THE APPLICATION OF GAS CHROMATOGRAPHIC TECHNIQUES IN THE DETERMINATION OF MONOSACCHARIDES, OLIGOSACCHARIDES AND VOLATILES

A Dissertation

Presented

to

the Faculty of the Department of Chemistry

College of Natural Sciences and Mathematics

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Maria del Socorro Martinez

December 1978

To my parents

ACKNOWLEDGMENT

I want to acknowledge Dr. Albert Zlatkis for his guidance, understanding and patience during the course of this work. Dr. David Nurok's invaluable help can not be acknowledged with words either. Both of them made my stay here a fantastic experience.

Everyone in Dr. Zlatkis' research group should be acknowledged too. They are too many to name but all of them have been of tremendous help. Their friendship and advice will always be remembered.

I also want to acknowledge Dr. J. Evans and Mr. D. McQuitty for their help in preparing the yeast samples used in this work and also for valuable suggestions for the direction of this research.

Lastly, I would like to thank my three families. Without their love, patience and support this work would have never been possible.

THE APPLICATION OF GAS CHROMATOGRAPHIC TECHNIQUES IN THE DETERMINATION OF MONOSACCHARIDES, OLIGOSACCHARIDES AND VOLATILES

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ABSTRACT

A concentration technique for the analysis of mono- and oligosaccharides by gas chromatography has been developed. The method increases the detection limit up to 500 times by using a double phase extraction of silylated derivatives and a splitless injection. The method has been found to be fast and reproducible for a variety of samples. The determination included liquid, solid and semisolid samples. Some samples of biological origin as cell cultures and yeasts were determined. The method was found particularly useful in the taxonomic classification of four strains of yeast.

Headspace sampling was applied to the analysis of volatiles from several yeast strains and the results compared. Some differences in the profiles were found. The transevaporation technique was applied to the analysis of volatiles from cell cultures.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (cont.)

INTRODUCTION

INTRODUCTION

A. OCCURRENCE OF CARBOHYDRATES

Carbohydrates are divided into monosaccharides or simple sugars, oligosaccharides containing two to ten monosaccharide units and polysaccharides that contain many monosaccharide units.[1] Monosaccharides are formed by plants during photosynthesis. Plant cells can combine monosaccharides to form oligo- and polysaccharides. Animals can not synthesize monosaccharides but they are able to convert them into polysaccharides, fat and proteins. Animals can catabolize proteins or polysaccharides to provide a source of monosaccharides. The simple sugars play, therefore, a very important role in metabolic processes. They are the starting blocks in the building of more complex molecules. Carbohydrates are also the main source of energy in animals and humans and they have very important structural functions in plants. The biological importance of carbohydrates can not be overstressed.

Carbohydrates are also very important from the industrial point of view. Food (bread, sweetners), fermentation (wine, beer), paper and wood are some of the industries in which carbohydrates are important.

B. ANALYSIS OF CARBOHYDRATES

The analysis of mono- and oligosaccharides has been carried out by several techniques. Traditional wet chemical methods[2,3], enzymatic analysis[4], paper chromatography[5], thin-layer chromatography[6-12], and liquid chromatography[13-18] are some of the methods used in their determination.

Gas chromatography has also been widely applied to this determination due to its high resolution power and extreme sensitivity. The analysis of sugars by gas chromatography can only be done if derivatives are used. The sugars themselves are not volatile enough. Several methods to prepare sugar derivatives are known. The first derivatives used were methyl ethers.[19-21] Other derivatives that have also been prepared are acetates[22-26], oximes that need further derivatization[27, 28] and trifluoroacetates.[29] The most widely used derivatives are the trimethylsilyl derivatives.[30] Sweeley and coworkers[31,32] derivatized about one hundred mono- and oligosaccharides and separated them by gas chromatography. Their work illustrated the wide application of silylation in the analysis of sugars. Their technique and modifications of it have been applied with success in the determination of sugars from several sources. The sugar composition of honey[33], fruits and foodstuffs[34-39], alcoholic beverages[40], syrups[41,42] and sugar cane juice[43] has been determined. Another important area of application is in the determination of sugars in biological fluids[44-48]. These derivatives have also been applied in the determination of various carbohydrates which occur in complex glycolipids[49,50] after hydrolysis or methanolysis have been employed to break the big molecules.

When derivatives have to be prepared, as it is the case for sugars, the sample is diluted in the derivatizing reagent. This usually means that the sample is diluted in 0.5 to 1.0 ml of the reagent. A concentration step is then very useful especially in the case where trace components are involved. A concentration step becomes increasingly

necessary when open tubular columns are used for the determination. Open tubular columns have very high efficiency and resolution power but they have very low sample capacity. The sample usually has to be split before being introduced onto the column. Concentration can be effected by evaporation but this can lead to sample losses. Nurok[51] overcame these problems in the analysis of the kestoses. He found that if the right amount of water was present in a sample to be derivatized a double layer would be formed when the derivatizing reagent used was trimethylsilylimidazole in pyridine. The top phase consisted of hexamethyldisiloxane, a non-polar solvent formed by the reaction of trimethylsilylimidazole and water. Ellis[52] also observed the formation of a double phase but it took 18 hours to form and it disappeared after another 18 hours. In the system described by Nurok.[51] The double phase forms instantaneously and it does not disappear or change volume. Both authors found that the sugar derivatives had high affinity for the top phase. Since the size of the top phase is about 5 to 10% that of the single phase system the sugars were concentrated 10 to 20 times. This technique is very important. There are very few reports on the silylation of aqueous samples.[53,54] This fact indicates the importance of the determination with trimethylsilylimidazole.

The application of this technique to various samples was carried out during the course of this work. The double phase can not only be formed in situ if the right amount of water is present, it can also be formed by adding hexamethyldisiloxane after saturating the single phase with imidazole. Other solvents such as pentane and hexane can also be used to form the double phase.

The method was first applied to a variety of samples to demonstrate whether it could be used for different materials. The method was applied successfully to liquid, semisolid and solid samples.

For biological samples in which the amount of oligosaccharides was very small it was found desirable to concentrate the sample even more. For this purpose a slow injection with split was used. Grob[55] used a technique similar to this one but in a splitless injection. By using this method in conjunction with the double phase technique, a further enhancement in sensitivity by a factor of 7 to 15 was achieved. This method was applied to the determination of sugars in cell cultures.

When using injection with split a great part of the sample is wasted. Most of the sample goes to the atmosphere. Several attempts have been made of using splitless injection.[56-58] During the course of this work a method that allows the splitless injection of several microliters of sample was developed. This allowed a further increase in sensitivity of between 50 to 500 times depending on the original split ratio.

The technique makes use of the low volatility of the sugar derivatives as opposed to the solvent used. The sample is injected into a short precolumn at low temperature. The solvent is allowed to evaporate while the sugar derivatives remain in the precolumn. The sample is then transferred to the analytical column and analyzed by gas chromatography.

This method was applied to the analysis of mono- and oligosaccharides in yeast. It was found possible to classify 4 different yeasts

according to their sugar components using this method.

C. APPLICATION OF GAS CHROMATOGRAPHY TO THE IDENTIFICATION OF MICROORGANISMS

Abel[59] suggested that chemical tests could be very useful in the classification of microorganisms. Mitruka[60] has used gas chromatography with this purpose in mind. Pyrolysis[61-62] and methanolysis of cell walls[63] has been used for the classification of some bacteria. All of this work has been done with packed columns. The analysis of mono- and oligosaccharides, per se, has not been done previously. It was found to be a fast and reproducible technique. Four strains of yeast were grown and compared at the same stage of growth. In some individual cases samples were also taken along the growth curve.

Gas chromatography of silylated derivatives of sugars has been used before to follow the consumption of nutrients by microorganisms.[64] During the course of this work the spent medium in which the yeasts were grown was also analyzed. Different yeasts are considered to give different metabolic products and this was found to be the case.

The method has proven useful in the taxonomic classification of some strains of yeast. It is believed, after analyzing such a wide variety of samples, that this technique can be applied to other complex biological problems. Its future use might be in the determination of the sugar composition of glycolipids, antigens, etc. It could also be used to elucidate the structure of polysaccharides that form part of cell walls. This could be of very high biological significance.

D. VOLATILE COMPONENTS IMPORTANCE

The so-called "metabolic profiles" are multicomponent analysis that have been used in a variety of ways. Zlatkis[65,66] found that the metabolic profile of urine from patients suffering from diabetis mellitus was different than the one obtained from normal subjects. Other workers[67-70] have also studied volatiles from human urine, breath, serum, etc. The work by Jellum[71-73] is also a very good example of the value of these metabolic profiles. He has identified several known and even some previously unknown metabolic disorders by using these profiles.

Fingerprinting is also important in a variety of samples. They have been very useful in air and water pollution studies[74-77], in flavor chemistry[78], in the identification of oil spills[79,80], in food quality evaluation[81], in the identification of tobacco smoke components[82] and in numerous other fields.

E. VOLATILE ANALYSIS

The concentration of volatile components on the samples mentioned above is very important. First of all trace components are usually present in very low concentrations. Thus, the complexity of the samples makes imperative the use of open tubular columns with their high efficiency and resolution but low sample capacity.

The first step in any concentration procedure is the isolation of the compounds of interest from the matrix in which they are present. This can be achieved by several methods that have been developed such

as: headspace, solvent extraction, vacuum distillation, steam distillation and transevaporation.

It was found that the headspace method combined with trapping on the porous polymer Tenax GC that was developed by Zlatkis and co-workers[83] was very adequate to analyze the volatiles from several strains of yeast. The different strains were thought to contain different volatiles due to their different aroma.

The amount of sample on the cell cultures prevented the use of headspace analysis that requires a few milliliters of sample. A more sensitive technique, transevaporation, developed also by Zlatkis and coworkers[84,85] that requires only a few microliters of sample was used in this case.

The determination of volatiles in the cell cultures is very important. The differences in the volatile composition might be later related to the volatile composition of biological fluids and tumors in the animals from which these cells are obtained.

EXPERIMENTAL

EXPERIMENTAL

A. OLIGOSACCHARIDE ANALYSIS

1. Reagents

The following were used:

Trimethylsilylimidazole (Ohio Valley Speciality Chemical, Marietta, Ohio).

Hexamethyldisiloxane (Fluka AG, Tridom Chemical Co., Haupauge, New York).

Pyridine (Certified ACS, Fisher Scientific Co., Fair Lawn, New Jersey), dried over Potassium Hydroxide (J. T. Baker Chemical Co., Phillisburg, New Jersey.

Imidazole (99%, Aldrich Chemical Company, Milwaukee, Wisconsin), dried in a desiccator over silica gel.

Dextrose (Certified ACS, Fisher Scientific Company (Fair Lawn, New Jersey).

β-D(-)Fructose (Crystalline, Sigma Chemical Company, St. Louis, Missouri).

Sucrose (Reagent ACS, Matheson Coleman and Bell Manufacturing Chemists, Norwook, Ohio).

