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Title GAS CHROMATOGRAPHY OF FERMENTATION PRODUCTS OF DIFFERENT YEAST STRAINS

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Many different strains of yeast have been isolated from the natural microflora on fruits. It has been known for some time that these strains of yeast have distinct fermentation characteristics and, when used in the production of wine, impart these characteristics to the wine. These characteristics may be evidenced in the flavor or aroma of the wine, the speed of the fermentation, the amount of ethanol produced and many other ways.

It was reasoned that these fermentation characteristics were involved with or were a result of the metabolic system of a particular yeast strain. Since fermentation products result from the metabolic pathways, these products were studied in the hope that a better understanding of the fermentation products would lead to a better understanding of the metabolic pathways and, in turn, of the fermentation characteristics of yeast strains.

The fermentations were conducted on blackberries with four

strains of yeast, chosen because of their different fermentation characteristics: Saccharomyces oviformis, Saccharomyces bayanus, Saccharomyces ellipsoideus and Saccharomyces cerevisiae.

Ethyl chloride was used to extract the wines after the fermentations were completed. The solvent was removed and the sample concentrated on a low temperature distillation apparatus, after which it was analyzed using gas chromatography. The gas chromatograph employed was a Wilken's Aerograph Hy-Fi with a hydrogen flame ionization detector. An eight foot X 1/8 inch stainless steel column packed with five percent Carbowax 400 on 80/100 mesh Celite 545 was used for the analyses.

Peak heights were determined by measuring the recorder response (in millivolts), and the percent contribution of each peak was calculated with the greatest percent deviation within a sample being less than four percent.

The initial five peaks, excluding ethanol and the solvent, accounted for approximately 99 percent of the sample and demonstrated significant differences between the yeast strains. The later peaks also aided in the differentiation although not in as pronounced a manner.

A tentative identification was made, using the enrichment technique, of peaks two, three, four, five, six, seven, nine, ten, thirteen and fourteen. These were believed to be ethyl acetate, ethanol,

propanol, isobutanol, butanol, isoamyl alcohol and active amyl alcohol, glycerol, 2, 3-butylene glycol or ethyl octanoate, linaloöl and ethyl decanoate.

GAS CHROMATOGRAPHY OF FERMENTATION PRODUCTS OF DIFFERENT YEAST STRAINS

by

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INTRODUCTION

Different strains of yeast have been isolated from the natural microflora on fruits by many workers. The classification and differentiation has been based upon morphological and physiological differences, such as modes of reproduction, fermentation and assimilation of carbohydrates, nutritional requirements and cell shape.

It has been known for some time that the strain of yeast used in the production of wine exhibits an influence on the final, finished product (1, p. 138; 2, p. 29; 21; 45; 82; 100; 103). This influence has been noted in different ways; the aroma, flavor, speed of fermentation and ease of clarification are some of the ways that yeasts can display their differences. The products of fermentation are involved with the flavor and aroma of wine; and it has been reported that these fermentation products, and the proportions that are present in the wine, are characteristic of the yeast strain and its particular enzyme system (43, p. 61; 82; 103).

Some yeast strains are noted for their ability to ferment to higher than normal alcohol content, some for their ability to ferment at refrigeration temperatures, and other strains are noted for other fermentation properties. Since it has been reasoned that these

fermentation characteristics are involved with, or are a result of, the particular metabolic process of a particular yeast strain, it was desired to study the fermentation products of different yeasts in the hope that a better understanding of the fermentation products would lead to a better understanding of why yeasts exhibit the properties they do and enable the selection of the proper yeast strain for a particular fermentation requirement.

Even though most of the compounds studied in this investigation were probably products of the yeasts' metabolic processes, they have not been designated as metabolities since some compounds, namely esters, were probably formed by the combination of metabolites (for example, ethanol and acetic acid forming ethyl acetate).

Since the advent of gas-liquid chromatography, this particular analytical tool has been utilized more and more for the analysis of organic compounds. It has been used to detect compounds in trace quantities, to separate compounds with less than $1^{o}C$ difference in boiling points and to give a qualitative and quantitative representation of aroma and flavor volatiles in a wide range of substances. It is a rapid means of separating a sample into its constituents, and the quantity of sample needed is very small (10ths of a microliter may be used).

A big advantage of gas chromatography is that there are many parameters and conditions which can be adjusted to fit the need of a

particular study. Column diameter, column length, solid support, liquid phase, percent of liquid phase, column temperature, gas flow rate and sample size are some of these.

Depending upon the instrument, other refinements can be used. If the compounds are not changed as they are detected, each can be collected for further analysis to determine its identity. Temperature programming, or raising the column temperature during analysis, gives conditions for optimum separation of compounds with wide boiling points (91).

Because of its versatility and effectiveness, gas-liquid chromatography was chosen for this analysis of the fermentation products of different yeast strains.

REVIEW OF LITERATURE

Effects of Yeast Strains

J. Ribereau-Gayon *et al.* (82) stated that the proportions of secondary products are determined by the level in the yeast cell of diverse enzymes responsible for their formation. The composition of the fermented liquid reflects in a certain measure the enzymic composition of the yeast and one can attempt to base a differentiation of yeasts, working under comparable conditions, upon this criterion.

That yeasts vary widely in their ability to ferment sugars, in the completeness of this fermentation and in the character of the by-products produced was noted by Amerine and Joslyn (2, p. 29). They found that various strains of *Saccharomyces cerevisiae* var. *ellipsoideus* (commonly called *Saccharomyces ellipsoideus*) differed in the amount and type of desirable vinous flavors they produced. Castelli (21) suggested that it should be possible to produce any desired type of wine by careful selection of yeast culture and regulation of fermentation conditions.

This was substantiated by Wahab *et al.* (100) who found that wines of different flavors and bouquets could be made by fermenting sterilized musts with pure cultures of ester-forming yeasts and completing the fermentations with *Saccharomyces ellipsoideus*.

Amerine and Cruess (1, p. 147) reported that, although a

wine-like flavor is produced in fruit juices with Saccharomyces ellipsoideus, different strains do not differ greatly in their effect on flavor. Mestre and Mestre (71) substantiated this with studies conducted on spontaneous fermentations[1] and pure cultures of Saccharomyces ellipsoideus from various regions. They also found that the wine yeasts did not differ greatly in their effect on the character of the wine, although the pure cultures gave more consistant and more satisfactory results than did the spontaneous fermentations with natural mixed cultures.

Amerine and Joslyn (2, p. 45) found that beer yeasts (commonly called Saccharomyces cerevisiae strains) gave a cereal or yeasty flavor to grape musts, whereas wine yeasts (commonly called Saccharomyces ellipsoideus strains) produced a fruity or vinous flavor even in sweet wort.

Castor (22), in an extensive comparison of the products of fermentation and flavors produced by yeasts in the fermentation of grape must, found considerable variation in the flavor of the fermented products. Amerine and Joslyn (2, p. 33) reported that products other than alcohol and carbon dioxide contributed to the flavors and aromas acquired by wines during fermentation.

There are many organic compounds reported in the literature

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[1] Ones in which the natural microflora on a fruit are allowed to bring about the fermentation.

that have been found in wines. Many are universally agreed to exist in wine, regardless of the yeast or fruit used, but as previously noted, there are differences due to the strain of yeast used. The chief products of vinous fermentation are ethanol and carbon dioxide (accounting for 94-95 percent of the sugar), glycerol, 2. 5-3. 6 percent, acids, 0.4-0.7 percent, an appreciable quantity of higher alcohols, some acetaldehyde and other aldehydes, and some esters (43, p. 31).

This is a generalization and allows for qualitative and quantitative differences in the products produced by different yeasts. For instance, some biochemists believe that certain yeasts form only 2, 3-butylene glycol and other yeasts only acetylmethylcarbinol. Others find both produced during active fermentation (1, p. 186). Amerine and Cruess (1, p.. 185) reported that yeasts differ markedly in their glycerol yield, whereas Ribereau-Gayon et al. (82) have established that the ratio of glycerol to alcohol at the end of fermentation varies but little from one yeast to another, and does not permit any differentiation.

Amerine and Cruess (1, p. 184) also reported that although it is not generally recognized that the higher alcohols of wines are of sensory importance, even at very low concentrations the higher alcohols (commonly called fusel oil) may play a desirable role in sensory quality. Webb and Kepner (103) determined that fusel oil contributes significantly to the aroma and flavor of wines and brandies.

They determined the main components of fusel oil as being n-propanol, isobutanol, active amyl alcohol and isoamyl alcohol. They suggested that variations in the proportions of these alcohols would result in variations in aroma in wines and brandies, even though other, trace, components would be of greater importance.

Amerine and Cruess (1, p. 188) reported that for normal wines, the conception that esters are an important part of the odor appears to be an exaggeration, not only because the esters normally present have little odor but because they are present in small amounts. Ethyl acetate is an exception and below about 200 mg/l has a desirable odor. Above this it appears to give a spoiled character to the wine.

Differentiation of Yeasts

Webb and Kepner (103) fermented a sterilized Sauvignon blanc must with three pure strains of yeast, chosen because they seemed to have somewhat different fermenting characteristics. The strains, obtained from the Department of Viticulture and Enology, University of California, Davis, California, were Montrachet (#522), Burgundy (#51) and Jerez (#519). Since the authors were only interested in the four main fusel oil alcohols, n-propanol, isobutanol, active amyl and isoamyl alcohols, the samples were prepared for gas chromatographic analysis by fractional distillation followed by extraction with ethyl ether. The differentiation between yeast strains was limited

to these four alcohols, but on this basis the authors were able to report highly significant variations between the yeast strains.

