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(Secale cereale)

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The volatile components of rye flour were isolated by steam distillation and trapped in cold traps under vacuum. After preliminary tests indicated the presence of carbonyl compounds, the distillate was reacted with 2,4 dinitrophenylhydrazine to convert the carbonyl compounds to their stable derivatives, 2,4 dinitrophenylhydrazones. Three classes of monocarbonyls were present as indicated by column chromatography. The identity of two classes was verified by ultraviolet spectra and a time fading study using ethanolic sodium hydroxide as a coloring agent. Tentative identification of the following compounds was achieved by paper chromatography of two classes of monocarbonyls: acetaldehyde, propanal, butanal and pentanal; prop 2-enal, but-2-enal, pent-2-enal,

hept-2-enal and oct-2-enal.

An ether extract of a rye flour distillate was concentrated to produce an essence which was subjected to gas-liquid chromatographic and mass spectral analysis. Several compounds were identified on the basis of the match of their relative retention times with those of authentic compounds on two columns, Carbowax and Apiezon, and their mass spectra. The compounds identified in rye flour volatiles were: acetaldehyde, hexanal, prop-2-enal, ethanol, 1-pentanol and ethyl acetate.

Some Components of the Volatiles from Rye Flour

(Secale cereale)

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# SOME COMPONENTS OF THE VOLATILES FROM RYE FLOUR

## (SECALE CEREALE)

### INTRODUCTION

Food flavor research has been largely concerned with the volatile fractions of various foods because the odor and flavor constituents are concentrated in this fraction. In common with many other foods, the odor constituents of rye, which would account for much of the flavor, are volatile. The isolation and identification of some of the volatile flavor and odor components of rye were the subjects of the research.

The flavor and odor of foods has been an area of intense scientific investigation in the last decade. Although expert taste testers and trained taste panels have been used for a long time in food analysis, the employment of instrumental techniques in odor and flavor research is a recent phenomenon. The development of instruments having a considerable degree of sophistication, such as the gas-liquid chromatograph, has facilitated the separation of complex mixtures of chemical substances that comprise the volatile flavor and odor fraction of foods. The mass spectrometer is among the instruments having value in their identification.

Knowledge about the nature of the chemicals that comprise the volatile flavor and odor fraction of foods is

considered valuable as this information can be used in many ways, for example in manufacturing synthetic flavor, adding flavor substances to fortify natural flavors, selecting lines of food plants or meat animals having the genetic capacity to produce the best flavor and in quality control during food processing.

A good deal of research has been concerned with the flavors of dairy products, vegetables, meat, poultry, fish and beverages. By contrast, the flavor components of cereal grains have been little explored. Yet the volatile flavor and odor fraction of cereals is an important area for investigation. Nutritionists, educators and others in the field of public health are becoming increasingly aware of the heavy reliance on cereal grains by many peoples of the world. The nutritional problems occasioned by such a dependence can be severe. The inadequate protein content of the diet of underdeveloped or overpopulated nations is of sore concern. Experiments aimed at developing a protein-rich simulated grain which might be used to supplement or substitute for other cereal grains in the diet have been conducted by Bhatia and Subrahmarayan (1959). However, without flavor acceptability the nutrients would go begging. Knowledge of the flavor and odor components of authentic cereal grains can contribute to the development of flavorful as well as nutrient-enriched simulated grains. In a more sophisticated

application, knowledge of the flavor and odor components of the cereal grains can contribute to the enhancement of bread or cracker type products.

Among the cereal grains, rye (*Secale cereale*) is the major source of food in northeastern Europe and parts of Asia. It is the principal cereal of Russia and the Scandanavian countries. It is the only cereal grain, other than wheat, which contains the kinds of proteins essential to making leavened bread. Many studies on the flavor of bread, especially wheat bread, have been conducted. However, but one study has thus far been reported on the flavor constituents of grains from which breads are made (McWilliams, 1968).

Although less used than wheat products at the present time in the United States, products made from rye flour are well liked by many people for their distinctive flavor and texture. The variety of products available commercially in which these attributes play a major role include rye bread, pumpernickel bread, Rye Crisp and prepared breakfast cereals.

## REVIEW OF LITERATURE

### Nature of Flavor Compounds

Equated with the natural flavor of foods are the volatile chemical compounds which are natively present in the food or are produced in the cooked food from these precursors. These flavor chemicals are usually small organic molecules, i.e. esters, alcohols, acids, and carbonyl compounds.

### Carbonyl Compounds

#### Importance as Components of Volatile Flavor Fraction

Many persons engaged in flavor research have devoted their efforts to isolating and classifying the carbonyl compounds in food volatile concentrates. A flavor concentrate having a very strong aroma may show an altered or reduced aroma after reaction with 2,4 dinitrophenylhydrazine which would remove the carbonyl compounds from the odor picture. From this observation it has been concluded that carbonyl compounds are important to the flavor, although it is recognized that they may not represent the entire picture.

### Odors

In his monograph on the interrelationship of chemical structure and odor Katz (1967) details the

odors associated with aldehydes and ketones as well as other chemicals. He discusses the fact that aldehydes are important flavor chemicals. The short chain aldehydes, like formaldehyde and acetaldehyde, tend to have sharp disagreeable odors. Increasing the chain length to more than five carbons results in a decreased intensity of aroma and a smoother, more oily character. The seven carbon and longer chain aldehydes are used in perfumery and in synthetic flavors.

The unsaturated aldehydes arouse different odor sensations. Acrolein ( $\text{CH}_2=\text{CH}-\text{CHO}$ ), for example, has a sharp irritating odor. The higher unsaturated aldehydes, such as citral, have lemon-like odors. The aromatic aldehydes containing the benzene ring nucleus are well known for their bitter almond odor.

Another group of compounds important in flavor because of their odor qualities are the ketones. Their odors are often characterized as pungent and sweet. Acetone smells like pickled green olives, acetophenone, like 'typical green floral.' Among the diketones diacetyl is well known for the frequency of its occurrence in dairy products. The ionones are powerfully odorous chemicals widely used in perfumery and synthetic flavors. Alpha and beta ionones exhibit a strong violet or raspberry odor. Another group of ketones, of which menthone is an example, have distinct peppermint odors.

Guadagni, Buttery and Okano (1963) studied the olfactory thresholds of 25 adults to aldehydes and found that the threshold of a homologous series of saturated or unsaturated aliphatic aldehydes decreased progressively with an increase in chain length from C-five to C-ten. No significant difference in olfactory threshold was detected in the aldehydes they tested from C-three to C-five. While propanal could be detected at 9.5 p.p.b. nonanal could be detected at one p.p.b. Branched chain aldehydes appeared to have much lower thresholds than the corresponding straight chain compounds. They emphasized that some compounds in very low concentration may contribute more to the odor of a food than compounds present in much higher concentration.

Kendall and Neilson (1968) showed that sensory perception of a combination of odors cannot be predicted. When an odorant is added to a mixture four possible results occur: 1) intensification of some odorants with resultant suppression of the other, 2) increase in intensity of odor added, 3) development of a different odor character, i.e. blending, and 4) no change. The odor intensity of any system is the result of minor as well as major components present in the system. Another effect these authors noted is that of a synergistic action whereby odorants, even at sub-threshold levels individually, can intensify the aroma picture.

Concentrated odors may have highly unpleasant aromas. Coffman (1967) reports that concentrated oven vapor from baking bread had objectionable aroma but it exhibited a pleasant odor when sprayed into a room, thus effectively decreasing its concentration.

