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QUANTITATIVE DETERMINATION OF CERTAIN CARBONYL COMPOUNDS IN PRE-FERMENTS¹

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ABSTRACT

A technique combining paper and vapor-phase chromatography was used to identify and determine the quantity of certain carbonyl compounds in bread pre-ferments. In addition to the previously reported carbonyl compounds, propionaldehyde and n-hexaldehyde were isolated from pre-ferments for the first time.

The concentrations of carbonyl compounds were determined by employing the ultraviolet absorption of their 2,4-dinitrophenylhydrazine derivatives. Where separation by paper chromatography was unsuccessful, the spots were eluted and the relative quantities of different components were determined by vapor-phase chromatography.

The effect of several different microorganisms on the amount of carbonyl compounds in pre-ferments was studied. Only *Pediococcus cerevisiae* had a pronounced effect by substantially increasing the quantity of acetone.

The pioneering work of Pence (8) on bread flavor showed that baking-oven vapors contained alcohols, acids, esters, aldehydes, and ketones. Baker *et al.* (1) distinguished three principal sources—ingredients, fermentation, and baking—for the origin of bread flavor. The importance of the fermentation process in flavor production has been emphasized by Robinson *et al.* (10) and Carlin (2). Robinson *et al.* obtained acceptable flavors by growing several selected microorganisms in pre-ferments.

Miller, Johnson, and Robinson (4) recently identified formaldehyde, acetaldehyde, acetone, iso-butyraldehyde, n-butyraldehyde, methylethylketone, iso-valeraldehyde, n-valeraldehyde, and 2-methylbutanal in bread pre-ferments. Quantitative data on the carbonyl com-

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ponents of pre-ferments, however, are meager. Cole and Pence (3) reported that the total carbonyls in pre-ferments were affected little by starting sugar concentration, but that amounts increased steadily with time and temperature of fermentation. Neish and Blackwood (5) measured acetoin quantitatively during dissimilation of glucose by yeast.

This paper describes the development of techniques designed to obtain quantitative data on the individual carbonyl compounds produced in pre-ferments. In addition, the effect of certain bacteria on the production of carbonyl compounds in pre-ferments was studied.

Materials and Methods

Pre-ferment. A pre-ferment of the following composition was employed.

Water	1,920	ml.
Glucose	252	g.
Malt	10.8	g.
Sodium chloride	42.0	g.
Yeast	200	g.
Brew improver ⁴	12.6	g.

Fermentation was allowed to proceed for 5 hours at 30°C. When the effect of organisms was being investigated, all ingredients but yeast were mixed with 1,500 ml. of water. The mixture was inoculated with the appropriate bacterial culture, if any, and incubated 5 hours at 30°C. This preliminary incubation was considered necessary to adapt the bacteria to fermentation conditions. Bacteria used were: *Pediococcus cerevisiae*, *Lactobacillus bulgaricus*, *L. plantarum*, *Streptococcus thermophilus*, *S. diacetylactis*, *Aerobacter aerogenes*, and *Bacillus brevis*. After preliminary incubation, yeast was added in suspension with the rest of the water, and fermentation was allowed to proceed for 5 hours at 30°C.

2,4-Dinitrophenylhydrazine Reagent. A 1% solution (w/v) of 2,4-dinitrophenylhydrazine (2,4-DNPH) in approximately 5N sulfuric acid was employed. Fifty-six milliliters of concentrated sulfuric acid were added to 4 g. of solid 2,4-DNPH. This mixture was added with stirring to 344 ml. of water.

Formation of 2,4-Dinitrophenylhydrazones from Carbonyl Compounds in Pre-ferment Gases. Fermentation gases were passed through 400 ml. of 2,4-DNPH reagent to trap the entrained volatile carbonyl compounds. The hydrazones thus formed were extracted three times with 20-, 15-, and 15-ml. aliquots of chloroform. The aliquots were

⁴Standard Brands, Inc., New York, N.Y. The mention in this publication of a trade product does not imply its endorsement by the U.S. Department of Agriculture over similar products not named.

combined and the volume adjusted to 50 ml.