The Burgundy strain was a high n-propyl and a low isoamyl alcohol producer, while the Montrachet strain produced small amounts of n-propyl and large amounts of isoamyl alcohol. The Jerez strain resembled the Burgundy in production of larger amounts of n-propyl and isobutyl alcohols. However, it only produced small amounts of active amyl alcohol.

Herstein and Jacobs (43, p. 61) made note of the fact that the structure of yeasts is exceedingly simple. Despite this fact, many different strains are known to exist. To illustrate, Peynaud and Domercq (76) isolated a total of 2,023 different strains of fermentative yeasts from 96 must samples taken over a period of three seasons.

The distinctions between strains are partly morphological, that is, in the physical form of the cells, and partly chemical, that is, in the enzymes elaborated by the cells. This means that the products resulting from the action of different yeasts on the same medium or substrate will be different (43, p. 61).

Various means have been employed to differentiate between yeast strains. Saccharomyces cerevisiae var. ellipsoideus is differentiated from Saccharomyces cerevisiae largely on the basis of cell shape and inability to ferment melibiose (1, p. 144). The above

mentioned strain, Saccharomyces cerevisiae, has been considered by some microbiologists as the name to be applied to most beer yeasts, bread yeasts and distillery yeasts (1, p. 148).

Wickerham and Burton (108) have advocated the use of as many as 38 different carbon compounds in assimilation tests to obtain a comprehensive picture of the yeast being studied. This type of testing relies on a difference in the enzyme systems present in a yeast. It is the enzymes that catalyze the complex chemical changes that take place in nutrient sugar solutions and whether a carbohydrate is fermented or assimilated depends on the nature of the enzymes elaborated by the yeast (79, p. 38). A considerable number of other compounds are used in assimilation tests, including alcohols, pentoses, polysaccharides and others (26, p. 27).

Cook (26, p. 27) noted that the fermentation of sugars is one of the important characteristics of a yeast and that a variety of combinations of fermentable carbohydrates exists to assist in the differentiation of species. He further noted that the assimilation of various carbohydrates can be determined by growing the yeast in question in a complete medium containing the carbohydrate in question as the sole source of carbon.

Efforts have been made to use the vitamin requirements of a yeast in classification. It appears that the need for different vitamins has more value for the differentiation of strains than for

separating species (26, p. 30), although Wickerham (107) employed the inability to grow in a synthetic medium without vitamins as a characteristic for separating species.

Ribereau-Gayon et al. (82) performed fermentations of grape must in a study on the influence of the type of yeast. The fermentations were performed under three conditions: 1) flasks closed with a cotton plug for "improvised aerobiosis", 2) flasks closed with a rubber stopper and a glass tube so penetration of air was prevented during active release of carbon dioxide, and 3) flasks provided with a mercury bubbler for complete anaerobiosis. Under these conditions, the authors accomplished a differentiation of yeasts based upon their oxidative power and upon the respective intensities of fermentation and of respiration, and consequently upon their level of respiratory enzymes.

Gas-liquid Chromatography

History and References

In a paper in 1941 dealing with a theory of chromatography, Martin and Synge (65) mentioned the use of gas-liquid chromatography for analytical purposes. But it wasn't until James and Martin (52) published their work in 1952 on the separation and micro-estimation of volatile fatty acids by gas-liquid chromatography that any work

was reported in this area. In this first paper, James and Martin wrote that the quantity of material used would be limited only by the efficiency of the method of detection (52, p. 679).

In that first analysis by James and Martin, the detection was by a continuous automatic acid-base titration (in aqueous media) of the vapors emerging in the gas stream from the column. Since that time, the limit of detection has been lowered by the development of the gas-density meter by A. J. P. Martin in 1956. This detector recorded the instantaneous concentration of material emerging from the chromatography column so that the more familiar Gaussian-shaped peaks could be recorded automatically.

There are various references one may consult for information on gas-liquid chromatography (commonly referred to as gas chromatography). For discussions of the many aspects of gas chromatography, Gas Chromatography by Littlewood (64), Gas Chromatography by Phillips (77) and Principles and Practices of Gas Chromatography by Pecsok (75) are suggested. For articles on specific areas of gas chromatography and its applications, Gas Chromatography Abstracts (57-61) and the literature surveys by Dal Nogare (29) and Preston (80) are recommended.

Principles

Gas-liquid chromatography (gas chromatography) is so named

because it effects a separation of solutes by their affinity to be absorbed in a liquid phase. As a solute passes through a gas chromatography column, it is continuously being absorbed into the liquid phase inside the column and then being swept away by the stream of gas flowing through the column. The degree of separation of two different solutes will depend on their solubility or affinity for the particular liquid phase inside the column. The factors of vapor pressure and solute-solvent effects are generally agreed to be highly important in determining the position of a compound on a chromatogram. James (51) reported molecular weight to be the main factor in controlling elution order and that other factors including van der Waal's interactions were important. The class of compound, whether it is polar or non-polar, the type of liquid phase, the percent of liquid phase, the column length, the column temperature, the rate of gas flow and the solid support also influence the elution order of a group of compounds.

The liquid to be used is coated on a solid support such as Celite or crushed firebrick and packed into a small tube. For analysis, the tube is coiled and placed in an oven. An inert gas, such as nitrogen, helium or argon, is then made to flow through the column in such a manner that when a sample is injected on to the end of the column, it will be swept through the column.

Ideally then, the different components of a sample will become separated as they pass through the column. As they emerge from

the exit end of the column their presence will be noted by some form of detection device which will transfer the signals to a recorder. The recorder will show a graphic representation of the compounds present in the sample.

As mentioned before, there are many parameters that may be adjusted to aid a particular gas chromatographic study. One of the most important is the type and percent of liquid phase used. As the amount of liquid phase is decreased, retention times become shorter and peaks become sharper without a significant change in resolution (44).

Martin (67) observed that changes in the ratio of liquid phase to solid support caused changes in the elution order of compounds. He proposed that this was due to solute adsorption on the liquid phase and that, with polar liquids, adsorption on the liquid phase was more important than adsorption on the solid support. Martin found adsorption was important when the liquid phase was highly polar and a poor solvent, when the surface area of the solid support was high, and when the ratio of liquid phase to solid support and temperature were low. An equation was formulated taking the adsorption factor into consideration and emphasizing its importance.

It has also become preponderantly apparent that besides the amount of liquid phase coating, the surface area and activity of the solid support significantly contribute to the overall gas chromatographic

behavior of polar solutes (9; 96).

The foregoing have been examples of the complexities of gas chromatography. For equations for determining optimum operating conditions, the article, "Principles of high-speed gas chromatography with packed columns", by Ayers et al. (5) is suggested.

Sample Preparation

Some workers have injected wine directly into a gas chromatograph. Kabot and Ettre (53) performed direct analyses on many alcoholic beverages using a Perkin-Elmer gas chromatograph equipped with a flame ionization detector. Although they only obtained ten peaks in their analysis of wine, their results indicate that direct analysis of alcoholic beverages by gas chromatography is possible, using the flame ionization detector and the proper column substrate material.

Obviously, the less preparation a sample has to be given, the less chance there is that the sample will be changed. The direct sampling and injection of a wine is the simplest and would theoretically be the most representative analysis of the volatile constituents of that wine. However, ethanol is in such great concentration in proportion to the other volatiles it makes direct sample injection very difficult.

Various workers have distilled their fermented samples to

concentrate the volatile constituents (6; 17; 36; 55; 98). Others have utilized solvents to extract the volatile constituents from alcoholic beverages (12; 70; 69; 86; 101). Bills et al. (11) reported an extraction procedure for determination of the free fatty acids of milk fat. They also described a low temperature distillation apparatus for removal of their solvent.

Mecke and de Vries (70) used a combination of ethyl ether and n-pentane (2:1) to extract alcoholic beverages. They extracted 300 ml of the beverage three times with 50 ml of solvent each time. In a later work, Mecke et al. (69) used essentially the same procedure with slight modification. They extracted 500 ml of the beverage three times with a total of 200 ml of the solvent. A further modification was the back extraction of the solvent phase six times with 50 ml of water to remove ethanol from the aroma fraction.

Singleton (86) employed a technique that suggests that Mecke and co-workers were losing aroma compounds with the back extraction with water to remove ethanol. Singleton's technique involved adding a salt (ammonium sulfate gave the best results) to a water-solvent mixture (such as wine) to separate the mixture into two phases. He stated that this system gave the advantage of an increased distribution coefficient. Thus a greater degree of concentration and a more efficient extraction of relatively polar substances resulted than with immiscible solvent systems.

Another extraction procedure involved working with the equivalent of 40 ml of 90 proof (45 percent) alcohol (12). This amount was added to a 100 ml volumetric flask, to which was added, with mixing, 10 ml of a two percent solution of polypropylene sebacate in ethyl ether. Water was added to bring up to the mark, and the mixture intimately mixed with a slow release of pressure. The phases separated after standing 15-20 minutes and the sample for injection was in the upper layer.

A few workers have combined distillation and extraction to concentrate wine volatiles (28; 68; 103). Webb and Kepner (103) and Crowell et al. (28), in studying fusel oil, distilled their wine samples prior to extraction with ethyl ether. Mathews et al. (68) used a rising film evaporator to concentrate wine volatiles five fold. Pentane, isopentane and ethyl chloride were tried as extracting solvents before ethyl chloride was chosen because of its ease of evaporation at room temperature and its higher yield of wine volatiles. A 1000 ml sample of wine was saturated with sodium chloride and 150 ml portions of this were extracted with 15 ml of ethyl chloride in a separatory funnel. The funnel was manipulated for one minute every five minutes for 30 minutes.

Another procedure tried by Mathews et al. was unsuccessful. This involved saturating the distilled sample with calcium chloride and bubbling nitrogen through it. The volatiles were trapped by

1) dry-ice and ethyl benzene and 2) dry-ice, alcohol and liquid oxygen. A similar procedure was used by Austin and Boruff (3) who bubbled helium through grain spirits and collected the volatile components in a dry-ice trap.

