FORMATION OF ALKYLPYRAZINE COMPOUNDS AND THEIR ROLE IN THE FLAVOR OF ROASTED FOODS

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CHAPTER I

INTRODUCTION

Even the simplest, most primitive animals developed likes and dislikes for certain types of foods. Until a comparatively recent time in history, man had learned only a very few chemical skills. These few, however, were largely in the area of food chemistry--the fermentation of wines, the making of vinegar, and the roasting of foods. Throughout almost all of history, traditional procedures handed down from generation to generation were followed in the production of foods. Any new methods were the result of accidental discovery, or at best an empirical trial and error method. The last century has seen a revolution in the methods of food processing. Orderly scientific methods are now used by virtually every food industry to develop new methods of processing their products that will result in a more desirable and a more stable product at the lowest possible cost.

The long-range goal of flavor research is to improve the flavor of new protein sources so that these foods can be made palatable and will be available for consumption as demanded by the ever-increasing world population. This can be accomplished by determining the flavor components of a food product. The flavor components can then be added to abundant protein sources in a synthetic form. Or else, by controlling conditions during curing, storage, or processing of the food product, reactions which created desired flavors or off-flavors can be either

enhanced or eliminated as desired. It is toward this latter goal that much of the work presented here is directed.

Determining the flavor constituents of food products is complicated by the large number of components present in natural products and the minute quantities in which many occur. Sensory evaluation studies are often necessary to determine which of the many components present are actively involved in the flavor of the product. Application of scientific methods to flavor research depended on the development of science to the point where accurate definition of the complex mixtures encountered in foods became feasible.

The development of gas-liquid chromatography by Martin and James (1,2) toward the end of 1952 did more than any other technique to make possible a concerted attack on the problem of food flavors. This technique permitted quick and easy separation of complex mixtures of volatiles obtained from natural food products. This method of assult on isolation of constituents of flavors has been so successful and so widely used that some investigators (3,4) now feel that some caution must be used before plunging ahead blindly identifying myriads of compounds present in a food product without making any attempt to show correlation between the substances identified and flavor. It is to this particular problem that the first part of this dissertation is directed.

A number of simple alkylated pyrazine compounds have been shown to occur among the volatile flavor constituents of roasted peanuts (5). Alkylated pyrazines have also been observed in the volatile aroma compounds from coffee, cocoa, and potato chips (6,7,8,9,10).

In view of the previously discussed goals of flavor research, the objectives of the work reported here are as follows:

1) To assess the role of pyrazine compounds in the flavor of roasted foods.
2) To survey quantitatively the distribution of pyrazine compounds in roasted food products.
3) To determine the method of formation of pyrazine compounds in roasted food products using a model system.
4) To initiate preliminary studies on the effects and metabolism of pyrazine compounds in the animal.

CHAPTER II

LITERATURE SURVEY

Four carbon atoms and two nitrogen atoms may be combined in three different ways to form the system of compounds known as diazines. These six-membered heterocyclic ring systems are divided into three classes. The most familiar class of diazines are the pyrimidines in which the two nitrogen atoms are meta to each other. The pyrimidines are of great importance because they occur in a wide variety of natural products such as purine and pyrimidine bases of nucleic acids, vitamins (thiamine, riboflavin), caffeine, etc. (11). The pyridazines, in which the nitrogen atoms are ortho to each other, are of considerably less importance. In the third class of diazines, the pyrazines, the nitrogen atoms are situated para to each other.

The first recorded synthesis of a pyrazine was the preparation of "amarone" in 1844 by Laurent (12). It was more than 50 years later, however, that "amarone" was identified as tetraphenylpyrazine (13). The name "pyrazine" was independently suggested in 1887 by Mason (14) and Wolf (15). Stoehr (16,17,18,19,20) and Wolf (21,22,23,24,25) were the first to conduct intensive studies on pyrazine compounds. Two extensive reviews of pyrazine chemistry have been published by Krems and Spoerri (11) and Pratt (26).

Alkylpyrazine compounds have occasionally been found to occur in natural products. In all instances, these compounds have been found in

roasted food products or occasionally as the result of microbial metabolism. A small amount of tetramethylpyrazine was isolated by Kosuge and Kamkya (27) from broths of a strain of Bacillus subtilis. This compound appears to be responsible for the characteristic odor of "natto", a fermented soybean beverage consumed in some Asian countries. These workers postulated (28) that tetramethylpyrazine is derived from 2 moles of acetoin (acetylmethylcarbinol) and 2 moles of ammonia. The only other reported occurance of an alkylpyrazine compound as a metabolite also involved tetramethylpyrazine. Demain and co-workers (29) reported a mutant of Corynebacterium glutamicum which accumulated high concentrations of tetramethylpyrazine. The tetramethylpyrazine was produced in such quantity that it crystallized and precipitated from the broth on cooling. This mutant is known to accumulate unusually large amounts of acetoin. Accumulation of tetramethylpyrazine was dependent upon addition of thiamine. Since diphosphothiamine has known activity as a cofactor for the formation of acetolactate (a precursor of acetoin) from pyruvate, this mutant presumable also converts acetoin to tetramethylpyrazine.

Pyrazine compounds have occasionally been found to occur in food products. Reichstein and Standinger (30) were the first to find pyrazine compounds in foods. They found 2,5-dimethyl- and 2,6-dimethylpyrazine in a coffee aroma fraction. In 1965, 2,5-dimethylpyrazine was isolated and identified as a volatile flavor component of potato chips by Deck and Chang (10). No indication of the amount of these compounds present was given. They reported an "earthy, raw potato" flavor for this compound at about 10 p.p.m. in oil. The following year, alkylpyrazine compounds were reported in two other foods, coffee and roasted

peanuts, both involving high temperature processing as do potato chips. Reymond and co-workers (31) identified pyrazine, 2-methylpyrazine, 2,3-dimethylpyrazine, and 2,5-dimethylpyrazine in the volatile aroma compounds of coffee using a headspace gas chromatographic analysis technique. Mason, et al. (5) published later in the same year a paper describing the isolation and identification of five alkypyrazines from roasted Spanish peanuts. Methylpyrazine, 2,5-dimethylpyrazine, trimethylpyrazine, methylethylpyrazine, and dimethylethylpyrazine were found in a mixture of volatile components isolated from roasted peanuts using high vacuum-low temperature trapping techniques. Again, no quantitative data were presented either for the work on coffee or roasted peanuts.

No other publication has appeared on pyrazine compounds in roasted peanuts; however, several groups have identified other pyrazines in coffee. Goldman, et al. (7) identified 16 additional alkylpyrazine compounds including those with ethyl, propyl, isopropyl, isobutyl, and vinyl side chains and various combinations thereof. Bondarovich and co-workers (9) listed 21 pyrazine compounds identified among the steam volatile components of cocoa beans (32). These workers attributed a "nutlike" odor to the alkylpyrazines.

In 1881, Etard (33) observed a new substance which he called "glycolin" that resulted from distilling a heated mixture of glycerol and an ammonium salt. Stoehr (18) in 1893 isolated 2,6-dimethylpyrazine from heated aqueous solutions of ammonia and glucose. A mixture of glucose or fructose with aqueous ammonia develops a brown color (34). Because of the similarity between this reaction and the "browing" reaction with amino acids, Hough and his co-workers (35) investigated the

products of glucose and ammonia reacted at 37°C. for two weeks. From these mixtures, 2-methyl-5- and 2-methyl-6-(D-arabotetrahydroxybutyl) pyrazine were isolated and identified. These authors hypothesized that these pyrazine derivatives are formed by the condensation of aldehydo-glucose with ammonia and methylglyoxal. Such a mechanism would give rise to both the 2,6- and 2,5-substituted pyrazines.

In the 1950's much interest developed in the ammoniation of carbohydrates, particularly molasses, as a possible route to protein substitutes for animal feeds. In the course of studies on the high temperature ammoniation of molasses, a mixture of small molecular weight heterocyclic bases were recovered by steam distillation (36,37,38). Among these, 2,5-dimethyl- and 2,6-dimethylpyrazine were identified.

The most commonly used method of determining the precursors of the flavor of roasted foods is to study the changes in concentration of various compounds during the roasting process. The studies of Pickett and Holley (39,40,41) on the peanut roasting process indicated that proteins were denatured, oil and starch remained unchanged, and sugars decreased. Total free amino nitrogen was found to decrease significantly during the course of the roasting process. Carbon dioxide, along with lesser amounts of aldehydes, ammonia, diacetyl, furfurals, and sulfur compounds were the major volatile compounds identified in the vapor above roasting peanuts. These workers (39) also studied carbon dioxide production and browning in aqueous sugar-amino acid mixtures. They found that aqueous mixtures of amino acids and sugars reacted to produce tetrahydrofuran derivatives along with considerable browning and formation of aromas. Mason and Waller (42) in 1964 attempted to isolate and identify the precursor of roasted peanut flavor. Flavor did not appear

to arise from the large globular proteins or from carbohydrate material per se. Rather, a single subcellular fraction of raw peanuts, the aleurone grain-protein body fraction, produced browning and typical peanut flavor when roasted. Mason and co-workers (5,43) also published two papers identifying a large number of volatile compounds isolated from roasted peanuts. A large number of carbonyl compounds, some low molecular weight pyrazines, and a pyrrole were identified. Since a flavor precursor, by nature, must be a substance of low volatility that can be converted to a more volatile compound during heating, Newell et al. (44) approached the peanut flavor problem by analyzing heated and unheated precursor fractions. Gross analysis of a soluble fraction of raw peanuts before and after heating showed that amino acids and sugars were the only classes of compounds that decreased significantly in concentration during heating. Thus, amino acids and sugars were implicated as precursors of typical roasted peanut aroma. Previous knowledge of the compounds produced during roasting (5,43) coupled with their precursor data permitted these authors to postulate that peanut flavor components (pyrazines) are produced from amino acid and carbohydrate precursors.

Cocoa beans, like peanuts, require roasting to develop their typical flavor. Pinto and Chichester (45) and Rohan and Stewart (46,47) employed an approach similar to that used by Newell (44) to determine cocoa flavor precursors. Pinto and Chichester (45) found that all amino acids except methionine were destroyed during roasting. Ammonia increased during roasting. Both Pinto and Chichester (45) and Rohan and Stewart (47) found nearly complete destruction of reducing sugars during cocoa roasting. Herz and Shallenberger (48) produced chocolate aroma by

heating leucine, glutamine, and threonine at 100°C. with glucose.

Evidently, one of the primary events occuring during the roasting of peanuts, cocoa, coffee, and other roasted food products must be a reaction between amino acids and carbohydrates which eventually leads to the formation of pyrazine compounds. The non-enzymatic browning reactions between amino acids and sugars have long been studied. Maillard (49) was the first to study such reactions systematically and his name is often applied to this type of reaction. The Maillard reaction is very complex and, as a result, much of the knowledge concerning it has been derived from work in simplified model systems which contain a single amino acid and carbohydrate. However, the work was almost entirely done in aqueous systems which are not representative of the conditions in the food products in which pyrazine compounds have been implicated. An enormous amount of literature concerning the non-enzymatic browning reactions is available. Since several reviews, some rather recent, have been published by Danehy and Pigman (50), Ellis (51), Reynolds (52, 53) and Hodge (54,55), this material will not be reported here.

Hodge (54,55,56,57,58) has made extensive studies of the sugar-amine non-enzymatic browning reactions and their relation to food flavors. Both glucosamine and fructosamine have long been known to undergo self-condensation to yield substituted pyrazines (35,54). In view of his work on Amadori-type rearrangements with both aldoses and ketoses in browning reactions, Hodge suggested that pyrazine and pyrrole compounds should be sought in sugar-amine reactions and their role in browning established (54).

Since the senses, particularly taste and smell, are intimately associated with food appreciation, and hence with consumption, the sen-

sory evaluation of foods and food flavor compounds is fundamental to food science. Strong acceptance or rejection responses are more pronounced for the senses of taste and smell than for sight and sound (59). That the sense of smell is more highly developed than the sense of taste is demonstrated by Parker and Stabler's (60) observation that the olfactory organ can detect dilution of ethanol 24,000 times greater than that required to stimulate the organ of taste. Bhargava's (61) studies showed that man has the least olfactory development of all the animals.

There is a problem in even defining "odor". The usual definition of odor as "that which can be smelled" needs qualification (62). Smelled by whom--man or animal? Some animals can detect odors that escape detection by man at any concentration (59). The problem is even more acute when the threshold levels of detection are discussed. For example, the male gypsy moth responds to as little as a trillionth of a microgram of the female's sex attractant (63). The same compound might be regarded as "odorless" by other animals or man. Generally, threshold of detection data are based on a sample population of man unless otherwise specified.

One of the earliest olfactory thresholds measured was that for ethylmercaptan by Fischer and Penzoldt (64) in 1886. Their value of 2.5×10^{-10} mg in 50 ml, obtained by simple sniffing tests, remains essentially unchanged in spite of the design and construction of many complex, sophisticated devices for the determination of odor sensitivity. Stone et al. (65) have prepared a critical review of olfactometric devices with special emphasis on food odors. The simplest technique, and still the most popular for odor determination is sniffing (59). Jones

(66,67) compared several methods and concluded that sniffing was an adequate technique for threshold studies. Adsorption of materials to the sides of the containers becomes important at threshold levels. Another difficulty with this method is finding a stable, inodorous, non-reactive dilution solvent for the compounds to be tested. However, individual subject variation is so great (68) that subjecting a larger number of individuals to the simple sniffing procedure may compare very favorably to data obtained from fewer subjects with more complex methods.

CHAPTER III

SYNTHESIS AND SENSORY EVALUATION

While many animals lack the ability to hear, and numerous others cannot see, all forms of life appear to respond to chemical stimuli (10). In man, the chemical senses are commonly referred to as taste and smell. Many modern techniques have been introduced in agriculture and the food industries which have drastically changed the methods of food harvest, preparation, storage, and distribution. Frequently these changes have altered the sensory appeal of foods. Thus, each new development has made more obvious the growing need for research in the sensory evaluation of foods and food products.

Much effort has been expanded by nutritionists in the development of new nutritionally-balanced prepared foods. However, these foods are often rejected, no matter how nutritious they are. The testimony of both World War II veterans and present-day astronauts confirms this. Certain appealing components of foods which are lost during processing or which were neglected by the nutritionist could be added to food products if their identities were known. Undesirable compounds created during storage might be more easily eliminated if they were identified. Also, by the addition of proper flavor-active chemicals, sources of abundant protein such as algae, seaweed, soybean, etc., could be made more appealing and therefore might then be utilized as inexpensive foods for the world's expanding population.

Only a few of the alkylpyrazine compounds are available commercailly. Synthesis of a number of the commercially-unavailable alkylpyrazines was undertaken for two principle reasons. In view of their presence in the volatile aroma fraction of peanuts and other roasted foods, it was deemed desirable to subject a series of alkylpyrazine compounds to sensory evaluation in order to determine their role, if any, in peanut flavor. It was also desirable to have available standard reference pyrazine compounds for use as retention time and quantitative standards in routine gas-liquid chromatographic analyses.

Materials

Methyllithium, 2M in ether, and ethyllithium, 1M in benzene, were purchased from Alpha Inorganics, Beverly, Massachusetts. White, heavy paraffin oil (Saybolt viscosity 335/350) was obtained from Fisher Scientific Co., Fair Lawn, New Jersey. Drierite was obtained from the W. A. Hammon Co., Xenia, Ohio, and Ascarite from the Arthur H. Thomas Co., Philadelphia, Pennsylvania. Carbowax 20M and Gas Chrom Q (100/120 mesh) were purchased from Applied Science Laboratories, State College, Pennsylvania. Pyrazine was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Methylpyrazine and 2,5-dimethylpyrazine were obtained from Wyandotte Chemicals Corp., Wyandotte, Michigan. Ethylpyrazine, 2-methyl-6-ethylpyrazine and n-pentylpyrazine were gifts of the research division of Wyandotte Chemicals. Deionized, distilled water was used in all experiments. All other chemicals and reagents were of reagent grade.

Procedures

Synthesis of Alkylpyrazine Compounds

The synthesis of alkylpyrazines for use in sensory evaluation and gas-liquid chromatographic standards was based on the procedure described by Klein and Spoerri (69,70). Because of the extreme reactivity of alkyllithium reagents with water vapor and carbon dioxide, it was necessary to utilize a closed reaction system with a continuous nitrogen flush. The reaction was conducted in a 250 ml round bottom flask with 3 necks having ground glass fittings. In one neck was placed a 30 ml dropping funnel, on top of which was placed a drying tube filled with Drierite and Ascarite to remove water vapor or carbon dioxide from the entering nitrogen stream. The second neck was fitted with a ground glass stirring rod and sleeve assembly which was connected to an overhead stirring motor. The third neck contained a 12 inch spiral-wound reflux condenser utilizing a tap water jacket. The top of this condenser was fitted with another drying tube containing Drierite and Ascarite to prevent diffusion of water vapor or carbon dioxide back into the system. The reaction vessel was then mounted in an ice-water filled battery jar which was resting on the top of a magnetic stirrer to circulate the ice-water.

In the synthesis of trimethylpyrazine, commercial 2,5-dimethylpyrazine was used as the starting material and was alkylated with methyllithium. Using a large syringe, 60 ml of a 2.3M solution of methyllithium in diethyl ether were cautiously transferred to the cooled ($0^{\circ}C.$), nitrogen-flushed reaction flask. A solution of 11 ml of 2,5-dimethylpyrazine in 20 ml of diethyl ether was then added dropwise through the dropping funnel while stirring. The apparatus was flushed with dry nitrogen throughout the course of the reaction.

A dark reddish-orange material precipitated as the 2,5-dimethylpyrazine was added. After about two-thirds of the 2,5-dimethylpyrazine was added, this gelatinous material caused the reaction mixture to become too viscous to stir. At this point, 50 ml of diethyl ether were added to the reaction flask to facilitate further stirring. The 2,5-dimethylpyrazine was added dropwise over a period of 40 minutes and the reaction was permitted to continue for 1 hour after the last addition. The reaction flask was then warmed to $20^{o}C$. and allowed to stand an additional hour. The reaction flask was then again cooled to $0^{o}C$. and any excess methyllithium was cautiously decomposed with water. The dark red precipitate dissolved as water was added and formed a yellow solution. The addition of about 50 ml of water was necessary to completely dissolve the precipitate.

The aqueous and ether layers were separated and the aqueous layer was extracted 5 times with 10 ml of diethyl ether. The ether extracts were combined with the original ether layer and reduced in volume prior to preparative gas-liquid chromatography by removing most of the ether on a rotary evaporator ($25^{o}C$.).

Preparative gas-liquid chromatographic purification of the trimethylpyrazine was effected with a F&M Model 500 gas chromatograph equipped with a four filament thermal detector. The column was a 3/8 inch x 12 foot aluminum column packed with 15 percent (w/w) Carbowax 20M on Gas Chrom Q (100/120 mesh) (71). The column was operated isothermally at $160^{o}C$. with a helium flow rate of 200 ml per minute. Repetitive injections of 0.1 ml were necessary to purify the entire yield of one reaction run. The trimethylpyrazine peak was collected in a U-shaped trap loosely filled with stainless steel shavings (Figure 1).

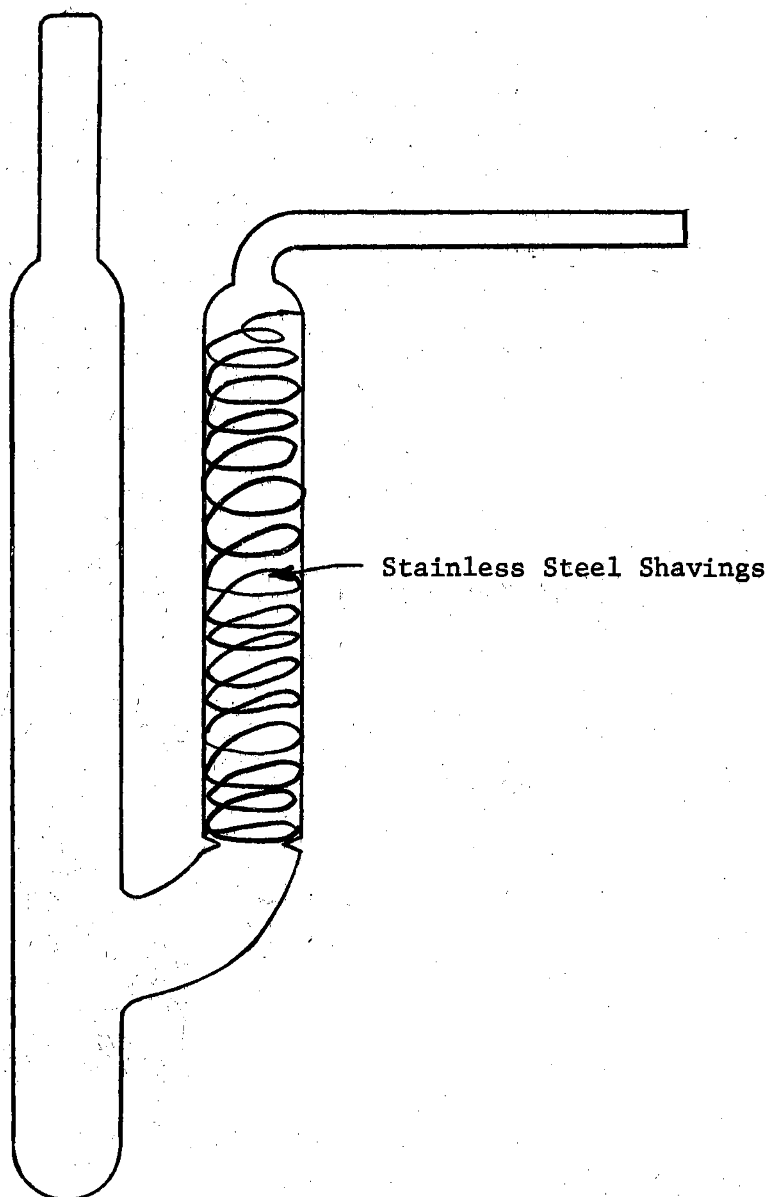

Figure 1. Preparative Gas-Liquid Chromatography Trap Used in the Collection of Chemically Synthesized Alkylpyrazines. Scale = 1:1

The efficiency of this trap for collecting pyrazine compounds was determined. Using ten 100 microliter aliquots, 1.0 ml of 2,5-dimethylpyrazine was injected on the column and collected. Recovery of 0.8 ml indicated a trapping efficiency of approximately 80 percent. The trap was warmed with a paper chromatogram drier since this appeared to help prevent aerosol formation and increased trapping efficiency. Purity of the collected alkylpyrazine was determined by analytical chromatography on a 20 foot x 1/4 inch glass column packed with 15 percent (w/w) Carbowax 20M on Gas Chrom Q.

Mass spectral analyses were obtained with a combination gas chromatograph-mass spectrometer. The instrument was a prototype of the LKB 9000 mass spectrometer. Infra-red spectra were obtained on both a Unicam SP200G and a Perkin-Elmer Model 457 grating infra-red spectrophotometers. The pyrazine compounds were run neat as thin films between IR-Tran plates if liquids, or as thin films of concentrated solutions in carbon tetrachloride if solids. Nuclear magnetic resonance spectra were obtained from a Varian 60 megacycle instrument using carbon tetrachloride solvent.

The same equipment and similar procedures were used for the synthesis of the other alkylpyrazine compounds. The synthesis of 2,5-dimethyl-3-ethylpyrazine involved alkylation of 2,5-dimethylpyrazine with ethyllithium. Alkylation of 2,6-dimethylpyrazine and 2,3-dimethylpyrazine with ethyllithium yielded 2,6-dimethyl-3-ethylpyrazine and 2,3-dimethyl-5-ethylpyrazine. The ethyllithium alkylations were all conducted in benzene rather than in diethyl ether which was used in the methylation reactions. The monomethylmonoethylpyrazines, 2-methyl-3-ethyl, 2-methyl-5-ethyl, and 2-methyl-6-ethylpyrazine were synthesized simultaneously

by alkylating 2-methylpyrazine with ethyllithium using the procedures described previously.

Sensory Evaluation of Pyrazines

The sensory evaluation tests were conducted in a special 10 x 18 foot room. This room had never been used as a chemistry laboratory and was relatively free of background odors. The room had a separate exhaust system. The samples to be tested were prepared in another laboratory to avoid building up background odors in the room. The air temperature was maintained at $74^{\circ} \pm 1^{\circ}$ F. during the entire course of the tests, which lasted several months. The relative humidity was normally about 50 percent with variation between 29 and 64 percent.

The sensory evaluation panel was composed of 20 members. Ten of these panel members were also members of a trained taste panel which scored peanut butter and roasted peanut samples. The other 10 members were drawn from among graduate students and technicians Department of Biochemistry. Most of the panel members were between the ages of 18 and 25. The panel was composed of 75 percent female members. No attempt was made to regulate the panel members' eating or smoking habits during the tests.

Each panel member was presented 20 samples in two separate groups. Each group contained the sample under study at eight different, randomly-ordered levels of concentration along with both an identified and a hidden blank containing only the dilution solvent. Heavy paraffin oil and deionized, distilled water were used as dilution solvents. The samples were presented in 16-ounce widemouth reagent bottles with groundglass stoppers. Each group of 10 samples was placed in a 20 x 13

x 12 inch styrofoam box coated with Carbowax 1500 to prevent the water from seeping through the pores of the styrofoam. Through these boxes was circulated water maintained at 25°C. in a temperature-controlled water bath. The bottles were about two-thirds submerged in the water with about one-third protruding through holes in the top of the box to allow easy grasping by the panel members.

All of the bottles were well shaken before the panel members started. Each panel member was then instructed to shake each bottle, remove the stopper, sniff the contents, and mark on the score sheet (Figure 2) whether or not any odor was detected. If an odor was detected, the panel member was requested to describe it on the score sheet if possible. The bottle was then shaken again before being returned to the styrofoam container. At least 15 minutes were allowed for the samples to re-equilibrate before they were introduced to the next panel member.

All sample bottles were carefully washed, rinsed with distilled-deionized water, and heated in an oven at 115°C. for 18 hours before being filled with new samples. All glassware used in preparing the samples was purchased new and stored separately from other laboratory glassware.