Formation of 2,4-Dinitrophenylhydrazones from Carbonyl Compounds in the Brew Residue. Two liters of the brew after 5 hours of fermentation were treated with 4 g. of mercuric chloride to kill yeast cells and saturated with sodium chloride. The treated brew was extracted with ethyl ether, without prior removal of yeast cells, in a liquid-liquid extractor for 12 hours. The receiver flask contained a mixture of 600 ml. of diethyl ether and 600 ml. of the 2,4-DNPH reagent. This mixture was stirred vigorously and heated during the extraction by means of a magnetic stirrer-heater. After completion of the extraction, the ether was distilled *in vacuo*, and the hydrazones were extracted with 100 ml. of chloroform.

Vapor-Phase Chromatography. A Perkin-Elmer Vapor Fractometer Model 154-C, equipped with thermal conductivity cell and a 1/4-in. by 6-ft. Celite 545 column, coated with diisodecyl phthalate, was employed throughout this study. The column temperature was 100°C. Helium gas flow rate was 32 ml. per minute.

A slightly modified technique of Stephen and Teszler (11) was used to release the free carbonyl compounds from their hydrazones prior to their passage through the fractometer. One milligram of a 2:1 (w/w) mixture of alpha-ketoglutaric acid and formaldehyde 2,4-dinitrophenylhydrazone was first placed in a capillary tube. Eight milligrams of Celite were weighed on a watch-glass, and approximately 250 γ of a hydrazone-in-methanol solution were added dropwise and dried under an infrared bulb. The mixture was finally dried for 15 minutes to remove traces of methanol. No methanol could be detected by gas chromatography in the dried mixture. Alpha-ketoglutaric acid (approximately 3 times the weight of the hydrazone) was added to the dry Celite-hydrazone mixture, mixed well, and the final mixture placed in the capillary tube on top of the mixture described above. For flash exchange the tube was heated using a silicone oil bath at 250°C. for 30 seconds.

The following retention times for known carbonyl compounds were obtained:

Compound	minutes	Compound	minutes
Acetaldehyde	2.8	n-Valeraldehyde	15.9
Acetone, propionaldehyde	4.7	2-Methylpentanal	23.6
iso-Butyraldehyde	6.3	2-Hexanone	29.0
n-Butyraldehyde	7.9	n-Hexaldehyde	31.5
Methylethylketone	8.4	Ethylpyruvate	38.2
iso-Valeraldehyde	11.9	Di-n-propylketone	49.0
2-Methylbutanal	12.2	n-Heptaldehyde	64.0
Crotonaldehyde	14.3	3-Heptanone	86.0

Paper Chromatography. Descending paper chromatography was used in conjunction with two principal solvent systems: *n*-heptane saturated with 2-phenoxyethanol (7) and cyclohexane saturated with *N,N*-dimethylformamide (9).

The first solvent system was used in conjunction with Whatman No. 1 paper which was spotted and then dipped into a 10% solution of 2-phenoxyethanol in acetone. The depth of immersion was within 0.5 cm. of the origin. After drying 20 minutes at room temperature, the paper was equilibrated in the presence of the solvent for approximately 5 hours, developed with *n*-heptane saturated with 2-phenoxyethanol (7) for about 30 hours, and subsequently dried at room temperature.