Quantitative Determinations

Gas chromatography has been used successfully to determine flavor and aroma constituents quantitatively (36; 54; 73; 74; 78). Gadsden and McCord (32) obtained quantitative data by referring to a standard curve based upon peak heights of standard compounds of varying concentrations. (Reproducibility, based upon their described technique, was within five percent). They reported that methods such as curve area analysis and those using an internal standard yielded no greater consistency of analysis. They further noted that comparison of peak heights would represent a fairly large error. In contrast, Özeris and Bassette (74) in their quantitative study on dilute aqueous solutions, obtained results using peak heights which varied no more than five percent with duplicate injections.

When tailing of peaks is encountered as a problem, the percent composition of samples can be calculated using the rectangular method of geometric approximation (78). With this method, commonly called retention volume, the retention time is multiplied by the peak height for each peak. The values are added and each peak's

percent composition can then be calculated.

In some instances an internal standard may be used to advantage. Kepner et al. (54) used this technique in the analysis of the volatile components of multicomponent aqueous solutions. They used 3- or 4-heptanone for their analyses at 55°C and 4-heptanone at 65°C. Calibration curves were used for quantitating their data. They used the rectangular method of geometric approximation but in a different manner than previously described. The value of peak height times retention time was calculated for each peak and for the internal standard. The value of the internal standard was divided into each peak's value. The quotient of this division was referred to a calibration curve where the concentration of each peak in parts per million was found.

Morrison (73), in his determinations of ethanol in wine with gas chromatography, used acetone as an internal standard and noted the method to be a very accurate one for ethanol determination.

Bills et al. (11) employed internal standards to improve the accuracy of their determinations of the free fatty acids of milk fat. They used heptanoic acid for their determinations at 94°C and heptadecanoic acid at 194°C.

Temperature Programming

Temperature programming has been used by numerous workers

in the area of gas chromatography (3; 24; 30; 31; 34; 91; 97). Workers in this area have reported an improvement in peak shape and in the distribution of peaks which results from programming the column temperature. A linear programmed temperature operation offers the advantages of 1) a relatively uniform resolution of peaks over the entire chromatogram, 2) its applicability to mixtures of wide boiling points, 3) speed and flexibility of operation (31). Retention time and peak height have been reproduced to better than two percent with careful manipulation (31).

Sullivan et al. (91) agreed that conditions for the optimum separation of compounds with wide boiling points were fulfilled when the column temperature was gradually increased. Since they felt that a linear programmed temperature operation necessitated the use of elegant temperature and flow controllers, they worked with non-linear programming. Their procedure involved having the column oven turned off and at room temperature, but with the oven thermostat turned on full scale. The sample was then injected and the oven switch turned on. They concluded that although better separations could be achieved with linear programming, non-linear programming gave comparable results and did so more conveniently.

EXPERIMENTAL PROCEDURE

Sample Preparation

Yeasts

The yeast cultures chosen for this study were: 1) a strain of Saccharomyces ellipsoideus used in the Enology Laboratory, Food Science Department, Oregon State University; 2) a strain of Saccharomyces oviformis (A-XVI) obtained from the Pasteur Institute, Paris, France; 3) a strain of Saccharomyces bayanus (A-36) also obtained from the Pasteur Institute; and 4) a strain of Saccharomyces cerevisiae obtained from a package of Fleischmann's yeast.

The yeasts were grown on agar slants with the following composition: two percent dextrose, one and one-half percent Bacto-Agar and one percent Bacto-Peptone.

Medium

The medium for the fermentations was blackberries. The berries had been commercially packed in 30# metal cans and frozen without the addition of sugar.

For each fermentation, one pound of berries was weighed into a 1000 ml erlenmeyer flask and set in hot water to thaw. After thawing, the flask was inoculated with a culture of yeast and placed

in a 34°C incubator for 24 hours to obtain an active fermentation. Fifty-three g of dextrose dissolved in 158 ml of water were added and the mixture left at room temperature to carry on its fermentation. Two days later an additional 53 g of dextrose dissolved in 158 ml of water were added. Two days later the last addition of 53 g dextrose was made. Berry juice, from the fermentation, was used to dissolve the last portion of dextrose prior to its addition to the fermentation flask. Once each day the flask was swirled to bring the berries in contact with the fermenting liquid. This was necessitated by the fact that the berries were buoyed up above the liquid by the release of carbon dioxide.

Depending upon the strain of yeast, the fermentation was completed in seven to eight days; it was considered complete when a Brix reading of -0.8° to -1.5° was obtained.

When this reading was obtained, the wine was poured through cheese cloth to separate it from the berry pulp and seeds. The pulp retained in the cheese cloth was squeezed slightly to ensure a yield of wine of approximately 500 ml.

Extraction

The wine was transferred to a 500 ml erlenmeyer flask and allowed to settle. After five hours the wine had clarified sufficiently so that 100 ml could be transferred to another flask. Twenty g of

sodium chloride were added to the 100 ml of wine to enhance the separation of the volatile constituents (59). The flask was rotated gently to help dissolve the sodium chloride and then allowed to stand for five hours. This time interval was chosen arbitrarily because it blended well with the overall extraction procedure. A flask was allowed to stand overnight to determine the effect on the wine of an extended period of time in contact with the salt. This resulted in a loss of volatiles and was discarded.

After the five-hour period, 100 ml of wine were decanted from the undissolved sodium chloride and poured into a 150 ml separatory funnel. The funnel and all other glassware needed in the extraction were taken to a cold room kept at or near $2^{\circ}C$. The wine and glassware were allowed to cool for one-half an hour before extraction was begun.

Forty ml of ethyl chloride were added to the cooled wine in the separatory funnel and the funnel rotated approximately 180°, 25 times. This was done gently since brisk rotation resulted in the formation of an emulsion between the wine and ethyl chloride. After mixing, the funnel was allowed to stand for ten minutes before it was rotated again. After another ten minute waiting period, the funnel was rotated for a third time. Then, after ten minutes, the aqueous phase was separated from the solvent phase. The solvent phase was collected in a small erlenmeyer flask and stoppered.

Forty more ml of ethyl chloride were added to the wine in the separatory funnel and the extraction procedure followed as above. This second 40 ml of ethyl chloride was added to the wine, and after extraction, was poured in the flask containing the previous solvent fractions. This flask, containing the solvent and its extracts, was stored in a refrigerator overnight.

Distillation

The ethyl chloride was removed using a low temperature distillation apparatus (see Bills *et al.* (11) for a schematic diagram). The solvent was poured into a 50 ml pear-shaped distilling flask immersed in a 21°C water bath. The vapors were refluxed with a Bantam-ware condenser maintained at a temperature of 11 1/2 to 12°C. With this apparatus, the extract was concentrated down to a 1/2 ml sample. This was transferred to a three ml conical centrifuge tube, stoppered, and stored in a refrigerator until it was chromatogrammed.

Gas Chromatographic Analysis

Operating Parameters

The instrument used in this study was an Aerograph Hy-Fi, model A-600-B, with a hydrogen flame ionization detector.

For the analyses of the blackberry wine extracts the following were standardized: an eight foot X 1/8 inch stainless steel column packed with five percent Carbowax 400 on 80/100 mesh acid-base treated Celite 545; column temperature was maintained at 100°C and injector port at 150°C; the nitrogen and hydrogen flow were both 25 cc/minute and the air flow was 320 cc/minute; the instrument was operated at 1/10th of its maximum sensitivity, with an input impedance of 10^9, an output sensitivity of 1X and an attenuation of 1; the sample size was 0.2 μl and the chart speed was 15 inches per hour.

The column described above and other columns used were prepared by dissolving the appropriate stationary phase in 40-50 ml of solvent (usually acetone, but chloroform and ethyl ether were used). This was poured onto acid-base treated Celite in a beaker. While air was blown across the surface to evaporate the solvent, the slurry was gently stirred and mixed to coat the stationary phase evenly on the Celite. When the mixture was drier, it was packed on the sides of the beaker and dried thoroughly by either being placed in a vacuum oven or by applying heat and continuing the stream of air.

One end of the desired length of stainless steel tubing was plugged with spun glass and connected to an aspirator. With the other end fitted with a funnel to receive the prepared column packing, the column was packed using the aspirator and a hand vibrator. About 0.38 g of material were used per foot of column.

The column was coiled around a glass bottle in such a manner that a tight coil, 2 1/2 inches in diameter, was obtained. This was installed in the gas chromatograph and purged, with the oven on and nitrogen flowing through the column. Wilkens Instrument and Research, Inc. recommended maintenance of an oven temperature 50°C above the expected operating temperature for at least 24 hours. This recommendation was followed except in those cases where the expected operating temperature was at or close to the decomposition temperature for a particular liquid phase.

Calculation of Percent Composition of Chromatographic Peaks

The recorder used in this study was a Honeywell Brown strip chart recorder, class 15, with a one millivolt (mv) full span sensitivity. This one mv full span sensitivity was the basis for calculation of the percent composition of the wine extracts.

The height or recorder response of each peak was measured in tenths of a mv. This value was multiplied by the attenuation factor (amount instrument sensitivity was reduced to keep peak on scale) to obtain the peak's full weighted value. The weighted values for all the peaks were added and the sum used as a divisor in determining the percentage contribution of each peak.

Tentative Identification of Fermentation Products Using the Enrichment Technique

Standards, chemically pure compounds, were added to the extract concentrate to tentatively identify the products produced by the fermentations. The identification was accomplished by noting which peak was increased in height after the addition of a standard compound.