Trehalose (Difco Laboratories, Detroit, Michigan).

Melezitose (Difco Laboratories, Detroit, Michigan).

PYG medium for growing yeast samples.

PYGA medium for growing yeast on petri dishes. (Composed of the same ingredients as PYG medium but solidified by the addition of 1.5% Agar).

The media were autoclaved at 110°C for 30 minutes.

2. Samples

1. Honey, sherry, beer, raisins and sugar cane molasses:

Honey was derivatized as a 20% solution in distilled water. Beer was derivatized as a freeze dried solid. Sherry and raisins were silylated without any prior treatment as were sugar cane molasses.

2. Cell Cultures:

The athymic mice cell cultures were obtained from Stahlin Cancer Research Center at St. Joseph's Hospital in Houston. Both normal and transformed cells were analyzed.

The transformed cells were produced by chemical action and both cells were grown under standard conditions. The cell count was 64×10^6 for normal cells and 100×10^6 for transformed cells. They were suspended in 1 ml of distilled water.

200 µl of the cell suspension were lyophilized.

3. Yeast:

a) Yeast grown on PYG medium;

Four strains of yeast were grown and harvested on the plateau observed after the growth curve reaches its maximum. The growth curve was determined by the Klett method. Growth curves of four micro-organisms are shown in Figure 1.

The yeast was separated from the spent medium by centrifugation. The yeast samples were treated in two different ways:

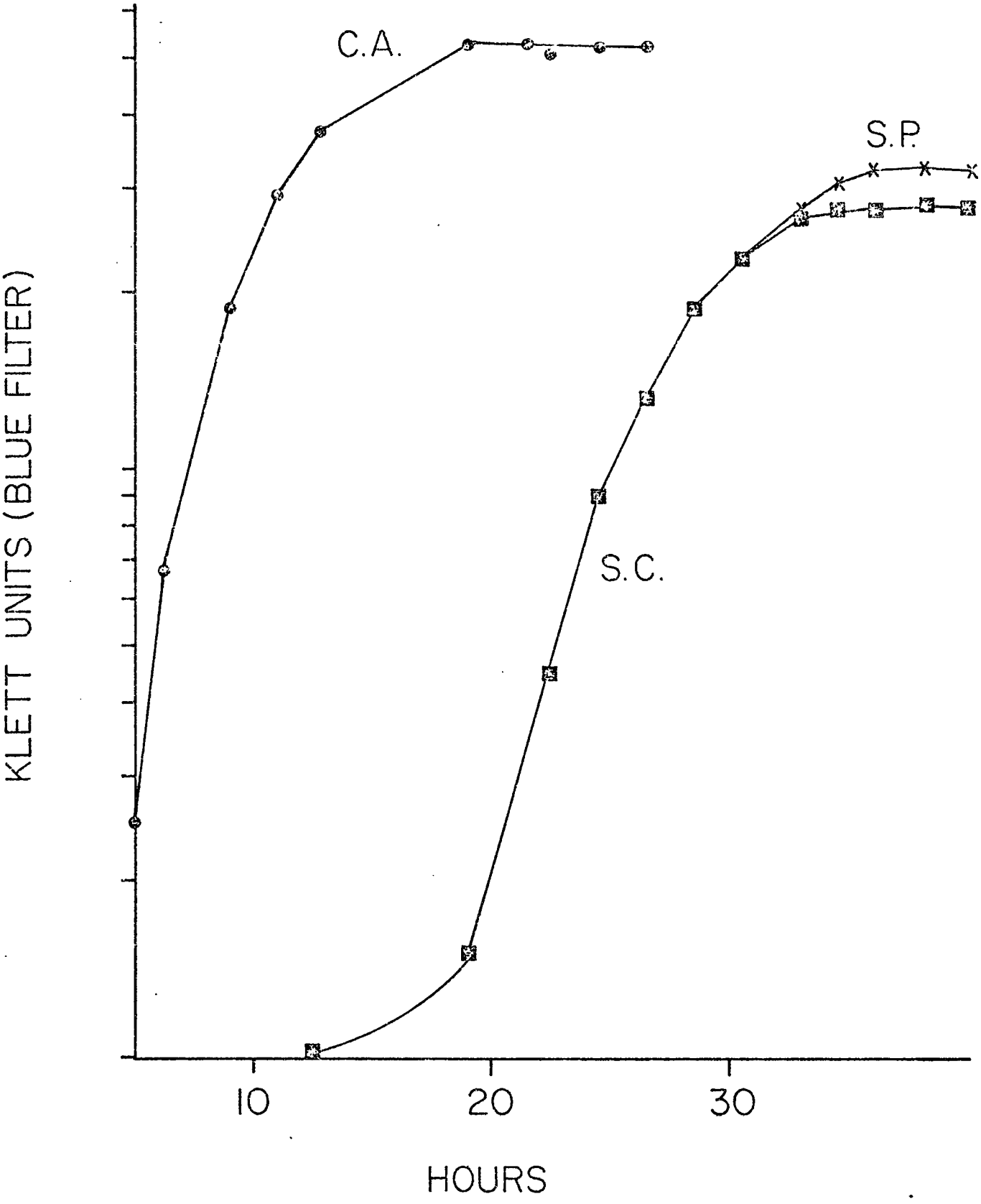
i) The yeast was frozen quickly in a mixture of acetone and dry ice and then lyophilized.

FIGURE 1. GROWTH CURVES OF:

C.A. - Candida albicans

S.P. - Schizosaccharomyces pombe

S.C. - Saccharomyces cereviseae


C.A.
S.P.
S.C.
KLETT UNITS (BLUE FILTER)
10
20
30
HOURS

ii) The yeast was washed one to five times in normal saline solution, frozen in acetone and dry ice and then lyophilized.

The spent medium was frozen quickly and then lyophilized.

The samples derivatized were:

Unwashed cells

Washed cells

New medium

Spent medium

The four strains of yeast are:

Candida Albicans 1

Saccharomyces cereviseae ATCC 18824

Schizosaccharomyces pombe ATCC 24930

Schizosaccharomyces pombe 972 h^-

C. Albicans 1 was obtained from S. M. H. Quadin, Hermann Hospital, University of Texas Medical School at Houston, Houston, Texas. S. Cereviseae ATCC 18824 and S. Pombe ATCC 24970 were obtained from American Type Culture Collection, and S. Pombe 972 h^- was obtained from Dr. J. E. Bailey, University of Houston, Houston, Texas.

The yeasts were grown by Mr. Donald McQuitty, from the Biology Department, University of Houston, Houston, Texas, to whom the author is deeply indebted.

b) Yeast grown on PYGA medium;

The same strains of yeast as discussed previously were grown and harvested after 48 hours of growing on a petri dish.

The samples were treated in the following way:

i) 50 mg of sample were scratched from the surface and derivatized directly.

3. Silylation Reaction

The silylating reagent used was four volumes of trimethylsilylimidazole (TMSI) and one volume of pyridine (the pyridine was dried over potassium hydroxide).

Trimethylsilylimidazole reacts selectively with hydroxide groups to form the silyl ether. TMSI can be used with either dry samples or aqueous solutions.[53] This gives it high flexibility in the determination of hydroxide containing molecules.

The reaction of TMSI with a carbohydrate is illustrated in Figure 2. The reaction of glucose with TMSI gives the completely silylated ether and imidazole as a side product.

The amount of reagent required varies from 3.89 mg of TMSI for 1 mg of a monosaccharide to 2.59 mg for each milligram of a decasaccharide.

4. Formation of the Double Phase System

Figure 3 illustrates the reaction of TMSI with water. The products of the reaction are the non-polar solvent hexamethyldisiloxane and imidazole.

In the case of honey, sherry and sugar cane molasses the double phase, in some instances, was allowed to form in situ by altering the size of the sample.

FIGURE 2. REACTION OF GLUCOSE WITH TRIMETHYLSILYLIMIDAZOLE

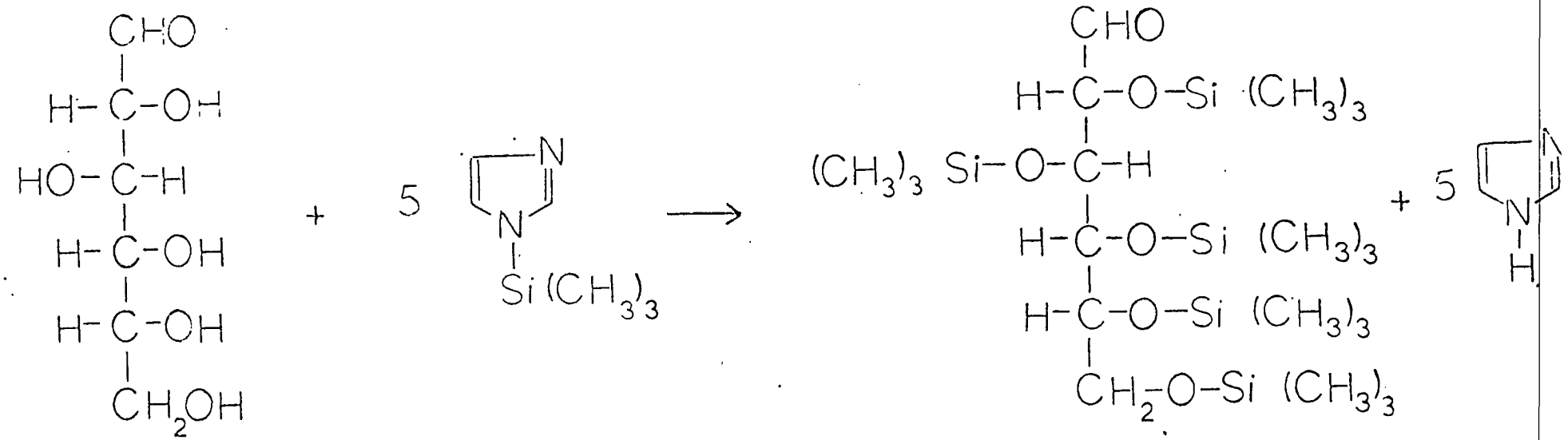
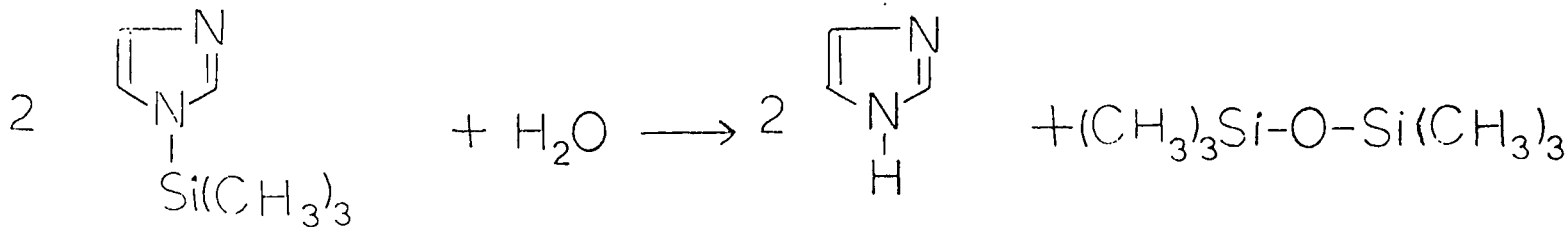


FIGURE 3. REACTION OF WATER WITH TRIMETHYL-SILYLIMIDAZOLE


2
N
N
Si(CH3)3
+ H2O
2
N
N
H
+(CH3)3Si-O-Si(CH3)3

For the samples that were dry or contained very little water the double phase was formed by saturating the reaction mixture with imidazole and adding an aliquot of hexamethyldisiloxane.