The second solvent system was used in conjunction with Whatman No. 4 paper. The paper was spotted and then dipped in a 1:1 (v/v) mixture of *N,N*-dimethylformamide in absolute ethyl alcohol, dried, and equilibrated as with the first solvent system. The chromatograms were developed with the upper layer of a 6:1 mixture of cyclohexane and *N,N*-dimethylformamide (9). Because of the extreme volatility of cyclohexane, an absolutely airtight chromatograph chamber must be employed. Even then, a few degrees' increase in room temperature may result in some evaporation of cyclohexane from the filter paper, with subsequent separation of the *N,N*-dimethylformamide phase before a new equilibrium is reached. This interfered severely with the resolution of the 2,4-dinitrophenylhydrazones, particularly at high room temperatures such as are frequently encountered during summer. It was found that this interference could be fully eliminated by lowering the room temperature by 2° to 4°C. following the equilibration and during development of the chromatogram. By this technique, sharp round spots were obtained even at room temperatures as high as 27°C. with 200 γ of a hydrazone mixture per spot.

Since not all of the 2,4-dinitrophenylhydrazones could be resolved by one solvent system alone, in some cases, two solvent systems were employed. This involved streaking as much as 7.5 ml. of the chloroform extract of the hydrazones obtained from the equivalent of 150 ml. of pre-ferment on a single chromatogram. The hydrazones were then resolved into seven distinct bands using the cyclohexane-*N,N*-dimethylformamide solvent. The hydrazones were extracted from the individual bands by absolute methyl alcohol, and the extracts were concentrated to near dryness *in vacuo* and adjusted to 1-ml. volume each. Aliquots of each extract concentrate were rechromatographed using the *n*-heptane-2-phenoxyethanol solvent.

Quantitative Determination of 2,4-Dinitrophenylhydrazine Derivatives of Carbonyl Compounds. Generally 250- μ l. aliquots of the chloroform solutions were chromatographed. The individual spots were cut from paper chromatograms and extracted 20 minutes with 5 ml. of 95% ethyl alcohol; absorbance was determined using a Beckman DU spectrophotometer. The quantity of the carbonyl compound in question was obtained by referring to a standard curve made by extracting known quantities of purified carbonyl 2,4-dinitrophenylhydrazones. The following wave lengths, as determined from the spectra of synthesized known 2,4-dinitrophenylhydrazones of carbonyl compounds were used to measure the absorbance of the 2,4-dinitrophenylhydrazones of various carbonyl compounds:

2,4-Dinitrophenylhydrazones	m μ
Formaldehyde	350
Acetaldehyde	356
Acetone	361
Propionaldehyde	358
iso-Butyraldehyde	358
Methylethylketone	360
iso-Valeraldehyde	358
n-Valeraldehyde	358
n-Hexaldehyde	358

Results and Discussion

Resolution of 2,4-Dinitrophenylhydrazones of Carbonyl Compounds. The separation of large quantities of the 2,4-dinitrophenylhydrazones from the pre-ferment by cyclohexane-N,N-dimethylformamide solvent is illustrated in Fig. 1. Seven bands were obtained with migrations relative to methylethylketone (R_{MEK}) as shown. Upon rechromatography of the bands shown in Fig. 1 with n-heptane-2-phenoxyethanol solvent, further resolution was obtained as shown in Fig. 2. Zone No. 3 contained other components in addition to formaldehyde. Possibly one is furfural although this was not confirmed. Zone No. 5 was composed of approximately 95% acetone and 5% propionaldehyde. The latter has not been previously reported in pre-ferments but has been found in bread.

The extract concentrates from each zone (Fig. 1) were subjected to gas chromatography for further resolution. The results are shown in Table I. Since retention time for acetone and propionaldehyde are the same, these two compounds could not be resolved by this technique. They separated well, however, with the use of paper chromatography in conjunction with n-heptane-2-phenoxyethanol solvent. Two other pairs of carbonyl compounds could not be resolved by gas chroma-