RESULTS AND DISCUSSION

Yeast Strains

The yeasts chosen for this study were selected because of their different fermentation characteristics. The culture of Saccharomyces ellipsoideus was selected because this is the species commonly used in wine production and because it is the standard strain used in the Oregon State University Enology Laboratory for the studies involving fermentation and for distribution to wineries and individuals interested in making wine.

Saccharomyces bayanus is a species noted for its ability to carry on fermentation at refrigeration temperature and for its use in the production of champagne.

Strains of Saccharomyces oviformis are present in very slight amounts at the beginning of most spontaneous fermentations and, as the fermentation proceeds, may increase to over one-third of the strains present, being only less dominant than Saccharomyces ellipsoideus (76).

Saccharomyces oviformis can be very helpful in white wine making or it can be very harmful. Since it has a high tolerance of alcohol, its contamination of a finished sweet wine can cause refermentation to occur. However, if a dry wine is desired, this species should be used, since it ferments to dryness, does it much

more quickly than other yeasts and gives the same fine fruity, pleasant odor as Saccharomyces ellipsoideus (76).

When red wines have stopped fermenting with fermentable sugar remaining, Saccharomyces oviformis is the only yeast that will complete the fermentation at the time the temperature rises in the spring (76).

Saccharomyces cerevisiae was chosen because it is commonly agreed to be the yeast used for beer. It has different fermentation characteristics than wine yeasts and has been found to give a yeasty flavor to grape musts (2, p. 45).

Gas Chromatographic Variations

The following liquid phases were tried and found less satisfactory than Carbowax 400 for this study. They failed to give as great a resolution or bled excessively at the temperatures desired for analysis. Carbowax 20M, Carbowax 600, Carbowax 1000, Carbowax 1540, Diethylene glycol adipate (DEGA), Diethylene glycol succinate (DEGS), Apiezon L, SE-30, Halcomid-18-M, Ucon polar 50-HB-2000, Versamid 900, b, b'-oxydipropanitrile, diisodecyl phthlate and 1, 2, 3-tris (2-cyanoethoxy) propane were those discarded.

Each of the above was not tried under all the variations of column packing, that is with respect to column length, percent of liquid phase, mesh size of Celite and solid supports. However,

even though an extensive study of column lengths and percent liquid phases was not carried out, some comparisons were made. It was noted that large differences in column length did effect resolution but not to a great extent. Large differences in percent liquid phase also effected the separation; larger percentages of coating retained the compounds more and were more likely to cause tailing.

The solid support, Celite 545, is not a totally inert substance. It has active sites which bind oxygenated compounds and cause them to tail.

Since considerable tailing of ethanol was encountered in this study, a compound for tieing up these active sites on the Celite was tried. The compound used was hexamethyldisilizane (HMDS) but its addition did not reduce the tailing of ethanol. Knight (62) reported tailing to be due to the nonideal adsorption of the sample and suggested the addition of a strongly adsorbed material continuously with the carrier gas to reduce tailing. Thus tailing in water and alcohol solutions can be reduced by passing the carrier gas through a tube of moistened C-22 (firebrick) according to Knight.

A technique similar to this was tried using steam as the carrier gas with a Versamid 900 column, and a steady baseline was obtained. The chromatograms contained a great number of peaks but they were not reproducible. It was thought that the steam caused the random formation of artifacts and was discontinued on this basis.

Sample Injection

Before the decision was made to extract the wine with ethyl chloride, efforts were made to obtain a chromatogram of the wine by injecting samples of the finished wine directly into the gas chromatograph. Since the samples contained pigment and any residual sugar, there was some concern that these non-volatiles might collect at the opening of the column and interfere with subsequent injections. To counteract this, a pyrex injector insert was fitted into the injector port to collect these non-volatiles before they reached the column.

These efforts to obtain a meaningful chromatogram by direct sample injection were unsuccessful. The best chromatograms showed only three peaks before and three peaks after ethanol and the peaks after ethanol were very short. Ethanol was in such great predominance in the sample that it tailed very badly and limited the sample size. Similarly alcohol dominance and a limited number of peaks was encountered with head space analysis.

It would be desirable to remove ethanol from wine prior to analysis. However, the methods of alcohol removal found in the literature were not selective to ethanol.

Ikeda et al. (47) removed all alcohols during gas chromatography by adding a small quantity of boric acid to the last few inches of their gas chromatographic column. Alcohols may also be removed

by using 3, 5-dinitrobenzoyl chloride; this forms a derivative which is soluble in water and crystalline in non-aqueous solvent. The electron capture detector is insensitive to alcohols and has also been used when it is not desired to record the presence of alcohols.

Quantitation of Chromatograms

Initially attempts were made to compare peak heights directly from one chromatogram to another. This would have removed any necessity of calculating the percent composition of each peak in a chromatogram. A ten μl syringe was first used to inject 0.2 μl samples. However, the needle on this syringe was able to retain one μl of sample. Since the needle was introduced into a 150°C injector port during sample injection, some of the sample normally retained in the needle was vaporized. This of course caused considerable variation between samples. Even with the injection technique repeated as precisely as possible, quantitative reproductions were not possible.

A one μl syringe with a wire plunger extending into the needle was also tried. This type of syringe did not leave any sample in the needle and fair success was achieved with this syringe in duplicating peak heights with subsequent injections. However, since the plunger was very thin and delicate the syringe was equipped with a metal, tubular plunger guide that prevented observation of the sample being drawn into the barrel of the syringe. This was a serious handicap when working with the concentrated wine extracts. Some ethyl

chloride was present even after distillation, and this was responsible for the formation of bubbles within the syringe. With the ten μl syringe, the plunger could be manipulated until it could be seen that the barrel was devoid of bubbles. This was not possible with the one μl syringe however.

The retention volume and calculation of peak areas methods were evaluated for use in this study but the previously described method of using recorder response was found to give the best results and did so with a minimum of calculation. A factor which contributed to the undesirability of calculating the peak areas was noted by Bills et al. (11). They found their highest standard deviations in peaks which eluted rapidly and had narrow bases and they felt this was partly due to the difficulty of measuring such peaks accurately. That difficulty was also encountered in this study.

Measurement of recorder responses was used for comparing peaks between chromatograms and not to give an absolute percent composition of a given wine extract. To do the latter would have required that all the peaks of the chromatogram had the same configuration. As it was, the initial peaks were very sharp, but as peaks continued to elute they became broader. Under this situation, a peak eluted one hour after injection could be of the same height as a peak eluted one minute after injection but the later peak, being broader, could represent twice as much compound.

A disc integrator was also used to record the area under peaks but because of the baseline shift, the closeness of the initial peaks and the failure of peaks to return to the baseline, its use was discontinued.

In using the recorder response method to calculate the percent composition of the chromatogram peaks, the ethanol peaks were not used in the calculations. Ethanol was present in such a large quantity in the extract concentrate that the signal sent from the detector saturated the electrometer tubes. This was evidenced by flat tops on the ethanol peaks, such that they were not being recorded in their true concentrations. To correct this, the input impedance would have had to have been changed from 10^9 ohms to 10^7 ohms, a reduction of sensitivity by 100 times. Since this was not possible during the analysis of a sample and since ethanol was a standard 13.0 percent in all wine samples, the ethanol peaks were not included in the calculations.

Evaluations of Chromatograms

Small variances in oven temperature and in sample injection resulted in changes in retention times. As a result, when comparing peaks from chromatogram to chromatogram, peaks with the same relative retention times and the same characteristic peak formation were assumed to be the same peaks.

Peak number one was undistilled ethyl chloride. Peaks number two, four, five, and seven comprised the main portion of the extract concentrate. Together they represented approximately 99 percent of the sample, excluding ethyl chloride and ethanol, as determined by the recorder response method.

The percents shown in Table I represent fermentations performed in triplicate and chromatographed in duplicate, with the absolute percent deviation within a sample being less than four percent.

Figure 1 is a graphic representation of the major peaks and illustrates their relative percent compositions. As can be seen from that figure and from the following discussion, the yeast strains have produced fermentation products in significantly different concentrations. These differences could serve as supplementary tests for the identification of yeast strains and might well be clues to the different fermentation properties of the yeasts.

Saccharomyces ellipsoideus (Figure 2) was a high producer of peak number two with 24.90 percent. This was a significant difference between the two intermediate strains, Saccharomyces bayanus (Figure 3) and Saccharomyces cerevisiae (Figure 4), at 19.93 and 18.86 percent respectively. Saccharomyces oviformis (Figure 5) was distinctive with a low production of only 10.86 percent.