### In Food Volatiles

Bernhard (1966) in a review article covering the research on vegetable flavor components remarked that many of the same low boiling volatiles occur in most cooked foods. He suggested that these common volatile substances, which may vary only in the relative quantities present, are produced by degradation of the metabolites which are normally present in all biological materials. He cited the work of Self (1963) who examined the low boiling volatiles from thirteen cooked vegetables, beef, coconut, coffee and tea by gas-liquid chromatography. It is interesting to note that the carbonyl compounds formaldehyde, propanal and acetone occur in the volatiles of Brussels sprouts, beans, beef, carrot, cauliflower, celery, coconut, coffee, corn, leek, lettuce, onion, parsnip, potato and tea. These volatile compounds were extracted from cooked foods that had been boiled for 30 minutes.

Ryder (1966) stated that low boiling alcohols, aldehydes, ketones and sulfur compounds are ubiquitous constituents of food volatiles although the ratios of their

occurrence may vary widely.

Damm and Kringstad (1963) reported the presence of carbonyls in barley and malts. They passed carbon dioxide gas over an agitated slurry of ground cereal and collected the volatiles in washing bottles containing 2,4 dinitrophenylhydrazine. The resulting hydrazones were identified by melting points and thin layer chromatography.

Lillard and Day (1961) were able to identify several n-alkanals and alk-2-enals in autoxidized milk lipids. After steam distillation under vacuum of the lipids, they reacted the distillate with 2,4 dinitrophenylhydrazine and made a hexane extract. Column chromatography of the hydrazones was employed for identification. They correlated sensory tests from trained taste panels and chemical results statistically.

Day and Libbey (1964) were able to separate 130 components of the flavor fraction of cheddar cheese by gas-liquid chromatography. Many identifications of aldehydes and methyl ketones were made on the basis of gas-liquid chromatographic retention times and mass spectral data.

Rothe (1960) investigated the volatile aldehydes produced in rye bread. He cut pieces of crust and distilled them with steam. By using 2,4 dinitrophenylhydrazine reagent he was able to bind the carbonyl compounds which were subsequently dissolved in carbon tetrachloride.



They were subjected to paper chromatography for separation. By spotting the papers with authentic hydrazones along with the hydrazones from the bread he was able to identify the following: methylglyoxal, oxymethylfurfuraldehyde, furfuraldehyde, acetaldehyde, methional, phenylacetaldehyde, isobutyraldehyde and methylbutyraldehyde.

Yasumatsu, Moritaka and Wada (1966b) found three carbonyl compounds to be responsible for the stale flavor of stored rice. They analyzed head space vapors by means of gas-liquid chromatography and correlated chromatographic data with sensory panel evaluation of flavor. They identified propanal, n-valeraldehyde and n-caproaldehyde. The same workers (1966a) examined the vapor of cooked rice by gas-liquid chromatography and infrared spectra and found five carbonyl compounds of which acetaldehyde and n-caproaldehyde were positively identified.

McWilliams (1968) in her study of wheat flavor also showed that carbonyl compounds were present. By the use of gas-liquid chromatography, paper chromatography and melting points of 2,4 dinitrophenylhydrazones she was able to identify several aldehydes and ketones in the volatiles of raw wheat flour.

Minyard et al. (1967) when analyzing the carbonyl constituents of the cotton bud claimed that it was very difficult to isolate volatile carbonyl compounds. The

quantities of individual aldehydes and ketones in essential oils from the cotton bud were often so small that trapping them by gas chromatography and later analysis by infrared or nuclear magnetic resonance was very difficult. They subjected cotton bud oil to gas-liquid chromatography and bubbled the effluent through vials containing 2,4 dinitrophenylhydrazine in phosphoric acid. The derivatives were subjected to thin layer chromatography for separation and identification.

Keith and Day (1963) also found it profitable to convert free monocarbonyls of oxidizing milk fat to dinitrophenylhydrazones. They passed a benzene solution of carbonyl compounds over an alumina dinitrophenylhydrazine column where the conversion of carbonyls to dinitrophenylhydrazones took place. The eluate was collected and the solvent removed by reduced pressure. Benzene, ethanolic potassium hydroxide and methanol were added to the derivatives and the absorbancies were read at 430 m $\mu$ , 460 m $\mu$  and 480 m $\mu$ . They were able to identify alkanals, alk-2-enals, and alk-2,4-dienals.

Spare and Virtenan (1961) extracted the volatile substances from onion by sucking air through a homogenate of onion, under vacuum. They led these volatiles directly to a dilute solution of 2,4 dinitrophenylhydrazine where a precipitate was formed. A solution of the hydrazones was subjected to a cellulose powder column for separation.

Many tests were employed for identification of the carbonyl compounds including melting points, elementary analysis, infrared spectra, paper chromatography, ultraviolet spectra and transderivation by levulinic acid.

Reymond, Chavan and Egli (1963) found many carbonyl compounds in the volatiles of roasted coffee. They swept helium gas through a slurry of ground coffee maintained at 80°C for 30 minutes and collected the volatiles in cold traps. The vapors were led directly to a gas-liquid chromatograph. Identification was based on retention times and infrared analysis.

Carbonyl compounds have also been associated with "off-flavors." An unusual carbonyl, oct-1-en-3-one, was found by Stark and Forss (1962) to be responsible for the metallic flavor of oxidized dairy products. Gas-liquid chromatographic data from three different columns, mass spectral analysis, infrared spectra and paper chromatography were all used to establish identity.

#### Methods Employed for Identification

Many techniques have been employed to separate and identify flavor compounds. Almost all researchers in the last decade have employed gas-liquid chromatography. In addition, more traditional methods are still being used. Methods employing adsorption chromatography (column) for separation of 2,4 dinitrophenylhydrazones

were developed or refined by Swartz, Parks and Keeney (1962), Day, Bassette and Keeney (1960) and Gaddis, Ellis and Currie (1959). Paper chromatographic methods for separating 2,4 dinitrophenylhydrazones were developed by Gaddis and Ellis (1959), Lynn, Steele and Staple (1956), Meigh((1952), Linko (1963) and Nonaka, Pippen and Bailey (1959). Standards for ultraviolet spectra for 2,4 dinitrophenylhydrazones were defined by the work of Jones, Holmes and Seligman (1956) and Stitt et. al. (1960).

#### Origin of Carbonyl Compounds in Foods

Much research has been directed toward uncovering the details of the reactions which lead to aldehydes and ketones in food products. Heat, oxidation and certain conditions of humidity may favor reactions which lead to the production of carbonyl compounds. Of course it is recognized that some carbonyl compounds represent normal metabolites present in the plant or animal tissue.

Hodge (1953, 1967) and Reynolds (1965) discuss the chemical reactions associated with browning reactions which are usually the result of drying, processing or cooking food. Non-enzymatic browning reactions have been shown to be responsible for the development of certain pigments and flavors. Reducing sugars and free amino groups can combine in a reaction (Maillard reaction) to form a Schiff base which can be converted in a series of

further reactions to a 1-amino, 1-deoxy, 2 ketose. This compound can enter many pathways, one of which, the Strecker degradation, leads to the production of aldehydes.

These reactions have been explored in model systems by Herz and Shallenberger (1960) and Johnson, Rooney and Salem (1966). The latter authors suggested certain amino acids as the precursors of certain carbonyl compounds as listed below, Table 1.

Table 1. Carbonyl compounds produced in Maillard reaction with specific amino acids

---

<u>Precursor</u>	<u>Carbonyl Compound</u>
Alanine	Acetaldehyde
Valine	Isobutyraldehyde
Leucine	Isovaleraldehyde
Isoleucine	2-methylbutanal
Phenylalanine	Phenylacetaldehyde
Methionine	Methional

---

These authors explored carbonyl compound production in bread systems also and concluded that most of these compounds are formed in the crust where heat and moisture conditions promote the Maillard reaction.

Kiely, Knowlin and Moriarty (1960) experimented with a bread system in an attempt to classify the odors produced by the reaction of amino acids and sugars. They concluded that leucine and dextrose could react to

replicate a toasted bread-like aroma and flavor.

Wiseblatt and Zoumut (1962) showed that, in a model system, employing dihydroxy acetone and proline, a cracker-like aroma was produced via the Maillard reaction.