The addition of other non-polar solvents such as pentane and hexane also caused the formation of a double phase, provided the system was presaturated with imidazole.

5. Sample Derivatization

a) Honey, sherry, raisins, beer and sugar cane molasses;

0.5 ml of the reagent are added to 8 mg of sample in a 1.0 ml mini-vial (Alltech Associates, Arlington Heights, Illinois) fitted with a Teflon-faced liner. The reaction is vigorous for samples containing appreciable quantities of water and care should be exercised. The mixture is shaken and allowed to stand for 10 minutes. The mixture is then saturated with imidazole and the double phase formed by adding 40 µl to 100 µl of either hexamethyldisiloxane, hexane, or pentane. The vial is shaken and then centrifuged to separate the two phases. The top phase contains the silyl derivatives and it is used for injection.

If 11 mg of honey solution or sherry are used instead of 8 mg, the amount of water present is enough to generate a double phase in situ. In this case the mini-vial must be shaken for 10 minutes to insure complete derivatization.

b) Cell cultures;

The derivatization was carried out in basically the same way as discussed in paragraph (a) but all the lyophilized product was used.

The visible cellular material was first transferred into a mini-vial and the container was rinsed twice with the derivatizing reagent. The first time 0.4 ml of the reagent was used and the second time 0.2 ml was used. Both times the reagent was also transferred to the mini-vial. The rest of the procedure was continued as in paragraph (a) in this section. The reaction mixture was allowed to stand for one hour before formation of the double phase.

c) Yeast samples grown on PYG medium;

50 mg of the lyophilized sample was weighed into a mini-vial. The rest of the procedure is as shown in paragraph (a) of this section. The reaction mixture was allowed to stand for 1 hour before forming the double phase as in paragraph (b).

d) New and spend PYG medium;

The samples scrapped from the plate and derivatized directly contained a very large amount of water. A very large double phase was consequently formed in situ.

6. Injection Techniques

The samples were injected in three ways:

a) 1 or 2 µl were injected with split. This method was used for honey, sherry, raisins, beer, sugar cane molasses and new and spent PYG medium.

b) 15 µl were injected with split at 150°C in 90 seconds to allow the solvent to travel down the column. This method was applied to cell cultures.

c) Splitless injection. A 2.5 m x 0.75 mm stainless steel precolumn coated with OV-17 was used. 5 µl of the top phase were evaporated for 1 minute at 60°C in a stream of nitrogen at 100 cc/minute. The precolumn was then connected to the analytical column. A septum swinger (Pierce Chemical Co., Rockford, Illinois) to minimize the amount of time the septum was in contact with the injection port at high temperature was used. A high temperature septum (Alltech Associates, Houston, Texas) was also used to minimize bleeding effects from a regular septum. This method was applied to the yeast samples.

7. Chromatography

A Perkin Elmer Model 900 gas chromatograph equipped with flame ionization detector was used. Injection port and interface temperatures were maintained between 300 and 330°C. Better control was not possible with the instrument used.

Two columns were used:

a) For the samples injected with split a 12 m x 0.25 mm glass open tubular column coated with SE-30 was used. The PYG media, both new and spent, are exceptions. They were run on column (b).

b) For the splitless injected samples a 27 m x 0.76 mm stainless steel open tubular column coated with OV-17 was used. Temperature programming was different for each of the three injection techniques. For 1 or 2 µl injection with split, the oven temperature was programmed from 150 to 270°C at 4°/minute. For 15 µl injection with split, the oven temperature was held at 150°C for 3 minutes and then programmed to 270°C at 4°/minute. For splitless injection, the oven temperature was programmed from 100°C to 270°C at 8°/minute. The carrier gas used was nitrogen.

8. Integration

The Autolab System I from Spectra-Physics was used.

B. VOLATILES

1. Headspace

a) Samples;

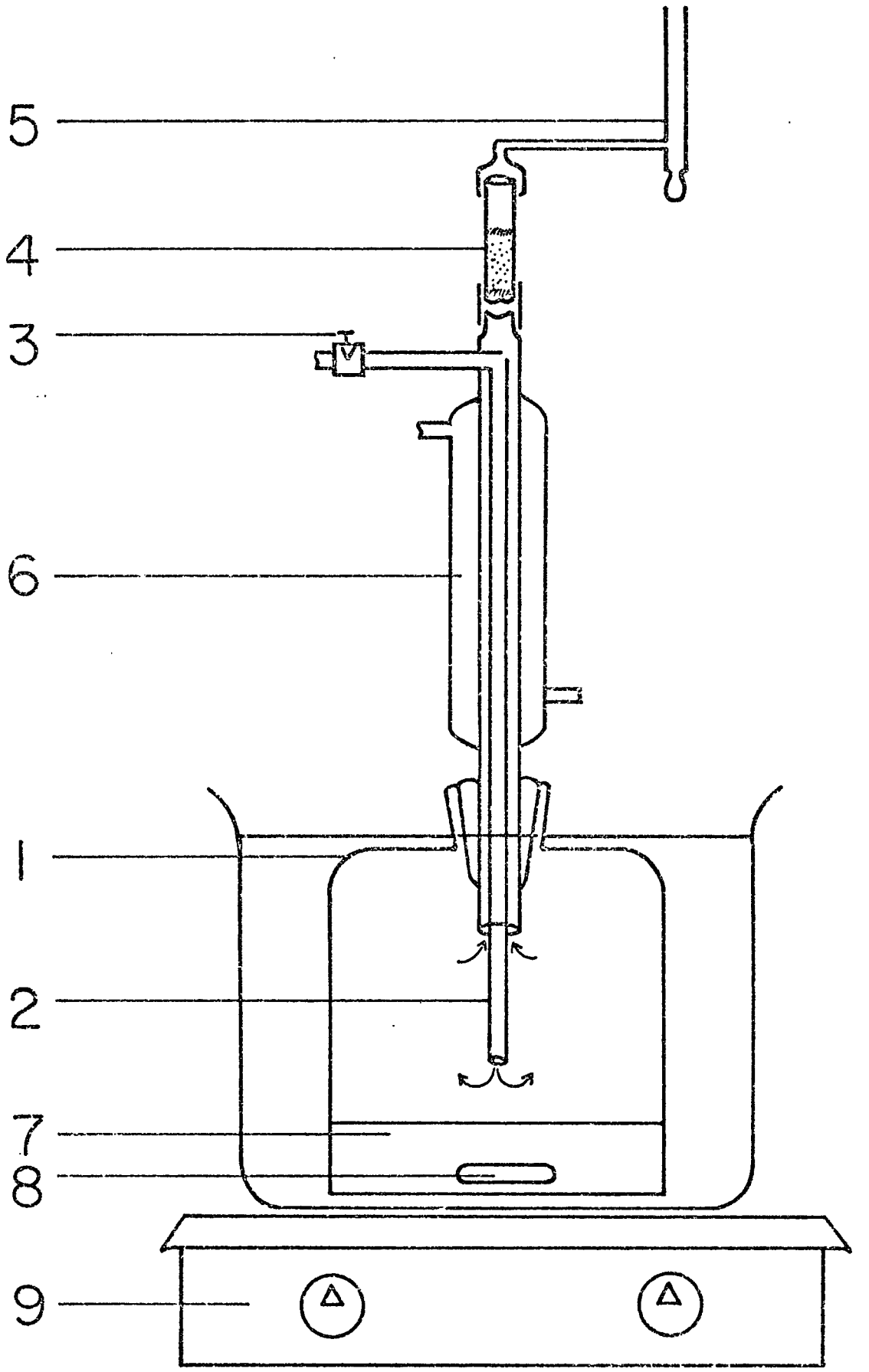
3 strains of yeast, S. Pombe "70", S. Pombe "M" and S. Cereviseae were used. The samples were harvested as shown in Part A of this chapter and washed five times to minimize the effect of the medium. 3 to 4 grams of each yeast were used for the determination.

b) Sampling apparatus;

The apparatus is described in Figure 4. The sample was put into a 100 ml container. The container was fitted with a tappered female joint and sealed with a specially designed condenser. The condenser

FIGURE 4. HEADSPACE APPARATUS

1. Pyrex container
2. Tube for helium introduction
3. Needle valve
4. Tenax G.C. trap
5. Flow meter
6. Condenser
7. Sample
8. Stirring bar
9. Magnetic stirrer


5
4
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1
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7
8
9

has an inside tube that allows the introduction of helium. Helium is the carrier gas for the volatiles present in the headspace. The helium flow was adjusted to 30 ml/min. The role of the condenser is to remove the water before it reaches the adsorbent. The volatiles are trapped on a Tenax GC trap joined to the condenser by a sleeve of Teflon tubing. The volatiles were collected for 30 minutes. The sample was maintained at 30°C with a water bath.

c) Sample transfer;