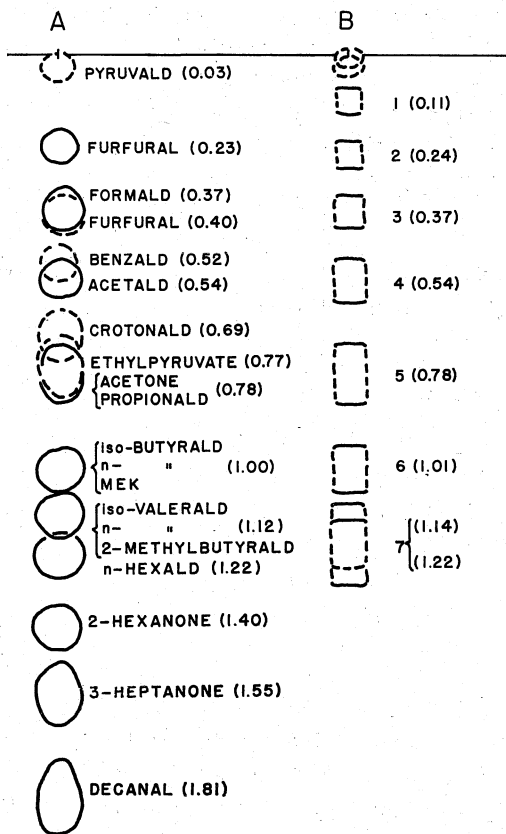


Fig. 1. Sample paper chromatogram of known and pre-ferment 2,4-dinitrophenylhydrazones on Whatman No. 4 paper, using cyclohexane saturated with N,N-dimethylformamide. A, 2,4-dinitrophenylhydrazones of known carbonyl compounds; B, zones of separated 2,4-dinitrophenylhydrazones of pre-ferment carbonyl compounds. $R_{\text{methylalketone}}$ values in parentheses.

tography: iso-valeraldehyde and 2-methylbutanal, as a mixture, emerging together after 12.00 minutes. Likewise, when n-butyraldehyde and methylethylketone were present in a mixture, they emerged together after 8.00 minutes. Zone 6 was resolved into iso-butyraldehyde and methylethylketone, the latter being the principal component. Likewise, zone 7 was resolved into iso-valeraldehyde (possibly with some 2-methylbutanal), n-valeraldehyde, and n-hexaldehyde.

The results clearly indicate that a separation of hydrazones can be achieved by a combination of paper and vapor-phase chromatography. The following carbonyl compounds were identified: formaldehyde,

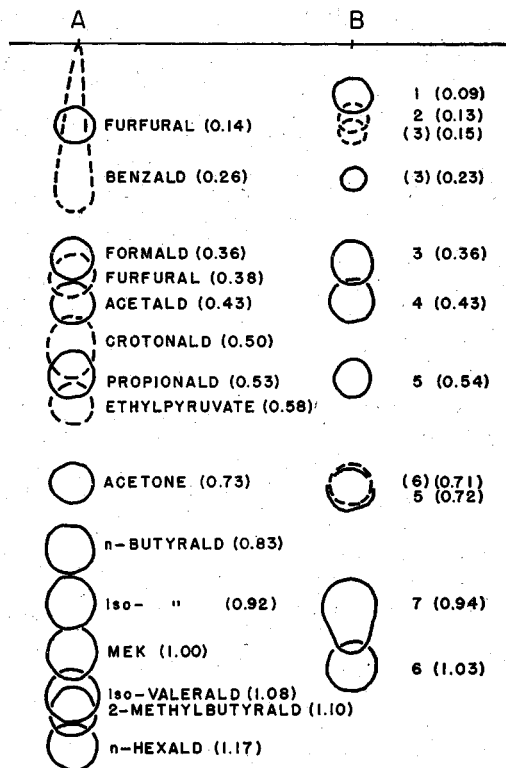


Fig. 2. Sample paper chromatogram of known and pre-ferment 2,4-dinitrophenylhydrazones on Whatman No. 1 paper using n-heptane saturated with 2-phenoxyethanol. A, 2,4-dinitrophenylhydrazones of known carbonyl compounds; B, zones of separated 2,4-dinitrophenylhydrazones of pre-ferment carbonyl compounds. R_{methyl}ethylketone values in parentheses.