Collyer (1964) in an extensive review article noted that both fermentation products and products formed from reactions during crust development are essential for bread flavor. She noted that the characteristic bread flavor owes little to the organic compounds present in the unaltered ingredients.

Coffman (1967) agreed with Collyer about the source of bread flavor and aroma and noted that no single compound could account for the entire aroma picture.

Reynolds (1965) discussed the importance of these reactions for flavor production. She stated,

"The Strecker degradation occurs under a wide range of conditions of pH and temperature...The quantity of a flavoring compound, as a percentage of the reactants is often so small that any type of reaction is conceivable....Condensation, dehydration or hydrolysis might proceed at an apparently negligible rate, yet still produce a significant flavoring compound."

Another source of carbonyl production in foods is that involving the reactions of oxidizing fats. Gaddis, Ellis and Currie (1961) found hept-2,4-dienal, propenal, heptanal and decanal in a steam distillate of mildly autoxidized mixed esters of oleic, linoleic and linolenic acids from animal and vegetable fats.

Badings (1959) used a model system to induce

autoxidation in a solution of ammonium linoleate and was able to show the presence of three aldehydes; 2,4-decadienal, 2-octenal, and n-hexanal. He detailed the chemical reactions involving peroxide formation that could lead to the production of these compounds.

Another possibility for production of carbonyl compounds in food products is discussed by Linko and Milner (1959). They showed that a cereal seed may undergo chemical reactions in storage which are not connected with germination or fungal action. They determined free amino acids and keto acid of wheat stored at varying humidities by means of quantitative paper chromatography. They proposed a series of chemical changes whereby the free amino acids in the germ of the cereal grain reacted with the sugars present and resulted in the production of aldehydes. A fairly high moisture condition, about 18%, was implicated in these reactions.

Enzymatic action has also been shown to be responsible for the production of carbonyl compounds. Fleming et al. (1968) showed that the characteristic flavor components of fresh cucumbers, which include several carbonyl compounds, are generated enzymatically as a consequence of cutting the fruit or otherwise mechanically rupturing it. Grosch (1968) used a model system to demonstrate the enzymatic production of carbonyl compounds. He incubated the enzyme, lipogenase, isolated from fresh

peas, with linoleic and linolenic acids and converted the carbonyl compounds formed to 2,4 dinitrophenylhydrazones.

### Alcohols, Esters and Acids

Studies have shown that alcohols, esters, and acids are essential to the flavor of many foods. Ethanol has been found in the flavor volatiles of such diverse foods as bread, meat and onions. It is not surprising to find this substance in bread (Coffman, 1967; Johnson, Salem and Rooney, 1966) since it is the primary product of fermentation. Despite the long baking time at elevated temperatures which might lead to its evaporation, some ethanol remains in baked bread, as revealed by steam distillation and subsequent gas-liquid chromatographic analysis. Although it may be less significant as a flavoring component than other alcohols which have stronger flavors, it does contribute a sweet, ethereal note.

Ethanol has been reported in the volatiles of raw beef (Hornstein, 1967). Nitrogen gas was bubbled through a water solution of beef extract and the volatiles collected in cold traps and subsequently analyzed by gas-liquid chromatography. Ethanol has been found in cooked beef also. Presumably the lactic acid present in muscle tissue can act as its precursor.



As reported by Bernhard (1966) onions have been studied and shown to contain ethanol as part of their volatile components. Onions were distilled under vacuum, the volatiles collected on activated charcoal and later extracted with ether. Gas-liquid chromatography and infrared analysis were employed for identification.

Esters too have been shown to be present in the volatiles of many foods. The ethyl esters are quite common in wine (Webb, 1967) where the high concentration of ethyl alcohol promotes their formation. The slow development of these esters may account for the bouquet of aged wines.

Many ethyl esters have been found in bread volatiles (Johnson, Rooney and Salem, 1966). These esters could be expected since both alcohols and organic acids are produced during fermentation.

Many esters are present in ripe banana (Wick et. al., 1966). Gas-liquid chromatography and infrared analysis were used to identify the volatiles which had been isolated by flash distillation from bananas.

Many organic acids have been found in the volatiles of foods such as meat (Hornstein, 1967), sauerkraut (Bernhard, 1966), bread (Johnson, Rooney and Salem, 1966) and wine (Webb, 1967).

### History and Characteristics of Rye Cereal Grain

The cultivation of rye, *Secale cereale*, has been known about 2,000 years and seems to have originated in the northern countries of Europe (DeCandolle, 1885 ). Rye is the only cross-fertilized plant among the major cereals. It is a wind pollinated crop, therefore rarely occurs as a pure strain. Because of this, few varieties have distinct characteristics. These characteristics are considered to be such factors as differences in winter hardiness, yield, tillering power and the protein content of the rye (Leonard and Martin, 1963).

The chemical composition of rye flour is similar to that of wheat, except that it is lower in protein content, Table 2 (Leonard and Martin, 1963).

Table 2. Comparison of Composition of Rye and Wheat

	Percentage		
	<u>Rye</u>	<u>Wheat</u>	<u>Wheat Germ</u>
Moisture	13.4	13.4	--
Protein	11.5	12.1	39.76
Oil	1.7	2.1	13.23
Carbohydrate	69.5	69.0	27.37
Crude fiber	1.9	1.9	2.44
Mineral matter	2.0	1.7	4.97
Lignin	--	--	6.82

The amino acids present in rye include alanine, arginine, aspartic acid, cystine, glycine, histidine, leucine, lysine, isoleucine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine (Shands, 1959).

One of the outstanding qualities associated with this cereal crop is its ability to overwinter under conditions of cold that would kill other cereal grains. Rye succeeds where wheat fails. It will grow in sandy infertile soil that will not support other crops. It flourishes as far north as the arctic circle and up to an altitude of 14,000 feet (Moore, 1960). Its greatest cultivation is in the Soviet Union where 30 million acres are devoted to this crop. The rye grown in the United States comes mostly from North and South Dakota, Minnesota and Wisconsin. The ratio of wheat to rye grown in the United States is 50 to one. In this country about one-fifth of the rye crop is used for flour for human food.

A similar quantity is used for making whiskey and other alcoholic beverages. Much rye is used for livestock feed or as seed. Farmers plant it as a cover crop to inhibit erosion. It is also used as green manure, i.e. allowed to sprout, and then plowed under as fertilizer.

In Europe the picture is quite different. High protein rye is still the major bread grain of north, central and eastern Europe and of northern Asia (Geddes, 1963).

## EXPERIMENTAL PROCEDURE

### Receipt and Storage of Rye Flour

The flour was received from Fisher Flour Mills of Seattle, Washington through a local supplier. Its origin was North and South Dakota, with the predominant variety being "Frontier", followed by "Caribou" and "Antelope." When received in the laboratory the flour was immediately transferred to clean glass jars with metal screw caps and stored at 4°C.

### Isolation of Rye Volatile Fraction

Concentration of the volatile components of rye flour was carried out by steam distillation of rye flour under vacuum. The concentrates from several distillations were individually reacted with 2,4 dinitrophenylhydrazine reagent to obtain derivatives. One concentrate was used to prepare an ether extract for analysis by gas-liquid chromatography and mass spectrometry.