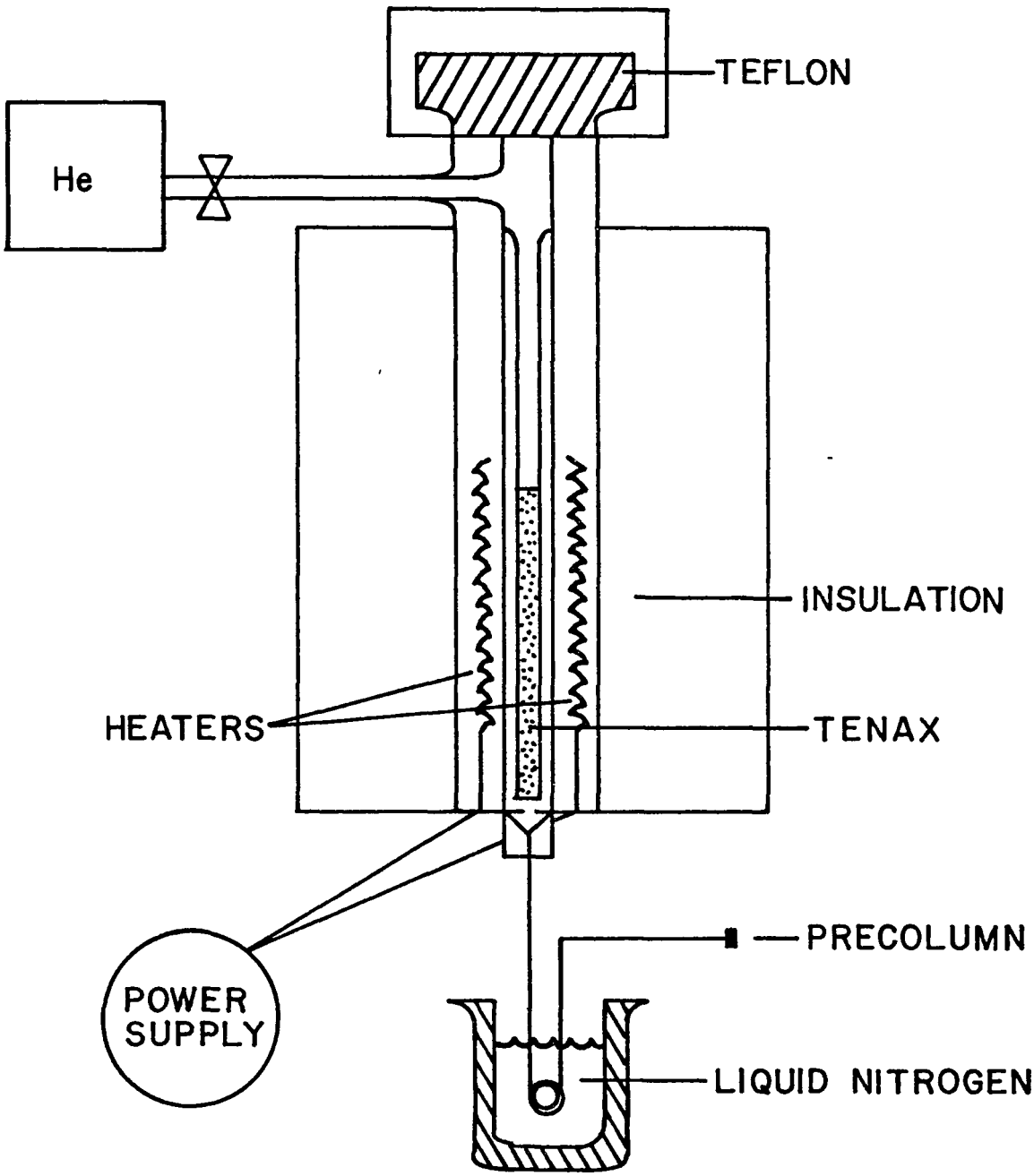
After the sampling was completed the volatiles trapped in the Tenax trap had to be transferred to the analytical system. With this purpose the apparatus described in Figure 5 was used.

The Tenax trap is put into a stainless steel heated block at 280°C. A 1 m x 0.5 mm I.D. nickel open tubular column is attached to it. In order to trap the sample as a plug, the precolumn is immersed into liquid nitrogen. A flow of helium of 10 cc/min is passed over it for 15 minutes. After that the precolumn is disconnected from the heating block and connected to the analytical system.

The Tenax trap can be reused by conditioning at 350°C for 30 minutes with a flow of either nitrogen or helium.

The importance of the condenser is very evident in the desorption system. If any water reaches the adsorbent it will be frozen in the liquid nitrogen trap. Since the internal diameter of the precolumn is very small it can very easily become plugged.

FIGURE 5. DESORPTION SYSTEM



DESORPTION SYSTEM

d) Chromatography;

A Perkin Elmer 900 with dual flame ionization detector was used. The injection port temperature was 250°C and the interface temperature was 270°C. A 100 m x 0.5 mm I.D. nickel open tubular column was used. The column was coated with 10% Witconol as the stationary phase. The oven temperature was programmed from 60°C to 150°C at 2°/minute. The carrier gas was nitrogen at a flow rate of 4.5 cc/min.

2. Transevaporation

The volatiles from the cell culture samples were determined by Mr. Kwan Lee of the University of Houston, Chemistry Department, Houston, Texas. The experimental details are contained in references 84 and 85.

C. COLUMN PREPARATION

The coating of open tubular columns is very important to the final results of any capillary chromatography analysis. The efficiency and resolution power of the column will finally dictate the results of the analysis

The coating of the non-polar Witconol phase was achieved by using the dynamic method in which a 10% w/w solution of Witconol in chloroform is pushed through the column in about 5 hours. The pressure is exerted by nitrogen and the flow rate is adjusted with a needle valve. The column is then conditioned by temperature programming from 40°C to its maximum operating temperature (150-170°C)

at 0.5°/minute. The column is allowed to stay overnight at the maximum temperature.

The coating of the silicone phases OV-17 and SE-30 on metal columns is more difficult. The reason for using these phases was their high temperature limit needed in oligosaccharide analysis.

The dynamic procedure used for non-polar columns was not effective in this case. Several attempts were made at coating both nickel and stainless steel columns. The static method in which the column is filled with the stationary phase in a given solvent and the solvent is gently evaporated with the aid of vacuum was tried unsuccessfully. This method is very common for glass columns. A procedure in which 0.1% Witconol was used as a wetting agent gave very good results at low temperature use but when it was conditioned to 270°C the results were also bad. A different approach in which the nickel surface was modified by complexing with a ketone was also tried with no success.

The method that gave the most acceptable results was the dynamic method with a very fast flow rate. A solution of 4.5% w/w OV-17 in chloroform to which 0.25% of benzyltriphenylphosphonium chloride as a wetting agent was added was introduced into the column at a very high pressure. The coating was completed in 15 to 20 minutes as opposed to the several hours in the regular method. After the coating was completed a small flow of nitrogen was passed through the column overnight to allow all the chloroform to evaporate. The column was then conditioned by temperature programming from 60°C to 270°C at

0.5°/minute. The column had to be maintained at 270°C for a week before it was ready to use.

RESULTS

A. DOUBLE PHASE SYSTEM. APPLICATION TO HONEY AND RELATED SAMPLES

The concentration effect of the two phase system is significant. It is illustrated by a consideration of chromatograms of a derivatized sample before and after the formation of a double phase system.

Figure 6(A) shows the chromatogram of a derivatized honey sample obtained by injecting 2 µl of a single phase system. Large monosaccharide peaks are present together with trace disaccharide and trisaccharide peaks.

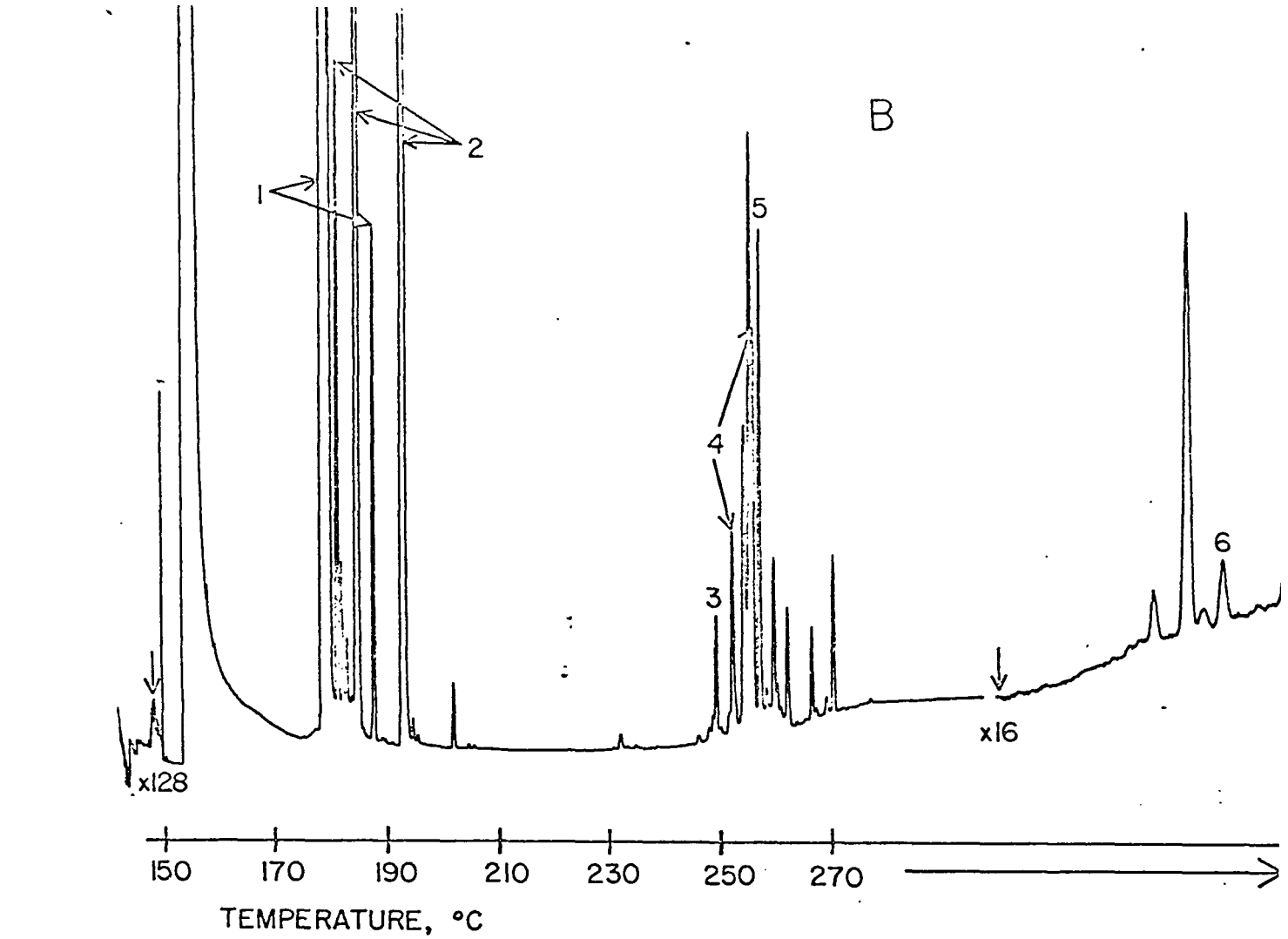
Figure 6(B) shows the chromatogram of the same honey sample after conversion to a two phase system. The monosaccharide peaks are off scale and can not be compared as an integrator was not used for this part of the work. The size of the disaccharide and trisaccharide peaks is about ten times greater as those observed for the single phase system.

The next step was to prove whether the size of the double phase had any influence on the extraction of the sugars into it. The shape of the chromatogram does not change if the top phase is made smaller or larger by using different amounts of hexamethyldisiloxane as it is illustrated in Figure 7. In (A) the double phase was formed by adding 60 µl of HMDSO. In (B) 100 µl of HMDSO were added while 400 µl were used in (C). Except for a uniform decrease in the size of the peaks, due to a dilution effect, no other effect was observed.