Results and Discussion

Synthesis of Alkylpyrazines

A typical gas chromatograph tracing of the diethyl ether extract of the trimethylpyrazine synthesis reaction mixture (Figure 3-I) shows that the ether extract contains only peaks corresponding to 2,3,5-tri-

ORGANOLEPTIC STUDIES

Date: ______________ Panel Members: ________

Sample: ______________

Series A	Odor	Evaluation: Nutty	Roasted	Burned	Other	Remarks
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						

Series B	Odor	Nutty	Roasted	Burned	Other
1.					
2.					
3.					
4.					
5.					
6.					
7.					
8.					
9.					
10.					

ODOR:

\+ indicates odor present
\- indicates no odor

EVALUATION:

1. Strong
2. Moderate
3. Weak
4. None

Figure 2. Sensory Evaluation Panel Score Sheet

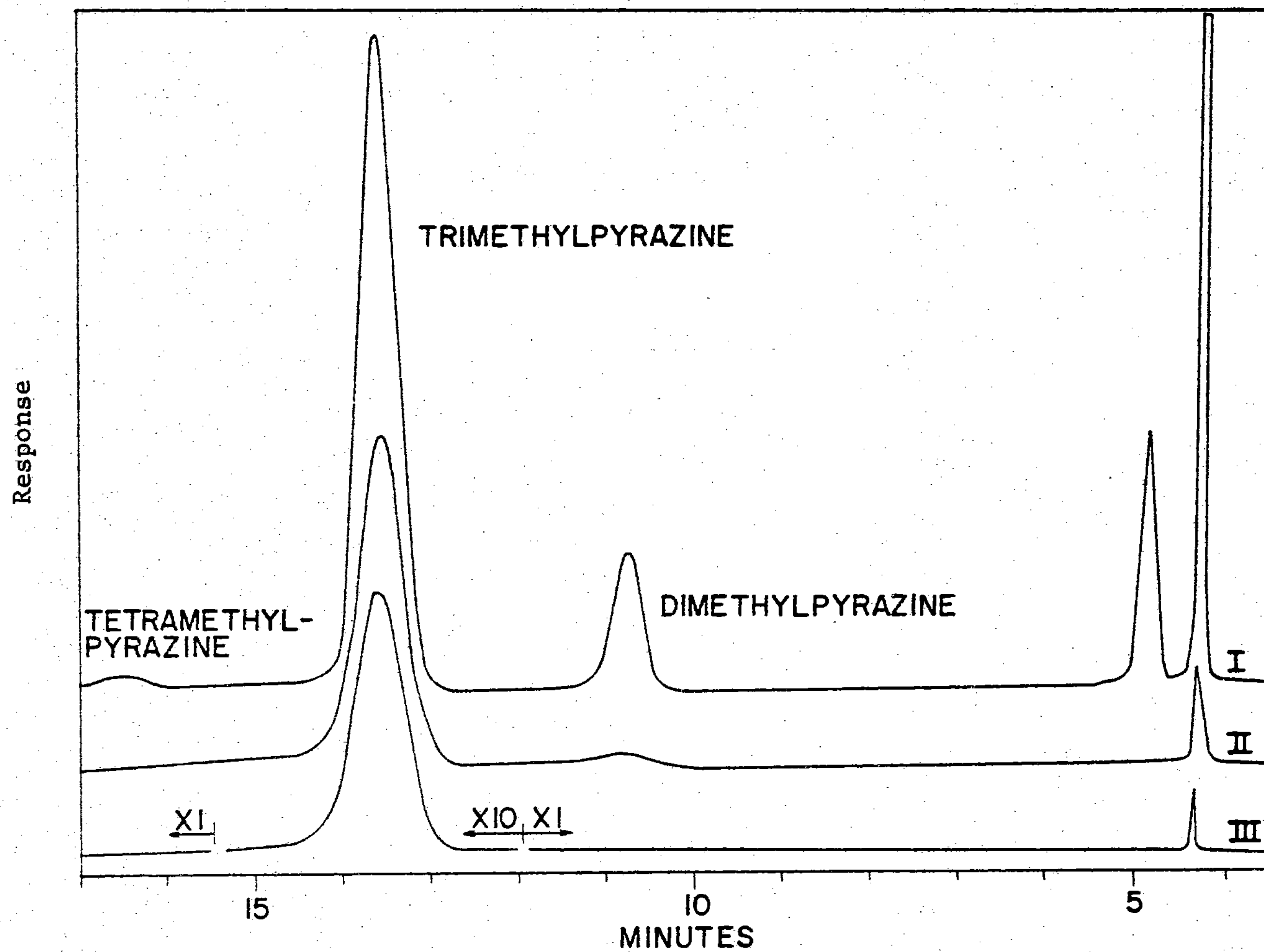

Figure 3. Purification of Synthesized Trimethylpyrazine on Repeated Gas-Liquid Chromatography.

methylpyrazine, unreacted 2,5-dimethylpyrazine, and a trace of 2,3,5,6-tetramethylpyrazine in addition to solvent peaks. Tetramethylpyrazine could probably be obtained using this procedure by reacting the methyllithium and dimethylpyrazine in a 2:1 ratio rather than a 1:1 ratio as was used to obtain trimethylpyrazine. The ether extract was reduced in volume and chromatographed preparatively on a 3/8 inch x 12 foot Carbowax 20M (15% w/w) liquid phase on Gas Chrom Q column (71).

Approximately 0.6 ml of trimethylpyrazine was collected in the preparative gas chromatographic trap (Figure 1). The gas chromatograph tracing after preparative chromatography (Figure 3-II) indicated that the only peak besides trimethylpyrazine was a very small 2,5-dimethylpyrazine peak caused by slight tailing of this compound. This impurity, however, amounted to less than 1 percent of the trimethylpyrazine peak. The entire isolate of trimethylpyrazine was rechromatographed preparatively using the same column. After the second preparative chromatography, no peak was seen in the tracing except 2,3,5-trimethylpyrazine (Figure 3-III). The yield of twice-chromatographed trimethylpyrazine was 0.5 ml, indicating an overall yield of approximately 4 percent. The synthesis was repeated 4 times in order to obtain a sufficient quantity of pure 2,3,5-trimethylpyrazine for use in the sensory evaluation experiments.

The nuclear magnetic resonance spectrum of the synthesized trimethylpyrazine (Figure 4) indicated that presence of only two types of hydrogen atoms. The large peak at 2.3 ppm is indicative of the methyl hydrogens. The smaller peak at 7.9 ppm represents the heterocyclic ring hydrogens. The measured ratio of these two peaks is 9.0 to 1.0 which may be predicted for 2,3,5-trimethylpyrazine which has 9 methyl hydro-

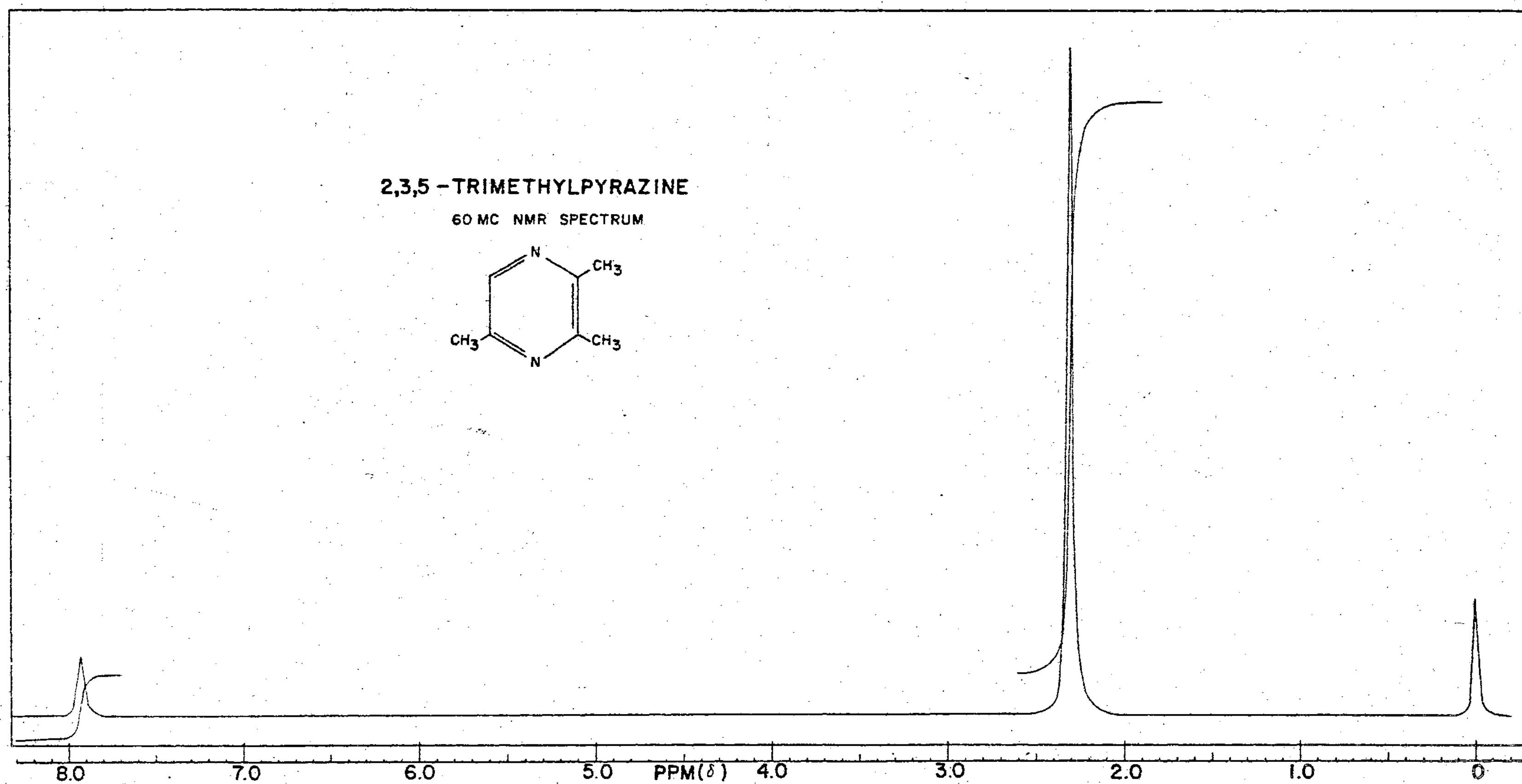

Figure 4. Nuclear Magnetic Resonance Spectrum of Synthesized 2,3,5-Trimethylpyrazine

gens and 1 ring hydrogen. The infrared spectrum of the synthesized trimethylpyrazine is shown in Figure 5. This spectrum appeared identical to a standard spectrum of 2,3,5-trimethylpyrazine obtained from the Stanford Research Institute (91). The mass spectrum (Figure 6) was obtained by combination gas chromatography-mass spectrometry. The peak at m/e 122 represents the molecular ion of trimethylpyrazine. The peak at m/e 81 is characteristic of the heterocyclic pyrazine nucleus. All peaks with relative intensities greater than 5 percent compare favorably with standard spectra obtained from the Stanford Research Institute (91). The GLC column was a 1/4 inch x 21 foot 15 percent (w/w) Carbowax 20M on Gas Chrom Q glass column. Ultraviolet spectrophotometric identification of the compound was established by Mr. B. R. Johnson (92).

Similar results were experienced in the synthesis of the dimethylmonoethylpyrazines, except that the yields of the alkylated pyrazine were considerably lower with much more unreacted dimethylpyrazine remaining in the ether extract of the reaction mixture. Yields were 1 percent or less. Identifications were made as for trimethylpyrazine. The infrared spectra are given in Figure 7. They compare favorably with standard spectra (91).

The synthesis of the methylethylpyrazines was conducted by reacting ethyllithium with 2-methylpyrazine, using the previously described precedures. However, in this case, the procedure results in the formation of three positional isomers, of methylethylpyrazine. Only partial resolution of the isomers was possible, even when the length of the preparative column was increased from 12 to 24 feet. While repetitive preparative gas chromatography with collection of the non-overlapping parts of each of the methylethylpyrazine peaks resulted in considerable enrich-

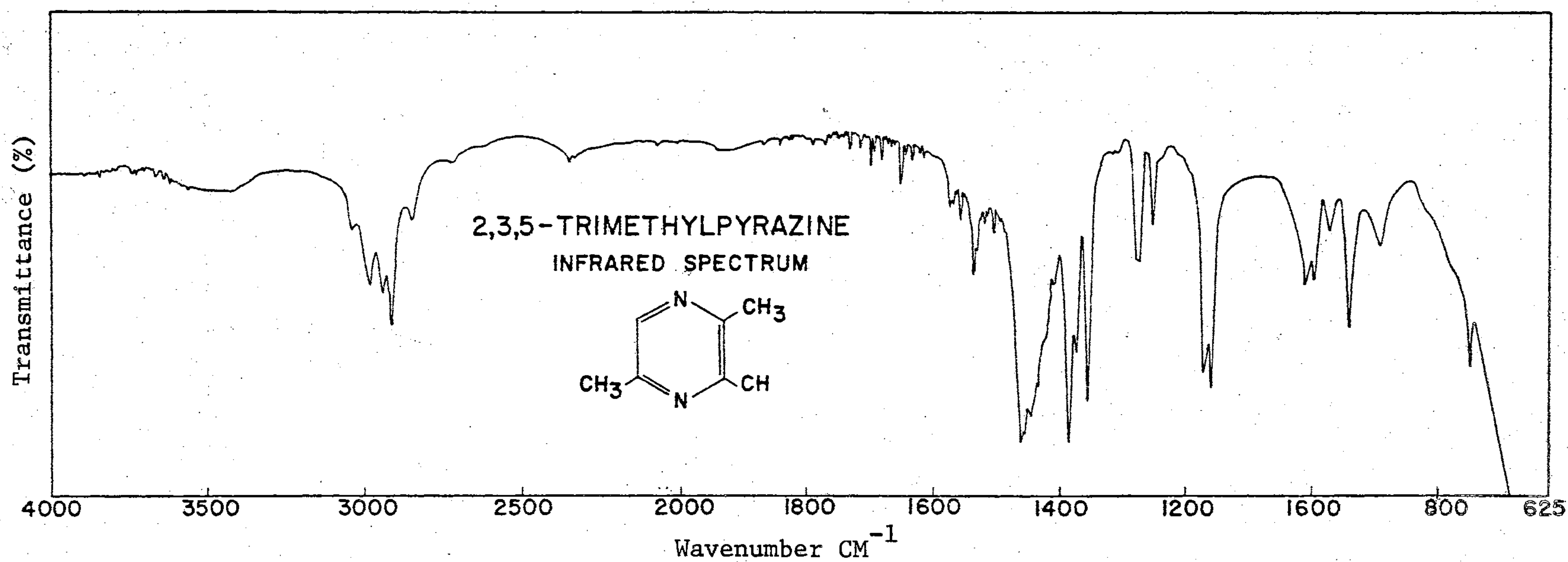

Figure 5. Infrared Spectrum of Synthesized 2,3,5-Trimethylpyrazine

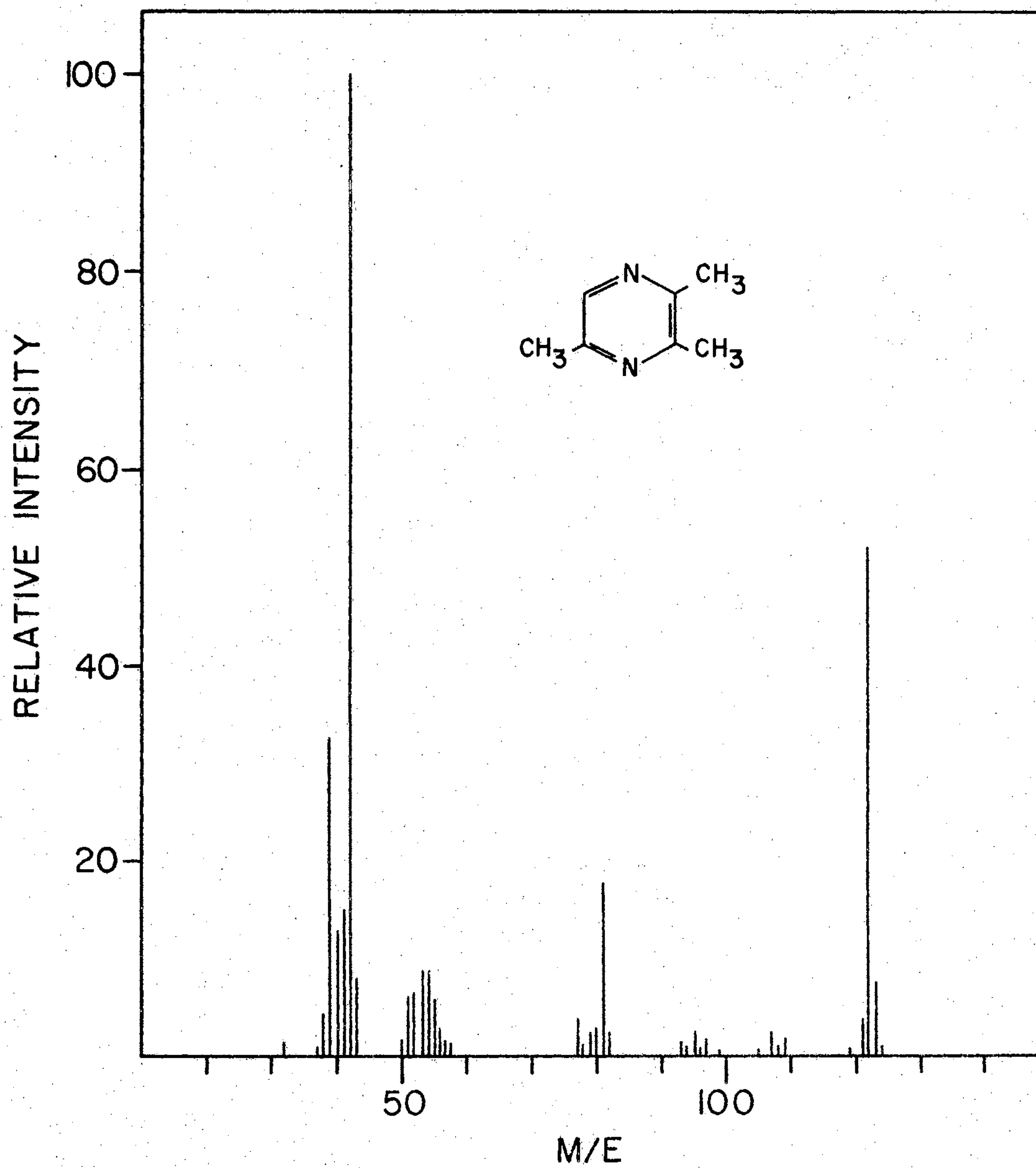

Figure 6. Mass Spectrum of Synthesized 2,3,5-Trimethylpyrazine.

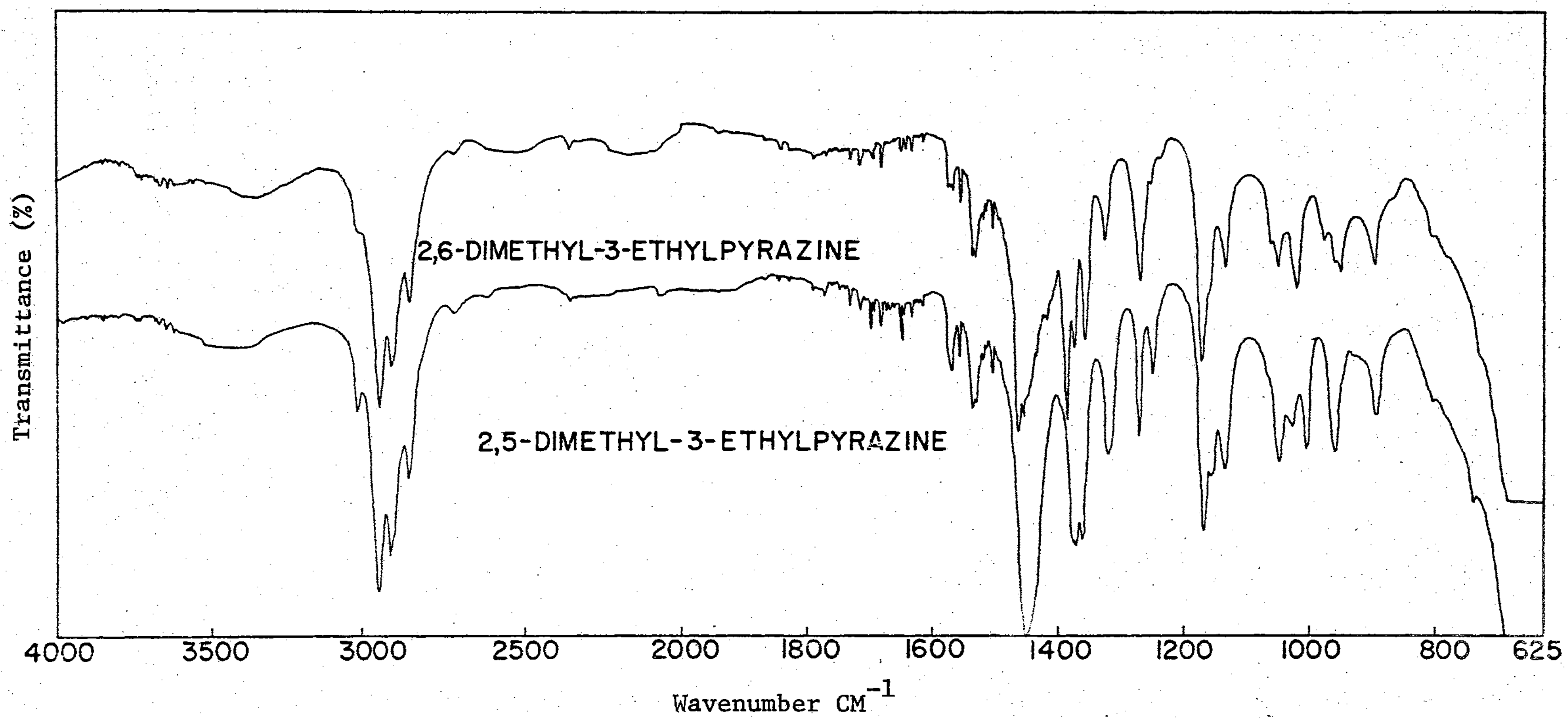

Figure 7. Infrared Spectra of Synthesized Dimethylethylpyrazines

ment for the individual isomers, a pure compound could not be feasibly obtained in sufficient quantities for sensory evaluation studies. These studies were therefore conducted on the entire methylethylpyrazine fraction.

Sensory Evaluation of Pyrazines

Two types of information were desired from the sensory evaluation studies. First, the odor thresholds of the alkylpyrazine compounds in aqueous and lipid media were obtained. This information, coupled with a quantitative knowledge of the alkylpyrazine content of roasted peanuts and other foods (Chapter IV), permits the assessment of the probable significance of the pyrazines in these food products. Second, a subjective response describing the nature of the odor was obtained. This allowed a specific evaluation of the role of pyrazine compounds in roasted peanut flavor.

The odor threshold level of detection for the compounds tested in this study was defined as that concentration which produced a positive (odor) response by the panel members 50 percent of the time. Each compound was presented to the 20 panel members on 3 different and isolated days. Thus, in every case, each point on the threshold profile curves reflects at least 60 responses. The threshold levels for each compound were determined in both water and mineral oil so that the results could be applied to many types of foods and food products. The threshold profiles of the two alkylpyrazine compounds found in the greatest amounts in roasted peanut aroma volatiles are shown in Figures 8 and 9. Ten pyrazine compounds were tested. The results are summarized in Table I along with 4 other compounds that were tested. The threshold

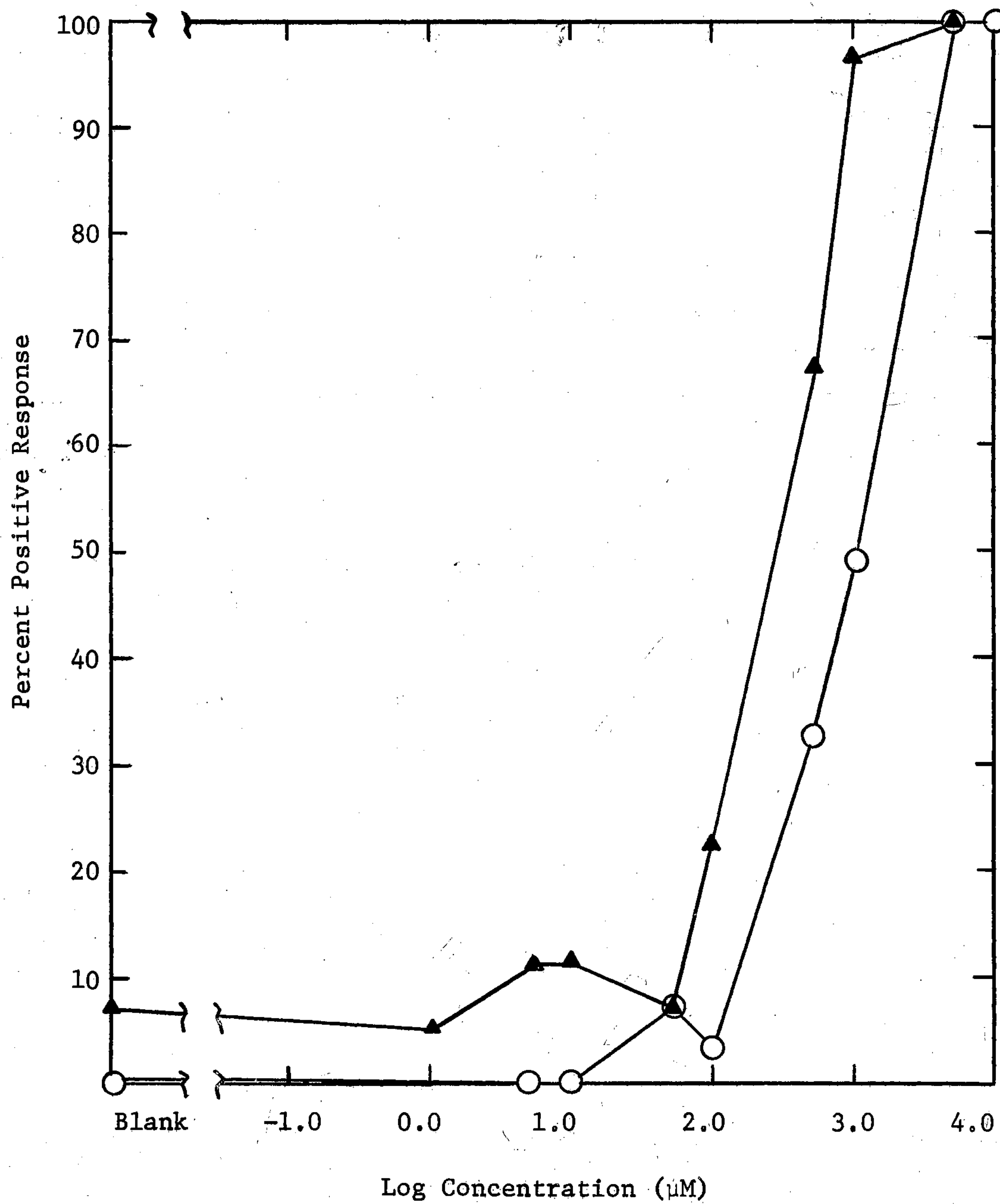

Figure 8. Odor Detection Threshold Profile for 2-Methylpyrazine in Water O and Oil ▲.