TABLE I
ESTIMATION OF CARBONYL COMPOUNDS IN PRE-FERMENTS BY GAS CHROMATOGRAPHY

ZONE No.	RETENTION TIME	COMPOUND	RELATIVE RECORDER RESPONSE PER LITER OF PRE-FERMENT
	<i>minutes</i>		
1	8.95	Unknown	2.6
2	4.40	Unknown	11.5
	8.80	Unknown	3.0
	17.0	Unknown	2.2
3	20.7	Unknown	2.1
4	2.80	Acetaldehyde	680
5	4.65	Acetone + propionaldehyde	370
6	6.35	iso-Butyraldehyde	4.5
	8.50	Methylethylketone	13.3
7	12.00	iso-Valeraldehyde + 2-methylbutanal	1.1
	15.95	n-Valeraldehyde	1.7
	32.4	n-Hexaldehyde	1.6

acetaldehyde, acetone, propionaldehyde, iso-butyraldehyde, methyl-ethylketone, iso-valeraldehyde, n-valeraldehyde, and n-hexaldehyde. Of this group, propionaldehyde and n-hexaldehyde are new to pre-ferments, although they have been reported in bread (6). n-Butyraldehyde and 2-methylbutanal, previously reported in bread pre-ferments (4), were not detected during the present study. These compounds are obviously present only in traces, since even by applying column chromatography repeatedly to 200 mg. of mixed hydrazones isolated from fermentation gases, Miller *et al.* (4) were able to obtain only micro quantities. Acetoin, which was found by Visser't Hooft and de Leeuw in bread (12), was not positively identified in the present study. However, the presence of acetoin and/or diketone(s) was indicated by the characteristic blue reaction with alkali of their 2,4-dinitrophenylhydrazones on paper chromatograms.

Quantitative Determination of Carbonyl Compounds. The recovery of known carbonyl compounds from liquid-liquid extractions was determined by adding 10 ml. of 1% (w/v) solution of aldehydes or ketones in water to 2 liters of pre-ferment saturated with sodium chloride. Enough ethyl alcohol was added to effect complete solution. The following recovery percentages, expressed as averages of seven replicates, were obtained:

	%
Formaldehyde	70 ± 5
Acetaldehyde	70 ± 5
Acetone	67 ± 5
Propionaldehyde	57 ± 6
Methylethylketone	80 ± 4
iso-Butyraldehyde	64 ± 7
iso-Valeraldehyde	45 ± 6
n-Valeraldehyde	58 ± 5
n-Hexaldehyde	41 ± 5

The quantitative determinations of pre-ferment carbonyl compounds were performed with two different batches of yeast, using five replicates in each case. The results are shown in Table II. These experiments were carried out several months after those described in Table III. Differences in the amounts of certain carbonyl compounds may be attributed to differences in the yeasts used in the pre-ferments. The variability in the data obtained for acetaldehyde is due to difficulty in handling this extremely volatile compound.

Effect of Certain Bacteria on the Amount of Carbonyl Compounds Produced during Fermentation. Table III illustrates that generally little effect by any of the bacteria used could be observed on the

TABLE II
QUANTITATIVE ESTIMATION OF CERTAIN CARBONYL COMPOUNDS IN PRE-FERMENTS BY
PAPER CHROMATOGRAPHY

CARBONYL COMPOUNDS	AMOUNT PRESENT IN FERMENTATION GASES	AMOUNT PRESENT IN LIQUID PRE-FERMENT
	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$
Formaldehyde	29 \pm 3 22 \pm 1	42 \pm 3
Acetaldehyde	745 \pm 129 701 \pm 54	844 \pm 250
Acetone + propionaldehyde	20 \pm 2 22 \pm 3	191 \pm 67
iso-Butyraldehyde + methylethylketone	8 \pm 1 8 \pm 1	21 \pm 1
iso-Valeraldehyde + n-valeraldehyde + 2-methylbutanal	26 \pm 2 26 \pm 1	90 \pm 20
n-Hexaldehyde	trace

amounts of the carbonyl compounds in bread pre-ferments, except that the presence of *Pediococcus cerevisiae* resulted in a marked increase in the amount of propionaldehyde-acetone. A further resolution by