### Distillation System

The distillation system was similar to that used by Lillard and Day (1961). Figure 1 illustrates the system. It consisted of a steam generator containing distilled water which led to a charge flask containing a slurry of rye flour in distilled water. These flasks

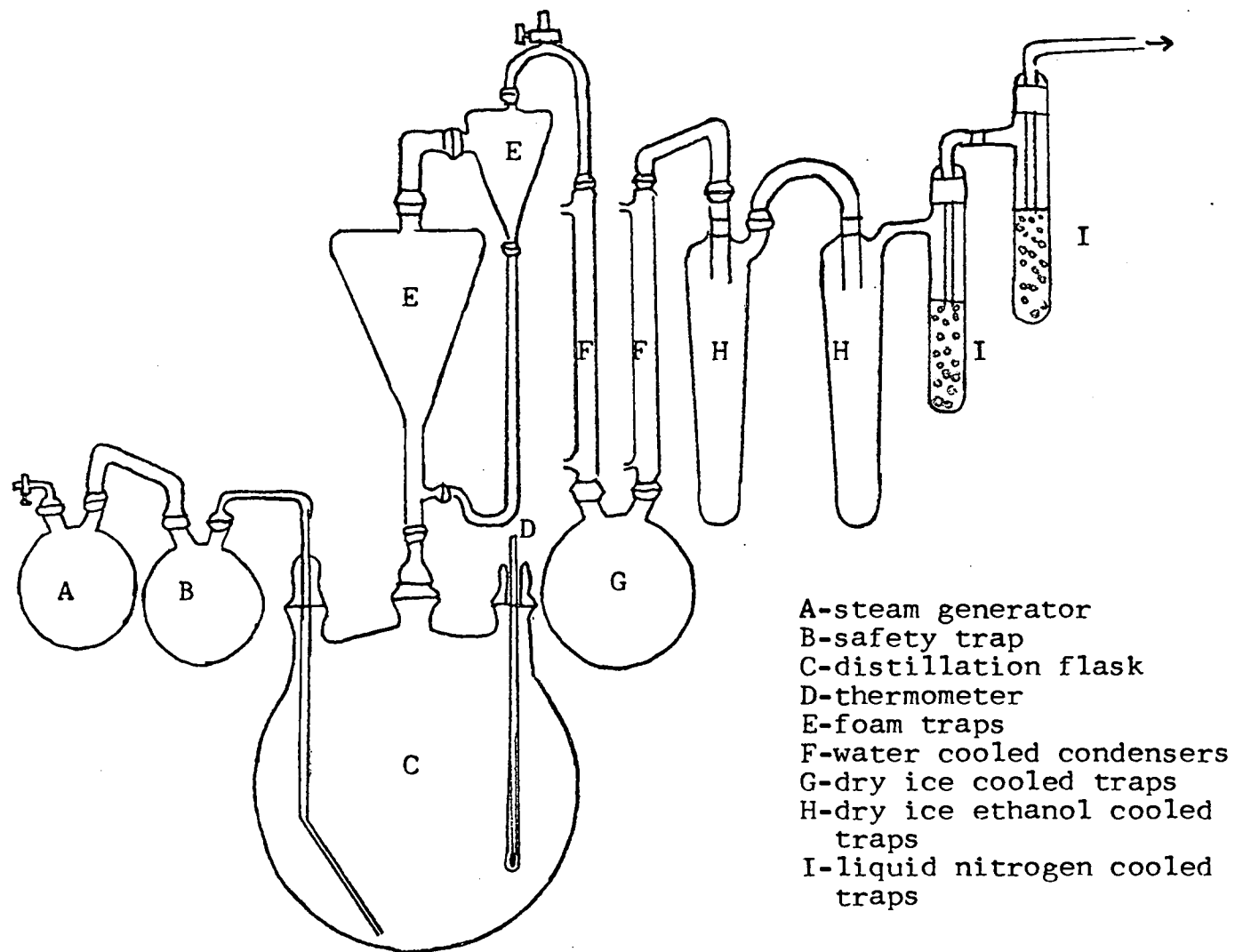


Figure 1. Vacuum steam distillation apparatus

rested in electric mantles. They were connected to a series of cold traps, the first packed with dry ice; the next two packed with dry ice and ethanol and the last encased in dewar flasks filled with liquid nitrogen. The final connection was made to a vacuum pump. Ground glass fittings, greased with Spectro-vac, secured by metal spring clamps, were employed throughout.

### Distillation Procedure

The distillations were accomplished on a slurry of rye flour and distilled water. The distillations destined for conversion to hydrazone derivatives employed a five liter flask containing 200 grams of flour in 950 ml. of saturated salt solution. The distillation destined for ether extraction employed 1500 grams of rye flour in six liters of distilled water. The system was degassed initially to reduce bumping. It was found that foaming could be controlled by intermittently closing the stopcock between the distillation system and the vacuum pump. The foam trap was found to be helpful as well.

After foaming ceased, heat was applied to the charge flask and the temperature maintained between 28 and 34°C. The distillation was carried out for four hours.

At the end of the distillation each connection on

the cold traps was severed and the opening immediately stoppered to prevent condensation of liquid oxygen. The traps which contained large amounts of aqueous distillate were defrosted rapidly in lukewarm water to prevent breakage. The other traps were allowed to defrost at room temperature and the openings carefully wiped free of grease with a tissue saturated with ether before the contents were poured out. Each cold trap was emptied into a glass stoppered bottle and rinsed two times with small quantities of diethyl ether. The distillate and ether washings were stored at 0°C.

A different procedure was followed to recover the condensate for preparation of 2,4 dinitrophenylhydrazine derivatives. As the volatiles vaporized from the defrosting cold fingers they were trapped in a series of two gas-washing bottles containing ethanol (20 ml.) set in dry ice. The aqueous distillate and the ethanol solution of low boiling volatiles were combined in glass bottles with plastic screw caps.

#### Ether Extraction

The aqueous distillate was saturated with sodium chloride and placed in a three liter capacity liquid-liquid extractor and extracted for 21 hours with 100 ml.

of anhydrous reagent grade diethyl ether.<sup>1</sup> The ether extract was dried over sodium sulfate and concentrated by distillation through a one cm. by 60 cm. column packed with glass helices. The rate of distillation was controlled so that three drops returned to the charge flask for every two drops of ether distilled. Two hours were required to remove the bulk of the ether. The extract was evaporated to 15-20 ml. and then transferred to two chromatographic tubes.<sup>2</sup> One portion was further concentrated under a slow stream of nitrogen to about 20  $\mu$ l. The samples were held at 5-6°C. until used for gas-liquid chromatographic analysis or mass spectrometry.

## 2, 4 Dinitrophenylhydrazine Derivatives

### Preparation

The distillate was reacted the same day as collected with 2,4 dinitrophenylhydrazine reagent. Two methods of preparing this reagent were used.

One method was that described by Shriner, Fuson and Curtin (1964) in which four grams of 2,4 dinitrophenylhydrazine were mixed with two ml. of concentrated sulfuric acid. Three ml. of distilled water were slowly added and the contents swirled to accomplish solution.

1 Allied Chemical, Morristown, New Jersey

2 Kontes Glass Company, Vineland, New Jersey



Ten ml. of 95% ethanol were then added. The hydrazine reagent was added to the distillate in a quantity equal to 50% of the volume collected.

Carbonyl-free ethanol was prepared by refluxing one liter of ethanol with three grams of trichloroacetic acid and four grams of 2,4 dinitrophenylhydrazine for one hour and then distilling as described by Gaddis (1966).

Another method of preparing the hydrazine reagent was employed, as described by Day (1965). For this method an equal volume of the aqueous distillate was added to 5N hydrochloric acid saturated with 2,4 dinitrophenylhydrazine. In both instances the reaction was allowed to proceed overnight.

#### Column Chromatography

The filtrate was then examined to determine the presence of low molecular weight aldehydes and ketones. It was extracted with GC Spectrophotometric quality hexane using five portions of hexane (200 ml. filtrate plus ten ml. hexane per each extraction). The use of a separatory funnel for these extractions enabled the ready separation of the aqueous phase from the hexane. The collected hexane extracts were washed with distilled water until the water was clear to remove traces of the 2,4 dinitrophenylhydrazine reagent (Day, 1965).