Saccharomyces oviformis distinguished itself again with a very high production, 19.78 percent, of peak number four. This was

TABLE I

The Average Percent Composition of the Major Peaks Obtained from Chromatographing the Ethyl Chloride Extracts of the Wines Made with Different Yeast Strains

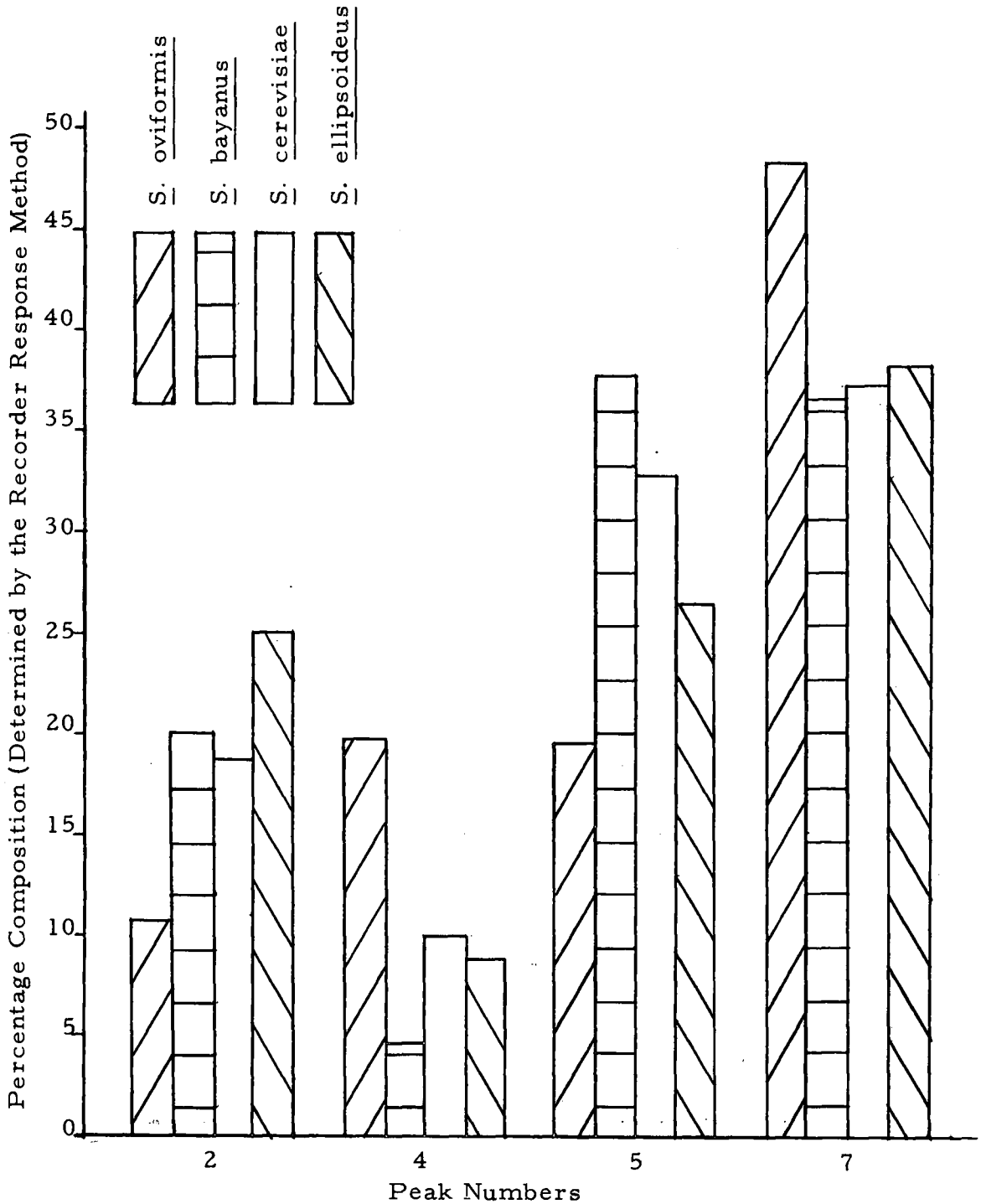
Peak Number	Retention Time (minutes)	S. oviformis	S. bayanus	S. cerevisiae	S. ellipsoideus
2	0.99	10.86	19.93	18.86	24.90
4	1.76	19.78	4.84	9.97	8.75
5	2.08	19.46	37.83	32.75	26.23
7	3.70	48.18	36.74	37.41	38.43

TABLE II

The Percent Composition, Expressed as a Range, of the Peaks Obtained from Chromatographing the Ethyl Chloride Extracts of the Wines Made with Different Yeast Strains

Peak Number	Retention Time (minutes)	S. oviformis	S. bayanus	S. cerevisiae	S. ellipsoideus
6	2.80	0.51-0.68	0.05-0.12	0.19-0.51	0.83-1.12
8	6.35	0.05-0.13	0.02-0.04	0.04-0.05	0.06-0.08
9	8.06	0.03-0.08	0.08-0.13	0.04-0.07	0.05-0.10
10	9.86	0.36-0.43	0.04	0.10-0.13	0.14-0.21
11	16.60	-----	-----	0.01	0.00-0.06
12	17.60	0.00-0.08	0.05-0.09	0.00-0.03	0.04-0.06
13	20.30	0.04-0.13	0.05-0.08	0.03-0.10	0.00-0.09
14	27.60	0.14-0.18	0.07-0.08	0.05-0.06	0.06-0.17
15	34.20	0.01-0.02	0.01	0.01-0.02	0.01-0.03
16	55.30	-----	-----	trace	-----
17	71.40	0.05-0.07	0.01-0.03	0.01-0.02	0.01-0.02
18	102.00	0.05-0.06	0.05-0.07	0.07-0.12	0.07-0.13
19	138.50	0.16-0.26	0.14-0.18	0.15-0.18	0.11-0.25

Figure 1



Peaks Comprising 99 Percent of the Ethyl Chloride Extracts of Wines Fermented with Different Yeast Strains