FIGURE 6. HONEY

A) Single phase

B) Double phase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1
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3
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6
x128
x16
150
170
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270
TEMPERATURE, °C

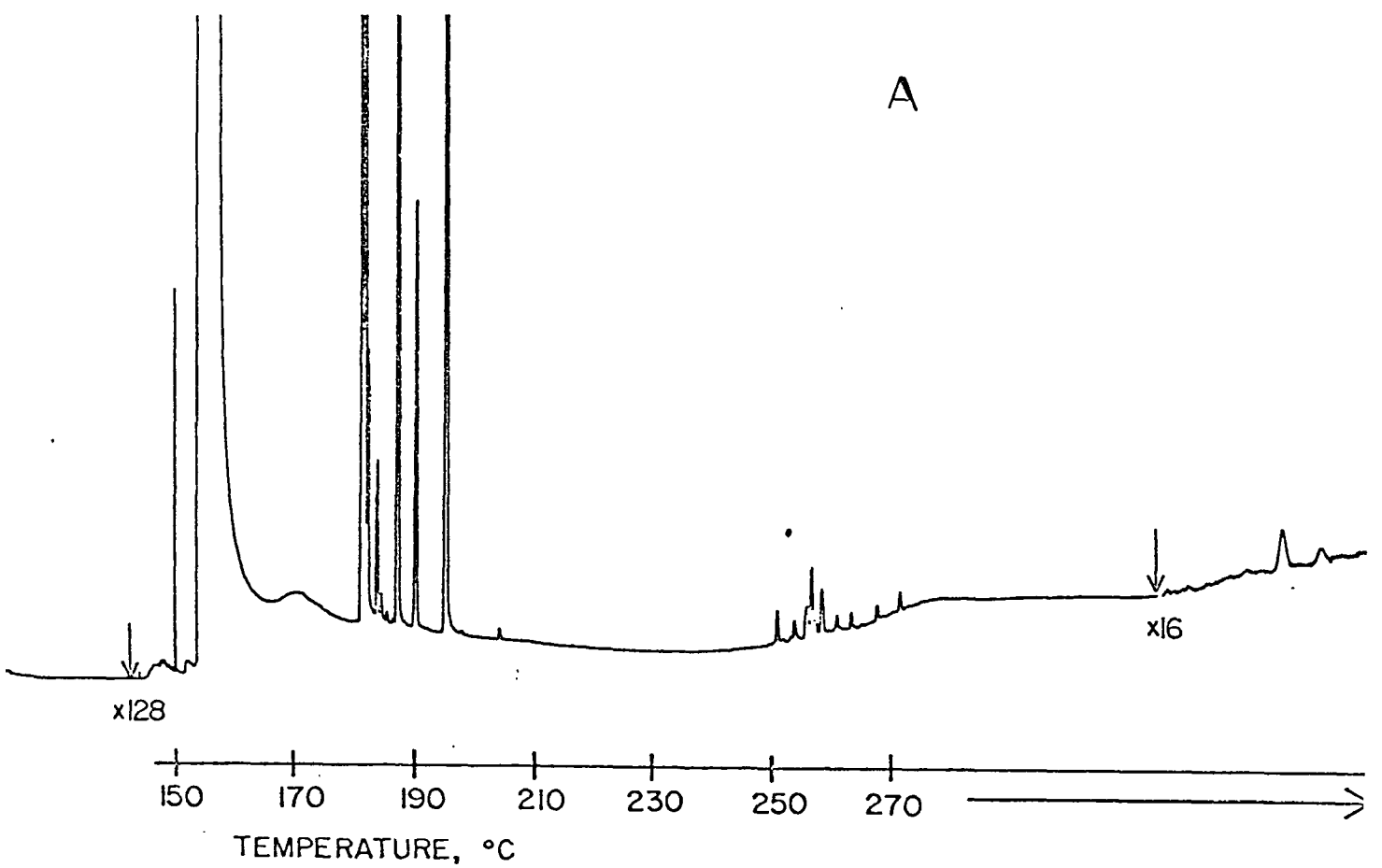
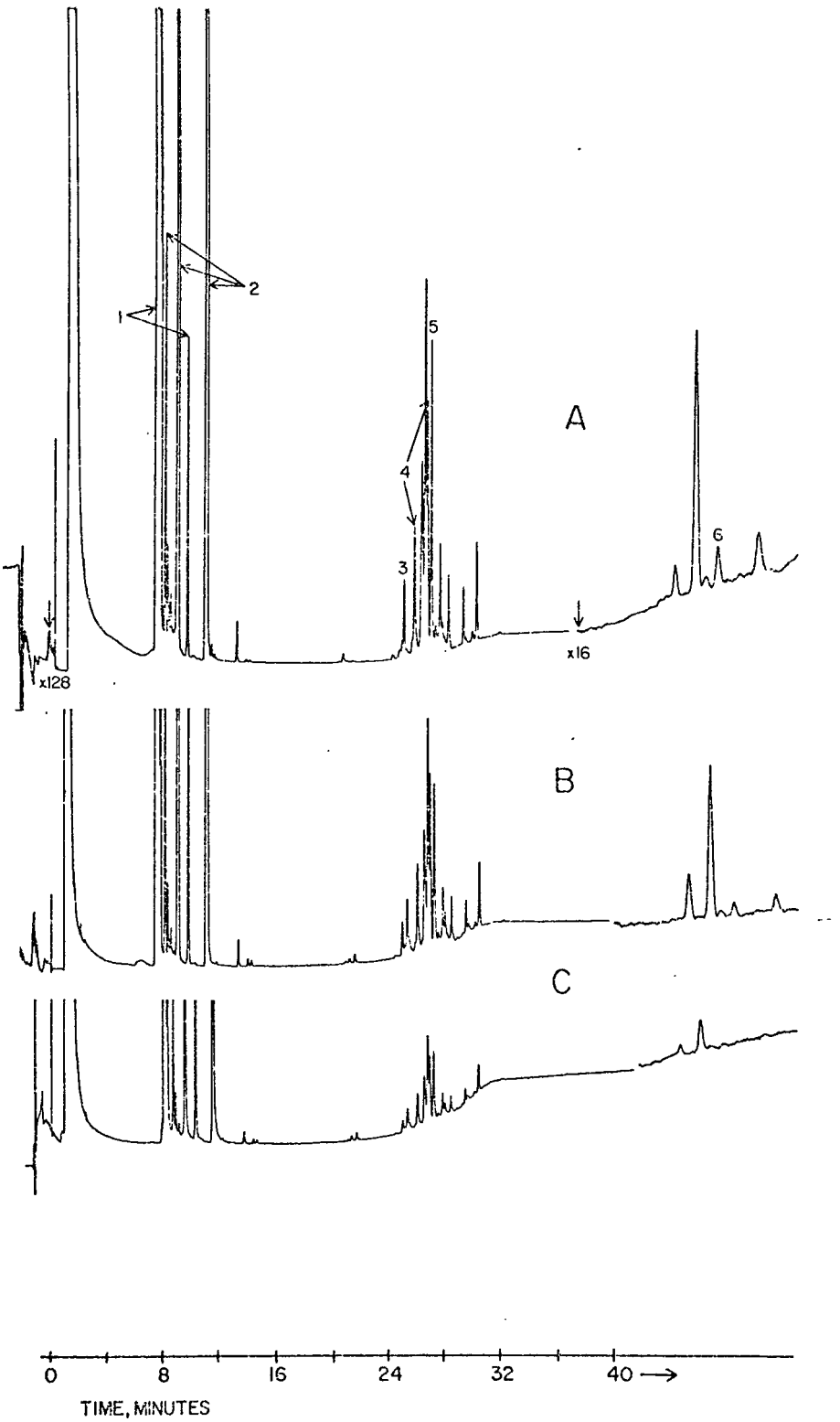

A
x128
x16
150
170
190
210
230
250
270
TEMPERATURE, °C

FIGURE 7. HONEY SAMPLE

A) Double phase formed with 60 µl of hexamethyldisiloxane

B) 100 µl

C) 400 µl


1
2
3
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6
A
x128
x16
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40 →
TIME, MINUTES

The identification of the peaks in Figures 6 and 7 was performed by comparing their retention times to standards. Peak No. 1 was identified as glucose, (2) as fructose, (3) sucrose, (4) trehalose and (5) melezitose. Some of the compounds that have not been identified might not be sugars. They could be other apolar compounds soluble in the top phase but the most likely candidates for the unidientified peaks are sugars. This is due to the nature of the sample and the reagent and also because of the clustering of the peaks in regions corresponding to mono-, di- and trisaccharides. The presence of several peaks for glucose and fructose is due to anomeric isomers.

The two phase system gave better chromatograms than the single phase system. The evaporation of the single phase system for a honey sample was also effected. The purpose was to find whether this method would achieve concentration without need of forming a double phase. The single phase system was prepared conventionally and evaporated under a stream of nitrogen to a sample size similar to that of a double phase. As it can be observed in Figure 8, there is a considerable loss of sample.

A blank was also an important consideration. For the purpose of a blank a single phase system was formed with distilled water. The double phase system was formed and the sample was run in the chromatogram under the same conditions as a regular sample. The results appear in Figure 9. Except for a small peak in the disaccharide region the blank is clean.