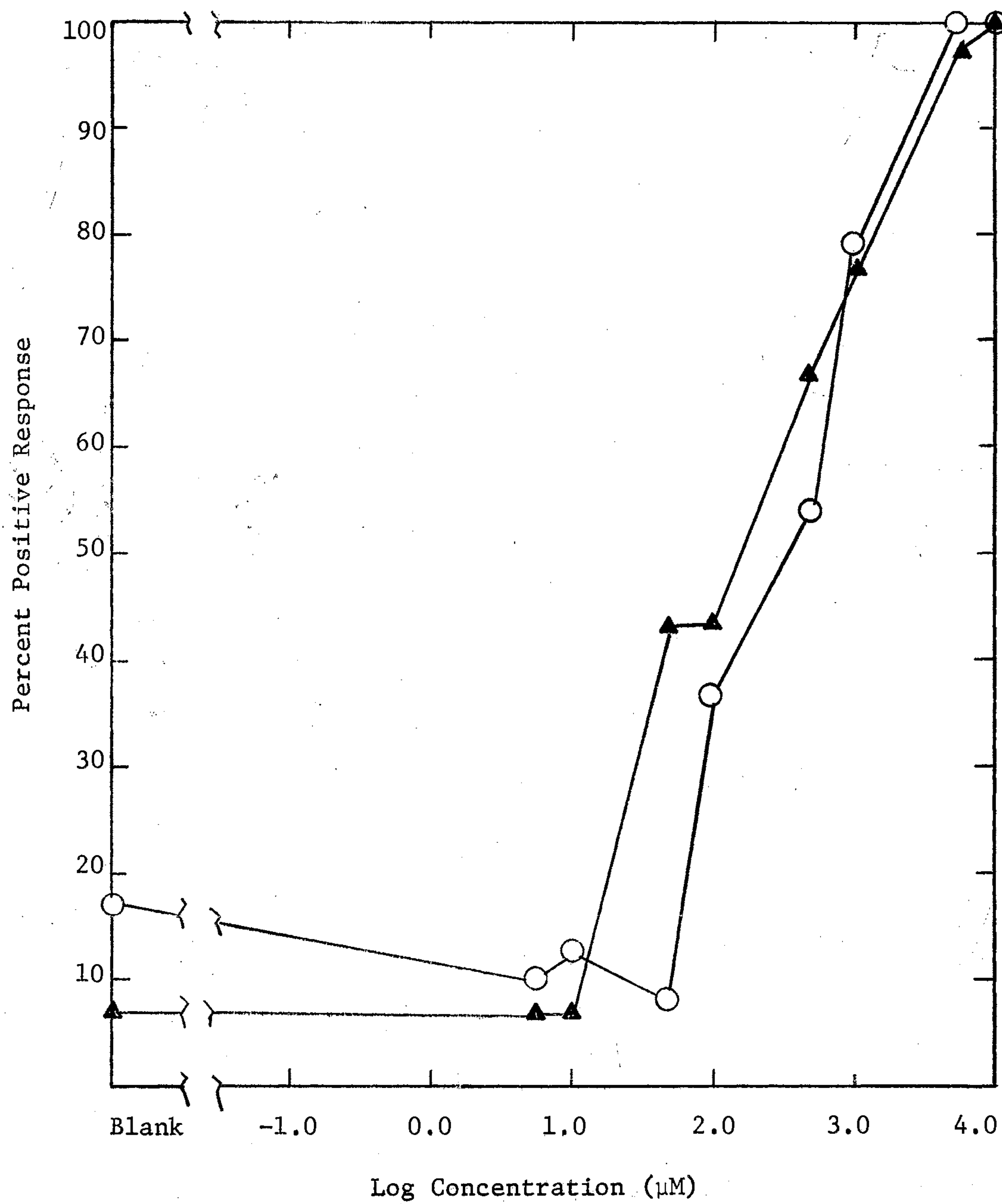

Figure 9. Odor Detection Threshold Profile for 2,5-Dimethylpyrazine in Water 0 and Oil ▲.

TABLE I

ODOR DETECTION THRESHOLD LEVELS IN WATER AND MINERAL OIL

Compound	Concentration in Water (Micromolar)	Concentration in Oil (Micromolar)
2-Methylpyrazine	1122	282
2,5-Dimethylpyrazine	320	159
2,6-Dimethylpyrazine	501	71
2-Ethylpyrazine	200	159
2,3,5-Trimethylpyrazine	71	224
Monomethylmonoethylpyrazine	4	7
2,3,5,6-Tetramethylpyrazine	71	282
2,5-Dimethyl-3-ethylpyrazine	316	180
2,6-Dimethyl-3-ethylpyrazine	112	178
2-n-pentylpyrazine	7	57
N-methylpyrrole	16	127
Phenylacetaldehyde	2	2
Benzaldehyde	6	355
Glyoxal	569	---

profiles of all of the compounds tested are given in Appendix A.

As would be expected, the subjective descriptions of the odor of the various alkylpyrazines encompassed a wide range of responses from the various panel members. The responses for a given compound were often much different in water than in mineral oil. In some cases, the nature of the odor of a single compound also changed dramtically with the concentration.

A sample of roasted peanut oil, freshly squeezed, caused a 100 percent "good peanut" response from the panel. The volatiles removed from the oil of one pound of peanuts were used to prepare a 1 percent solution of the volatiles. When the volatiles were made up to a 1 percent solution in paraffin oil, the response was again 100 percent "peanut odor". However, a 1 percent solution of the volatiles in water elicited responses ranging from "disagreeable" to "burned or over-roasted peanut". This again points out the very critical nature of sensory evaluation work and the necessity of testing compounds in a media which is similar to the media in which they occur naturally.

The great diversity of responses prevented statistical or even tabular presentation of the subjective results. It is probably sufficient to note that none of the compounds evaluated gave a true peanut odor at any concentration. There was a trend toward a "roasted", "burned", or even "nutty" response for a few of the alkylpyrazines, notably 2-ethylpyrazine, 2-methylpyrazine, and methylethylpyrazine.

That synergistic effects between two or more alkylpyrazine compounds at some concentration might be responsible for roasted peanut aroma cannot be ruled out. However, it seems more likely that the alkylpyrazine compounds contribute the roasted or nutty character to

roasted peanut aroma. The odor threshold values for the various alkylpyrazines will be compared with the actual amount of pyrazines occuring in several roasted food products in Chapter IV.

CHAPTER IV

QUANTITATIVE SURVEY OF PYRAZINE COMPOUNDS IN FOOD PRODUCTS

Alkylpyrazine compounds have been identified in the volatile aroma fractions of peanuts, coffee, cocoa, and potato chips (5,6,7,8,9,10). However, no quantitative work has been done on the pyrazine content of any of these foods. Since the odor-detection threshold concentrations of the alkylpyrazine compounds are now known (Chapter III), a quantitative survey of the alkylpyrazine levels in these and other foods would make possible an assessment of the probable significance of pyrazine compounds in roasted food flavor. Also, since pyrazine compounds may be destined for use as food flavorants (72,73,74), quantitative data on their natural levels in food products assumes particular importance because little is known of their toxicological properties.

Procedures

Roasted ground coffee (350 gm of Cain's Hotel and Restaurant Coffee-fine grind) was placed in a large (90 mm x 200 mm) Soxhlet extraction thimble. The coffee was extracted for 12 hours with 1500 ml of redistilled dichloromethane. The coffee was then removed from the extraction thimble and ground twice in a Wiley mill using a fine (0.5 mm) screen. The coffee was then returned to the Soxhlet and extracted for 18 hours using the same dichloromethane.

The 1500 ml of dichloromethane extract were then reduced to 150 ml using a Buchi rotary evaporator with a 25° C. water bath. This reduced extract was then passed slowly 3 times down a falling film evaporator. The falling film evaporator used in these studies was similar to that described by Herz and Chang (75). The extract passed slowly down the walls of two 50 cm bubble condensers connected in series and surrounded by a steam jacket. The volatile materials were trapped on a single liquid nitrogen (-196° C.) cold-finger trap. A smaller cold-finger trap, placed between the larger trap and the vacuum pump, prevented volatile compounds from the vacuum or oil diffusion pumps from contaminating the isolated volatiles. The volatile material collected on the large cold-finger trap was transferred under vacuum to a detachable 100 ml round bottom flask by removing the liquid nitrogen from the large trap and heating the trap with a paper chromatogram drier. As the large cold-finger trap was warmed, the trapped volatile materials were transferred to the liquid nitrogen-cooled round bottom flask. When this transfer was complete, the small flask was removed from the cold-finger trap and stored at -20° C. prior to further steps.

The collected volatiles, about 50 ml, were thawed, placed in a small separatory funnel, and extracted 7 times with 7 ml portions of dichloromethane. The dichloromethane extract was reduced to a small volume by removing the dichloromethane on a rotary evaporator. The evaporated extract was transferred quantitatively to a 5 ml volumetric flask and the contents diluted to exactly 5 ml with redistilled dichloromethane. Aliquots of this material were stored at -20° C. prior to gas-liquid chromatographic analysis.

Quantitative gas-liquid chromatographic analyses were performed on

a Perkin-Elmer 801 equipped with a dual hydrogen flame ionization detector. A 20 foot x 1/4 inch o.d. glass column containing 15 percent (w/w) Carbowax 20M on Gas Chrom Q (100/120 mesh) was used with a nitrogen carrier gas flow rate of 60 ml per minute. The column temperature was varied between 120° C. and 150° C. to obtain the best separation. Isothermal chromatography at 150° C. gave the best resolution and most reproducable retention times. Gas chromatographic peaks were quantitated by comparison of peak areas of samples with those of known concentrations of standards chromatographed the same day under the same conditions. Three injections of each of the sample and standard were made and the results averaged to obtain the data presented in this chapter.

Extraction of the alkylpyrazines from 550 gm of roasted peanuts was conducted by a similar procedure. The peanuts were roasted whole and ground in a food grinder before being placed in the Soxhlet for extraction for 18 hours with dichloromethane. The extracted peanuts were reground twice in the Wiley mill as described previously and then extracted for 24 additional hours. The remainder of the extraction and analyses were effected as described for the coffee sample.

A 281 gm bag of potato chips (Kitty Clover) was crushed, placed in a large Soxhlet extraction thimble, and extracted 12 hours with redistilled dichloromethane. A second 281 gm batch was subjected to the same procedure. Both of the above batches were finely ground, and because of the smaller volume after grinding, both were returned to a single extraction thimble and extracted for 30 hours with the same dichloromethane used for the previous extraction. The remainder of the extraction procedure followed that used for the coffee sample.

Results and Discussion

Although it has been definitely established that pyrazine compounds occur in a number of roasted food products, no quantitative data on the amount of these compounds actually present have ever been published. The common technique of isolating aroma constituents of foods has been to pump off the volatile compounds under a high vacuum and isolate them by freezing on cold-finger traps (75,5,76). While this technique is satisfactory for isolation of compounds from the volatile aroma fraction, it by no means removes all of the aroma compounds from the food. Experience in this laboratory has proven that even prolonged vacuum degassing of homogenized roasted peanut slurries over a period of 4 or 5 days does not completely remove the pyrazine compounds and other flavor constituents from the system.

An exhaustive procedure of extraction and grinding was employed to obtain the quantitative data presented in this chapter. After the foods had been exhaustively extracted as described in the preceeding section, they were again subjected to the same extraction procedure. No pyrazines were detected in the second extracts, indicating that the extraction procedure was sufficient to remove the pyrazine compounds from the foods completely.

The level of some pyrazine compounds found in four common foods is presented in Table II. These amounts are certainly significant in view of the threshold of detection levels presented in Chapter III. At least in the case of coffee and roasted peanuts, the levels are such that the metabolic properties of these compounds assumes importance. The two pyrazines occurring in the largest amounts in roasted food pro-

TABLE II

PYRAZINE CONTENT OF SELECTED FOODS

	Pyrazine		2-Methylpyrazine		Dimethylpyrazine	
	μmoles/kg	mg/kg	μmoles/kg	mg/kg	μmoles/kg	mg/kg
Coffee	57	5	695	65	178	19
Coffee (2nd extraction)	ND		ND		ND	
Roasted Peanuts	ND		66	6	97	11
Potato Chips	ND		ND		1.6	0.2
Gain's Dog Meal	ND		ND		ND	

ND = Not Detected

ducts are 2-methylpyrazine and the dimethylpyrazines (Figure 10). Methylpyrazine was found in coffee at levels of 65 mg per kg. The positional isomers of dimethylpyrazine were not separated by the gas chromatographic procedures used in this study. Many higher molecular weight alkylpyrazines are known to be present in coffee and roasted peanut volatiles. However, quantitative work indicates that these other pyrazines are very minor components.

It is interesting to note that of the four foods surveyed, coffee is the most heavily roasted product. Coffee beans are roasted until extremely dark brown, nearly black, a condition that might actually be calssified as "burned" in other foods. Peanuts are roasted at temperatures of about 130° C. to 150° C. (39). While they are well browned, they are roasted to a considerably less extent than coffee beans. Potato chips obtain only a very slight surface browning. The extent of heating of the dog meal product was not known. The level of pyrazine compounds in these roasted food products is proportional to the extent of browning which the food undergoes. Coffee, clearly the most darkly roasted product, has the highest concentration of pyrazine compounds. Potato chips, which are only very lightly browned, contain only very small amounts of alkylpyrazines. The intermediate levels of pyrazines in roasted peanuts are consistent with this hypothesis.

Not only do the pyrazine levels as a whole vary from food to food, but also the ratio of the various pyrazines varies. In coffee, for instance, the ratio of dimethylpyrazine to 2-methylpyrazine is 0.3. However, in roasted peanuts the ratio is 1.7. Some possible explanations for these variations in the amounts of the various pyrazines produced will be discussed in the following chapters which deal with possible

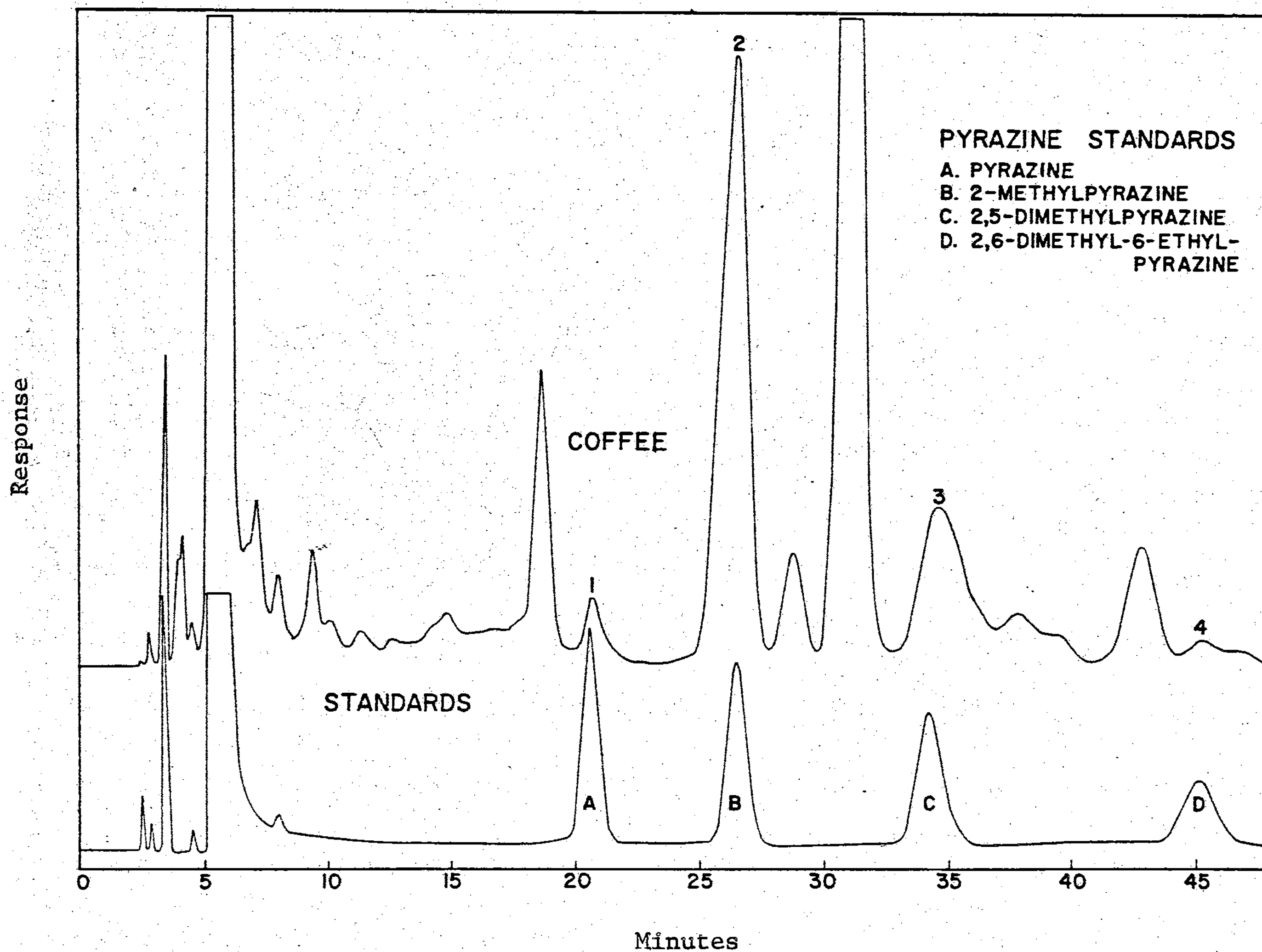

Figure 10. Gas Chromatograph Tracing of Coffee Extract

pathways for the formation of pyrazine compounds.

A comparison of the odor-detection threshold levels of 2-methylpyrazine and dimethylpyrazine with the concentration of these pyrazines in some food products is presented in Table III. Peanuts have a high lipid content, about 50 percent, and low water content, about 5 percent (39). The water content is further decreased to a very small value during the roasting process. Thus, for the roasted peanut, the odor-detection threshold values in a lipid medium should be more representative than the values for aqueous solutions.

The concentration of pyrazines in coffee is considerably above the odor threshold values in oil for both 2-methylpyrazine and dimethylpyrazine (Table III). The probability that pyrazine compounds play a role in the aroma of roasted coffee is thus rather high. The concentration of 2-methylpyrazine in the peanut is somewhat below the odor threshold level while the dimethylpyrazine concentration is about at the threshold level. The very low concentration of dimethylpyrazine in potato chips makes doubtful its contribution to the aroma of the product. It should be kept in mind, however, that synergistic effects between two or more compounds in solution can cause the threshold of detection of the mixture to be considerably lower than that of either component individually (59). Antagonistic effects can likewise cause the opposite effect. Also, the odor of a mixture of two non-reactive compounds can produce an odor which bears no similarity to either of the pure components (59). These effects make the evaluation of flavor compounds extremely difficult. However, in summary, pyrazine compounds very likely may be involved in coffee aroma. The levels of alkylpyrazines, especially the dimethylpyrazines, are such that these compounds should not

TABLE III

COMPARISON OF ODOR THRESHOLD LEVELS OF ALKYLPYRAZINE COMPOUNDS WITH THEIR CONCENTRATION IN FOODS

<table>
<tr><th></th><th>2-Methylpyrazine</th><th>2,5-Dimethylpyrazine</th><th>2,6-Dimethylpyrazine</th></tr>
<tr><td>Threshold in Water
(moles/liter)</td><td>1,122</td><td>320</td><td>501</td></tr>
<tr><td>Threshold in Oil
(moles/liter)</td><td>282</td><td>159</td><td>71</td></tr>
<tr><td>Coffee Concentration
(moles/liter)</td><td>695</td><td colspan="2">178*</td></tr>
<tr><td>Roasted Peanut Concentration
(moles/kg)</td><td>66</td><td colspan="2">97*</td></tr>
<tr><td>Potato Chip Concentration
(moles/kg)</td><td>-----</td><td colspan="2">1.6*</td></tr>
</table>

*Represents total dimethylpyrazines, isomers not separated

be neglected in considering the make-up of roasted peanut aroma. This is especially true in view of the characteristic "roasted" or "nutty" aroma of some of these compounds (Chapter III). Their significance in potato chip aroma is very doubtful.

CHAPTER V

CARBON-14 LABELING STUDIES ON PYRAZINE FORMATION PATHWAYS

Newell et al. (44) suggested that amino acids and carbohydrates are among the precursors of typical peanut flavor. A number of simple alkylated pyrazine compounds have been shown to occur among the volatile compounds isolated from roasted peanuts (5). It has been suggested that these heterocyclic compounds are responsible for the "roasted-nutty" character of roasted peanuts. Alkylated pyrazines have also been found among the volatile aroma compounds from potato chips, coffee, and cocoa (6,7,8,9,10). All of these foods are characterized by treatment at high temperatures during their processing. Thus it becomes important to determine if amino acid and carbohydrate precursors give rise to alkylated pyrazines on heating and to investigate the pathway by which this occurs, a pathway which is at present unknown. The purpose of this study was to obtain data from a model system using carbon-14 amino acids and sugars which would demonstrate the sources of the carbon and nitrogen atoms of the pyrazine molecule and suggest the probable pathway for their incorporation.

Materials

Diethylene glycol (reagent grade) and anhydrous glucose were purchased from Fisher Scientific Co., Fair Lawn, New Jersey. L-Asparagine

(anhydrous) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. A variety of unlabeled amino acids and sugars were purchased from Calbiochem, Los Angeles, California. D-glucose-1-^{14}C, D-glucose-2-^{14}C, D-glucose-3,4-^{14}C, and D-glucose-6-^{14}C were obtained from New England Nuclear Corp., Boston, Massachusetts. L-Asparagine-UL-^{14}C monohydrate was obtained from International Chemical and Nuclear Corp., City of Industry, California. Alanine-2-^{14}C was purchased from Calbiochem, Los Angeles, California.

Procedures

Amino acid-carbohydrate model systems were routinely prepared by adding 10 mmoles each of an amino acid and a sugar to 20 ml of deionized water and 200 ml of diethylene glycol. For radioactive studies, 2 microcuries of either a radioactive amino acid or glucose labeled in a known position was added to the reaction mixture. The model system reaction mixture was refluxed for 24 hours at $120^{o} \pm 1^{o}$ C. in an oil bath. The temperature of the paraffin oil bath was maintained with a Thermocap relay connected to a 1000 watt immersion heater placed in the oil. Both the oil bath and the reaction mixture were stirred by a magnetic stirrer.

The reacted mixture was passed twice slowly down the falling film evaporator described in Chapter III. The material collected on the liquid nitrogen-filled cold-finger trap was extracted with dichloromethane. This extract was subjected to preparative gas-liquid chromatography using a 20 foot x 1/4 inch aluminum column packed with 15 percent (w/w) Carbowax 20M on Gas Chrom Q in an F & M Model 500 gas chromatograph equipped with a four filament thermal detector. The column was main-

tained at 120°C. The helium flow rate was 60 ml per minute. The 2-methylpyrazine and dimethylpyrazine peaks were collected in traps filled with dichloromethane (Figure 11). A large (number 18) needle attached to the exit port of the gas chromatograph was used to puncture the rubber septum and collect the desired fraction in the trap. The helium carrier gas containing the compound bubbled up the first, dichloromethane-filled, column of the trap displacing the liquid into the second column. As soon as the collection of a single peak was complete, both valves on the trap were closed and trap shaken to dissolve any of the compound which might not have dissolved on the first pass through the liquid. The trap was placed in an ice bath to reduce evaporation of dichloromethane.

Each of the collected compounds was then quantitatively reassayed by gas-liquid chromatography. The peak areas of the samples were compared with the areas of known weights of standards chromatographed the same day under the same conditions. The radioactivity of each compound was determined by counting duplicate aliquots of each with a Packard Tricarb Model 3003 liquid scintillation spectrometer. Quenching corrections were made by adding a standard of known disintegrations per minute to each sample and recounting. Corrections were also made for any gradual bleed of radioactivity from the column by trapping a 5 minute sample of column bleed background. From this information, the specific activity of each alkylpyrazine was determined.