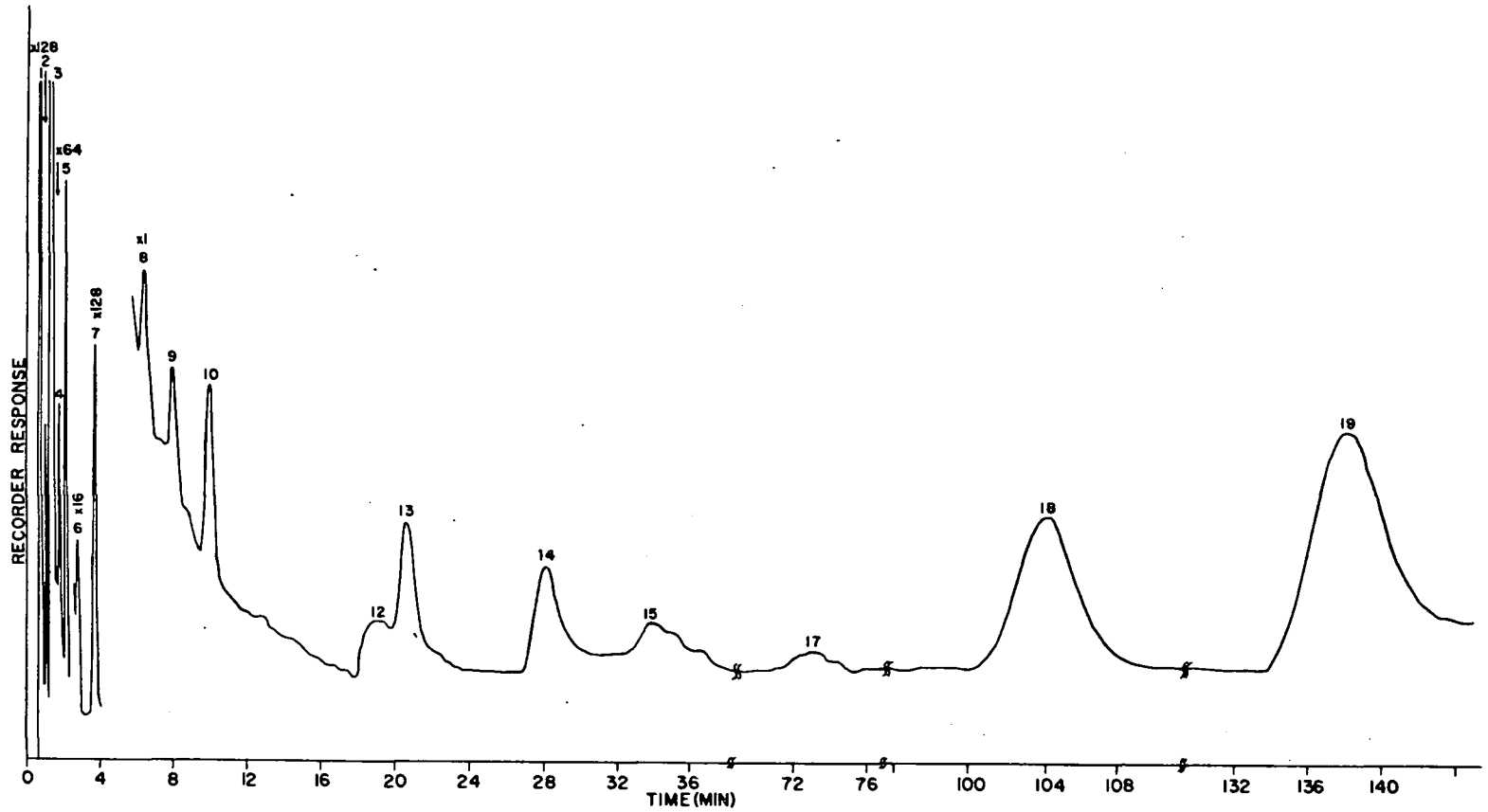


Figure 2. Gas chromatogram of wine fermented with Saccharomyces ellipsoideus

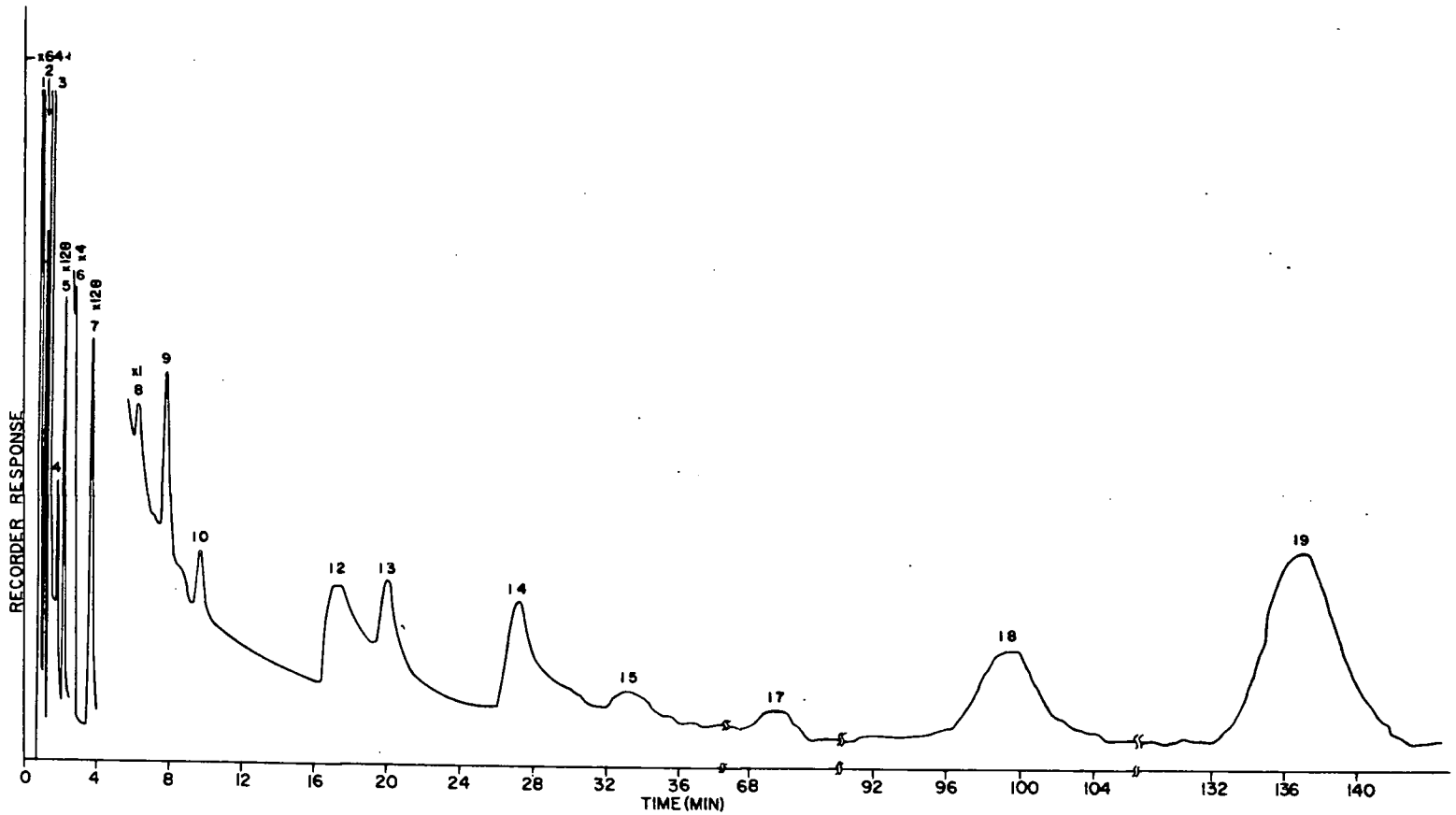


Figure 3. Gas chromatogram of wine fermented with Saccharomyces bayanus.

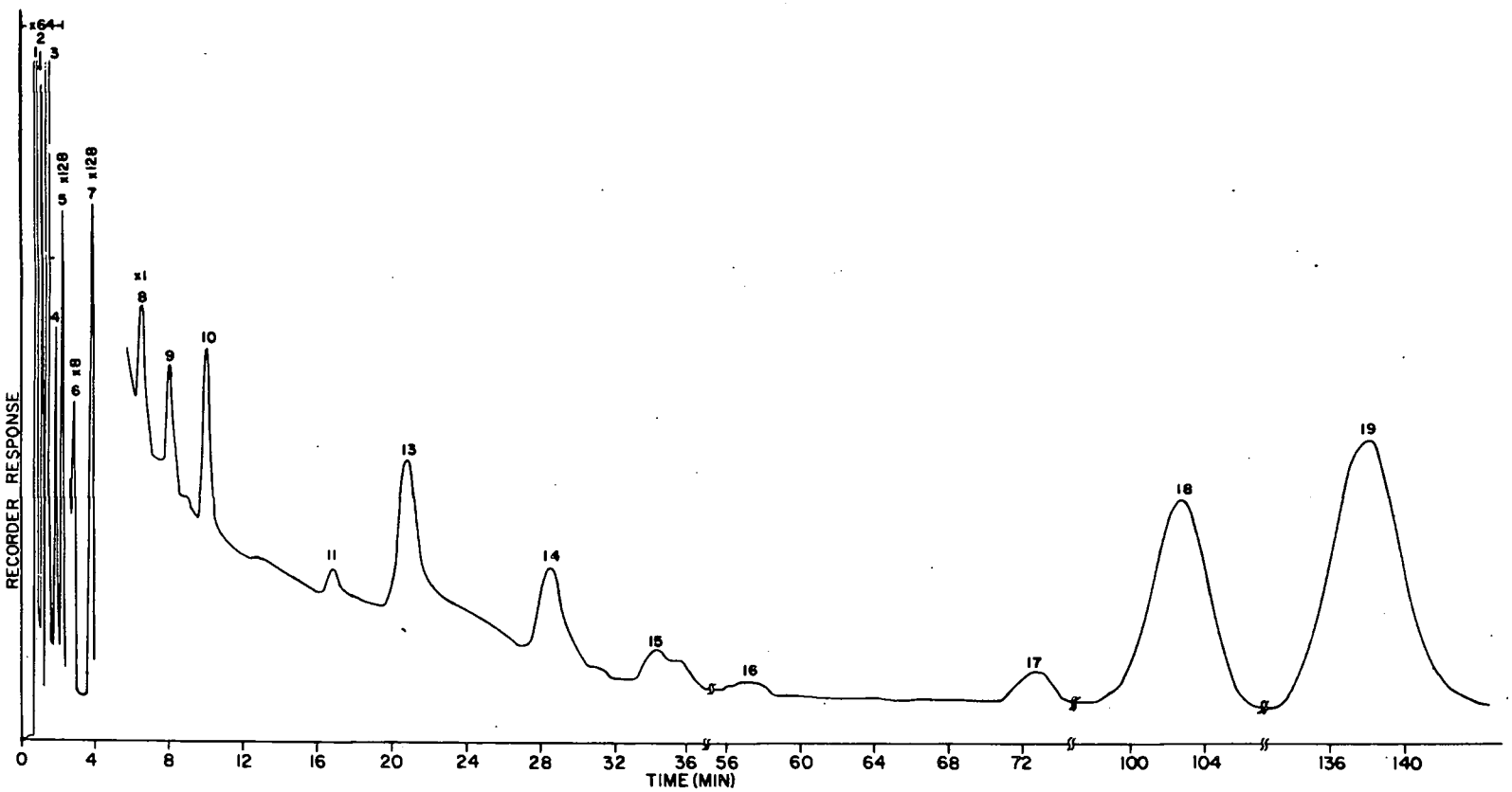


Figure 4. Gas chromatogram of wine fermented with Saccharomyces cerevisiae.

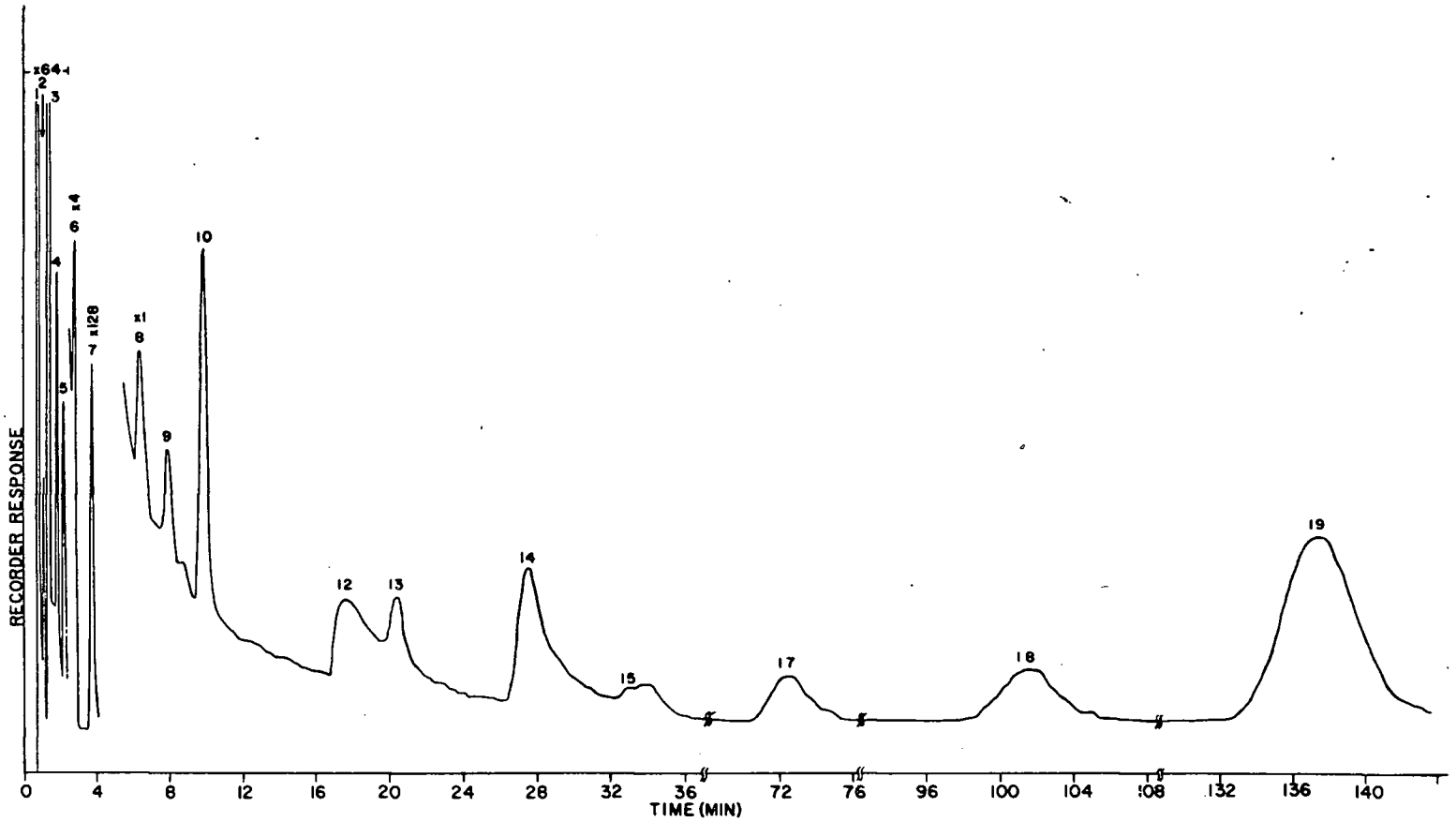


Figure 5. Gas chromatogram of wine fermented with Saccharomyces oviformis

very meaningful since the next highest production of peak number four was 9.97 percent by Saccharomyces cerevisiae. Saccharomyces ellipsoideus was next with 8.75 percent. Saccharomyces bayanus, with a production of 4.84 percent, was fairly well separated from these latter two strains and widely separated from Saccharomyces oviformis.

