *Cereal Chem.* **35**: 295-305 (1958).
12. SHRINER, R. L., FUSON, R. C., and CURTIN, D. Y. *The systematic identification of organic compounds* (4th ed.). Wiley: New York (1956).
13. SMITH, D. E., and COFFMAN, J. R. Analysis of food flavors by gas-liquid chromatography. Separation and identification of the neutral components from bread pre-ferment liquid. *Anal. Chem.* **32**: 1733-1737 (1960).
14. VISSER't HOOFT, F., and DE LEEUW, F. J. G. The occurrence of acetylmethylcarbinol in bread and its relation to bread flavor. *Cereal Chem.* **12**: 213-229 (1935).
15. WISEBLATT, L., and KOHN, F. E. Some volatile aromatic compounds in fresh bread. *Cereal Chem.* **37**: 55-66 (1960).