The side chains were removed by oxidizing the alkylpyrazine to the corresponding pyrazinoic acid and then decarboxylating the acid to yield the intact pyrazine ring. Dimethylpyrazine was oxidized by dissolving 2.7 gm of dimethylpyrazine and 0.5 gm of potassium hydroxide in 15 ml

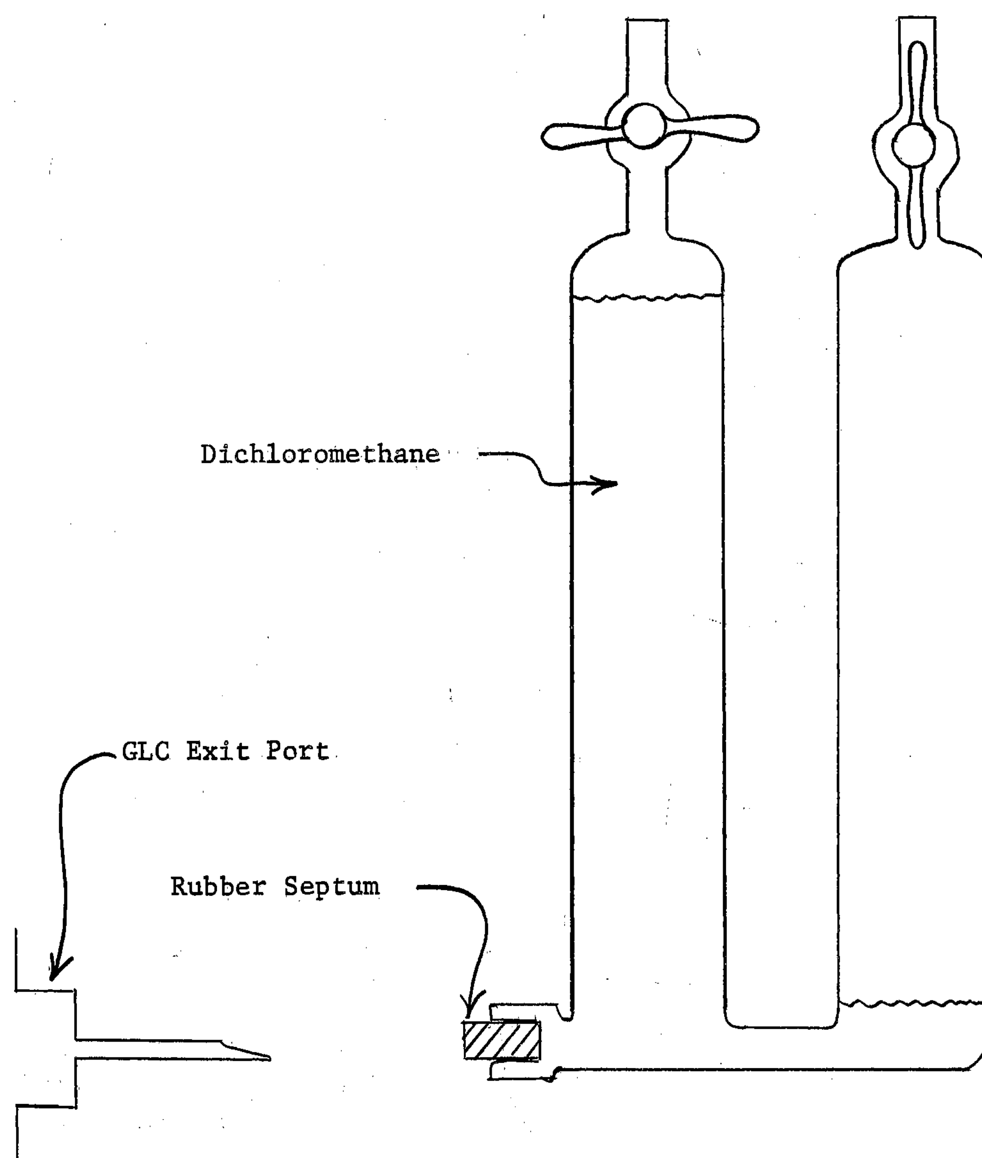

Figure 11. Trap for Collecting ^{14}C-Labeled Alkylpyrazines Separated by Gas-Liquid Chromatography (Scale = 3/4:1).

of deionized water (77). This mixture was then placed in a 250 ml, 2-neck round bottom flask held at 75° C. in a thermostatically-controlled oil bath. Both the oil bath and the contents of the flask were stirred by a magnetic stirrer. One neck of the round bottom flask contained a water-cooled reflux condenser. Through the other was slowly added 76 ml of a hot 0.1 M aqueous solution of potassium permanganate. Stirring and heating at 75° C. were continued for 30 minutes after all of the potassium permanganate solution had been added. The mixture was allowed to cool to room temperature and stand overnight.

The precipitate was then filtered and washed several times with boiling water. The filtrate was reduced to a small volume on a Buchi rotary evaporator. The filtrate was made acidic with dilute nitric acid (2N) and allowed to stand for one hour. The dipyrazinoic acid precipitated and was filtered and then washed several times with hot water. The precipitated diacid was then dissolved in dilute ammonium hydroxide (2N) and reprecipitated with dilute nitric acid. The reprecipitated pyrazine-2,5-dicarboxylic acid was filtered and dried in a dessicator over anhydrous calcium chloride.

Methylpyrazine was oxidized (78) by refluxing 0.88 gm of 2-methylpyrazine, 10 ml pyridine, and 2.2 gm of selenium dioxide dissolved in 1 ml of water at 105° C. for 16 hours. The mixture was filtered and the precipitate washed with pyridine. The filtrate was then reduced to dryness on a rotary evaporator. The residue was dissolved in 2N sodium hydroxide and the pyrazinoic acid reprecipitated with 7N hydrochloric acid.

The pyrazinoic acids were decarboxylated by placing them in sealed, evacuated glass tubes and heating for 12 hours at 200° C. in a Perkin-

Elmer 800 gas chromatograph oven. A study of decarboxylation yields at various temperatures between 100° C. and 250° C. and heating times between 2 and 48 hours was conducted, but the highest yields were obtained using the conditions described. After cooling, the tubes were opened and the pyrazine formed was removed by washing the tubes with dichloromethane. This solution was reduced to a small volume by evaporating the dichloromethane on a rotary evaporator. From this, pure pyrazine was obtained by preparative gas-liquid chromatography using the same column conditions and liquid traps described previously for obtaining the pure alkylpyrazines. The dichloromethane solution from the trap containing pure pyrazine was reduced to a small volume and made up exactly 1 ml prior to quantitative gas-liquid chromatography and radioactivity determination by liquid scintillation spectrometry. From these data, obtained as described previously, the specific activity of the pyrazine nucleus was calculated and compared with the specific activity of the starting alkylpyrazine from which it was derived.

Results and Discussion

Diethylene glycol containing 9 percent water was chosen as the solvent for the study of pyrazine formation pathways in an effort to provide a reaction medium somewhat more representative of the roasted peanut than the strictly aqueous systems commonly used in studies of the browning reaction. Earlier work in this laboratory established that heating of sugar-amino acid mixtures in this media would give rise to a wide distribution of alkylpyrazine compounds (79). Figure 17 clearly demonstrates that the water content of the system has dramatic effects on both the yield and the distribution of the various alkylpyrazines

formed.

To determine the origin of the carbon atoms of alkylpyrazine molecules, companion experiments were conducted, first utilizing ^{14}C-labeling in the glucose molecule with an unlabeled amino acid, and then repeating the experiment using a ^{14}C-amino acid with unlabeled glucose. The ratio of the specific activities of the product alkylpyrazine (P) to the labeled reactant (R) are compared in Table IV for two different amino acid-glucose mixtures. In this table, the values in Experiment 1 represent the average of six independent experiments. Those in Experiment 2 are the average of two experiments. The average deviation from the mean is also given in Table IV. Regardless of the position at which the glucose molecule was specifically labeled, the radioactivity incorporated into the product alkylpyrazines was much greater when the ^{14}C-label was in the sugar molecule. In either mixture, the labeling of the product alkylpyrazines was dramatically lower when the ^{14}C-label was in the amino acid. Thus, the major source of the carbon atoms of the pyrazine molecule was the sugar. The amino acid served primarily as the nitrogen source.

Probable pathways for the incorporation of carbon atoms into pyrazine molecules were also investigated. The formation of all of the alkylpyrazines can be thought of as involving condensation of two and three carbon fragments from sugars with nitrogen from amino acids (79). Pyrazine itself could arise from condensation of two 2-carbon fragments with nitrogen from amino acids, methylpyrazine from condensation of one 2-carbon and one 3-carbon units, and dimethylpyrazine from condensation of two 3-carbon fragments. Previous results from this laboratory (79) have established that 2-carbon fragments do indeed take part in the pyr-

TABLE IV

ORIGIN OF PYRAZINE CARBON ATOMS IN SUGAR-AMINO ACID MODEL SYSTEMS

Labeled Reactant	Unlabeled Reactant	Ratio of Specific Activities (P/R) Methylpyrazine	Dimethylpyrazine
Experiment 1:			
Glucose-1-^{14}C	Asparagine	1.290 ± 0.010	0.970 ± 0.010
Glucose-6-^{14}C	Asparagine	0.630 ± 0.002	1.140 ± 0.020
Glucose-3,4-^{14}C	Asparagine	0.630 ± 0.005	1.100 ± 0.001
Asparagine-UL-^{14}C	Glucose	0.002 ± 0.001	0.007 ± 0.003
Experiment 2:			
Glucose-1-^{14}C	Alanine	1.200 ± 0.012	0.950 ± 0.010
Alanine-2-^{14}C	Glucose	0.026 ± 0.006	0.083 ± 0.020

azine formation reactions.

Two possible pathways by which a hexose or hexose intermediate could fragment into 2 and 3-carbon units, which would then combine with nitrogen to form pyrazines, were considered. In either pathway, the hexose can break into two equivalent 3-carbon fragments which on recombination and incorporation of nitrogen yield dimethylpyrazine. The two pathways differ as follows: Pathway I, shown in Figure 12, assumes the breakdown into only one 2-carbon fragment per hexose, with this fragment being formed from carbon atoms 1 and 2 of the hexose. The possible recombinations of Pathway I hexose fragments with nitrogen are shown by lines which indicate the alkylpyrazine that would be produced. The predicted specific activity of the resultant alkylpyrazine molecule is shown in the figure for the case of specifically labeled glucose-1-^{14}C. In the alternate pathway (II), Figure 13, three equivalent 2-carbon units would be produced per hexose molecule. The predicted specific activity for the alkylpyrazines produced by this fragmentation pathway are indicated in this figure, again for the case of glucose-1-^{14}C which is illustrated. The specific activity of the resulting alkylpyrazine molecules was also predicted for glucose labeled specifically in the 6 or 3,4 positions and is shown in Table V.

Radioactive pyrazine compounds were synthesized by heating glucose labeled specifically in either the 1,6, or 3-4 positions with unlabeled asparagine in the model system. The alkylpyrazines formed were separated by preparative gas-liquid chromatography and the specific activity of each was determined. The predicted and the experimentally-determined values for the ratio of specific activity of product (alkylpyrazine) to reactant (glucose labeled specifically in the 1, 3-4, or

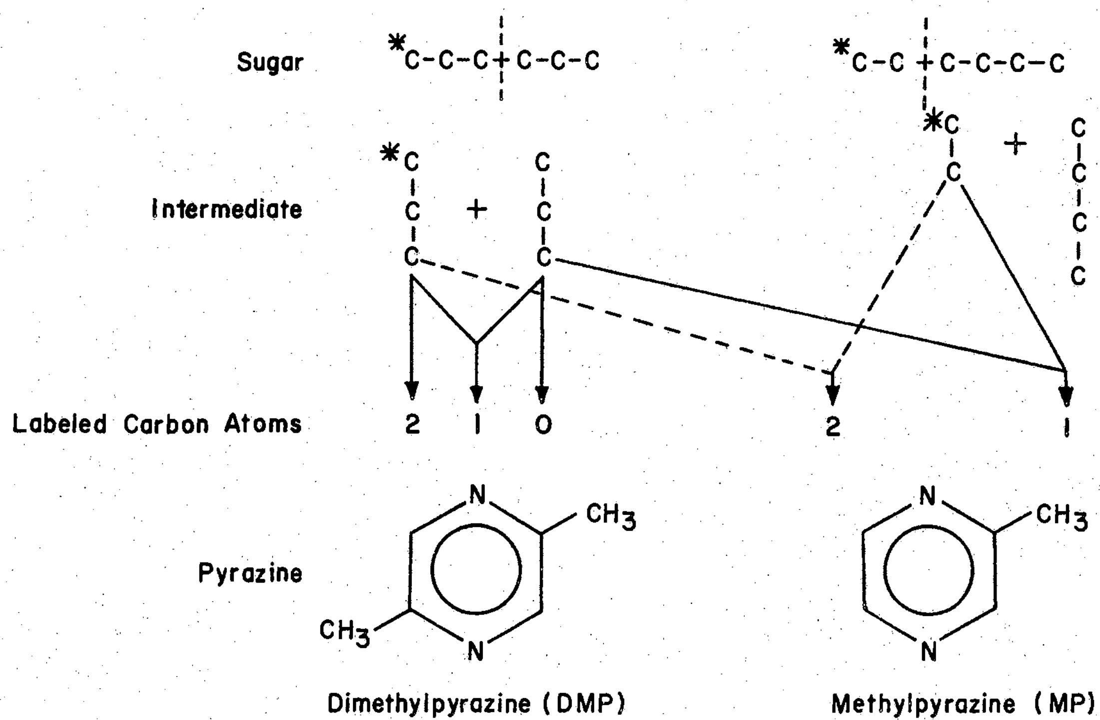

Figure 12. Hexose Fragmentation Pathway I

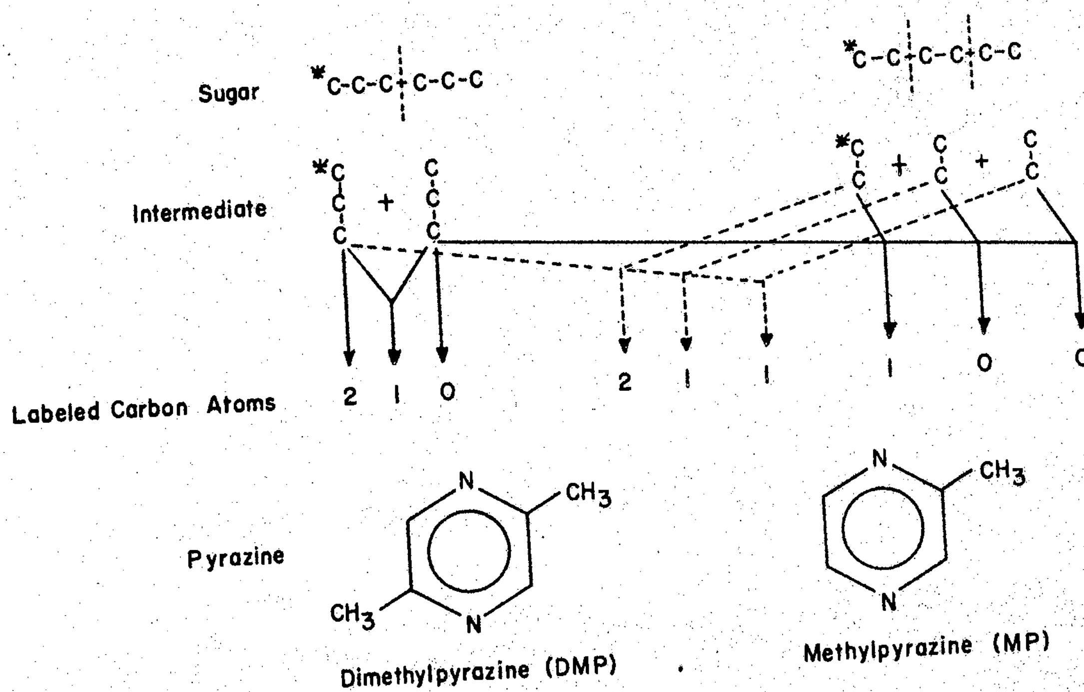

Figure 13. Hexose Fragmentation Pathway II

TABLE V

RATIO OF SPECIFIC ACTIVITY OF PRODUCT (PYRAZINE) TO REACTANT (GLUCOSE) FOR HEXOSE FRAGMENTATION PATHWAYS I AND II

Labeled Reactant:	Glucose-1-^{14}C		Glucose-6-^{14}C		Glucose-3,4-^{14}C	
Product:	DMP	MP	DMP	MP	DMP	MP
Pathway I (Theoretical)	1.00	1.50	1.00	0.50	1.00	0.50
Experimental Results	0.97±.01	1.29±.01	1.14±.02	0.63±.002	1.10	0.63±.005
Pathway II (Theoretical)	1.00	0.83	1.00	0.83	1.00	0.83

6 position) are compared in Table V for each of the two hexose fragmentation pathways considered. Each of the experimental values in Table V represents an average of either 2 or 3 experiments. The average deviation from the mean value is shown in the table.

It is evident from the data in Table V that the breakdown into a single 2-carbon unit per hexose (pathway I) gives a better correlation of predicted and experimental labeling values than pathway II. The slightly lower than predicted ratio for methylpyrazine from glucose-1-^{14}C in the preferred pathway (I) could be explained by assuming a small amount of cleavage of the remaining 4-carbon unit (Figure 12) into two 2-carbon units, thus diluting the activity of the 2-carbon unit pool. The slightly higher than predicted Pathway I values for methylpyrazine from glucose-6-^{14}C and glucose-3,4-^{14}C tend to confirm this explanation, since further breakdown of the remaining 4-carbon unit in these situations would add ^{14}C-labeled 2-carbon units to the otherwise unlabeled 2-carbon unit pool. Slight fragmentation of this 4-carbon unit into a 3-carbon and a 1-carbon unit could explain the slightly higher than predicted specific activity ratios for dimethylpyrazine from glucose labeled in the 3-4 and 6 positions.

The data presented thus far in this chapter provide experimental support for the hypothesis of Dawes and Edwards (80) that the carbon atoms of pyrazines arise from sugar degradation products. The results obtained from the ^{14}C-labeling studies are difficult to resolve in terms of compounds known or proposed in the pathway for amine catalyzed breakdown of sugars in non-enzymatic browning reactions (55,58). The fact that both 1- and 6-^{14}C-glucose labeled dimethylpyrazine to the same extent (Table V) seemed to indicate that fragmentation into two

3-carbon fragments occurs through a symmetrical intermediate such as diacetylformoin (I) shown in Figure 14 (55). However, the dissimilar labeling of methylpyrazine from glucose-1-^{14}C from that of methylpyrazine from glucose-6-^{14}C and glucose-3,4-^{14}C would rule against this intermediate, since splitting into a 2 and 4-carbon fragment could occur with equal facility from either end of the tautomeric forms of diacetylformoin. The results of these experiments indicate that the split into 2 and 4-carbon fragments takes place almost exclusively between carbons 2 and 3 of the hexose or hexose intermediate suggesting a dissymetrical intermediate.

Knowledge of the position of the labeling in the alkylpyrazine molecule when glucoses labeled specifically in a single position are used as precursors could provide an essential step in formulating a logical mechanism for pyrazine production. The pyrazine nucleus is an aromatic structure and therefore is extremely difficult to degrade in any orderly fashion. It was possible, however, to determine whether the label was in the alkyl side chain or in the ring (exocyclic or endocyclic). Oxidation of the side chain with either potassium permanganate or selenium dioxide converts the alkylpyrazine to the corresponding pyrazinoic acid. The potassium permanganate oxidation procedure gave better yields in the oxidation of dimethylpyrazines (77). Yields of pyrazinedicarboxylic acid from 2,5-dimethylpyrazine by this procedure were about 6 percent. When this procedure was applied to the oxidation of 2-methylpyrazine, only very low yields of about 1 percent could be obtained, even though a number of temperatures and reagent concentrations were tried. Oxidation of 2-methylpyrazine by the selenium dioxide procedure (78) produced pyrazinoic acid in yields of 25 to

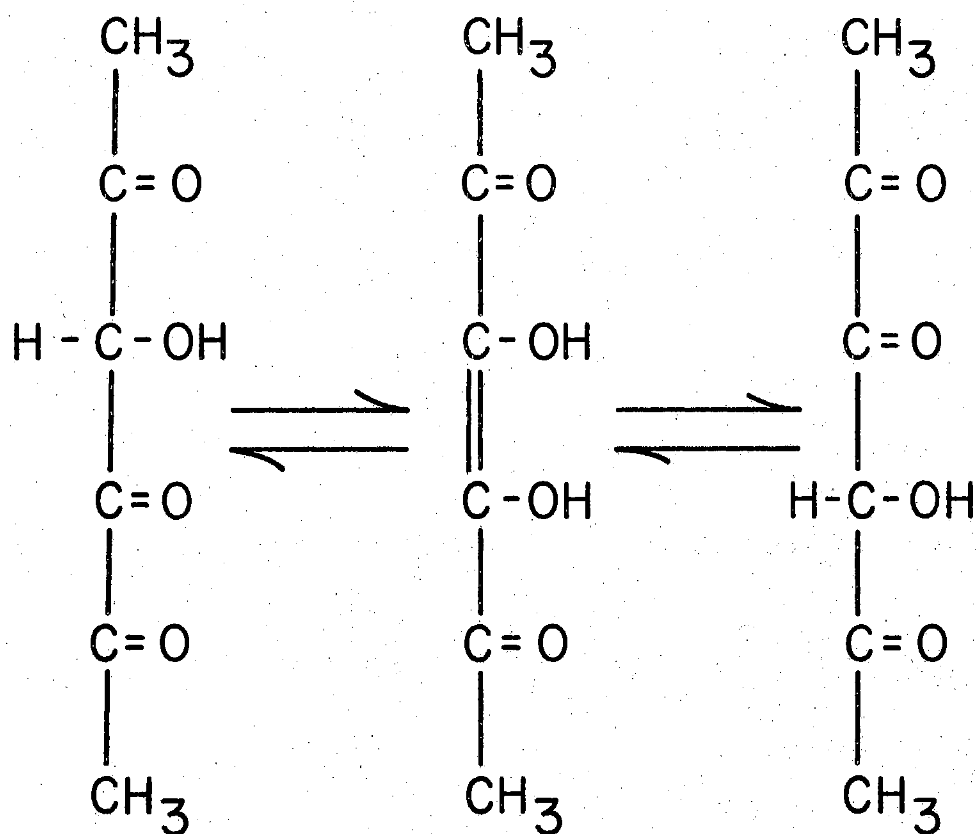

Figure 14. Diacetylformoin: A Possible Symmetrical Intermediate in the Pyrazine Formation Pathway (55).

35 percent. However, this procedure would not work for the oxidation of 2,5-dimethylpyrazine.

The purity of the synthesized pyrazinoic acids was determined by titration of known weights of the isolated acid with 0.5 N sodium hydroxide. Assuming the equivalent weight of pyrazine-2,5-dicarboxylic acid to be 84 (MW = 168) and that of pyrazine-2-carboxylic acid to be 124 (MW = 124), the purity of the recovered pyrazine-2,5-dicarboxylic acid was 84 percent and the purity of the pyrazine-2-carboxylic acid was 100 percent. The reason for the lower purity of the dicarboxylic acid was that partial oxidation of 2,5-dimethylpyrazine could cause some 2-methyl-5-pyrazinoic acid to be formed which would precipitate along with the pyrazine-2,5-dicarboxylic acid. Any impurity of this type would be removed during the later preparative gas-liquid chromatography purification step.

Decarboxylation of pyrazine-2-carboxylic acid was accomplished with 20 to 28 percent yields. Yields of pyrazine of up to 59 percent were obtained from the decarboxylation of pyrazine-2,5-dicarboxylic acid. Thus, a large number of steps, some of rather low yields, were involved in the overall procedure necessary to determine the position of labeling in the alkylpyrazine molecule. In order to have sufficient radioactivity remain in the pyrazine nucleus, the amount of radioactivity in the starting, specifically-labeled glucose was increased from 2 microcuries, used in the previous experiments, to 50 microcuries.

If the labeled atoms of the alkylpyrazine molecule were exclusively in the pyrazine ring (endocyclic), removal of the alkyl groups would leave the specific activity of the resulting pyrazine molecule unchanged. Thus, the ratio of the specific activity of the pyrazine molecule to the

specific activity of the alkylpyrazine from which it was derived would be 1.0 for endocyclicly-labeled molecules. If the labeled atoms of the alkylpyrazine were all in the alkyl side chains (exocyclic), the removal of the alkyl groups would produce a pyrazine molecule with no radioactive atoms. In this case, the ratio of specific activities of the pyrazine produced to the alkylpyrazine from which it was derived would be zero.

Methylpyrazine synthesized from glucose labeled specifically in the 6 position was oxidized and then decarboxylated to pyrazine. The ratio of the specific activity of pyrazine to that of 2-methylpyrazine from which it was derived was found to be 0.54 ± 0.04 (Table VI). These results indicate that about 50 percent of the carbon atoms in the 6 position of glucose go into the ring and about 50 percent into the methyl side chain of 2-methylpyrazine. Evidently, either end of the 3-carbon fragment formed from the last 3 carbon atoms of glucose or hexose intermediate can react with equal facility with the amino acid nitrogen. The end reacting with the nitrogen atom will be incorporated into the ring with the other end automatically becoming a part of the alkyl side chain. The same ratio might be expected for dimethylpyrazine synthesized from glucose-6-^{14}C. However, the yields at the end of the long procedure were so low that a consistant specific activity ratio could not be obtained even after seven attempts.

The ratios of the specific activities of pyrazine to 2-methylpyrazine synthesized from glucose-1-^{14}C (Table VI) were not clearly as precise as in the last case, but the average value of 0.68 ± 0.07 is significantly higher than the corresponding value for glucose-6-^{14}C. The two ends of the 3-carbon fragment formed from the first 3 carbon

TABLE VI

LOCATION OF RADIOACTIVE LABEL IN METHYLPYRAZINE

	Specific Activity (μCurie/μmole)		Ratio of Specific Activities of Pyrazine to 2-Methylpyrazine
	Methyl Pyrazine	Pyrazine	
Glucose-6-^{14}C:			
1.	3.29×10^{-6}	1.66×10^{-6}	0.51
2.	2.81×10^{-5}	1.37×10^{-5}	0.49
3.	1.32×10^{-5}	1.43×10^{-5}	0.59
4.	6.72×10^{-6}	3.99×10^{-6}	0.59
5.	1.01×10^{-5}	5.26×10^{-6}	0.52
			Avg. 0.54 ± 0.04
Glucose-1-^{14}C:			
1.	2.29×10^{-5}	1.55×10^{-5}	0.68
2.	1.51×10^{-5}	1.07×10^{-5}	0.71
3.	9.97×10^{-6}	6.46×10^{-6}	0.65
4.	3.43×10^{-4}	2.64×10^{-4}	0.77
5.	7.44×10^{-6}	5.79×10^{-6}	0.78
6.	2.83×10^{-5}	1.40×10^{-5}	0.50
			Avg. 0.68 ± 0.07

fragment formed from the first 3 carbon atoms of glucose or its hexose intermediate evidently do not react with equal facility with amino acid nitrogen. The specific activity ratio of 0.68 indicates that there is a significant tendency of the amino acid nitrogen to react with the carbon atom from the number 1 position of glucose. This means that the carbonyl carbon of glucose is most apt to be incorporated into the ring of the alkylpyrazine molecule while the number 3 carbon atom goes into the alkyl side chain. This seems reasonable, since the carbonyl group at the 1 position of glucose is immediately available for reaction with an amine group while some type of rearrangement or cleavage of the hexose molecule must occur before reaction could occur at the other positions. However, since the value is still considerably less than 1.0, there must be considerable equivalency between the 1 and 3 positions of glucose of the 3-carbon fragment derived from it. This equivalency could be brought about by rearrangement of glucose producing a hexose intermediate with the carbonyl either shifted to the 3 position, or else by cleavage of glucose forming a 3-carbon intermediate with equivalency of the 1 and 3 positions.