When treated in the following ways the 2,4 dinitro-

phenylhydrazones in the hexane extract may be separated into four classes of monocarbonyls; saturated ketones, saturated aldehydes, alk-2-enals, and alk-2,4-dienals. The hexane extract was dried using a rotary flash evaporator. A warm water bath, 45-60°C, was employed to hasten the evaporation. Hexane boils at 69°C, hence care was taken to maintain a lower temperature. The residue in the flask was dissolved in a minimum of chloroform, spectrophotometric grade, and removed to a five ml. beaker. The chloroform solution was then subjected to column chromatography for separation into the four classes of monocarbonyls, following the method described by Schwartz, Parks and Keeney (1962). The chloroform solution of the 2,4 dinitrophenylhydrazone compounds was allowed to evaporate in a five ml. beaker until about one ml. remained. This solution was drawn into a graduated five ml. syringe to the three-tenths mark and hexane drawn into the syringe until the volume totaled two ml. Thus, the proportion of chloroform to hexane in which the unknowns were dissolved was equivalent to the solvent used for development, i.e. 15% chloroform in hexane.

#### Preparation of Column

The column used was three cm. inside diameter by 60 cm. tall with a Teflon Univalve closure and a coarse

fritted glass disc. The adsorbent was Magnesia 2665<sup>3</sup> which was activated at 400°C for 48 hours as recommended by Wyatt and Day (1963). Celite 545<sup>4</sup>, dried for 24 hours at 150°C, was used in combination with the magnesia to improve flow qualities. Upon removal from the oven these materials were stored in a vacuum desiccator, evacuated by means of an aspirator.

Ten grams each of magnesia and Celite 545 were placed in a 500 ml. volumetric flask together with 90 ml. of the solvent, 15% chloroform in hexane, spectrophotometric grade. The solvent was prepared by mixing appropriate quantities of each solvent in a 500 ml. volumetric flask. The mixture of adsorbents and solvent was shaken, then added slowly through a long stem glass funnel to the chromatographic column which was half filled with solvent. The Univalve was closed. As the material settled slowly by gravity, the column was shaken slightly to remove air bubbles. When the material had settled a Vibra Graver was used to compact the materials until bubbles ceased coming to the top and the packing seemed to be uniform. The Univalve was then opened to allow the extra solvent to run out until the level above the bed was two mm. A Stanley nine inch Torpedo level was used to assist in adjusting the column to a vertical position.

3 Fisher Scientific Co., Fairlawn, New Jersey

4 Johns Manville Corp., New York, New York

### Application of Sample to Column

The sample was applied rapidly to the top of the column by rotating the needle of the syringe around the circumference of the column while slowly pushing the plunger down. Additional solvent used for washing the syringe, was applied to the column in a similar fashion.

After two minutes, to allow adsorption of the sample, a glass wool plug was applied near the top of the bed. This prevented disturbing the surface as solvent was added. Development was as follows: 150 ml. 15% chloroform in hexane, 150 ml. 30% chloroform in hexane, 100 ml. 60% chloroform in hexane. New solvent was applied when the previous solvent had run to within one half inch of the top of the bed, to minimize changes in composition of the previous solvent. At no time was the column allowed to become dry.

Known compounds, representative of the four classes which this method is designed to separate, were prepared as 2,4 dinitrophenylhydrazine derivatives and applied to the column in a chloroform-hexane solution. They were the derivatives of butanone, heptaldehyde, oct-2-enal, and hept-2,4-dienal. The column separated these compounds effectively. The first band, methyl ketone was gray, the second band, a saturated aldehyde was pink, the third band, an alk-2-enal was a redder pink and the fourth band, an alk-2,4-dienal was a bright lavender.

The chloroform-hexane solution of the unknown 2,4-dinitrophenylhydrazine derivatives was applied to the column and separation of colored bands occurred.

As the colored bands were eluted they were collected in 125 ml. beakers which were allowed to stand at room temperature until evaporated to dryness. The residue in the beakers was dissolved in a minimum amount of chloroform and stored in small glass vials with screw caps. These solutions were then subjected to paper chromatography in order to identify the homologues within each class as described by Nonaka, Pippen and Bailey (1959). The bands faded as they moved down the column, making separation by elution difficult. Further distillates were prepared and the 2,4 dinitrophenylhydrazine derivatives separated on a column. The column was broken and the bands recovered while still distinctly separated.

#### Ultraviolet Spectra of Bands

A chloroform solution of the individual classes separated by the column was subjected to ultraviolet analysis on a Model Cary 11 Recording Spectrophotometer using a continuous span from 400 to 300 mμ. The wavelength at the maximum absorption was compared to reference values for authentic 2,4 dinitrophenylhydrazones (Jones, Holmes and Seligman 1956; Stitt et. al. 1960).

### Time Fading Study of Bands

A time fading study as described by Jones, Holmes and Seligman (1956) was used. Chloroform solution of the individual classes together with sodium hydroxide (2:1 v/v sodium hydroxide:hydrazone) were placed in small test tubes with the aid of a syringe. At the end of 15 min., 45 min., one hour and two hours fresh tubes were prepared and the color compared with the earlier samples.

### Paper Chromatography

A glass chromatographic jar, 45 cm. tall by 14.5 cm. I.D., for descending chromatography was employed. Whatman no. one paper, three inches wide by 17 inches long was first pre-treated by impregnation with 10% 2-phenoxy-ethanol in acetone. Papers were handled carefully with a metal clamp and the machine direction marked with graphite pencil on each strip. They were hung to dry for ten minutes before spotting.

A one-tenth ml. microsyringe was used to spot the papers. Two spots were applied to the starting line on each paper; one, a known reference compound, the 2,4 dinitrophenylhydrazone of heptaldehyde (prepared in this laboratory), the other, the unknown. Twenty drops of the unknown were required to make the spot as dark as needed.

Less was required of the reference compound. The analysis was carried out in quadruplicate. Known compounds and mixtures of known compounds were spotted on paper also.

Heptane, saturated with 2-phenoxyethanol was the developing solvent. The heptane was shaken with enough 2-phenoxyethanol in a separatory funnel to cause a cloudy appearance. After a few minutes, when the heptane became clear, it was drawn off and used in the development trough. The 2-phenoxyethanol was discarded. Papers were hung in the machine direction and development took place for sixteen hours.

The papers were removed after development, the spots circled with pencil and the distance from the starting line to the center of the spot measured in mm. Because the solvent runs off the paper, the  $R_h$  is taken as the ratio of the distance moved by the unknown hydrazone to the distance moved by the reference compound. This  $R_h$  value is an almost linear relationship for each class of monocarbonyls treated in this manner and helps established identity (Nonaka, Pippen and Bailey, 1959). In some cases, when the spots were too faint, the papers were sprayed with 10% potassium hydroxide to reveal the location of the spots.

#### Gas Chromatography and Mass Spectrometric Analysis of Ether Extract

Samples of the ether extract prepared from the rye

flour distillate were chromatographed on each of two different columns.

#### Gas Chromatographic Conditions

Instrument	Aerograph 1200
Detector Temperature	Flame ionization 160°C.
Injector Temperature	250°C.
Apiezon Column	20% Apiezon L on 80-100 mesh acid-base washed Celite 545, 12 feet long by 1/8" O.D. Flow rate 25 ml./min.
Carbowax Column	20% Carbowax 20M on 80-100 mesh Celite, 12 feet long by 1/8" O.D. Flow rate 25 ml./min.
Temperature program:	Carbowax-isothermal for one hour at 75°C., then programmed at 2°/min. to 150°C.  Apiezon-isothermal for 22 min. at 100°C., then programmed at 2°/min. to 185°C.

Many of the carbonyl compounds tentatively identified by paper chromatography were selected as authentic compounds for gas chromatography and were injected on each column under conditions duplicating those of the sample. The known compounds included were saturated aldehydes from C-two to C-eight, but-2-enal and hex-2-enal. Following the mass spectral analysis which indicated the presence of alcohols and a lactone the following authentic compounds were tested on both columns: ethanol, 1-pentanol, 1-hexanol and gamma valerolactone. Ethyl acetate was also injected each day as the reference compound and



relative retention times calculated for the peaks shown by each authentic compound and the sample. A semi-log plot was prepared using two authentic alk-2-enals to locate the line relating retention time and number of carbon atoms for this type of compound. This plot was used to establish retention times of other members of the series.