Saccharomyces oviformis was further differentiated by peak number seven. Saccharomyces cerevisiae, Saccharomyces ellipsoideus and Saccharomyces bayanus yielded a concentration of this peak within two percent of each other, 36.74 to 38.43 percent, whereas Saccharomyces oviformis was ten percent in excess of these three with 48.18 percent.

The strains differed completely in their production of number five, but varied fairly uniformly from a low of 19.46 percent with Saccharomyces oviformis to a high of 37.83 percent with Saccharomyces bayanus. Intermediate were Saccharomyces ellipsoideus at 26.23 percent and Saccharomyces cerevisiae at 32.75 percent. Since this peak was produced by all four strains in different concentrations, it is especially significant as a means of differentiating yeast strains and might be very meaningful to a better understanding of their fermentation characteristics.

Peaks number six and eight through 19 only represented approximately one percent of the extract concentrate. Their percentage

composition has been reported as a range (Table II) and except for isolated values, offers little as a means of differentiating yeast strains.

Saccharomyces bayanus was a low producer of peak six with a range of 0.05 to 0.12 percent. This was much lower than the 0.51 to 0.68 percent production by Saccharomyces oviformis and the 0.83 to 1.12 percent production by Saccharomyces ellipsoideus. Saccharomyces cerevisiae was higher too but with a much broader range, 0.19 to 0.51 percent.

Saccharomyces oviformis produced peak number ten in a significantly greater amount, 0.36 to 0.43 percent, than any other strain, especially in comparison to Saccharomyces bayanus with 0.04 percent. and even with Saccharomyces cerevisiae at 0.10 to 0.13 percent and Saccharomyces ellipsoideus at 0.14 to 0.21 percent.

Peak number 11 was absent in Saccharomyces oviformis and Saccharomyces bayanus. It was present in very small concentration in Saccharomyces cerevisiae and was present in one of the three preparations of wine made with Saccharomyces ellipsoideus. Its absence or presence would serve to differentiate Saccharomyces oviformis and Saccharomyces bayanus from Saccharomyces cerevisiae and even Saccharomyces ellipsoideus from Saccharomyces oviformis and Saccharomyces bayanus if enough preparations of wine were made.

Peak number 12 was very elusive. It was found in fair concentration in all samples of Saccharomyces bayanus and Saccharomyces

ellipsoideus but only in two of three samples of Saccharomyces oviformis and in only one of Saccharomyces cerevisiae. On this basis it would seem to be of little value as a means of differentiating between yeast strains.

Peak number 13 was found in all samples of all strains. It was reported as 0.00 percent in one sample of Saccharomyces ellipsoideus but that was because it did not distinguish itself as a peak in that sample. Rather, it was present only as a shoulder to peak 12, which distinguished itself as a rather large peak in that sample.

Peak number 16 was evident only in Saccharomyces cerevisiae and was present in trace quantities, 0.002 to 0.007 percent. Even in trace amounts this peak may represent a distinguishing characteristic of the cerevisiae species, since this species has been found to give a cereal or yeasty flavor to grape musts.

In peak number 17, Saccharomyces oviformis again distinguished itself from the other strains. The other strains produced number 17 within a range of 0.01 to 0.03 percent whereas Saccharomyces oviformis produced 0.05 to 0.07 percent of this compound.

Saccharomyces bayanus, 0.02 to 0.04 percent, and Saccharomyces cerevisiae, 0.04 to 0.05 percent, had peak number eight in low concentration as versus the relatively higher concentration of 0.05 to 0.13 percent in Saccharomyces oviformis. Saccharomyces ellipsoideus was intermediate with 0.06 to 0.08 percent.

Saccharomyces oviformis and Saccharomyces bayanus, both within a range of 0.05 to 0.07 percent, were distinguished slightly from Saccharomyces cerevisiae and Saccharomyces ellipsoideus, both within 0.07 to 0.13 percent, in peak number 18.

It should be noted that the percentage composition for some peaks was a very narrow range for some yeast strains and wide for other strains. This was particularly evident in peaks number 14 and 19. Saccharomyces bayanus, Saccharomyces cerevisiae and Saccharomyces oviformis had a very narrow range of peak number 14, 0.07 to 0.08, 0.05 to 0.06 and 0.14 to 0.18 percent respectively; Saccharomyces ellipsoideus had a range encompassing all three of the other strains, 0.06 to 0.17 percent. It was not known whether this range represented a variance due to the nature of the compound, or was a characteristic of the yeast strain.

This same situation was evident in peak number 19. Saccharomyces bayanus and Saccharomyces cerevisiae had narrow ranges, 0.14 to 0.18 and 0.15 to 0.18 percent respectively; Saccharomyces oviformis was broader with 0.16 to 0.26 percent but Saccharomyces ellipsoideus encompassed them all with a range of 0.11 to 0.25 percent.

Peaks number nine and 15 offered little as means of differentiating yeast strains. Number 15 was especially true in this respect with a range for all four strains of 0.01 to 0.03 percent.

In this study the samples of wine were extracted right after fermentation was completed. To gain some idea of the effects of letting the wine stand for a period of time, one sample of wine fermented with Saccharomyces ellipsoideus was allowed to stand for 27 days before it was extracted. After this period of time it was treated in the same way as the previous samples; sodium chloride was added, the wine was extracted and then concentrated on the low temperature distillation apparatus.

A study of Table III shows that there is very little difference between the percentage compositions of the wine extracted immediately after fermentation was completed (0 days) and of the wine extracted after standing 27 days.

The greatest difference was in peak number nine. After 0 days, this peak represented 0.08 to 0.10 percent of the extract concentrate, while after standing 27 days it represented 0.51 to 0.53 percent.

Two other changes were in peaks number eight and 15. Peak number eight increased from a range of 0.06 to 0.07 percent to a percentage of 0.20, and number 15 increased from 0.01 percent to a range of 0.06 to 0.07 percent.

Another change, of less magnitude, was in peak number 18. Here a decrease in size was noted, from a range of 0.07 to 0.08 percent down to 0.04 to 0.05 percent.

The other peaks did not differ from one another to a significant

TABLE III

The Percent Composition of the Peaks Obtained from Chromatographing the Ethyl Chloride Extracts of Wine Fermented with Saccharomyces ellipsoideus and Aged 0 to 27 Days*

Peak Number	Retention Time (minutes)	0 Days	27 Days
2	0.99	24.30	23.60
4	1.76	10.18	9.97
5	2.08	27.08	27.20
6	2.80	0.86	0.66
7	3.70	37.00	37.43
8	6.35	0.06-0.07	0.20
9	8.06	0.08-0.10	0.51-0.53
10	9.86	0.19-0.21	0.21
12	17.60	0.05-0.06	0.06-0.07
13	20.30	0.00-0.004	0.00-0.00
14	27.60	0.06	0.07-0.08
15	34.20	0.01	0.06-0.07
17	71.40	0.02	0.02
18	102.00	0.07-0.08	0.04-0.05
19	138.50	0.11-0.12	0.12-0.13

*The percentage of peaks number one through seven was expressed as an average; the percentage of peaks number eight through 19 was expressed as a range.

extent. The larger peaks, number two, four, five, six and seven, were especially true in this regard with the greatest difference between the 0 days and 27 days samples being only 0.70 percent.

The sample of wine allowed to stand for 27 days was the same sample in which peak number 13 was only a shoulder to peak number 12 and therefore reported as 0.00 percent. It can be seen from Figure 6 that peak number 13 is still present as a shoulder to peak number 12.

Aging is generally agreed to effect the flavor and aroma of wine. However, few differences were noted in the wine aged 27 days and the wine analyzed right after fermentation was completed. Since the variances were small, a longer aging period might produce more and greater differences and it is possible that these differences, even though small, effected changes in the aroma and flavor of the wine. It is also likely that flavor and aroma were influenced by compounds not detected in this study.

Tentative Identification of Some Fermentation Products

Utilizing the enrichment technique, a tentative identification was made of peaks number two, three, four, five, six, seven, nine, ten, thirteen and fourteen (see Table IV).