CHAPTER VI

MODEL SYSTEM REACTION STUDIES

Previous studies on the reactions between amino acids and reducing sugars focused their attention on the browning that occurs when these compounds react. The increased interest in the development of dehydrated food products during and immediately after World War II stirred investigation of the non-enzymatic browning reaction. In all of these studies, interest was in the browning pigments formed and emphasis was usually directed toward preventing the reactions that form these pigments from occurring during the processing and storage of food products. The progress of the reaction was followed in these studies by observing the increase in the brown coloration as the reaction progressed. The studies were usually carried out in aqueous solutions.

The identification of pyrazine compounds in the aroma fraction of coffee, cocoa, and peanuts (5,6,7,8,9), along with the proof that these compounds are formed by the condensation of carbon atoms from sugars with nitrogen from amino acids, make it necessary to investigate the reactions between sugars and amino acids from a new direction. Since the formation of alkylpyrazine compounds may be viewed as a favorable reaction than one to be eliminated, the sugar-amino acid reaction should now be studies with pyrazine compounds as the subject of interest.

Materials

Acetaldehyde, propionaldehyde, 2,3-butanedione, and glucosamine hydrochloride were purchased from Eastman Organic Chemicals, Rochester, New York. Glyoxal (30 percent aqueous solution) and reagent grade diethylene glycol were obtained from Fisher Scientific Company, Fair Lawn, New Jersey. Acetol (hydroxyacetone) was purchased from Aldrich Chemicals Company, Milwaukee, Wisconsin. A variety of amino acids and sugars were obtained from Calbiochem, Los Angeles, California.

Methods

The model system used in the previous studies in this work utilized 0.01 mole each of glucose and asparagine heated 24 hours at $120^{o} \pm 1^{o}$ C. in 200 ml of diethylene glycol solvent containing 9 percent deionized water. The studies in this chapter are based around the same system except that the amount of the reactants was increased to 0.1 mole each. This basic system was then altered by varying one of the "normal" reaction parameters while holding the others constant.

The pyrazine compounds produced were isolated from the reaction mixtures by passing them very slowly twice down the falling-film evaporator described previously. The volatiles which were collected by freezing on a liquid nitrogen-filled cold-finger trap were extracted 5 times with 5 ml of dichloromethane. The extract volume was reduced by removing some of the dichloromethane on a rotary evaporator. After dilution to exactly 5 ml with dichloromethane, an aliquot of this extract was chromatographed on a 21 foot x 1/4 inch O.D. glass column packed with 15 percent (w/w) Carbowax 20M on Gas Chrom Q in a Perkin Elmer 801 gas chromatograph. Each pyrazine was determined quantitatively by comparing the peak areas of the sample with the peak areas of

standards chromatographed the same day under the same conditions.

Results and Discussion

The "normal" model system used and described thus far in this research utilized equilimolar ratios of glucose and asparagine reactants heated 24 hours at 120° C. in diethylene glycol solvent containing 9 percent water. In this study, the effects of changing the various reaction parameters on the yields and the distribution of the pyrazine compounds were investigated and are reported.

Before initiating this quantitative work, it was necessary to insure that the various pyrazine compounds could be recovered quantitatively from the complex reaction mixture. Table VII shows the amount of each pyrazine that could be recovered from the reaction mixture solvent (200 ml diethylene glycol plus 20 ml water) by a single pass down the falling film evaporator. In the actual experiment, two passes were made to insure complete removal of all of the pyrazines. The next step in the recovery procedure involved extraction of the pyrazines from the mixture of volatiles (mostly water) collected on the cold-finger trap of the falling-film evaporator. Figure 15 shows the relative efficiencies of three solvents in extracting a mixture of pyrazine compounds from aqueous solutions. Dichloromethane was found to be considerably better than the other solvents. The efficiency of dichloromethane in extracting pyrazine, 2-methylpyrazine, and 2,5-dimethylpyrazine from aqueous solutions is shown in Figure 16.

The effect of increasing the water content of the model system solvent was quite apparent (Figure 17). The yield of both alkylpyrazines dropped quite rapidly. Since the increased amounts of water

TABLE VII

EFFICIENCY OF FALLING FILM EVAPORATOR

Compound	Initial Amount (μmoles)	Amount Recovered (μmoles)	Recovery (percent)
Pyrazine	2500	2395	96
2-Methylpyrazine	2120	2150	101
2,5-Dimethylpyrazine	1850	1750	95

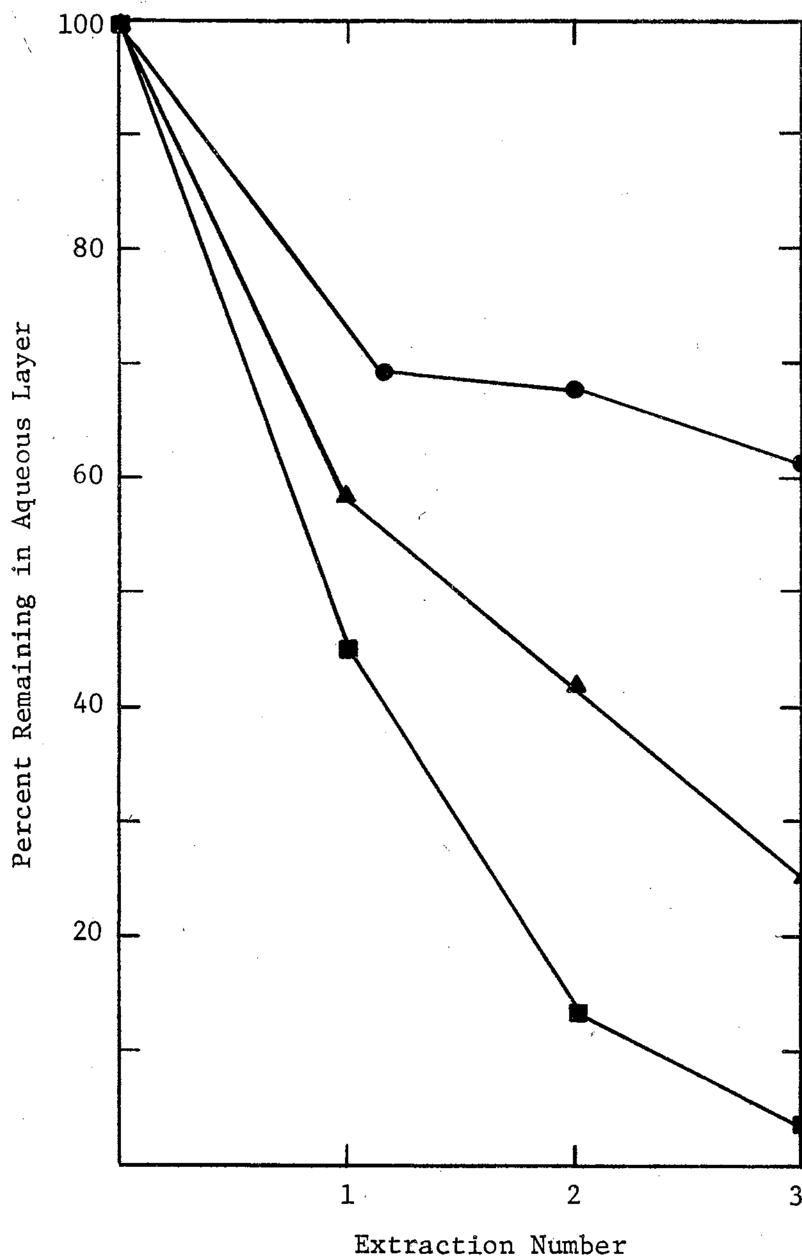

Figure 15. Efficiency of Solvents in Extracting a Mixture of Pyrazine Compounds From Aqueous Solutions. (● = pentane, ▲ = ether, ■ = dichloromethane)

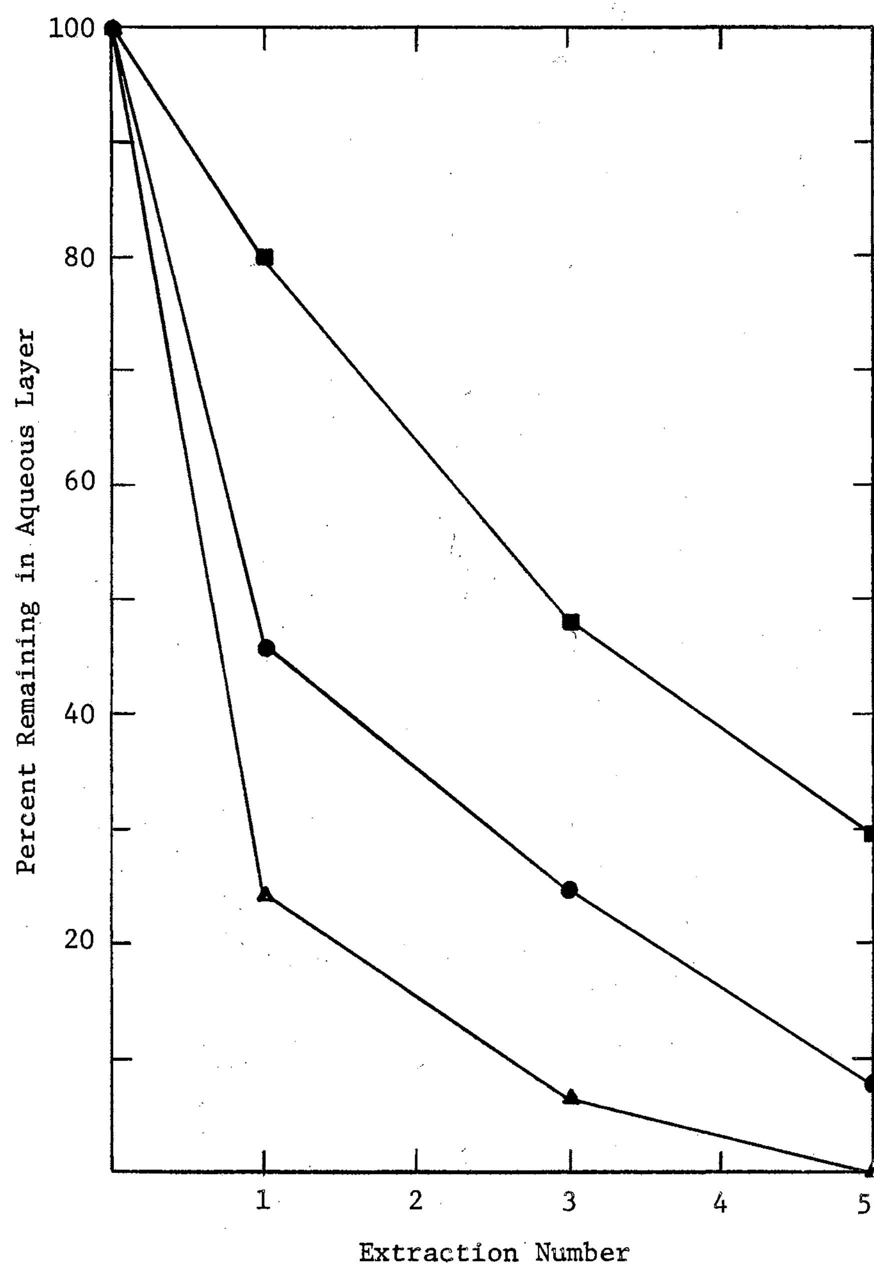

Figure 16. Efficiency of Dichloromethane in Extraction of Pyrazine Compounds From Aqueous Solutions. (■ = pyrazine, ● = 2-methylpyrazine, ▲ = 2,5-dimethylpyrazine)

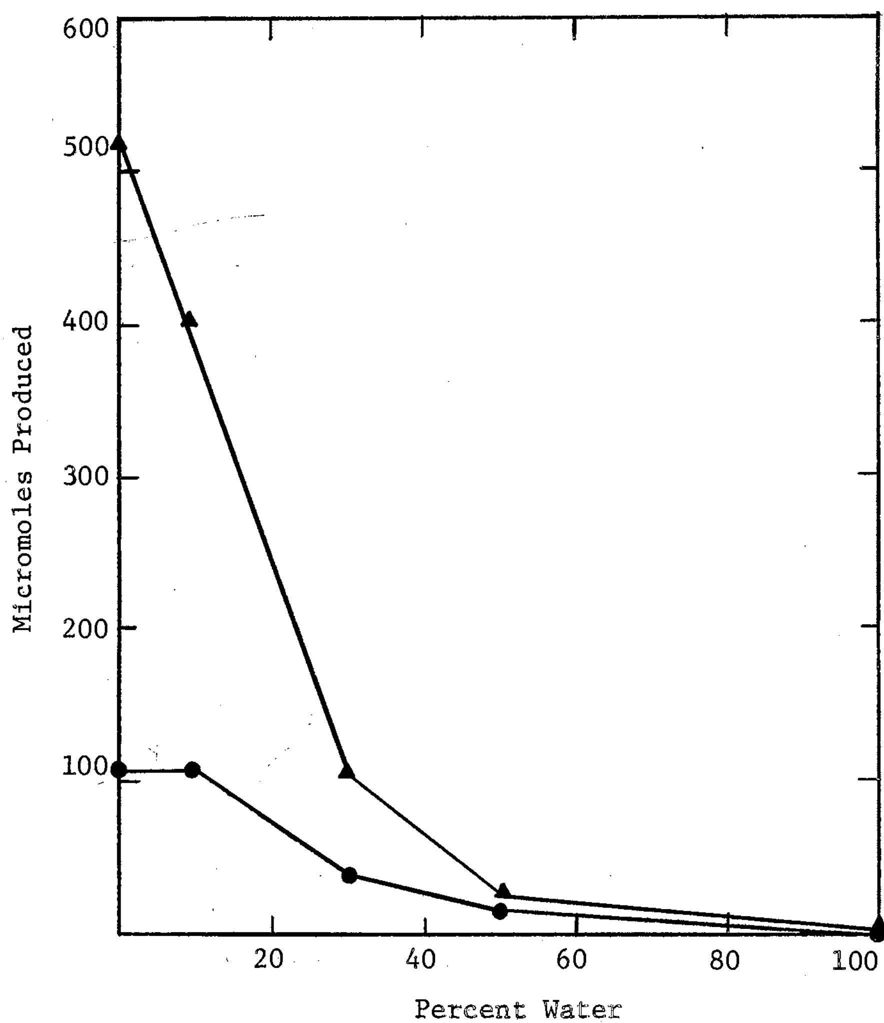

Figure 17. Effect of the Addition of Water on the Production of Alkylpyrazine Compounds in the Model System. (▲ = Dimethylpyrazines, ● = 2-Methylpyrazine)

lower the reflux temperature somewhat, these effects reflect both solvent and temperature effects. The ratio of dimethylpyrazine to methylpyrazine decreased as the water content increased. This might indicate that the more aqueous solvent favors cleavage of the sugar or hexose intermediate into 2-carbon units while the less polar diethylene glycol solvent favors cleavage into 3-carbon units.

The production of pyrazine compounds at 120°C. (Figure 18) increased rapidly as the length of the heating period was increased until about 24 hours where pyrazine formation quickly begins to level off with only a minor increase in yield for periods of up to 72 hours. While methylpyrazine is the major product in the early stages of the reaction, less than 3 hours, the ratio of dimethylpyrazine produced to methylpyrazine produced continues to increase until about 9 hours (Figure 19). After this time, the ratio remains essentially constant, approximately 3, regardless of the length of the heating period.

The amount of alkylpyrazines produced by the model system in a 24 hour period at a variety of temperatures between 80° C. and 150° C. was determined (Figure 20). At temperatures below 100° C., essentially no pyrazine compounds (less than 0.1 micromole) are produced. At 100° C., pyrazine formation begins and the yield increases rapidly as the temperature increases. At temperatures above 150° C., the yields were very variable. This may have been due either to the destruction of the pyrazines after formation, or else to the loss of the compounds from the reaction mixture through the reflux condenser at the high temperatures. The ratio of dimethylpyrazine to methylpyrazine produced also varies with the temperature (Figure 21). The ratio passes through a maximum of about 4.5 at 120° C. This information could be of consider-

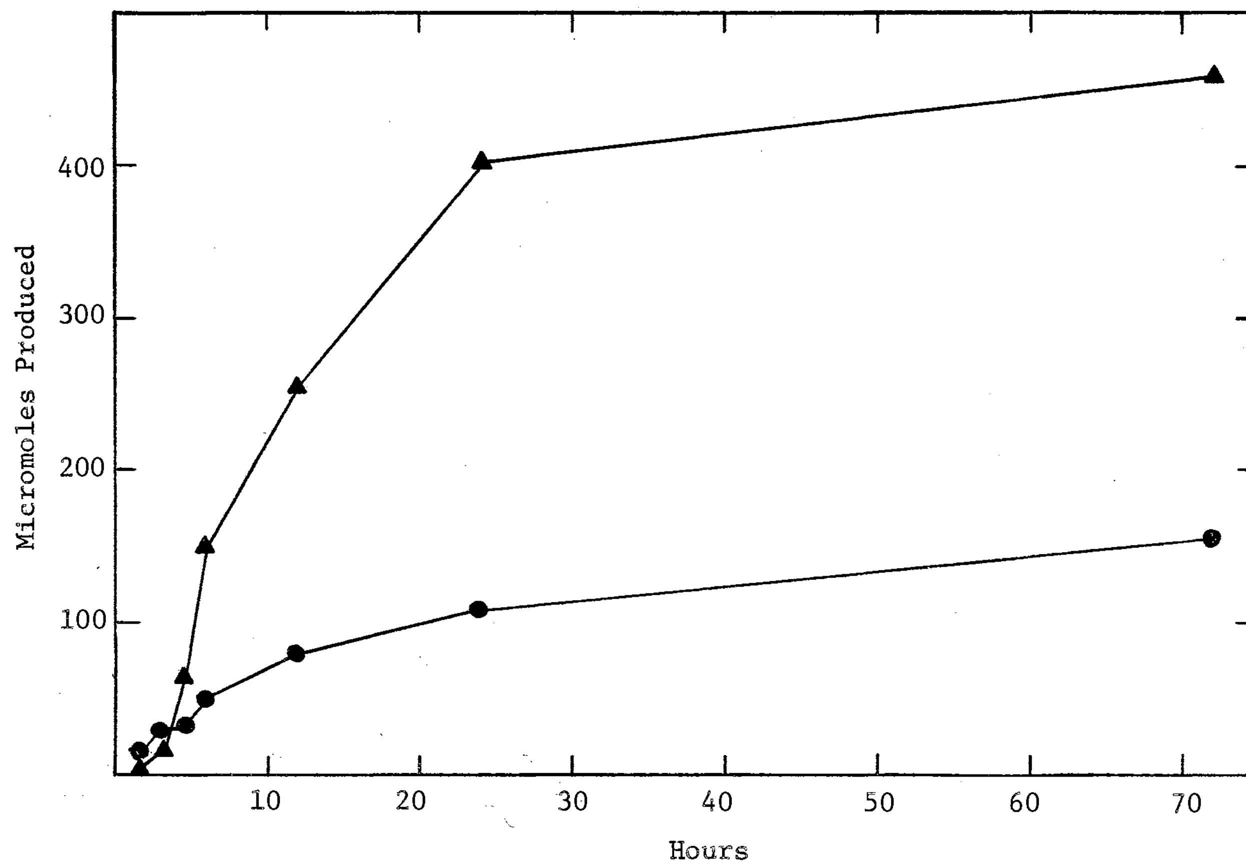

Figure 18. Alkylpyrazine Yields From the Model System After Various Heating Intervals. (▲ = Dimethylpyrazine, ● = 2-Methylpyrazine)

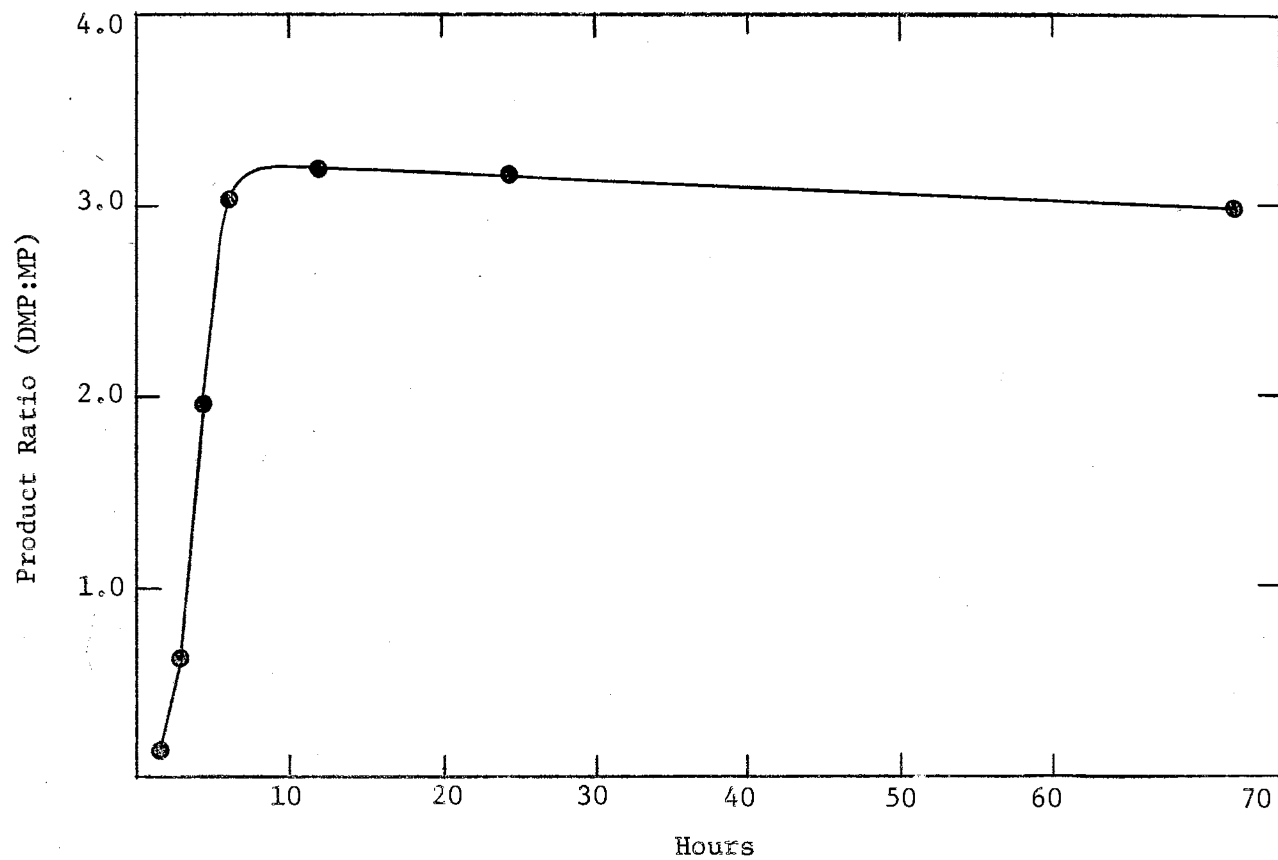

Figure 19. Variation of the Ratio of Dimethylpyrazine to Methylpyrazine Produced in the Model System With the Length of the Heating Period.