Mass spectrometry data were obtained by means of an Atlas CH 4 single focusing mass spectrometer which is connected to a gas-liquid chromatograph. The Carbowax column described previously was used with a temperature program of 75°C. for 30 min. and then a 2°/ min. rise to 150°C. Spectra were taken at each peak apex and where possible, on the leading and tailing edges to help pinpoint incompletely resolved components. Spectra were also taken where no peaks were visible in order to monitor and subtract the background mass peaks and column bleed.

## RESULTS AND DISCUSSION

### Concentration of Rye Volatiles

Following vacuum steam distillation the odor of the trapped volatiles was noted. The first trap had a mild cereal odor while the later traps which were chilled in liquid nitrogen had an intense rye odor. The charge flask also had a cereal-like odor, but its intensity was considerably less than that of the traps. As the cold fingers were allowed to defrost some odorous material escaped. Hydrogen sulfide, formaldehyde, an amine-like odor and a sweet fragrant odor were detected.

The smaller charges of rye flour yielded distillates totaling about 800 ml. each, of which 600 ml. were condensed in the first two flasks, the remainder in the liquid nitrogen cooled traps. The larger quantity of rye flour yielded a distillate totaling about 2500 ml. The latter distillate was extracted with ether. To test the odor of this extract, a strip of filter paper was dipped therein, the ether allowed to vaporize and the odor noted. The odor was extremely strong, indicating a good concentration of odorous volatiles. The odor was reminiscent of sulfur compounds, strong cereal and rye flour.

## Analysis of Distillate

### Odor after reaction with specific functional group reagents

The distillate was tested for the presence of two classes of compounds by reacting small aliquots with reagents specific for carbonyl compounds and amines. A change in odor of the reacted material would indicate that these compounds no longer contributed to the aroma, hence, they had reacted with the specific reagent.

An aliquot of the distillate which was allowed to react with 2,4 dinitrophenylhydrazine reagent lost most of its aroma, indicating the importance of carbonyl compounds in the rye aroma volatiles.

Although the odor of an amine was noticeable in the distillate, no amine was detected after reaction with dilute sulfuric acid (Weurman, 1963) or with phenyl isothiocyanate (Shriner, Fuson and Curtin, 1964).

### Conversion to 2,4 dinitrophenylhydrazones

Because of the evident importance of the carbonyl compounds in the aroma volatiles of rye flour, attention was first directed toward their 2,4 dinitrophenylhydrazine derivatives. The hydrazones of the concentrated volatiles when prepared by the method described by Shriner, Fuson and Curtin (1964) using sulfuric acid, yielded numerous crystals when allowed to stand overnight. By morning several types of shapes and colors

were observed, black, pale yellow, orange and dark maroon. The supernatant was deep orange.

When the reagent was prepared using hydrochloric acid, (Day, 1965) no crystals were detected the next day and the supernatant was pale yellow. This solution was carried through hexane extraction to column and paper chromatography.

The crystals in the first instance may have been due to the fact that more hydrazine is available for reaction, as eight times as much 2,4 dinitrophenylhydrazine is employed in this method as in the second method. Certain carbonyls, such as formaldehyde and acetaldehyde may polymerize in the presence of small amounts of sulfuric acid, thus preventing them from reacting with the 2,4 dinitrophenylhydrazine (Feiser and Feiser, 1956).

#### Hexane Extraction

Hexane extraction of the hydrazone solution is designed to retrieve the monocarbonyl derivatives which are more soluble in hexane than water. When the extract was reduced to dryness a thin yellow-colored film appeared on the inside of the flask. The amount of monocarbonyl derivatives recovered from the distillate was estimated to be in the microgram range.

### Column Chromatography

The first step in analysis of the unknowns was identification of the classes of carbonyl compounds in the hexane extract.

Authentic hydrazones, representing all four classes of monocarbonyl compounds, were first separated on a magnesia column. The colors as described by Swartz, Parks and Keeney (1962) were observed except for the alkanal band which was pink instead of tan. The order in which the bands occur as described by the authors is unchanging; ketones the fastest moving, followed by aldehydes, alk-2-enals and finally alk-2,4-dienals.

The hydrazones recovered from the hexane extract were dissolved in 15% chloroform in hexane and applied to the column as previously described. They were adsorbed as a pink band at the top of the column within five minutes of application. Within 15 to 30 minutes three different colored bands could be distinguished. The first band, the largest, was a grayish brown, the second band was pink and the third band (the smallest) was a deep intense lavender.

In order to verify the identity of the class of compounds in each band, ultraviolet absorption spectra were obtained. This was done on separated bands from two distillations. In the first instance the column bands

were eluted and in the second instance the column was cut, the colored bands removed physically along with the adsorbent and recovered by filtration. The latter method may insure a more complete separation of classes.

The maximum absorbence of ultraviolet light of four classes of hydrazones is given by Jones, Holmes and Seligman (1956) and Stitt, et. al. (1960) as follows:

No. of Carbons	Absorbence - $\mu$			
	<u>Alka-</u> <u>nones</u>	<u>Alka-</u> <u>nals</u>	<u>Alk-2-</u> <u>enals</u>	<u>Alk-2,4-</u> <u>dienals</u>
1		348		
2		354		
3	363	356	366	
4	363	358	373	
5	363	358	374	384

The ultraviolet spectra from the first band from the column showed a maximum absorbence at 358  $\mu$  and contained alkanals. The identity of the second band was considered to be alk-2-enals because its maximum absorbence occurred at 360 and 363  $\mu$ , close to that reported for this class. Some alkanals, i.e. acetaldehyde and formaldehyde may travel with the alk-2-enals (Swartz, Parks and Keeney, 1962).

The time fading study described previously (Jones, Holmes and Seligman, (1956) helped to confirm the identification of the band. A solution of the hydrazones from the first band with 0.25N sodium hydroxide showed a marked depletion of color after one hour and a shift from cherry red to light pink. The hydrazones of

these aldehydes have an ultraviolet absorbance peak at 520-535 m $\mu$  which is completely lost after one hour and therefore a slight change in hue might be expected. The second band showed no change in color. This is additional evidence that the first band was composed of alkanals and the second alk-2-enals.

#### Paper Chromatography

R<sub>h</sub> values for carbonyl compounds, calculated from the distances moved on paper are given in Tables 3 and 4. Four to six papers were spotted for each distillation and developed 16 hours. The R<sub>h</sub> value was calculated as the distance moved by the unknown/distance moved by the hydrazone of heptaldehyde. On the basis of these R<sub>h</sub> values the following compounds were tentatively identified from the alkanal band: acetaldehyde, propanal, butanal and pentanal. The following compounds were tentatively identified from the alk-2-enal band: prop-2-enal, but-2-enal, pent-2-enal, hept-2-enal and oct-2-enal.

#### Gas-liquid Chromatography and Mass Spectral Analysis

The sample was injected on Apiezon and Carbowax columns as previously described. The gas-liquid chromatograms are shown in Figure 2 and 3.

Authentic alkanals and alk-2-enal compounds were

Table 3. Paper Chromatography of 2,4 dinitrophenylhydrazine derivatives of alkanals in rye distillate.

The Rh value is the distance moved by the unknown/distance moved by the hydrazone of heptaldehyde.