FIGURE 8. HONEY SAMPLE EVAPORATED SINGLE PHASE

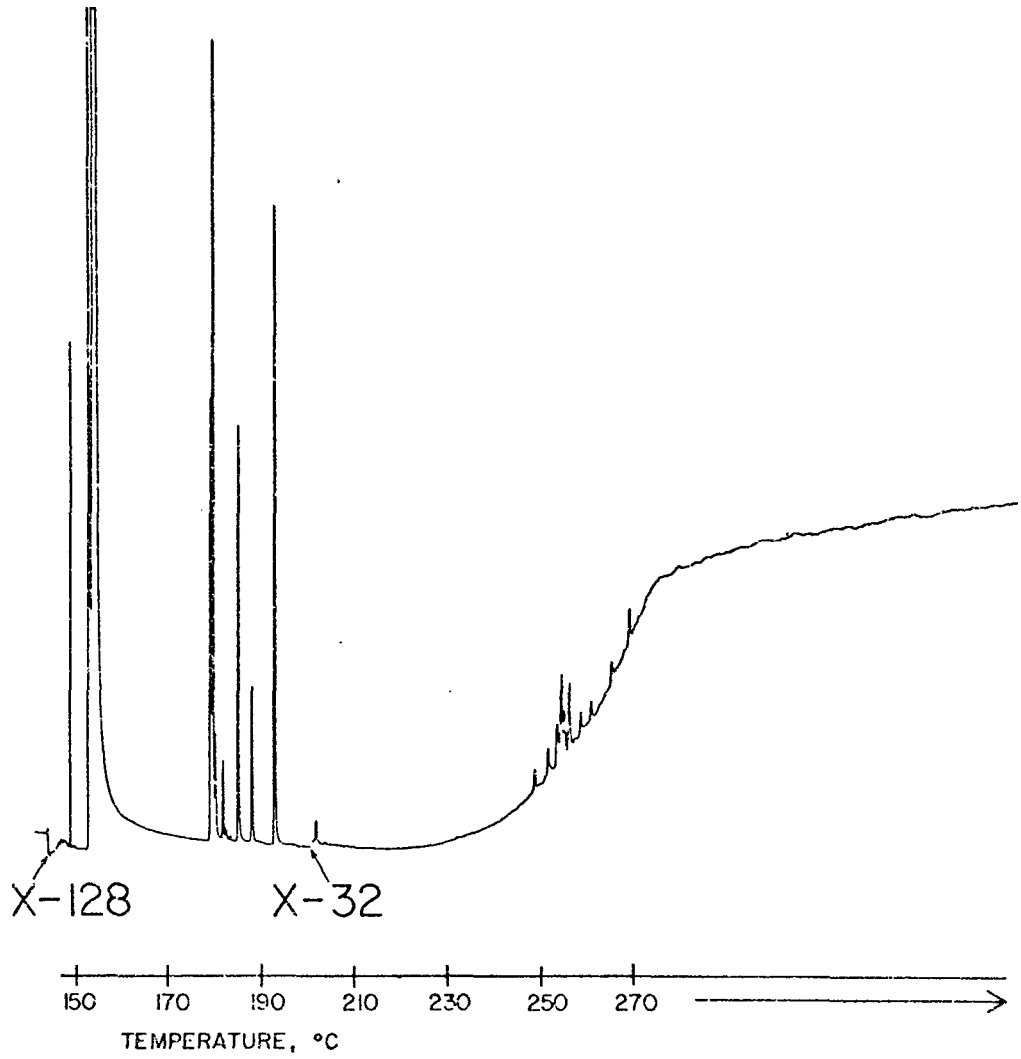
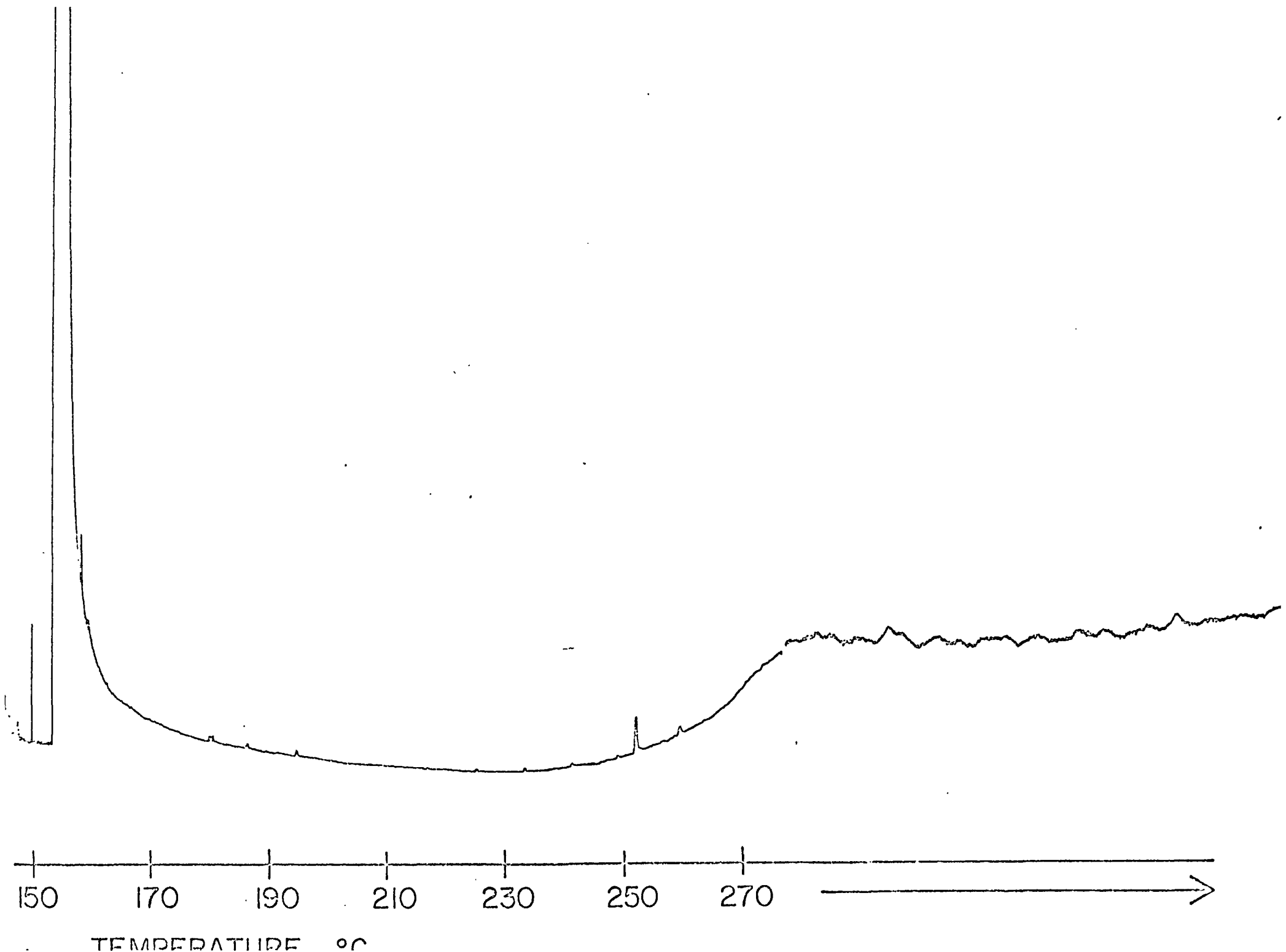

X-128
X-32
150
170
190
210
230
250
270
TEMPERATURE, °C

FIGURE 9. BLANK


150
170
190
210
230
250
270
TEMPERATURE °C

The feasibility of preparing the double phase system with solvents that do not contain silicon is very important from the detector point of view. When silicon containing molecules are burned in the flame ionization detector (FID) a deposit of silicon dioxide is formed. This deposit decreases the sensitivity of the detector making it necessary to clean up the detector very often. By eliminating the silicone containing solvent the problem is markedly reduced.

Figure 10(P) shows the chromatogram of a honey sample with the double phase formed with pentane. Figure 10(H) is the chromatogram of a honey sample with the double phase formed with hexane. The chromatogram obtained with either of the three solvents are almost identical.

The method had to be tested for reproducibility which was found to be the case. Figure 11 shows the chromatograms that were obtained from freeze dried beer samples. The samples were independently dried, derivatized and analyzed. The runs were nearly identical. They show very small peaks in the mono- and trisaccharide region and several large peaks in the disaccharide region.

The silyl derivatives have been assumed to be stable for up to several weeks. Due to the high detection limit attained by this technique the presence of artifact peaks have been detected. These peaks appear at random and consequently are very difficult to track. The peaks are very easily recognized because they present a very regular pattern as can be seen in Figure 12. They are shown in a beer sample. These peaks took a few days to appear after the preparation of the silyl derivatives but in a few cases they appeared