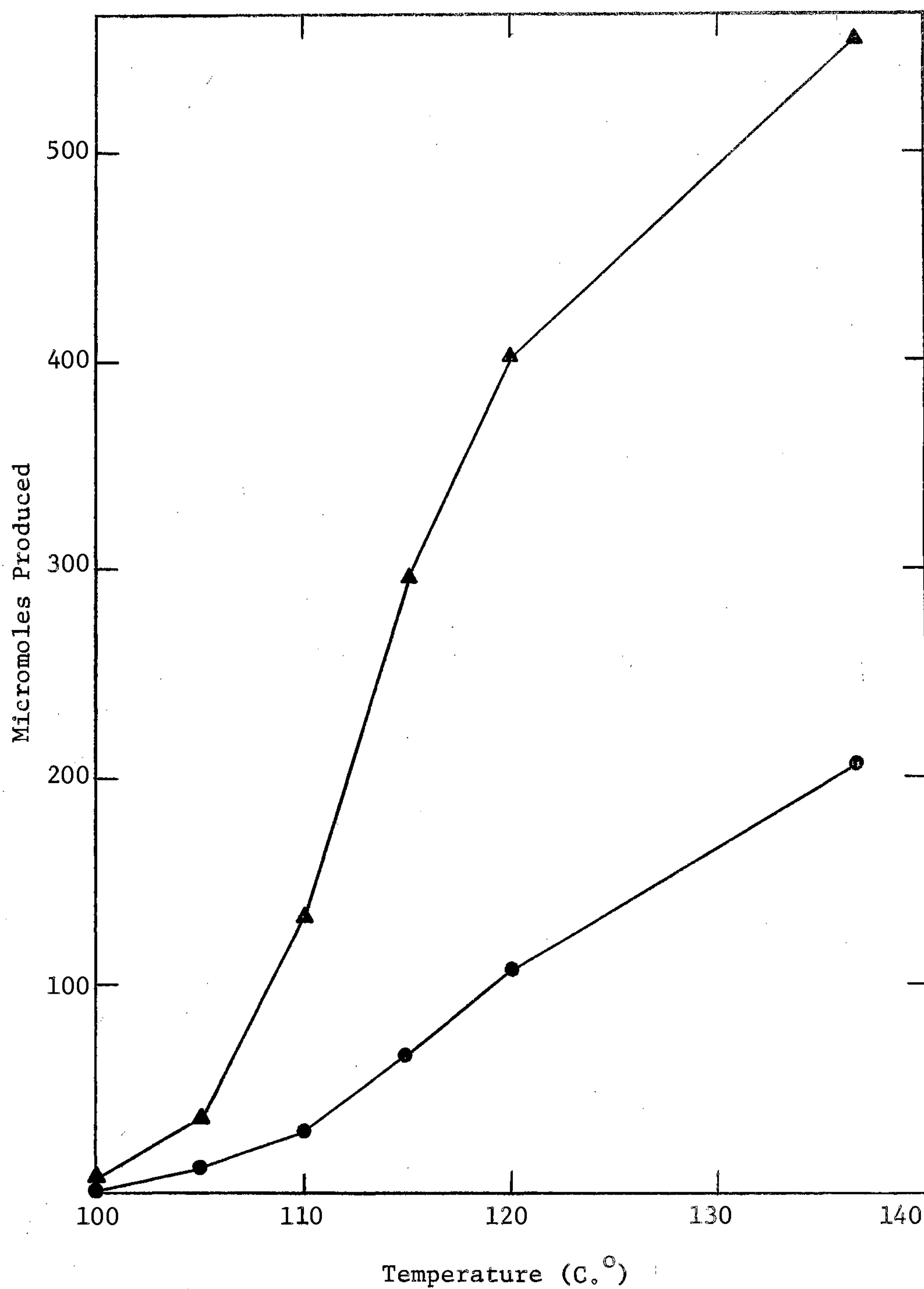

Figure 20. Temperature Effect on Pyrazine Yields in the Sugar-Amino Acid Model System. (▲ = Dimethylpyrazine, ● = 2-Methylpyrazine

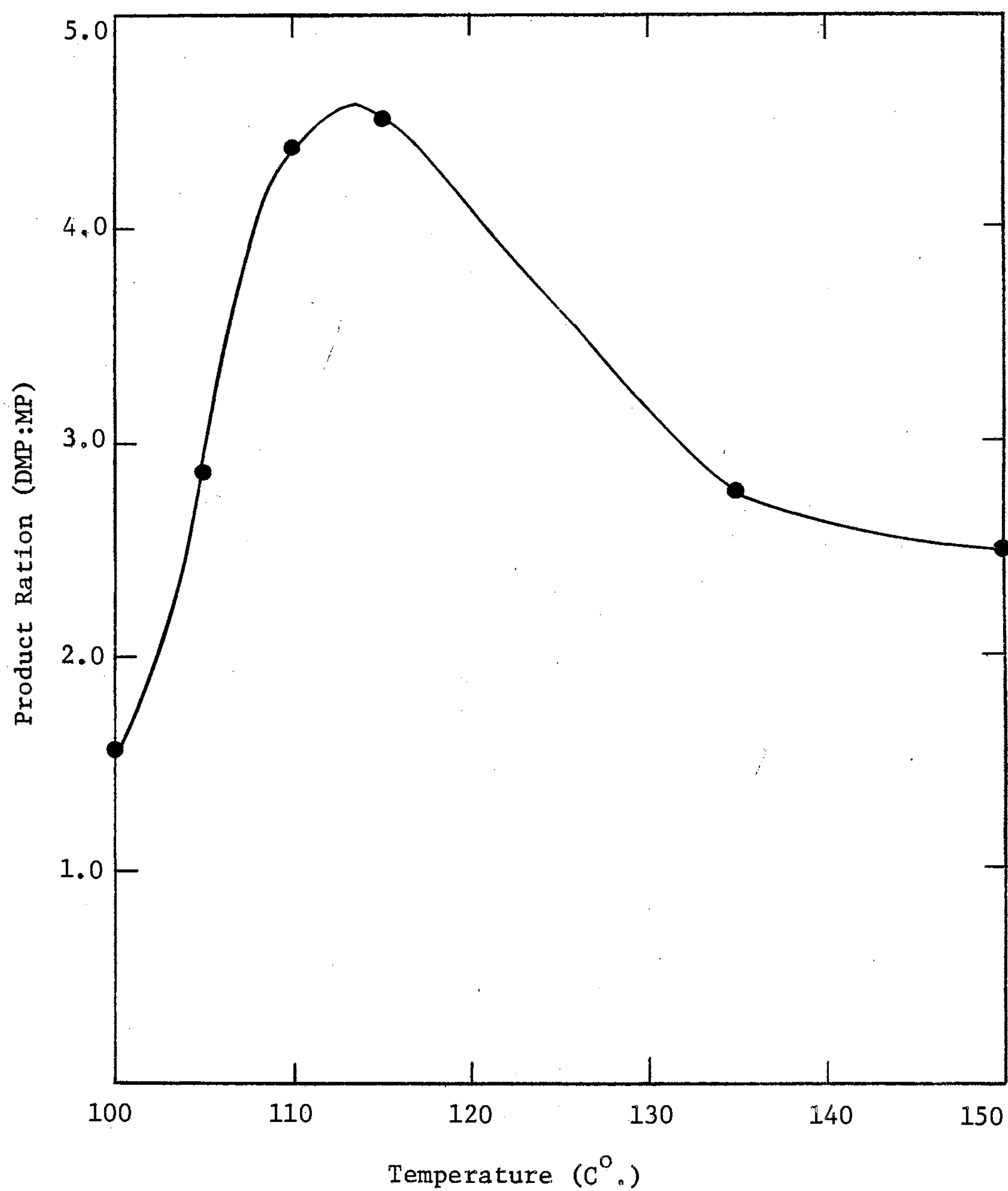

Figure 21. Temperature Effect on the Ratio of Dimethylpyrazine to Methylpyrazine Produced in the Model System.

able importance to the food processing industry, especially coffee and peanut producers, since it indicates a possible means of controlling the aroma constituents of the finished product. Flavor panel studies correlating the desirability of roasted peanut or coffee samples to the pyrazine content of the samples would furnish data that coupled with information on the effects of reaction parameters presented here would allow processing conditions to be regulated in such a manner as to maximize favorable components and minimize undesirable compounds.

The normal model system utilized the asparagine and glucose reactants in a 1:1 ratio. Tripling the amount of glucose decreased the yield of 2-methylpyrazine 10 fold and that of the dimethylpyrazine about 125 fold (Figure 22). Tripling the amount of asparagine had very little effect on the dimethylpyrazine yields and decreased the methylpyrazine yield only about 25 percent.

Since the work was done in a nearly non-aqueous solvent, it is difficult to specify the pH of the reaction media. However, the addition of acid (H_2SO_4) or base (NaOH) has a dramatic effect on alkylpyrazine formation (Figure 23). Both the glucose and asparagine reactants were present in amounts of 0.1 mole. Addition of an equal number of equivalents of acid lowers pyrazine formation to practically zero. Addition of an equal amount of base increases the yield of methylpyrazine 10 fold and the yield of dimethylpyrazine 5 fold. The base catalysis is probably due both to the increased nucleophilic reactivity of the amino group of the amino acid toward the carbonyl of glucose in basic solutions and to the increased rearrangement of fragmentation of sugars in the basic media. Also, it is thought that the equilibrium between the lactal form of sugar and the free aldehyde form is shifted

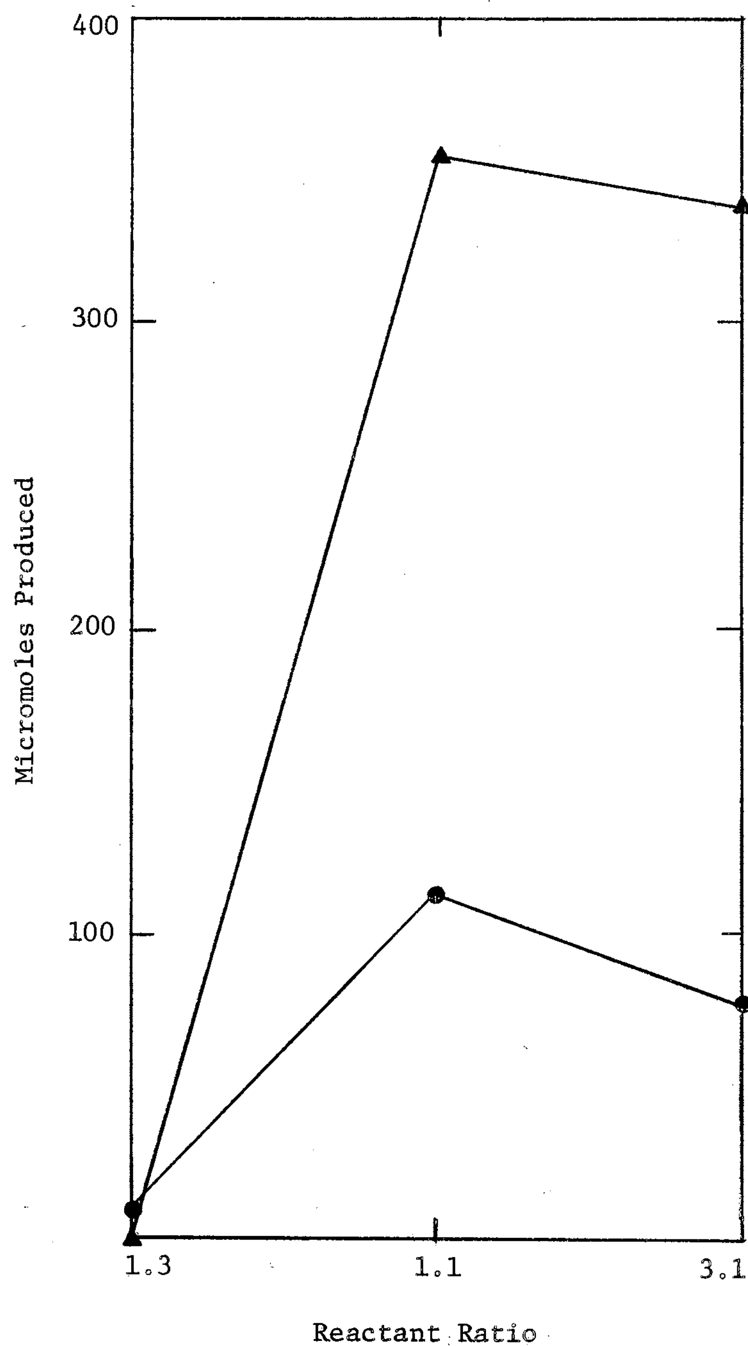

Figure 22. Effect of the Asparagine: Glucose Reactant Ratio on the Yield of Pyrazines in the Sugar-Amino Acid Model System. (▲ = Dimethylpyrazine, ● = 2-Methylpyrazine)

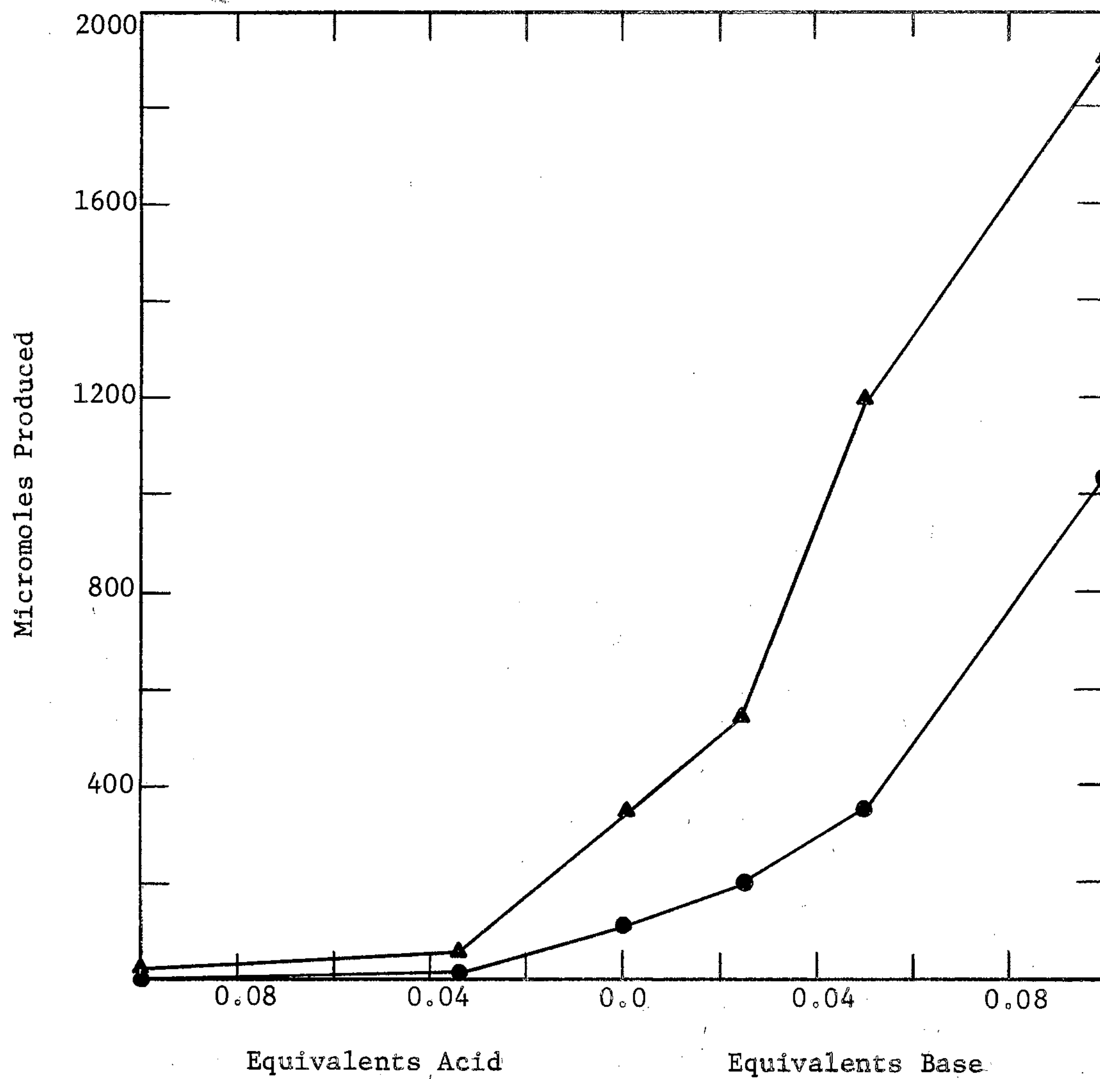

Figure 23. Acid-Base Effects on Pyrazine Production in the Sugar-Amino Acid Model System. (▲ = Dimethylpyrazine, ● = 2-Methylpyrazine)

toward the latter as the alkalinity increases (81,82). Considerable evidence favors the view that it is the free aldehyde of a sugar which reacts with amino acids (83).

In order to study the metabolism of pyrazine compounds, it will be necessary to synthesize radioactively-labeled alkylpyrazines. In view of the rather high cost of the labeled precursors, it would be highly desirable to obtain maximum yield from the synthesis. The knowledge gained thus far from the effects of time, temperature, solvent, reactant ratio, and acidity-basicity on the reaction yields was used to establish conditions which would result in the best possible yield of alkylpyrazines. Using diethylene glycol solvent with no water, equimolar quantities (0.1 mole) of glucose, asparagine, and sodium hydroxide, the reaction was carried out at 130° C. for 48 hours. From this system, 190 mg of dimethylpyrazine and 75 mg of 2-methylpyrazine were obtained. This represents yields of 1.8 and 0.8 percent respectively. These yields represent an increase of 7 fold for methylpyrazine and 4 fold for dimethylpyrazine over the normal model system used previously.

In addition to asparagine, other amino acids, glycine, alanine, aspartic acid, and lysine were used, but the yields were much lower in all cases (Table VIII). Table IX compares the yields of methyl- and dimethylpyrazine from glucose reacted with asparagine, aspartic acid, and aspartic acid plus base under the normal model system conditions. Equivalent amounts (0.1 mole) of all reactants were used. The results seem to indicate that the differences between asparagine and aspartic acid were due to differences in acidity-basicity rather than to the type or number of nitrogen atoms. Similar results are found when ammonium hydroxide and ammonium chloride are compared. Ammonium hydroxide

TABLE VIII

PRODUCTION OF PYRAZINES DURING THE REACTION OF GLUCOSE WITH VARIOUS AMINO ACIDS IN THE MODEL SYSTEM

Nitrogen Source	Methylpyrazine (μmoles)	Dimethylpyrazine (μmoles)	DMP/MP
Asparagine	110	402	3.7
Glycine	ND	14	---
Alanine	23	82	3.6
Aspartate	16	81	5.1
Lysine	11	20	1.8

ND = Not Detected

TABLE IX

PYRAZINES PRODUCED DURING THE REACTION OF GLUCOSE WITH NITROGENOUS COMPOUNDS IN THE MODEL SYSTEM

	Methylpyrazine (μmoles)	Dimethylpyrazine (μmoles)	MP/DMP
Asparagine	110	402	3.7
Aspartate	16	81	5.1
Aspartate + NaOH	92	282	3.1
Ammonium Hydroxide	1415	56	0.04
Ammonium Chloride	ND	ND	----

ND = Not Detected

when reacted with glucose gave excellent yields of 2-methylpyrazine and 2,5-dimethylpyrazine. Ammonium chloride did not produce signficiant amounts of any of the pyrazine compounds (Table IX).

Table X compares the pyrazines produced when asparagine is reacted with either glucose, fructose, sucrose, or arabinose. The alkylpyrazine yields are higher with fructose than with glucose and the ratio of dimethylpyrazine to methylpyrazine is much higher. The higher yields could be due to the fact that fructose forms smaller carbon fragmentation units more readily than does glucose (84). Also, a communication by Erickson (85) indicates that ketoses react more readily with primary amines than aldoses in alcoholic aqueous solutions. This could also explain the higher alkylpyrazine yield from fructose as well as the higher dimethylpyrazine to methylpyrazine ratio. The carbonyl in the number 2 position in fructose is immediately available for reaction with the amino acid nitrogen causing the number 1 carbon of fructose to become a one carbon side chain on the pyrazine ring. Thus alkyl side chains are a more likely occurance with fructose than with glucose where a rearrangement of the carbonyl position must occur before alkyl side chains could be formed.

Sucrose (Table X) produces fairly good yields of alkylpyrazine compounds although less than either glucose or fructose. Arabinose also produces pyrazines when reacted with amino acids. In this case, more methylpyrazine than dimethylpyrazine is produced, and the dimethylpyrazine to methylpyrazine ratio is very low. This is reasonable since a 5-carbon sugar must produce one 2-carbon fragment for each 3-carbon fragment produced, whereas a hexose can produce two 3-carbon units on fragmentation. Thus one might expect more methylpyrazine from a pentose.

TABLE X

YIELD OF ALKYLPYRAZINE COMPOUNDS ON REACTION OF ASPARAGINE WITH VARIOUS SUGARS IN THE MODEL SYSTEM

Carbon Source	Methylpyrazine (μmoles)	Dimethylpyrazine (μmoles)	DMP/MP
Glucose	110	402	3.7
Fructose	137	1142	8.4
Sucrose	85	166	2.0
Arabinose	94	48	0.5

ND = Not Detected

In addition to sugars, several smaller carbon fragments were examined for effectiveness in forming pyrazine compounds (Table XI). Propionaldehyde is known to be formed in amino acid-sugar reactions (86). No pyrazines were detected when propionaldehyde was reacted with asparagine in the model system. Glycerol gave small amounts of each of the first three members of the alkylpyrazine series. Acrolein was very likely the species actually responsible for the condensation reaction with nitrogen to give the pyrazines. Acetaldehyde also occurs in amino acid-sugar reaction mixtures (86). Acetaldehyde, being a 2-carbon unit, might be expected to yield only pyrazine if it reacts. Indeed, when reacted with ammonium hydroxide, large amounts of pyrazine were produced and none of the alkylpyrazines were detected. However, when reacted with asparagine, all of the first three members of the pyrazine series were detected with dimethylpyrazine being formed in the greatest amounts (Table XI). This indicates that either acetaldehyde causes breakdown of the amino acid to give 3-carbon units or else acetaldehyde itself can react to give 3-carbon units necessary for the formation of methyl- and dimethylpyrazine. Since in the normal glucose-amino acid system no carbon atoms of the pyrazines arise from the amino acid, the latter case is favored. The pH differences in the media brought about by the different nitrogen compounds may cause this effect. Glyoxal, another 2-carbon unit, gives the highest yields of pyrazine of all. It does, however, give some methylpyrazine also. Again, indicating that some 3-carbon units must be formed. The presence of 7-carbon furan compounds in the volatiles from heated glucose suggests that secondary recombinations and rearrangements of sugar fragments does indeed occur (87).

TABLE XI

COMPARISON OF PYRAZINE YIELD FROM VARIOUS CARBON SOURCES

	Pyrazine	Methylpyrazine	Dimethylpyrazine	Tetramethylpyrazine
Propionaldehyde-Asparagine	ND	ND	ND	ND
Glycerol-Asparagine	2	7	2	ND
Acetaldehyde-Asparagine	29	51	291	ND
Acetaldehyde-NH_4OH	399	ND	ND	ND
Glyoxal-Asparagine	527	91	ND	ND
Glyoxal-NH_4OH	253	16	ND	ND
2,3-Butanedione-Asparagine	ND	ND	ND	2662
2,3-Butanedione-NH_4OH	ND	ND	ND	640
Hydroxyacetone-Asparagine	ND	ND	9725	ND
Hydroxyacetone-NH_4OH	ND	ND	2208	ND
Glucosamine	107	252	27	ND

ND = Not Detected; Yield in μmoles.

The 4-carbon dicarbonyl, 2,3-butanedione, might be expected to produce the 8-carbon alkylpyrazine, 2,3,5,6-tetramethylpyrazine. Diacetyl (2,3-butanedione) has been reported as a degradation product of sugars and amino acid mixtures (88). As seen in Table XI, tetramethylpyrazine is produced in very large quantities when 2,3-butanedione is heated either with asparagine or ammonium hydroxide. No other alkylpyrazines were detected, indicating that fragmentation into smaller units probably does not occur. Again, pH differences may be responsible for the differences in the yields, particularly in view of the large pH effects shown in Figure 13. Pyrazine compounds have been found only rarely as products of microbial metabolism. Tetramethylpyrazine is believed to be responsible for the characteristic odor of fermented soybean or "natto" and is produced by a strain of Bacillus subtilis (27). This same compound has also been found in concentrations sufficient to crystallize from the broth media containing a mutant strain of Corynebacterium glutamicum (29). In both of these cases, the condensation of acetoin with ammonia was believed to be responsible for the formation of tetramethylpyrazine. Metabolic blocks in the organisms cause formation of unusual amounts of acetoin. It is not known if the condensation of acetoin with ammonia to form tetramethylpyrazine is an enzymatic step.

Lento et al. (89) studied the formation of some 3-carbon carbonyl compounds from glucose and fructose heated in buffer solutions (pH 4-11.8). It was suggested (90) that acetol (hydroxyacetone) might be an important intermediate in the browning reaction at high pH levels. When used as the carbon source in the model system reaction study, the 3-carbon compound, hydroxyacetone, produced only dimethylpyrazine as

might be predicted (Table XI). The yield of dimethylpyrazine was very high, indicating that hydroxyacetone might be a good intermediate in the pyrazine formation reaction as well as in the browning reactions.

Glucosamine hydrochloride neutralized with an equivalent amount of sodium hydroxide produced pyrazine, 2-methylpyrazine, and dimethylpyrazine when heated in the model system (Table XI). Fragmentation of glucosamine between carbon atoms number 2 and 3 would produce a 2-carbon unit with both an aldehyde and an amine group. Condensation of two of these units could easily form pyrazine. Fragmentation of glucosamine between carbons atoms 3 and 4 would produce a 3-carbon unit with adjacent carbonyl and amine groups. Condensation of two of these units could produce dimethylpyrazine. Condensation of one of these 3-carbon units with one of the 2-carbon fragments could produce methylpyrazine. Thus glucosamine is capable of serving as the source of both the carbon and nitrogen atoms of a variety of alkylpyrazine molecules.

There are apparently several pathways and many different compounds which can lead to the formation of alkylpyrazines in the model system and in natural food products. Probably, in the natural food product, two major routes could be defined. In one, the sugar molecules would first react with amino acids and then this glucosylamine product would condense to form a di-tetrahydroxybutylpyrazine (35) intermediate. This intermediate would then undergo rearrangement and cleavage to form alkylpyrazines. Alternatively, at high temperatures, the sugar may immediately undergo rearrangements and cleavage into numerous smaller hydroxy-carbonyl and dicarbonyl fragments. Any two of such fragments could then condense with nitrogen from amino acids to form the many alkylpyrazine compounds found in roasted food products. The pathway

through the tetrahydroxybutylpyrazine intermediate (35) may be important in the slow formation of alkylpyrazine compounds at low temperatures over longer intervals of time. At higher temperatures which favor rearrangement and fragmentation of the sugars, the condensation of smaller hydroxycarbonyl and dicarbonyl fragments may be of the most importance.

CHAPTER VII

METABOLISM OF PYRAZINE COMPOUNDS

Alkylpyrazine compounds occur at varying levels in a variety of roasted food products (5,6,7,8,9,10). They are also known to occur in at least one uncooked food product, "natto", a fermented soybean product consumed in some Asian countries (27,28). The use of pyrazine compounds as artificial food flavorants (72,73,74) may mean that in the near future these compounds will be consumed in increased amounts, probably at levels considerably above those encountered in natural food products. In this chapter, the results of a preliminary study of the metabolism of alkylpyrazines will be reported and discussed.

Materials

White, female laboratory rats weighing approximately 160 gm from the Biochemistry Department rat colony were used. The 2,5-dimethylpyrazine was purchased from Wyandotte Chemicals Corporation, Wyandotte, Michigan. Dichloromethane obtained from Fisher Scientific Co., Fair Lawn, New Jersey, was redistilled before use. Carbowax 20M and Gas Chrom Q (100/120 mesh) were purchased from Applied Science Laboratories, State College, Pennsylvania. Rockland Mouse/Rat Diet was obtained from Rockland, Inc., Winfield, Iowa.

Methods

The 2,5-dimethylpyrazine was introduced by intraperitoneal injection into female rats weighing approximately 160 gm. For doses of less than 100 mg, the 2,5-dimethylpyrazine was administered as a 10 percent aqueous solution containing 0.8 percent sodium chloride. The 250 mg dose was administered as a 25 percent solution containing 0.8 percent sodium chloride.

The rats were placed in cages suspended over large funnels which permitted collection of the urine and feces. A layer of dichloromethane was added to the urine collection bottle to inhibit bacterial growth. The urine and feces samples were collected at 24 hour intervals for a period of 2 weeks following each injection. The samples were obtained from 2 experimental and 1 control animal. The rats were allowed free access to both food and water.