Tentative Identi- fication	Paper No.	Rh Value of Unknown	Close to Rh Value reported by Nonaka, Pippen and Bailey (1959)
Acetaldehyde	5	0.28	0.25
	6	0.33	
	7	0.24	
	8	0.27	
Propanal	3	0.38	0.40
	8	0.46	
Butanal	1	0.50	0.52
	4	0.48	
	5	0.54	
Pentanal	2	0.60	0.65
	3	0.64	
	6	0.64	



Table 4. Paper Chromatography of 2,4 dinitrophenylhydrazine derivatives of alk-2-enals in rye distillate.

The Rh value is the distance moved by the unknown/distance moved by the hydrazone of heptaldehyde.

Tentative Identi- fication	Paper No.	Rh Value of Unknown	Close to Rh Value reported by Nonaka, Pippen and Bailey (1959)
Prop-2-enal	4	0.18	0.20
	5	0.20	
	6	0.20	
	7	0.18	
But-2-enal	1	0.32	0.25
	2	0.28	
	3	0.29	
	4	0.31	
Pent-2-enal	7	0.38	0.40
	8	0.46	
	9	0.47	
	10	0.47	
	12	0.44	
Hept-2-enal	5	0.70	0.70
	6	0.70	
Oct-2-enal	11	0.81	0.80
	12	0.84	

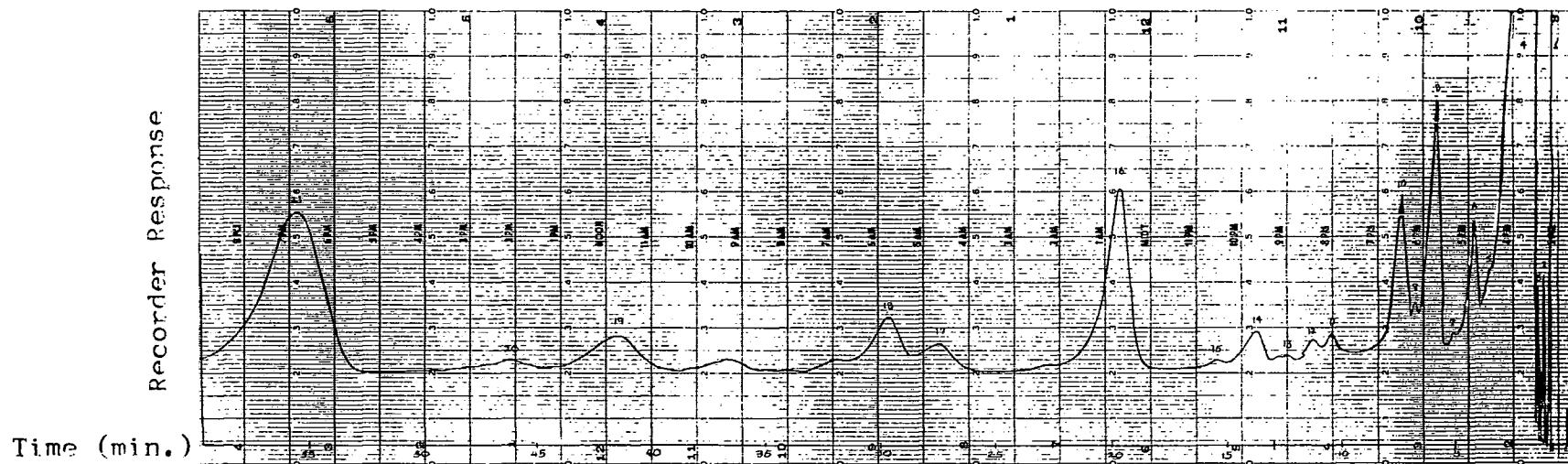


Figure 2. Chromatogram of volatile components of rye flour (*Secale cereale*), using a Carbowax 20M column. Conditions were: column 12 ft. long, 1/8 in O.D., 20% Carbowax 20M on 80-100 mesh Celite, N<sub>2</sub> flow rate-25 ml./min., isothermal at 75°C.

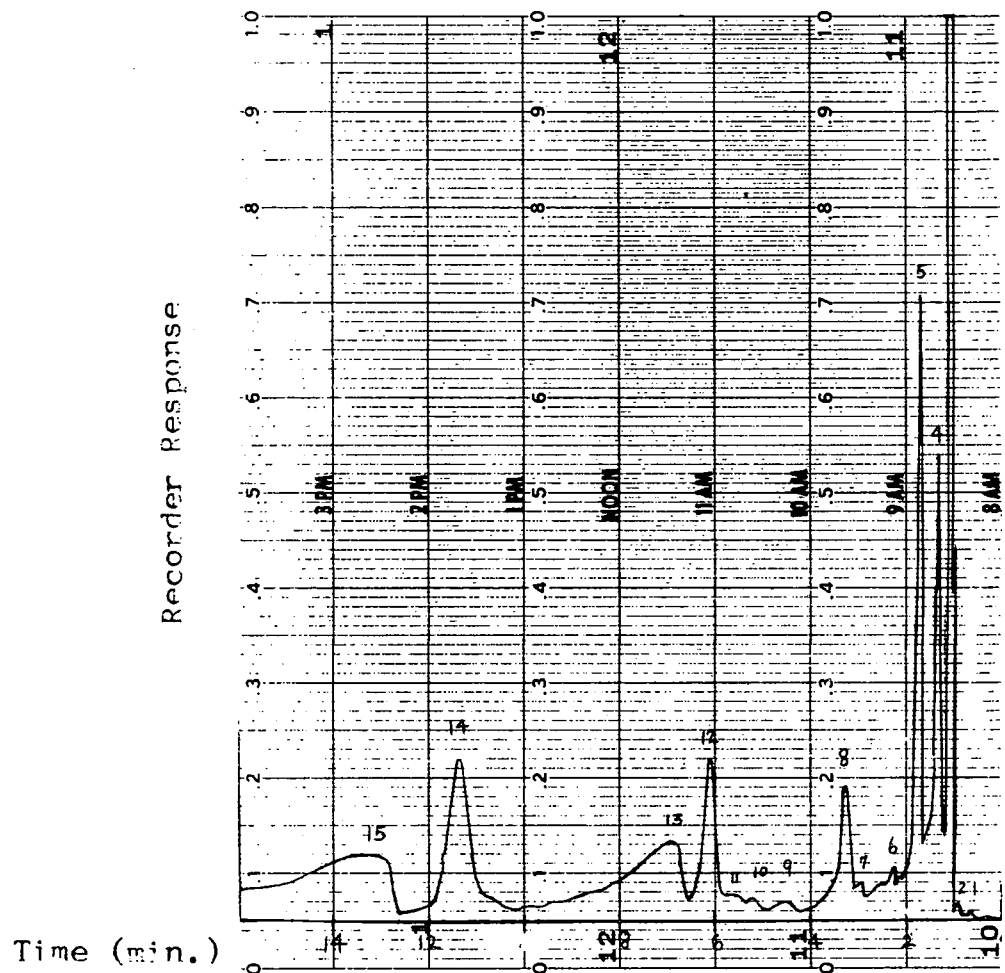


Figure 3. Chromatogram of volatile components of rye flour (*Secale cereale*) using an Apiezon column. Conditions were: column 12 ft. long, 1/8 in. O.D., 20% Apiezon L. on 80-100 mesh acid-bas washed Celite 545. N<sub>2</sub> flow rate-25 ml./min. isothermal at 100°C.

injected on both columns under conditions duplicating those for the rye volatiles and relative retention times were calculated using ethyl acetate as the reference compound. Retention times of these compounds matched those of a number of peaks in the chromatograms of rye volatiles.

As described in the Experimental Procedure the tr's of two alk-2-enal compounds were used for the construction of semi-log plots for both columns, Figure 4. Among the unknowns the tr values from both gas chromatographic columns fell on the semi-log plot at the three carbon position, indicating the presence of prop-2-enal. Only those carbonyl compounds present in rye volatiles that were corroborated by mass spectral analysis are reported here. These compounds were acetaldehyde, hexanal and prop-2-enal, Table 5.