TABLE III
EFFECT OF MICROORGANISMS ON THE PRODUCTION OF CARBONYL COMPOUNDS IN
PRE-FERMENTS
(μg . per 100 ml. of pre-ferment)

PRE-FERMENT MADE WITH VARIOUS MICROORGANISMS PLUS YEAST	FORMAL- DEHYDE	ACETAL- DEHYDE	PROPIONAL- DEHYDE + ACETONE	ISO-BUTYR- ALDEHYDE + METHYLETHYL- KETONE	ISO-VALERAL- DEHYDE + 2-METHYL- BUTANAL + N-VALERAL- DEHYDE
Control	21	460	41	63	39
<i>P. cerevisiae</i>	36	490	1010	94	84
<i>L. bulgaricus</i>	27	510	51	63	39
<i>S. thermophilus</i>	27	500	41	49	29
<i>A. aerogenes</i>	27	490	41	49	29
<i>S. diacetylactis</i>	36	490	41	57	32
<i>B. brevis</i>	trace	430	39	54	49
<i>L. plantarum</i>	trace	560	41	60	36
Liquid Pre-ferment					
Control	41	750	420	60	94
<i>P. cerevisiae</i>	49	800	770	60	120
<i>S. thermophilus</i>	77	820	290	20	91
<i>A. aerogenes</i>	66	790	260	29	78
<i>S. diacetylactis</i>	71	800	250	17	71
<i>B. brevis</i>	26	690	115	40	123
<i>L. plantarum</i>	30	910	300	26	162

n-heptane-2-phenoxyethanol chromatography revealed this to be due to an increase in acetone.

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Literature Cited

1. BAKER, J. C., PARKER, H. K., and FORTMANN, K. L. Flavor of bread. *Cereal Chem.* **30**: 22-30 (1953).
2. CARLIN, G. T. The fundamental chemistry of bread making. I and II. *Proc. Am. Soc. Bakery Eng.* **1958**: 55-61; **1959**: 136-146.
3. COLE, E. W., HALE, W. S., and PENCE, J. W. Effect of processing variations on the alcohol, carbonyl, and organic acid contents of pre-ferments for bread baking. *Cereal Chem.* **39**: 114-122 (1962).
4. MILLER, B. S., JOHNSON, J. A., and ROBINSON, R. J. Identification of carbonyl compounds produced in pre-ferments. *Cereal Chem.* **38**: 507-515 (1961).
5. NEISH, A. C., and BLACKWOOD, A. C. Dissimilation of glucose by yeast at poised hydrogen-ion concentrations. *Can. J. Tech.* **29**: 123-129 (1951).
6. 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).
7. NONAKA, M., PIPPEN, E. L., and BAILEY, G. P. 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).
8. PENCE, E. A. A study of baking oven vapors. Master's thesis, Kansas State University (1952).
9. PIHA, P., KITUNEN, M., HOLMBERG, A. M., and SUOMALAINEN, H. Aliphatische Aldehyde des Sulfit- und Getreidesprits. *Z. Lebensm. Untersuch. u. -Forsch.* **113**: 134-143 (1960).
10. 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).
11. STEPHEN, R. L., and TESZLER, A. P. Quantitative estimation of low boiling carbonyls by a modified alpha-ketoglutaric acid-2,4-dinitrophenylhydrazone exchange method. *Anal. Chem.* **32**: 1047 (1960).
12. VISSER'T HOOFT, F., and DE LEEUW, F. J. G. The occurrence of acetyl methylcarbinol in bread and its relation to bread flavor. *Cereal Chem.* **12**: 213-229 (1935).