Each urine sample was extracted 7 times with one-tenth volume dichloromethane. The extract was reduced to a small volume by removing most of the dichloromethane on a Buchi rotary evaporator (25° C.). The extract was then diluted with dichloromethane to exactly 2 ml in a small volumetric flask. Each sample was then quantitatively analyzed for its pyrazine content by injecting triplicate aliquots onto a Perkin-Elmer 801 gas chromatograph equipped with dual hydrogen flame ionization detectors. A 20 foot x 1/4 inch O.D. glass column packed with 15 percent (w/w) Carbowax 20M on Gas Chrom Q (71) was used with a nitrogen flow rate of 60 ml per minute and a constant temperature of 150° C. The pyrazine peaks were quantitated by comparing the peak areas of the samples with the peak areas of standards chromatographed the same day under the same conditions.

Feces were placed in Soxhlet extraction thimbles and extracted con-

tinuously with 250 ml of refluxing dichloromethane for 24 hours. The feces were then macerated and extracted with dichloromethane for a second 24 hour period. The dichloromethane extracts were reduced in volume and analyzed by the same methods used for the urine samples.

The extent that pyrazine compounds can be eliminated through the lungs was determined by placing the rat in an enclosed metabolic chamber. The air in the chamber was continuously drawn from the chamber and bubbled through a series of four large (20 cm x 3 cm) tubes filled with dichloromethane. The dichloromethane solutions were combined, reduced in volume, and analyzed for pyrazines by gas-liquid chromatography.

Results and Discussion

Little information on the metabolism or toxicity of alkylpyrazine compounds has been reported in the literature. The present work was begun by determining what dosages could be tolerated by the rat. Dosages beginning at 17 mg (106 mg/kg) and continuing up to 250 mg (155 mg/kg) were injected interperitoneally into laboratory rats. Injections of 17, 25, and 50 mg had no visible immediate effects on the experimental animals. The 75 mg (469 mg/kg) dose caused a "drowsiness" of the rat beginning about 1 minute after injection and lasting about 1 hour. The two rats receiving 250 mg injections both went into an unconscious state within 1 minute of injection. One of these rats died after 3 hours and the other after 8 hours. Neither rat regained consciousness. An autopsy by the Oklahoma State University Veterinary Medicine College indicated that the cause of death from these large doses was probably internal hemorrhaging in the abdominal cavity. These

effects are observed on the injection of many organic solvents. If alkylpyrazines are to be included in food products as flavoring agents, long term studies on the possible effects of repeated subtoxic doses would be of great value.

The extent to which alkylpyrazine compounds might be eliminated from animals directly, without chemical modification, was determined. After injecting rats interperitoneally with 2,5-dimethylpyrazine, the urine, feces, and exhaled vapors were collected and assayed to determine the rat's ability to eliminate these compounds without alteration. The elimination of dimethylpyrazine in the urine (Table XII) occurs primarily during the first 48 hours. When 75 mg of dimethylpyrazine were injected, 1.9 mg (2.5 percent of the injected dosage) was eliminated through the urine during the first 24 hours. Another 1.0 percent (0.73 mg) was eliminated during the second 24 hour period. Very little more was eliminated during the third and fourth days. Over a 4-day period, 3.5 percent of the 75 mg injected dose was eliminated in the urine. No dimethylpyrazine was detected in the feces samples from the same rat at any time during the first 4 days after injection (Table XIII). From a rat given 70 mg of dimethylpyrazine intraperitoneally, 0.3 mg was collected in the vapors exhaled by the rat during the first 24 hours (Table XIII). This represents elimination of 0.4 percent of the injected dose through the lungs during this interval.

Nearly 4 percent of a 75 mg dose of dimethylpyrazine administered intraperitoneally was eliminated unaltered through the urine and lungs of rats within 48 hours. None was eliminated in the feces. Whether the remainder was eliminated after chemical alteration or was metabolized and utilized by the rat remains unknown. Stolte (93), in 1908,

TABLE XII

ELIMINATION OF DIMETHYLPYRAZINE IN THE URINE

	Total mg Eliminated	Percent of Dose
75 mg Intraperitoneal Dose		
0-24 hours	1.90	2.5
24-48 hours	0.73	1.0
48-72 hours	0.01	0.01
72-96 hours	0.00	0.00
4 Day Total	2.64 mg	3.51 percent
50 mg Intraperitoneal Dose		
0-24 hours	0.82	1.1
24-48 hours	0.03	0.04
48-72 hours	0.00	0.00
72-96 hours	0.00	0.00
4 Day Total	0.85 mg	1.14 percent
Control		
4 Day Total	0.0 mg	0.0 percent

TABLE XIII

ELIMINATION OF DIMETHYLPYRAZINE THROUGH LUNGS AND FECES

	Total mg Eliminated	Percent of Dose
Lungs		
(70 mg intraperitoneal dose)		
0-24 hours	0.30	0.43
Feces		
(75 mg intraperitoneal dose)		
0-24 hours	0.0	0.0
24-48 hours	0.0	0.0
48-72 hours	0.0	0.0

reported feeding a pyrazine derivative, 2,5-ditetrahydroxybutylpyrazine to rabbits. The oxidation product, 2-methylol-5-pyrazionic acid was isolated from the urine. Using reaction conditions presented in this thesis, radioactively-labeled alkylpyrazine compounds can be synthesized which should be of great value in investigating the metabolism of these food-flavor compounds.

SUMMARY

The concentration of alkylpyrazine compounds in four common roasted food products was determined quantitatively. Synthesis of a number of the commercially unavailable alkylpyrazine compounds was accomplished. These compounds were purified by gas chromatographic techniques and identified by mass spectrometry, infra-red spectrometry, and nuclear magnetic resonance spectrometry. Ten alkylpyrazine compounds were subjected to sensory evaluation studies. Threshold detection levels as well as subjective evaluations of the odor of these compounds were obtained.

The pathway of formation of alkylpyrazine compounds in roasted food products was studied using sugar-amino acid model systems of low water content. Using carbon-14 labeling studies, it was determined that, in the model system, the sugar furnishes all of the carbon atoms of the alkylpyrazines, while the amino acid serves primarily as a source of nitrogen atoms. A possible pathway for the fragmentation of sugars into smaller fragments and subsequent condensation of these fragments with nitrogen to form alkylpyrazines is proposed. The position of the carbon-14 label in alkylpyrazine molecules derived from glucose labeled specifically in known positions was determined. The effects of various reaction parameters on the yields and distribution of alkylpyrazine compounds in the sugar-amino acid model system were studied. Some possible intermediates in the alkylpyrazine formation pathway are discussed.

A preliminary investigation of the metabolism of alkylpyrazine com-

pounds by animals was conducted. The tolerance of rats to various levels of dimethylpyrazine by intraperitoneal injection was observed. The extent of elimination of alkylpyrazine compounds through the urine, feces, and lungs without chemical alteration was determined.

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APPENDIX A

ODOR DETECTION THRESHOLD PROFILES

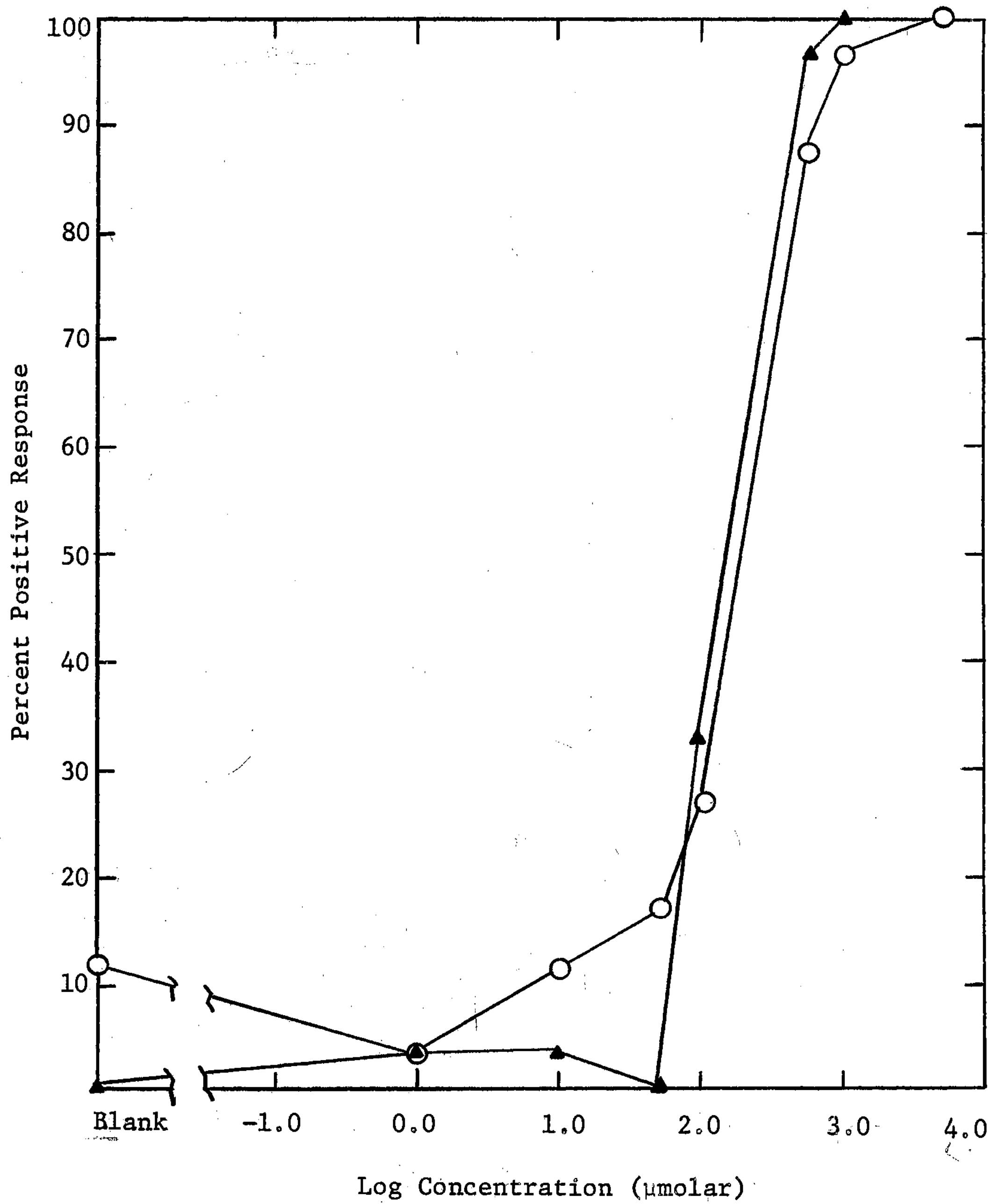

Figure 24. Odor Detection Threshold Profile for 2-Ethylpyrazine in Water O and Oil ▲.

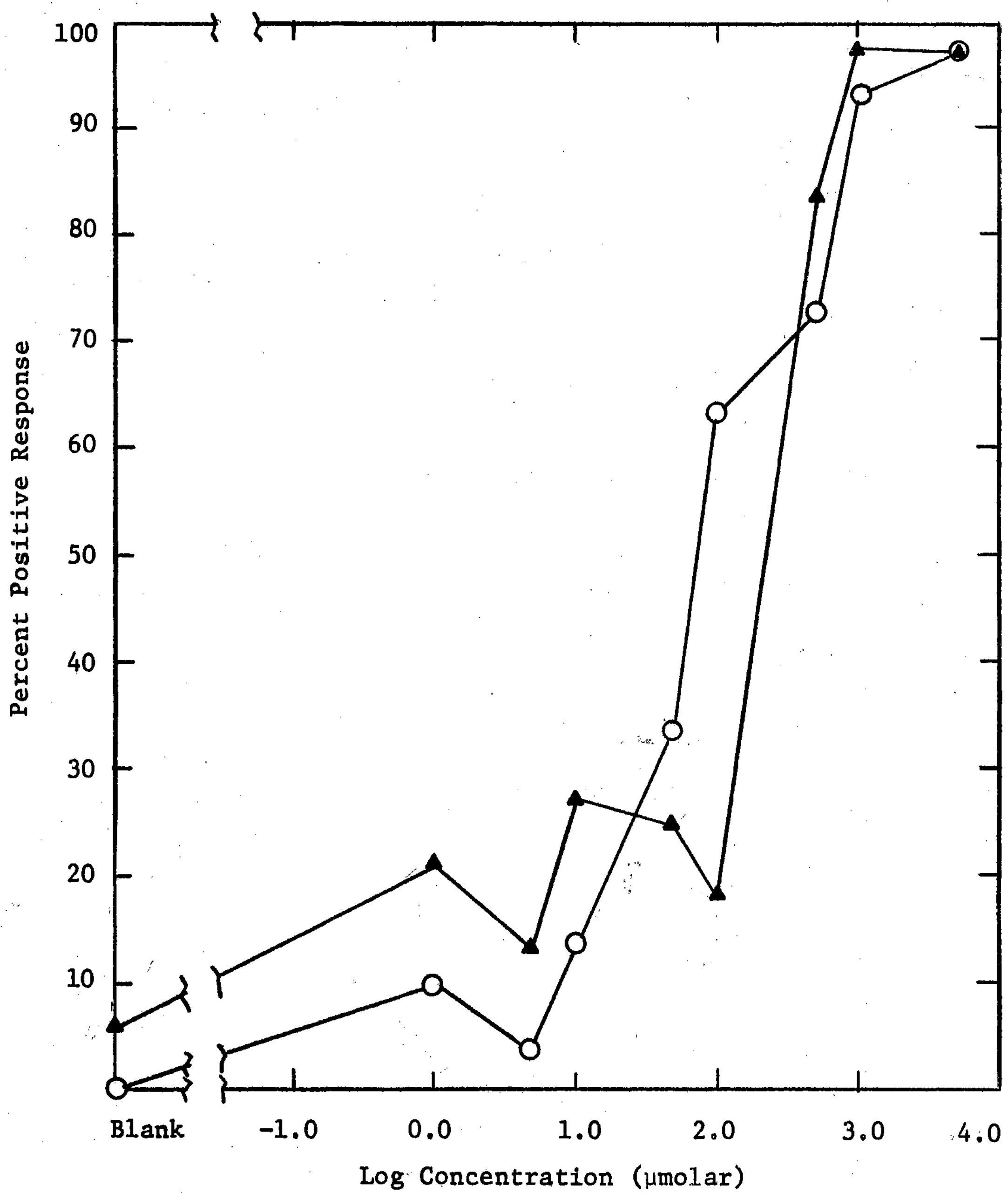

Figure 25. Odor Detection Threshold Profile for 2,3,5-Trimethylpyrazine in Water O and Oil ▲.

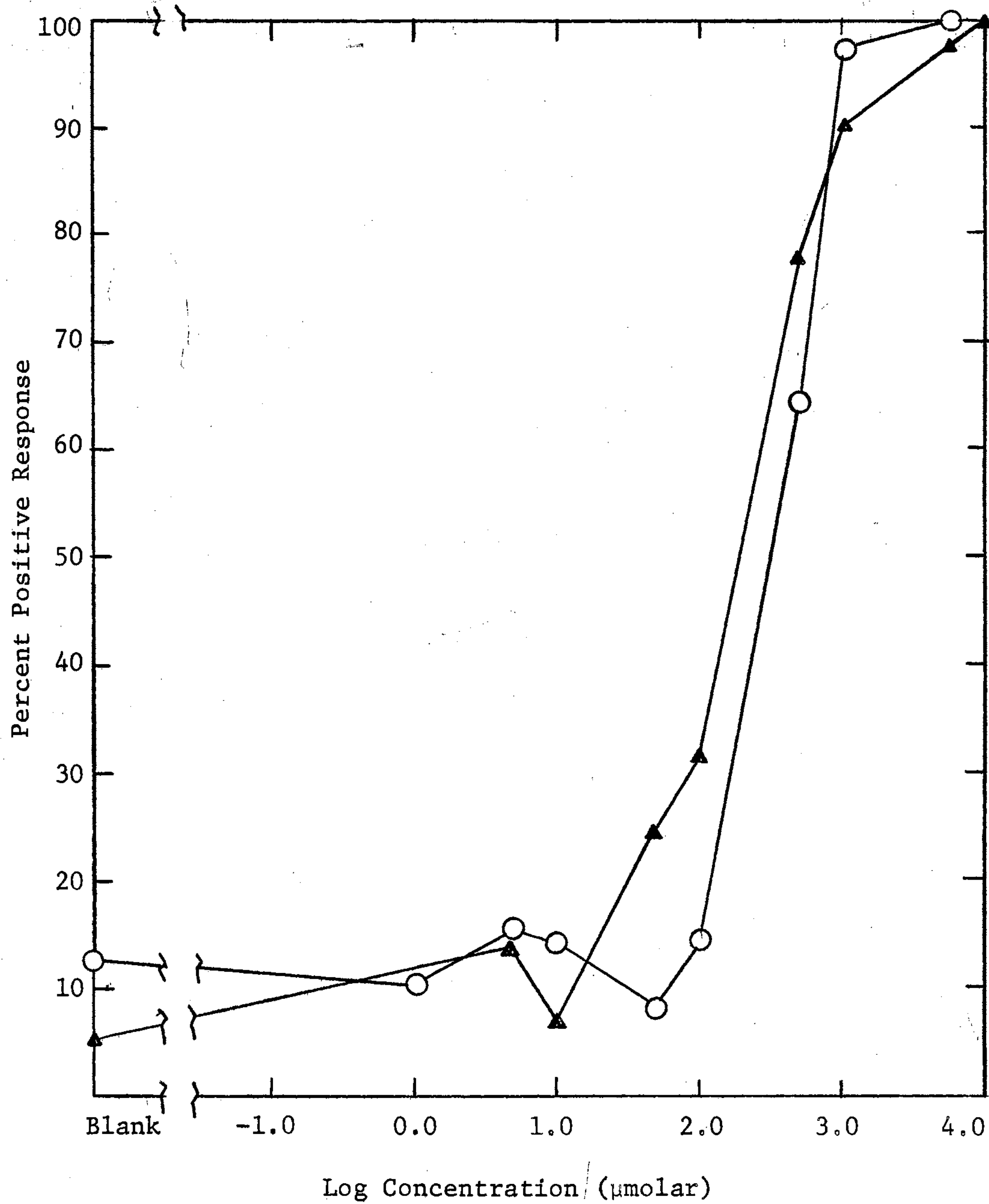

Figure 26. Odor Detection Threshold Profile for 2,5-Dimethyl-3-Ethylpyrazine in Water O and Oil ▲.

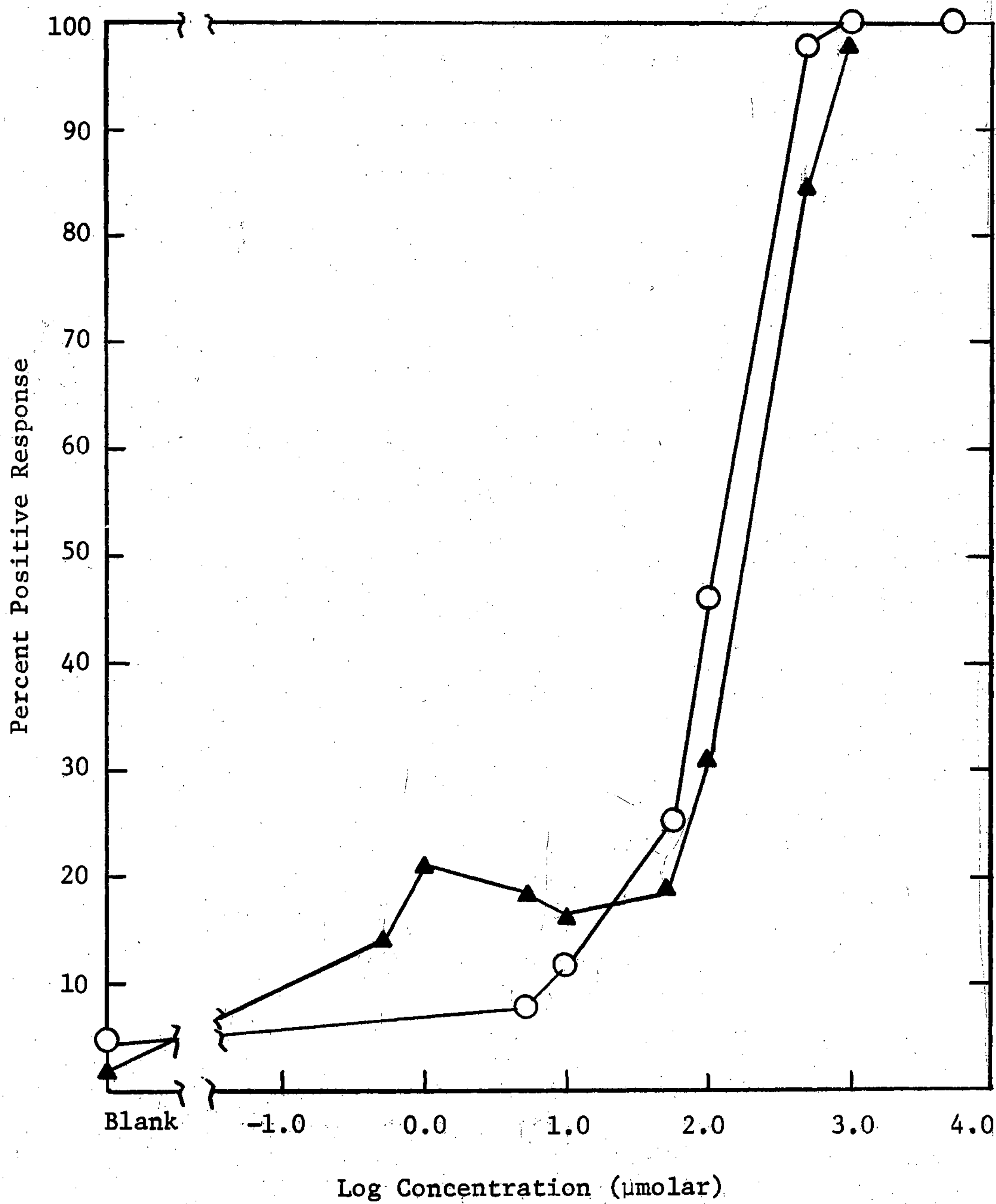

Figure 27. Odor Detection Threshold Profile for 2,6-Dimethyl-3-Ethylpyrazine in Water O and Oil ▲.

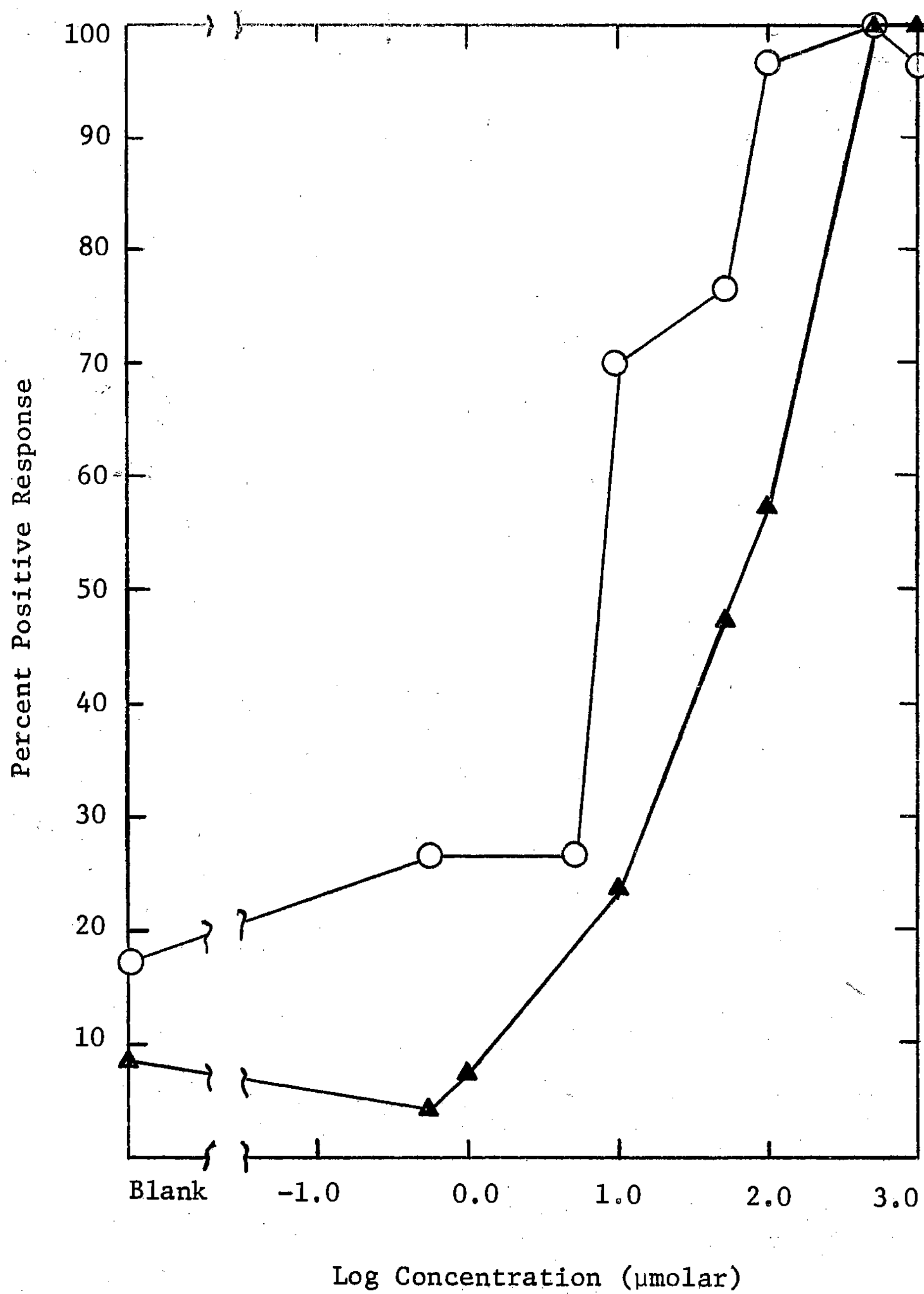

Figure 28. Odor Detection Threshold Profile for 2-n-Pentylpyrazine in Water O and Oil ▲.