Mass spectral analysis also demonstrated the presence of ethanol and ethyl acetate. The presence of ethyl acetate was further corroborated by tr values on both chromatographic columns. Ethanol was further corroborated by its tr value on the Carbowax column. It was obscured by the ether peak on the Apiezon column since the tr of ether coincided with ethanol. In addition, mass spectral data indicated the presence of 1-pentanol, 1-hexanol and gamma valerolactone. The presence of 1-pentanol was corroborated by gas chromatographic relative

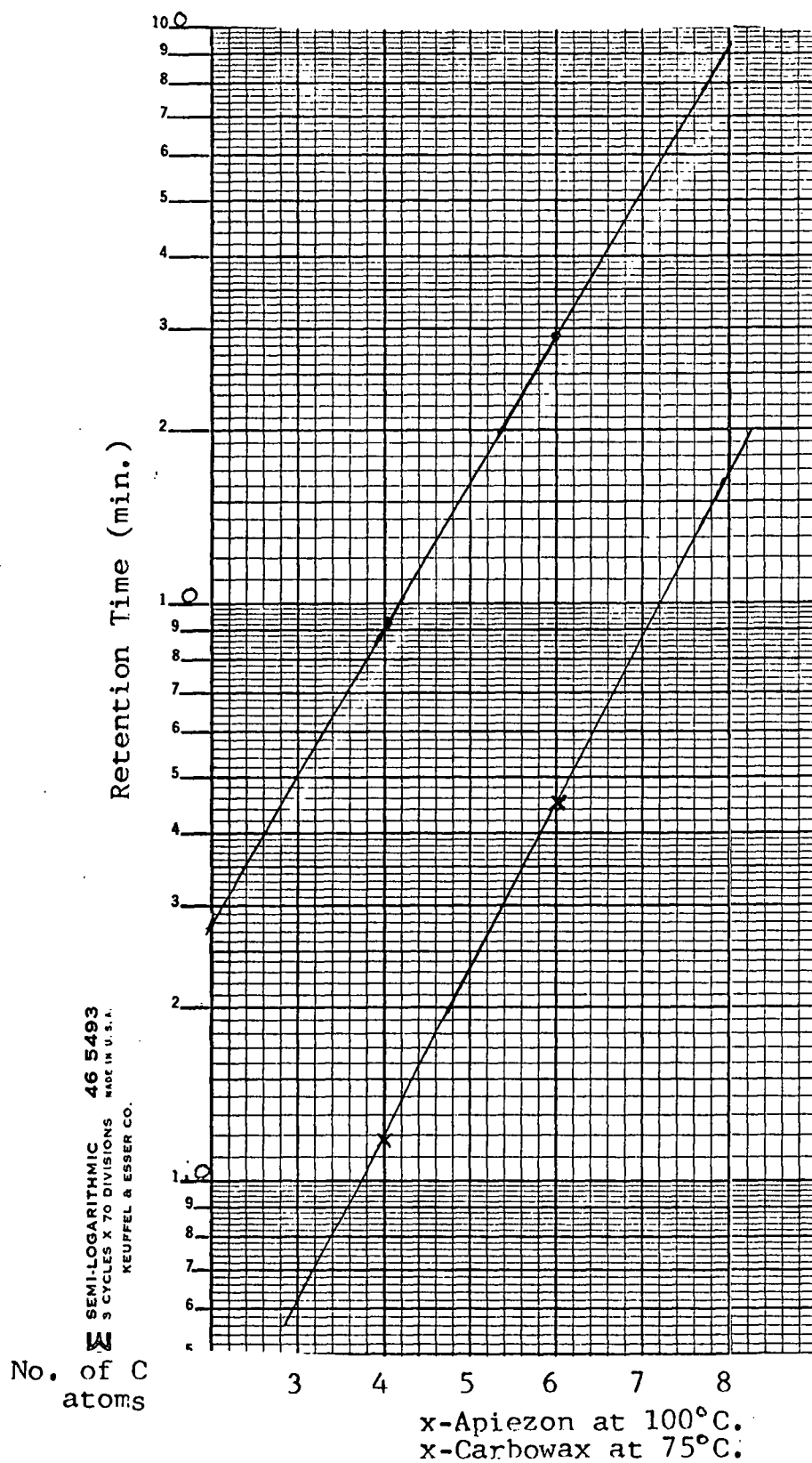


Figure 4. Semi-log plot of alk-2-enals on Carbowax 20M and Apiezon columns

Table 5. Identification of compounds based on relative retention times and mass spectral analysis.

<u>Compound</u>	<u>Carbowax column</u>			<u>Apiezon column</u>			<u>Mass Spectra</u>
	<u>Peak No.</u>	<u>tr/tr Ethyl Acetate</u>		<u>Peak No.</u>	<u>tr/tr Ethyl Acetate</u>		
		<u>Unknown</u>	<u>Known</u>		<u>Unknown</u>	<u>Known</u>	
Acetalde- hyde	3	.22	.25	2	.54	.49	X
Ether	4	.41	.31	3	.63	.63	X
Ethyl Acetate	8	1.00	1.00	5	1.05	1.00	X
Ethanol	9	1.19	1.27	3	.63	.63	X
Prop-2-enal	11	1.63	1.67	4	.80	.72	X
Hexanal	17	3.32	3.41	12	3.53	3.30	X
1-Pentan- ol	21	9.12	8.56	10	3.03	3.08	X

retention times.

Following is a list of compounds present in the rye flour volatiles demonstrated by matching relative retention times on polar and nonpolar gas chromatographic columns and by mass spectral analysis: acetaldehyde, hexanal, 1-pentanol, ethyl acetate, and prop-2-enal, Table 5. The presence of ethanol was demonstrated by mass spectral analysis and tr on a Carbowax column.

## SUMMARY AND CONCLUSIONS

The volatile fraction of light rye flour (*Secale cereale*) grown in North and South Dakota was isolated for study by steam distillation under vacuum. Preliminary testing for carbonyl compounds and amines revealed the presence of the former and the absence of the latter. The odors from the distillate included formaldehyde, hydrogen sulfide, an amine-like odor and a sweet, fruity odor.

A concentrate of rye volatiles was reacted with 2,4 dinitrophenylhydrazine reagent and then extracted with hexane. The hexane extract was subjected to column chromatography for separation into classes of 2,4 dinitrophenylhydrazine derivatives of monocarbonyl compounds. Three classes were visible, as colored bands on the column. The first band was identified as alkanals by means of ultraviolet spectroscopy and a time-fading study which differentiated alkanals from alk-2-enals. The second band was tentatively identified as alk-2-enals because of its position on the column, its color and its ultraviolet spectrum. The third band was tentatively identified as alk-2,4-dienals on the basis of its distinctive color on the column.

Paper chromatography was employed to tentatively identify the homologues within each class. The following carbonyl compounds were tentatively identified by the



R<sub>f</sub> values of the spots, i.e. the ratio of the distance moved by the unknown/distance moved by the reference compound (the 2,4 dinitrophenylhydrazone of heptaldehyde): acetaldehyde, propanal, butanal, and pentanal from the alkanal band and prop-2-enal, but-2-enal, pent-2-enal, hept-2-enal and oct-2-enal from the alk-2-enal band.

An ether extract of one distillation was prepared in a liquid-liquid extractor, then concentrated by distillation and nitrogen gas to produce an essence. The essence was subjected to gas chromatographic analysis on polar (Carbowax) and nonpolar (Apiezon) columns and relative retention times were calculated for the peaks using ethyl acetate as a reference compound. Mass spectral analysis was performed on the essence. Matching retention times of chromatographic peaks of the rye volatiles with authentic compounds on two columns combined with the mass spectral data revealed that the following compounds were present in rye volatiles: acetaldehyde, ethyl acetate, hexanal, prop-2-enal and 1-pentanol. The presence of ethanol was demonstrated by mass spectral analysis and by tr on the Carbowax column.

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