FIGURE 10. HONEY

P) Double phase formed with pentane

H) Double phase formed with hexane

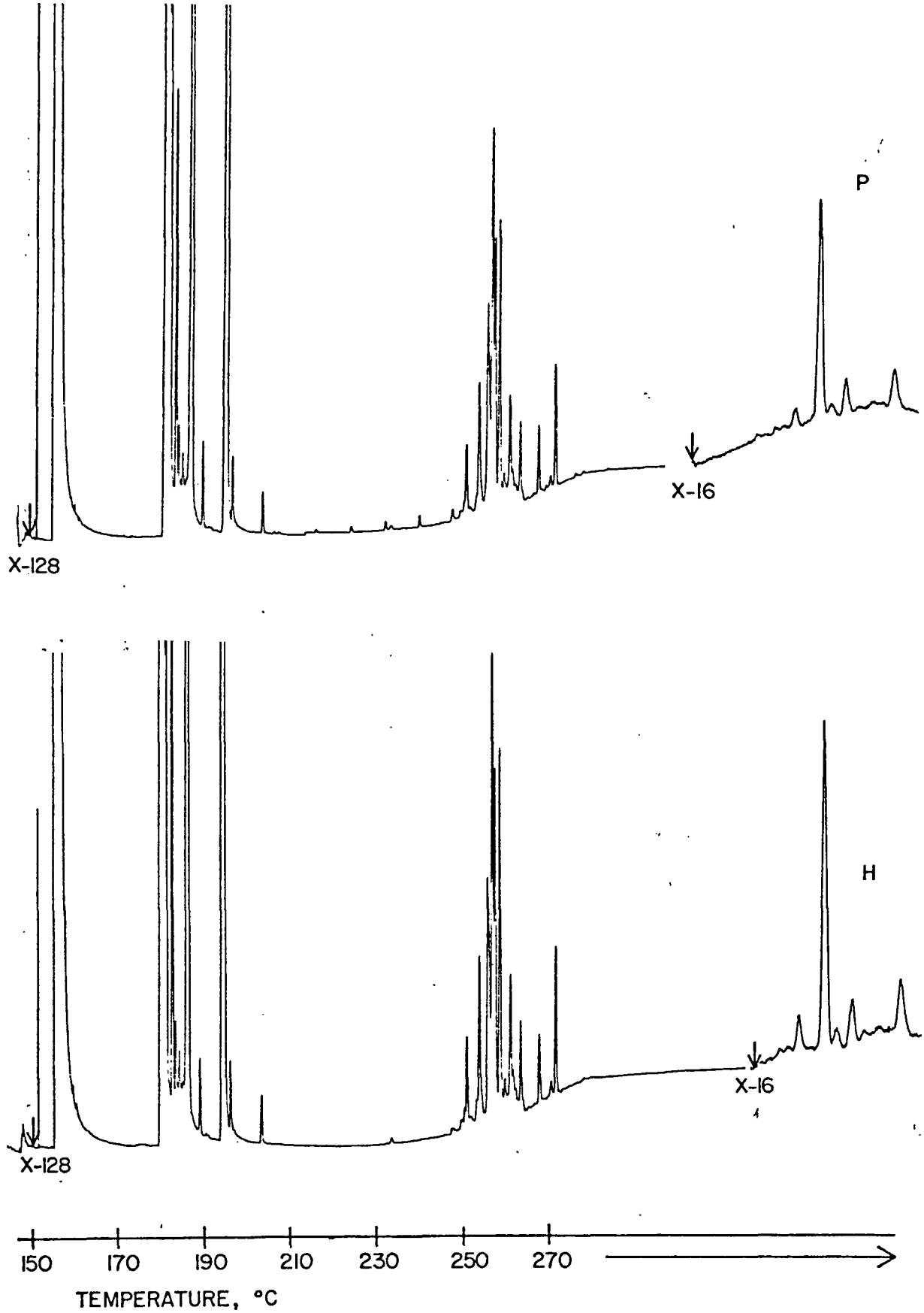

P
X-16
X-128
H
X-16
X-128
150
170
190
210
230
250
270
TEMPERATURE, °C

FIGURE 11. BEER. REPRODUCIBILITY

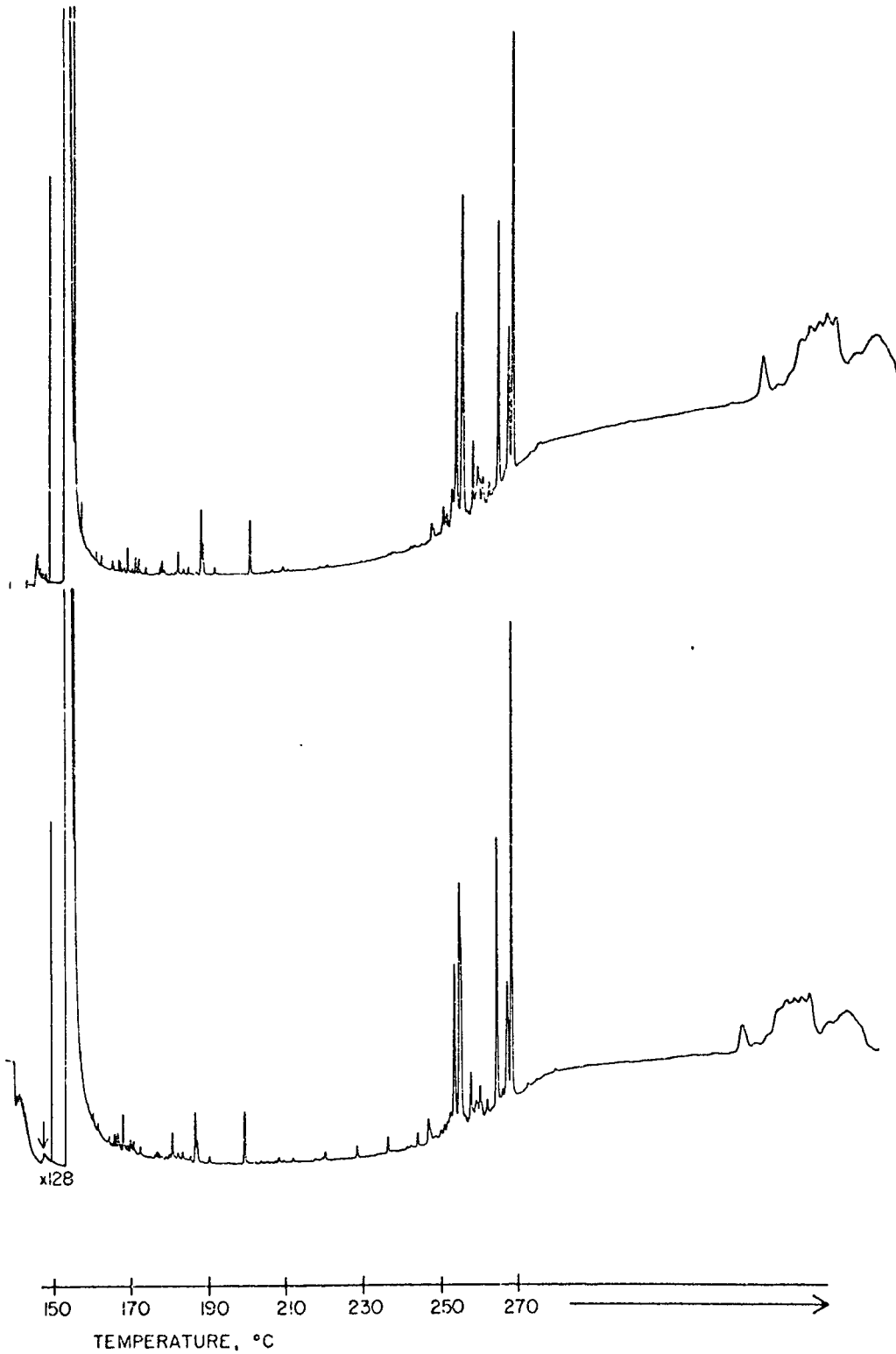
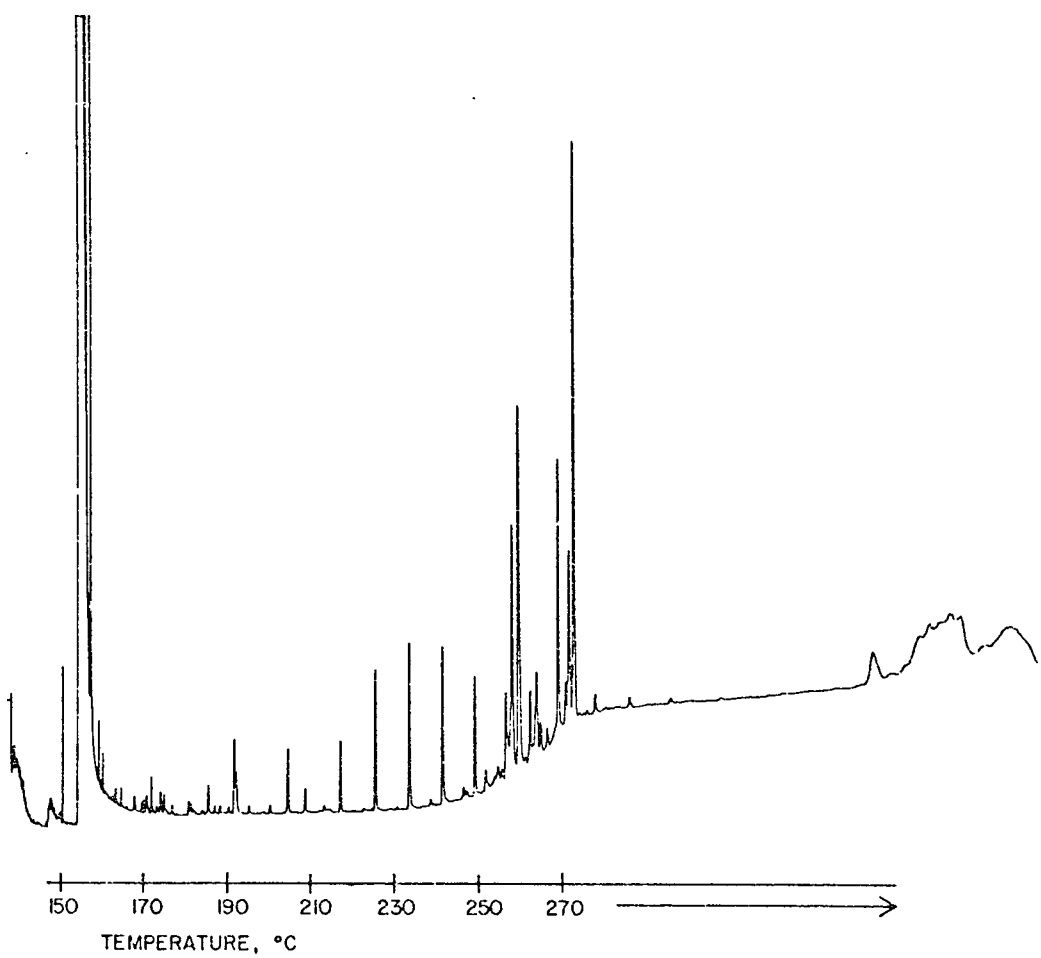

x128
150
170
190
210
230
250
270
TEMPERATURE, °C

FIGURE 12. ARTIFACT PEAKS IN A BEER SAMPLE


150
170
190
210
230
250
270
TEMPERATURE, °C

as soon as 24 hours after derivatization. In no case had the artifact peaks appeared before 24 hours or before the sample cap was punctured for injection even if several days elapsed between derivatization and chromatographic run. The peaks appeared on a variety of samples including a water blank, standards, yeast, etc.

The method was applied to a variety of samples to demonstrate its wide application. Figure 13(A) shows the chromatogram of sugar cane molasses. There are considerable amounts of several monosaccharides, one disaccharide and one trisaccharide besides traces of all of them. The large trisaccharide peak was identified as one of the kestoses. In sherry, Figure 13(B), several peaks are present in the mono- and disaccharide region and only traces of trisaccharides. In raisins there are several large peaks present in the monosaccharide region, about twelve disaccharides are also present but there are no trisaccharides. The chromatogram is shown in Figure 13(C).

The double phase system was also prepared in situ in a honey sample by augmenting the amount of sample and consequently the amount of water. When 11 mg of sample were used the double phase was formed spontaneously. The resulting chromatogram is shown in Figure 14.

B. SLOW INJECTION METHOD. APPLICATION TO CELL CULTURES.

The top phase was injected in the chromatograph very slowly to allow the solvent to travel down the column while the derivatives were retained in the first part of it. The method was first tried for reproducibility, which is good, as illustrated in Figure 15 for