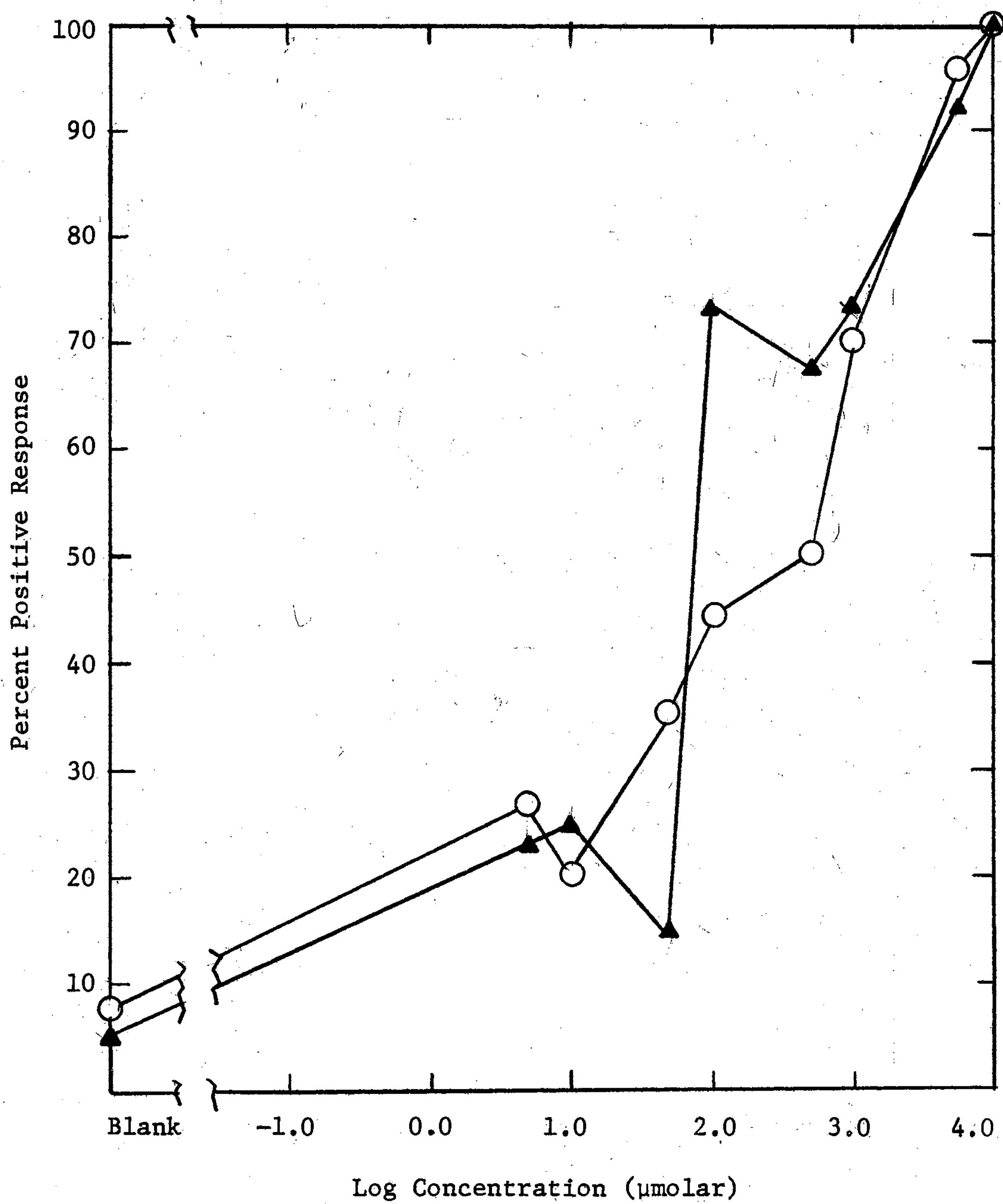

Figure 29. Odor Detection Threshold Profile for 2,6-Dimethylpyrazine in Water O and Oil ▲.

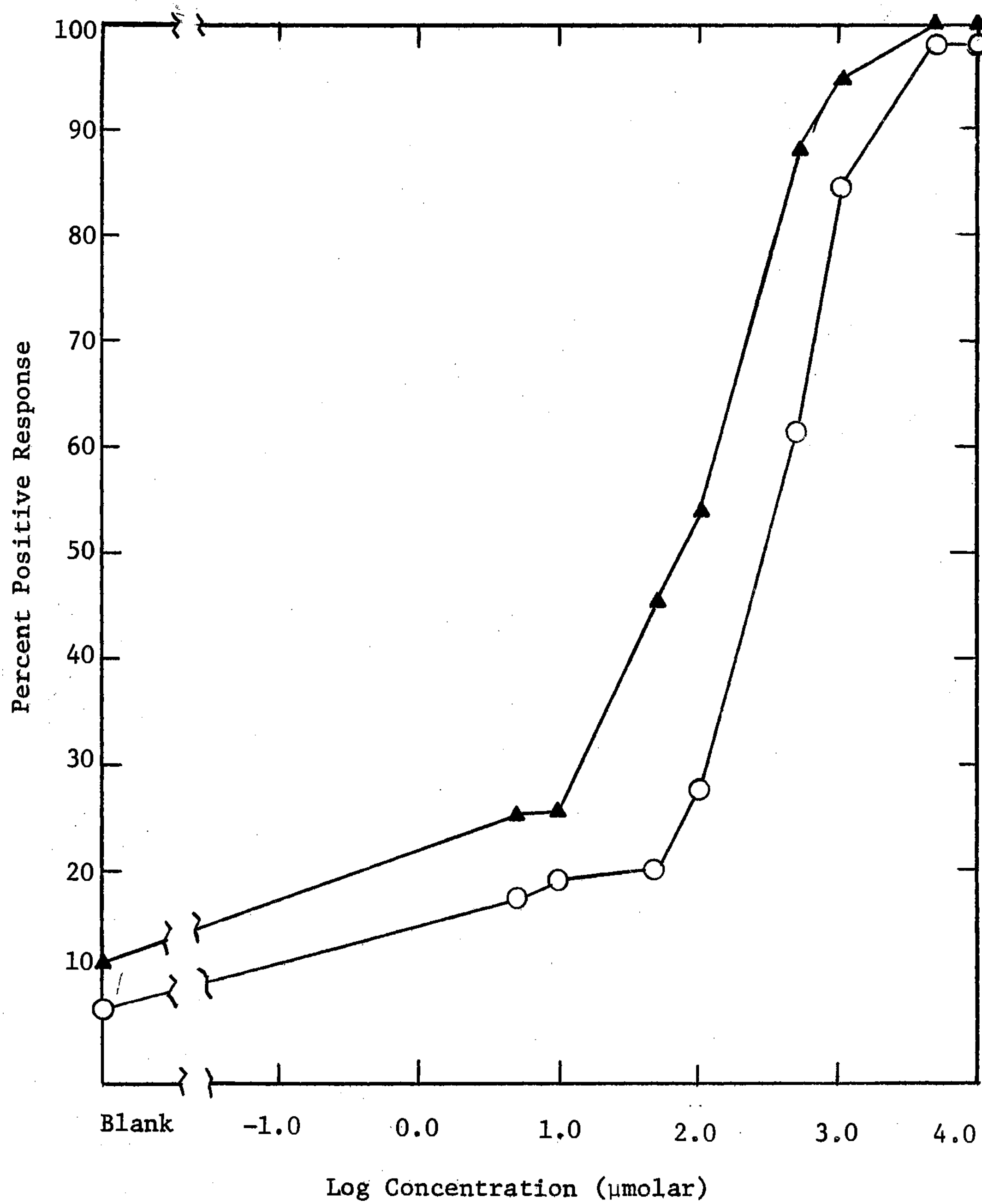

Figure 30. Odor Detection Threshold Profile for 2,3,5,6-Tetramethylpyrazine in Water O and Oil ▲.

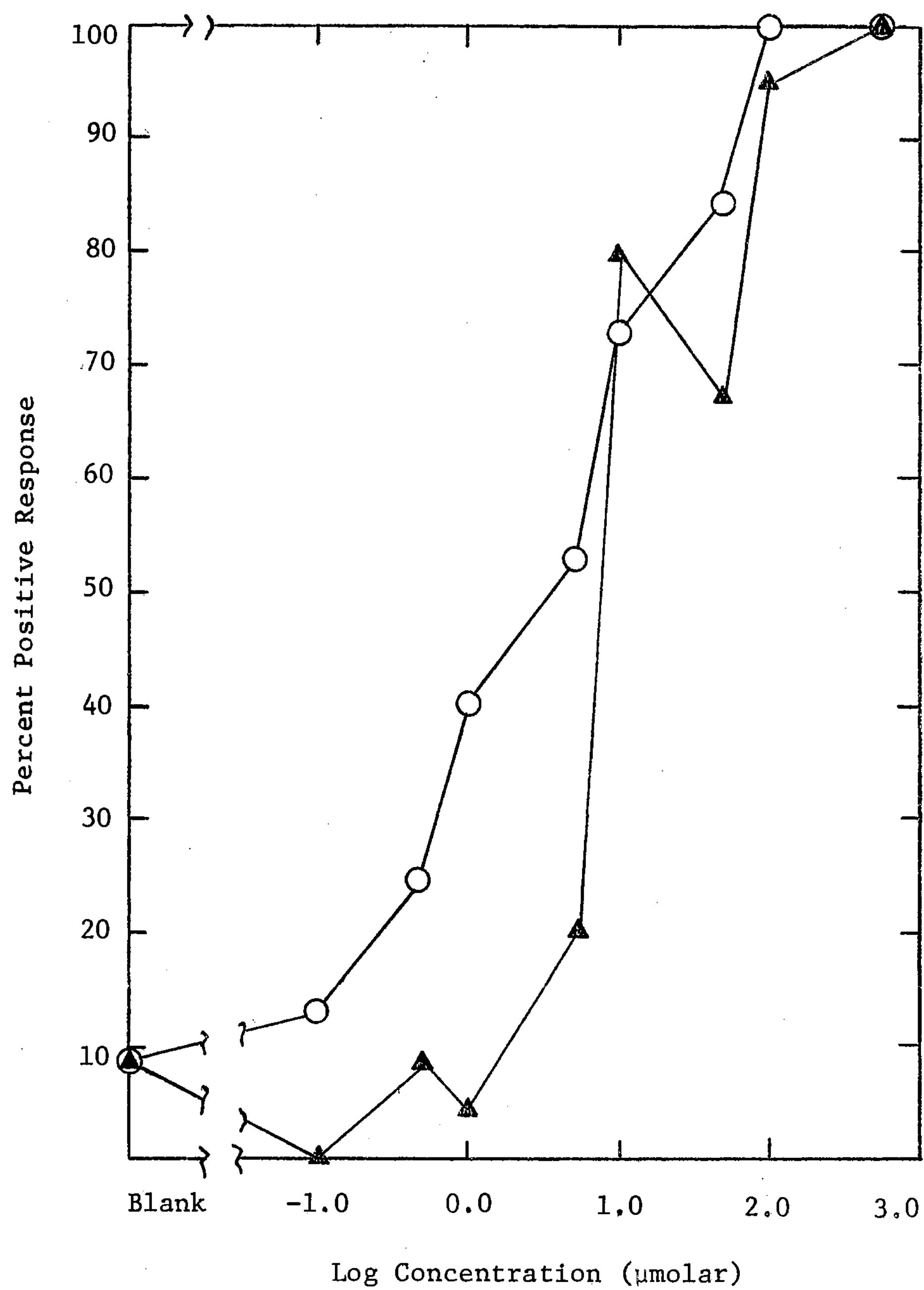

Figure 31. Odor Detection Threshold Profile for Monomethylmonoethylpyrazines in Water O and Oil ▲.

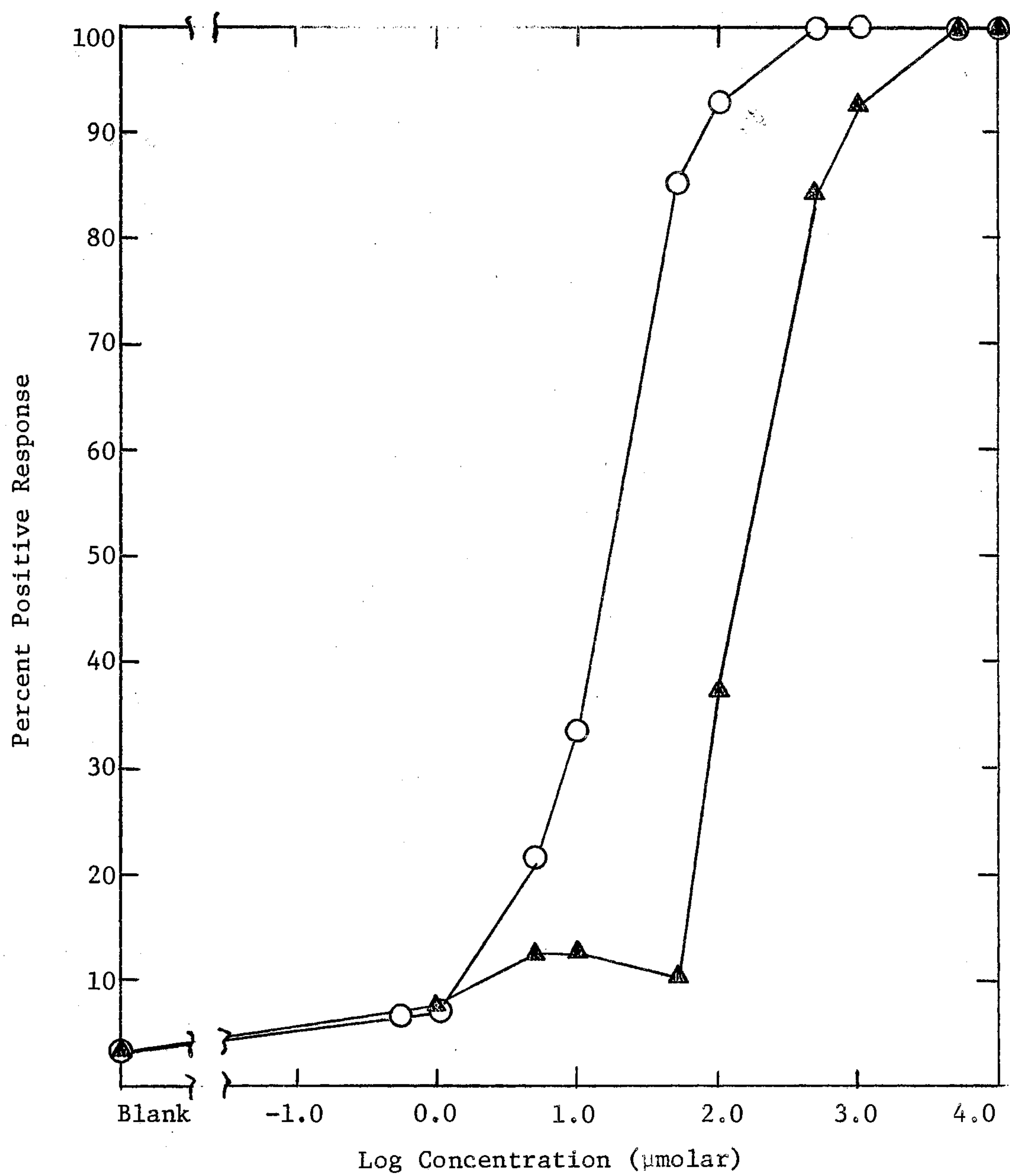

Figure 32. Odor Detection Threshold Profile for N-Methyl-pyrrole in Water O and Oil ▲.

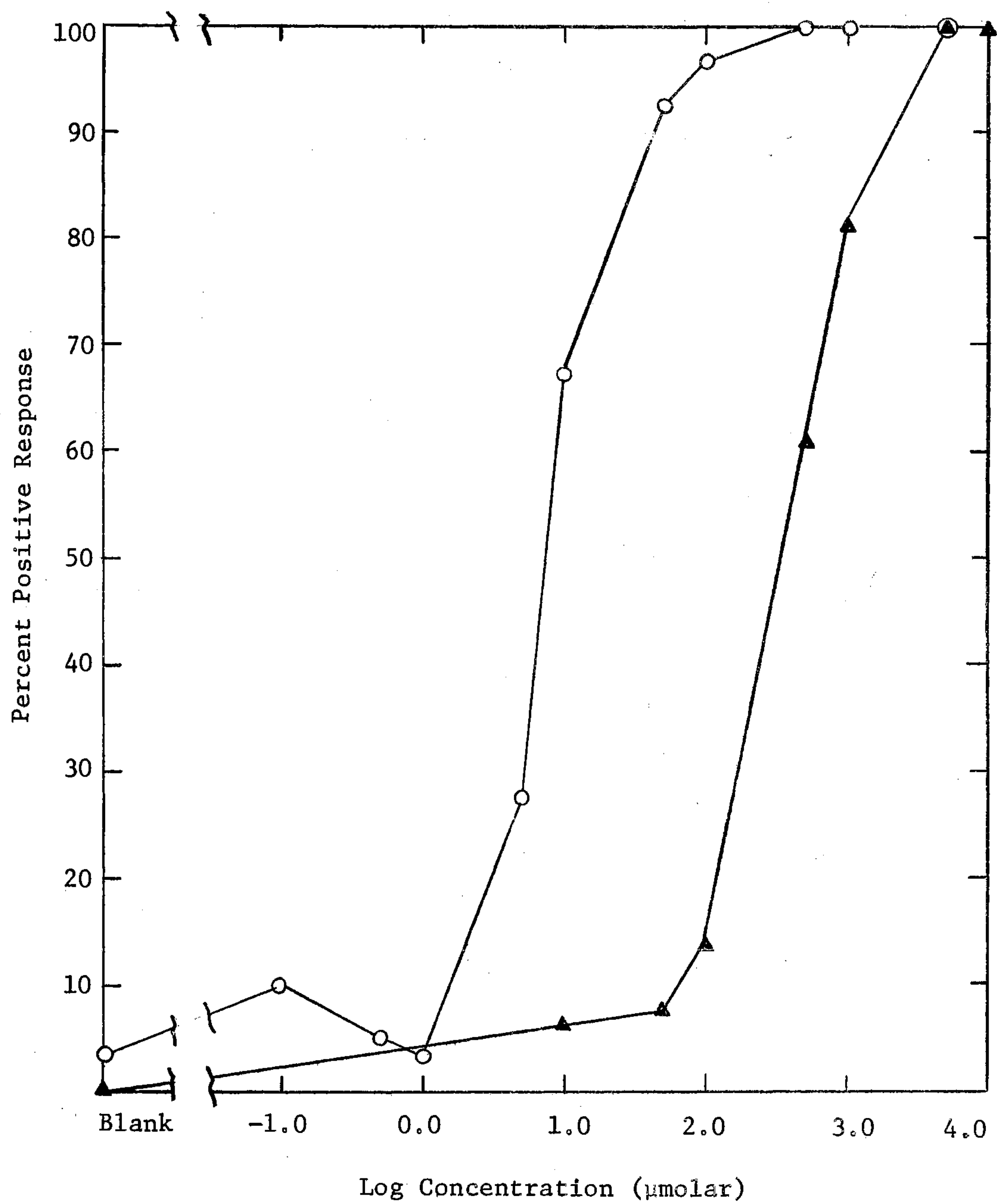

Figure 33. Odor Detection Threshold Profile for Benzaldehyde in Water O and ▲.

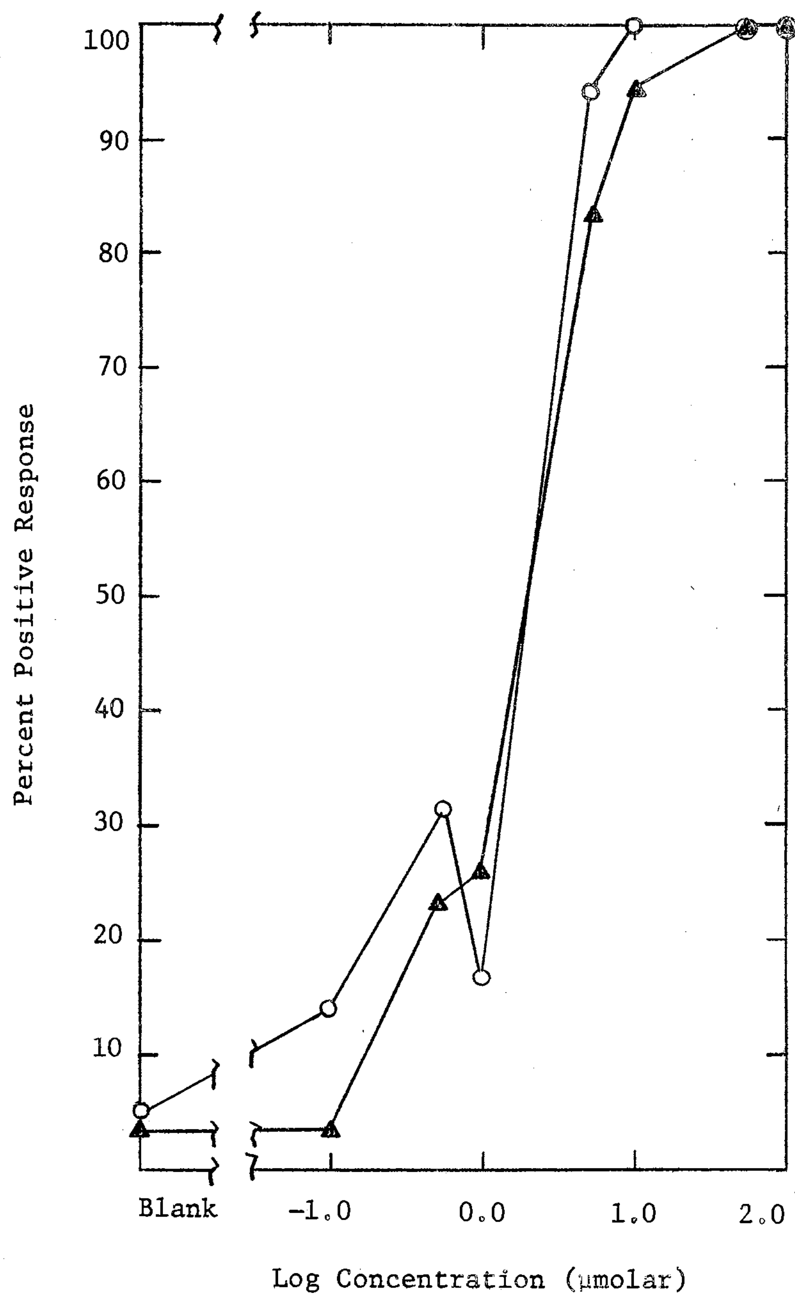

Figure 34. Odor Detection Threshold Profile for Phenylacetaldehyde in Water O and in Oil ▲.

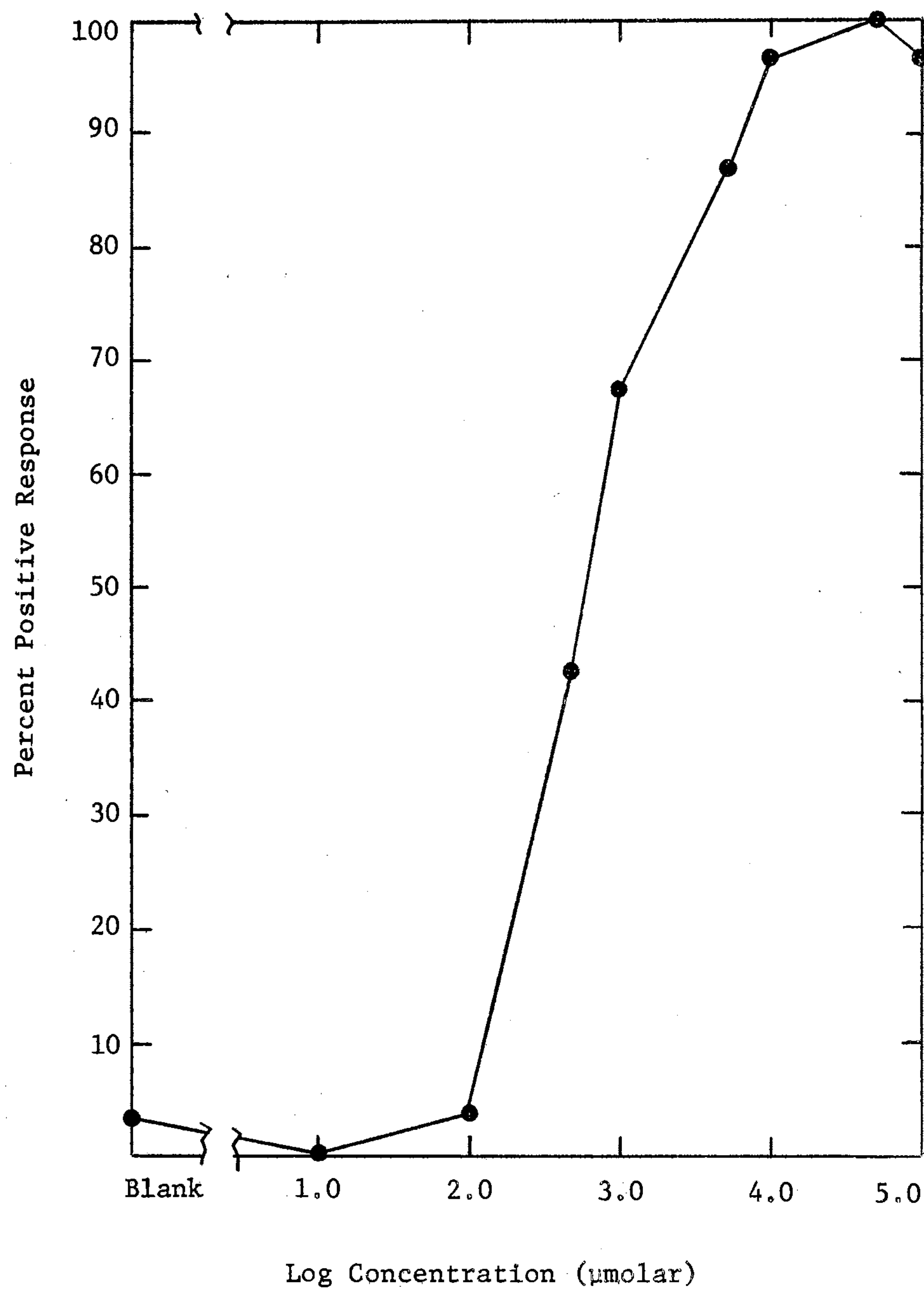

Figure 35. Odor Detection Threshold Profile for Glyoxal in Water.

VITA

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