Reference was made to the literature to find what compounds had been previously identified as fermentation products and as many

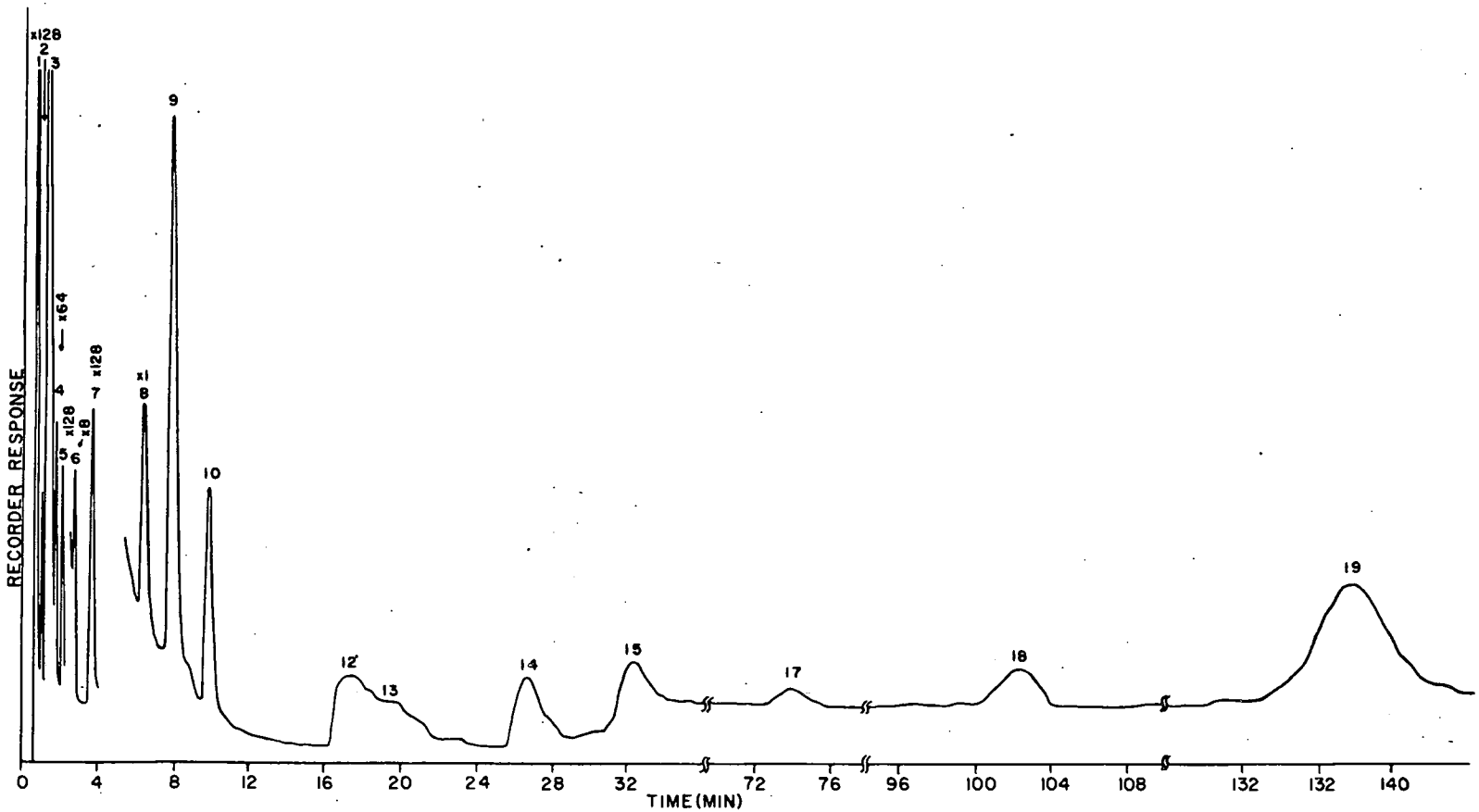


Figure 6. Gas chromatogram of wine fermented with Saccharomyces ellipsoideus and aged 27 days.

TABLE IV

Tentative Identification of Some of the Fermentation Products of the Yeast Strains Using the Enrichment Technique

Peak Number	Retention Time (minutes)	Standard Compounds Found to Increase Peak Heights	Compounds Probably Present in Wines
2	0.99	Ethyl acetate	Ethyl acetate
3	1.31	Ethyl alcohol	Ethyl alcohol
4	1.76	Propyl alcohol Ethyl isovalerate	Propyl alcohol
5	2.08	Isobutyl alcohol	Isobutyl alcohol
6	2.80	Butyl alcohol Amyl acetate	Butyl alcohol
7	3.70	Isoamyl alcohol Ethyl hexanoate Active amyl alcohol	Isoamyl alcohol Active amyl alcohol
9	8.06	Glycerol Methyl octanoate	Glycerol
10	9.86	2, 3-butylene glycol Ethyl octanoate	2, 3-butylene glycol
13	20.30	Linaloöl	Linaloöl
14	27.60	Ethyl decanoate	Ethyl decanoate

of these as possible were obtained in the chemically pure state to use as standards for identification. These standards were added, one to three at a time, to the previously chromatographed extract concentrates and the peak height increases noted.

In four cases, peaks number four, six, nine and ten, two of these standard compounds caused an increase in the same peak. Peak number four had the same retention distance as propanol and ethyl isovalerate; peak number six the same as butanol and amyl acetate; peak number nine the same as glycerol and methyl octanoate; and peak number ten the same as 2, 3-butylene glycol and ethyl octanoate. Since Carbowax 400 did not resolve these compounds it is possible that both compounds were present. However, after consulting previous studies, one of the two, in three cases, was a preferred choice.

Mathews et al. (68) reported butanol in their analysis of the volatile fraction of apple wine but did not find amyl acetate. Sihto et al. (85) found the same to be true in separate analyses on Finnish red berry wine, Finnish white berry wine and French red wine. Propanol and no ethyl isovalerate was found in the Finnish red berry wine and the French red wine. From these reports, peak number four was proposed to be propanol and peak number six to be butanol.

Peak number seven was increased by isoamyl alcohol, active amyl alcohol and ethyl hexanoate. In this case, both isoamyl alcohol

and active amyl alcohol were preferred over ethyl hexanoate since the two alcohols are usually present in large quantities in alcoholic beverages (85).

Herstein and Jacobs (43), as reported previously, found glycerol to account for 2.5 to 3.6 percent of the sugar utilized in a fermentation, third only to ethanol and carbon dioxide. Ribereau-Gayon et al. (82) reported that the concentration of glycerol was equal to the total concentration of the following secondary products: succinic acid, acetic acid, 2, 3-butylene glycol, acetylmethylcarbinol and acetaldehyde. It therefore seems probable that peak number nine was glycerol and not methyl octanoate.

2, 3-butylene glycol and ethyl octanoate have both been reported to the same extent as fermentation products in previous studies so neither was preferred over the other.

Mathews et al. (68) isolated and identified 16 compounds in their analysis of the volatile fraction of apple wine. In their use of gas chromatography to separate and purify the compounds, the same liquid phase was used as was used in this study, Carbowax 400, although their conditions for analysis were different. They used a six foot X 1/4 inch stainless steel column packed with 30 percent Carbowax 400 on acid-washed 40/60 mesh firebrick and a column temperature of 130°C. Thus their chromatograms and evaluations were helpful in tentatively identifying the fermentation products of the

yeasts in this study.

They found that isopropanol was not resolved from ethanol on Carbowax 400, but was eluted in the same position and therefore underneath the ethanol peak. An observation made in this study of the same phenomenon confirmed their findings. Similarly, methanol was not thought to be resolved from ethanol.

Peak number two had the same retention time as ethyl acetate and was proposed to be that compound. On that same basis, isobutanol was suggested to be number five, linaloöl to be number 13 and ethyl decanoate to be number 14. None of these last four peaks were increased by any other compound than the one proposed.

SUMMARY AND CONCLUSIONS

It was hoped at the outset of this study to demonstrate that yeast strains differed in the kind and quantity of products they formed during alcoholic fermentation. It was already known that yeasts had different fermentation characteristics but it was not known why strains fermented differently under identical conditions or why one strain was more suited to a particular fermentation than another.

Four strains of yeast with different fermentation properties were used in the study to ferment blackberries. The wine that resulted was extracted with ethyl chloride, concentrated by distillation and analyzed by gas chromatography.

This study has shown that yeasts differ in the quantity and kind of products they form during fermentation. Since enzymes within the yeasts are responsible for the formation of these fermentation products, the results of this study open the way to a better understanding of the enzyme systems in individual yeast strains and to an understanding of why yeasts have characteristic fermentation properties. Coupled with a better understanding of the flavor and aroma compounds, and the proportions and concentrations most desirable, the results of this study and further studies will enable the selection of the most suitable yeast strain or combination of yeast strains for a particular fermentation requirement.

Gas chromatography was found to be an effective method for detecting the fermentation products of yeasts and consequently for differentiating between yeast strains. However, a greater instrument sensitivity is desirable to detect the compounds present in trace quantities.

Since the compounds are eluted from the column over such a wide time interval, temperature programming is recommended to give a greater separation of the initial compounds and to improve the formation of the later peaks. Compounds present in trace quantities, that would normally appear late in the chromatogram, might not distinguish themselves as peaks except under the conditions of temperature programming.

A tentative identification, using the enrichment technique, was made of peaks number two, three, four, five, six, seven, nine, ten, thirteen and fourteen. The peaks, in order, were believed to be ethyl acetate, ethanol, propanol, isobutanol, butanol, isoamyl alcohol and active amyl alcohol, glycerol, 2, 3-butylene glycol or ethyl octanoate, linaloöl and ethyl decanoate.

At their high concentrations, peaks number two, four, five and seven may be expected to contribute to the flavor and aroma of wine. Certainly other minor constituents contribute too and it is possible that certain of these minor constituents, besides being responsible for the organoleptic differences in wines fermented by different

yeast strains, are clues to the enzymatic behavior of yeasts.

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