FIGURE 13. DOUBLE PHASE APPLICATIONS

A) Sugar cane molasses

B) Sherry

C) Raisins

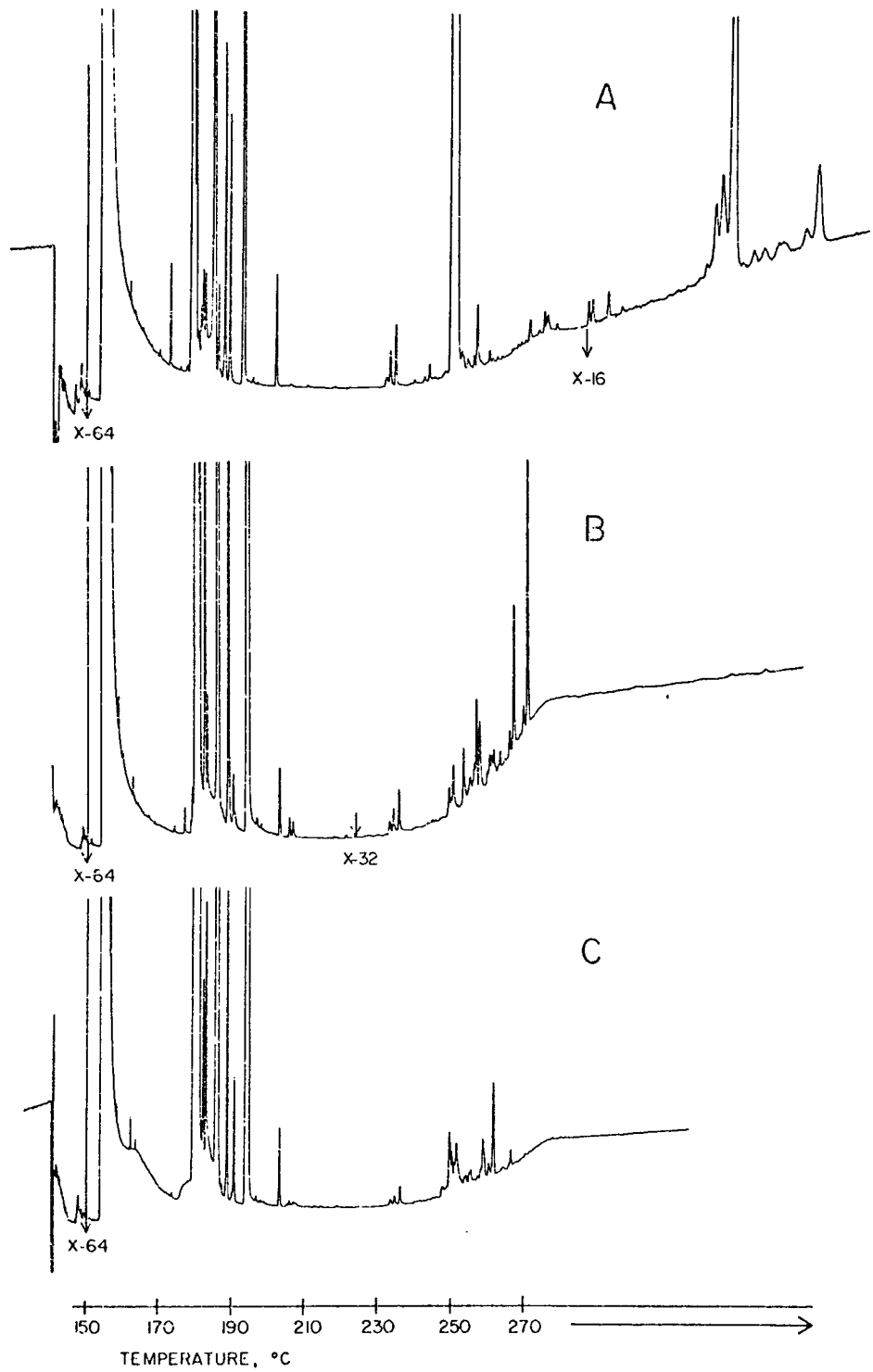
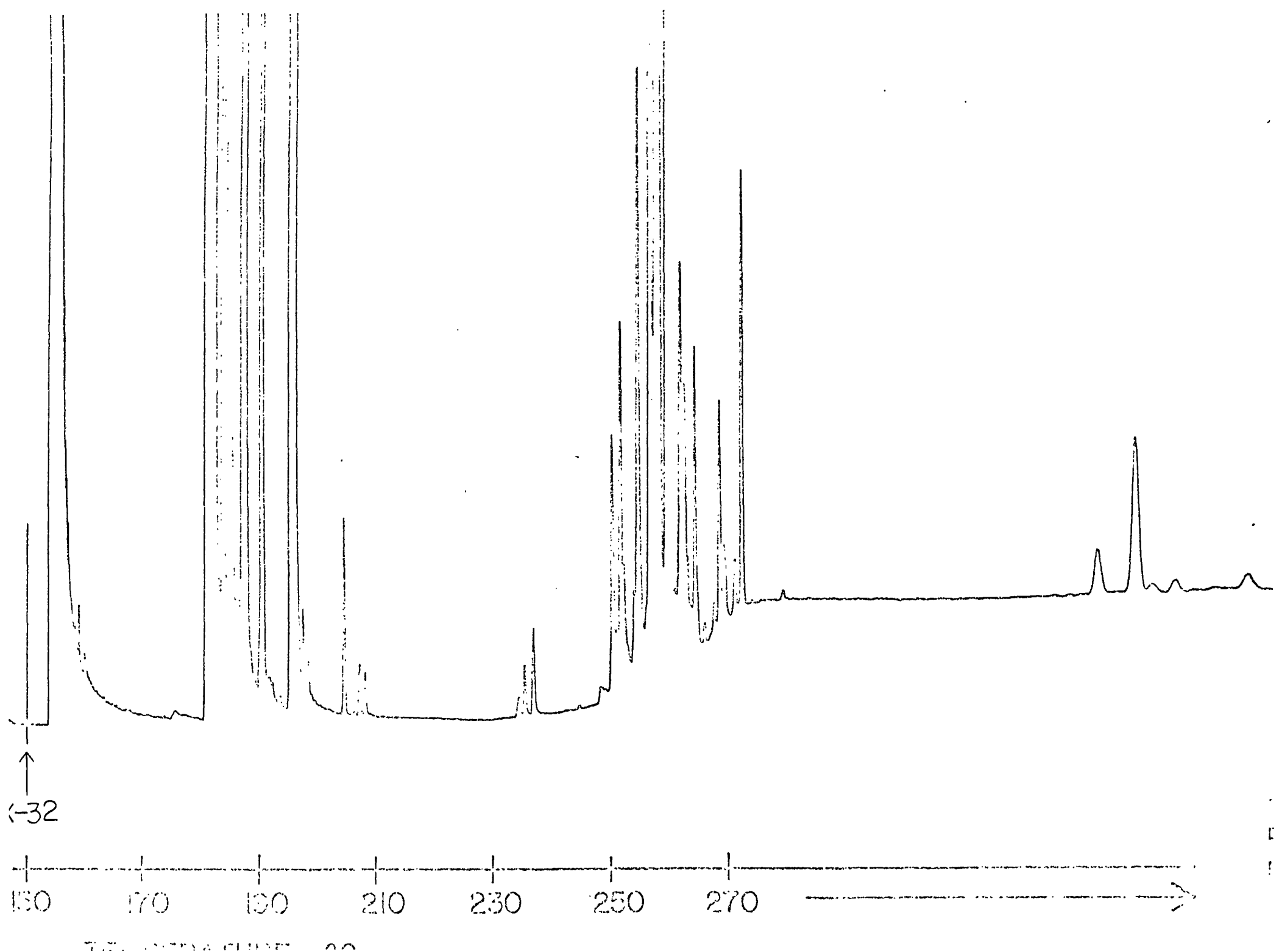

A
X-64
X-16
B
X-64
X-32
C
X-64
150
170
190
210
230
250
270
TEMPERATURE, °C

FIGURE 14. HONEY SAMPLE. DOUBLE PHASE FORMED IN SITU.


-32
150
170
190
210
230
250
270

transformed cells. The reproducibility from several samples lyophilized independently was also found to be good.

The difference between transformed and normal cells was very noticeable. It is found in the trisaccharide region as it is illustrated in Figure 16. In Figure 16(B) a chromatogram of normal cells is shown. The peak marked 1 in the trisaccharide region is completely absent in Figure 16(A) which is the chromatogram of transformed cells.

No attempt has been made to identify this compound. It is assumed to be an oligosaccharide due to the selectivity of the trimethylsilyl-imidazole for hydroxide groups, the extraction procedure and the retention times in the chromatogram. Identification of this compound might be necessary in the future. The absence of this peak in transformed cells might be very important from the biological point of view but more work is necessary in the way of confirmation.

C. SPLITLESS INJECTION. APPLICATION TO YEAST SAMPLES.

The splitless injection of 5 µl of the top phase has a very marked effect in the amount of sugars that can be detected. 250 to 500 times times more sample is introduced into the column. Sugar derivatives that did not appear at all when 1 µl with split was injected are now very easily detected.

The conditions for solvent elution mentioned in the experimental part are critical. The use of higher temperature, higher flow rate or longer time of elution causes the peaks to be lost. This affects

FIGURE 15. TRANSFORMED CELLS-REPRODUCIBILITY

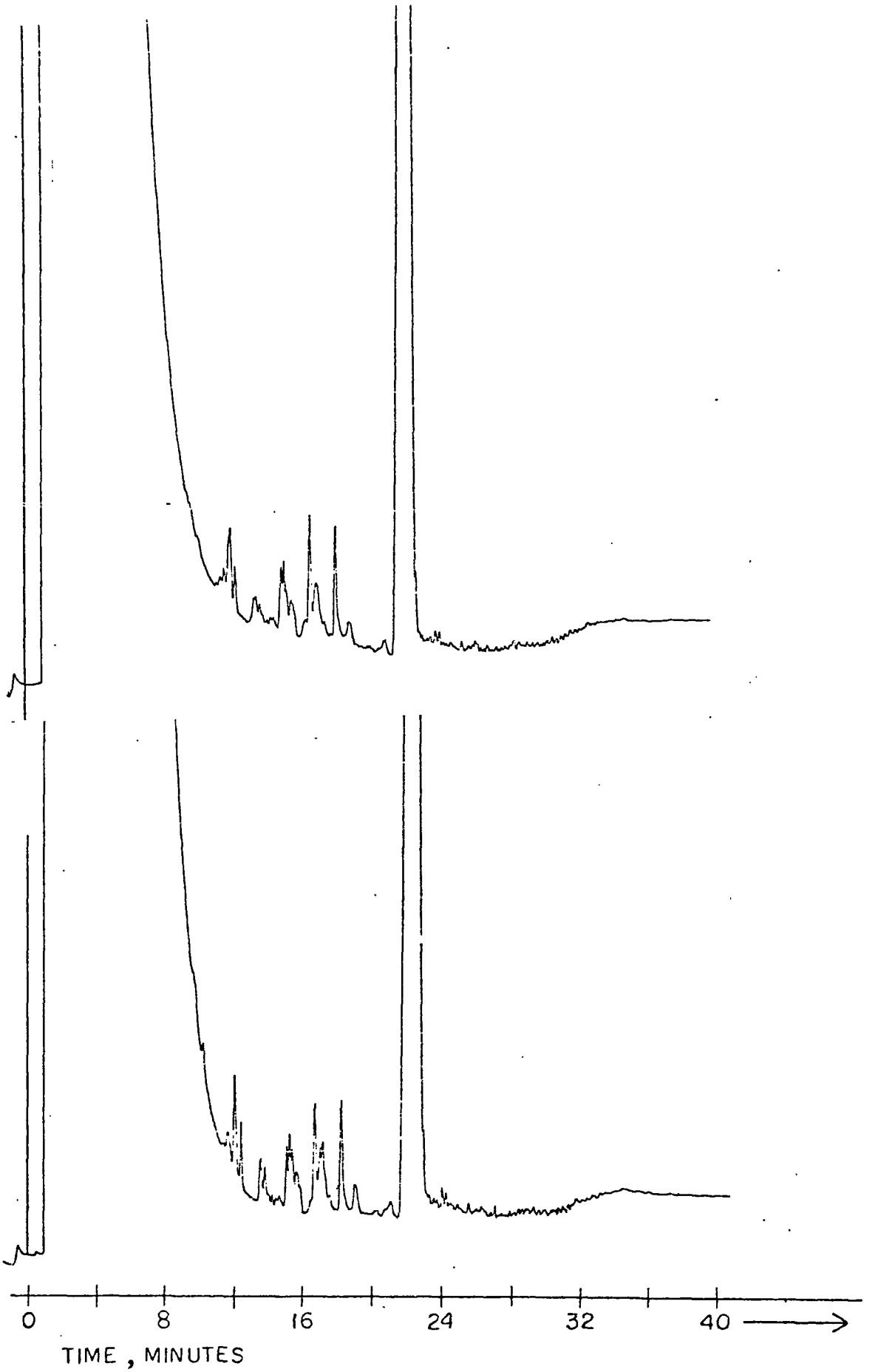
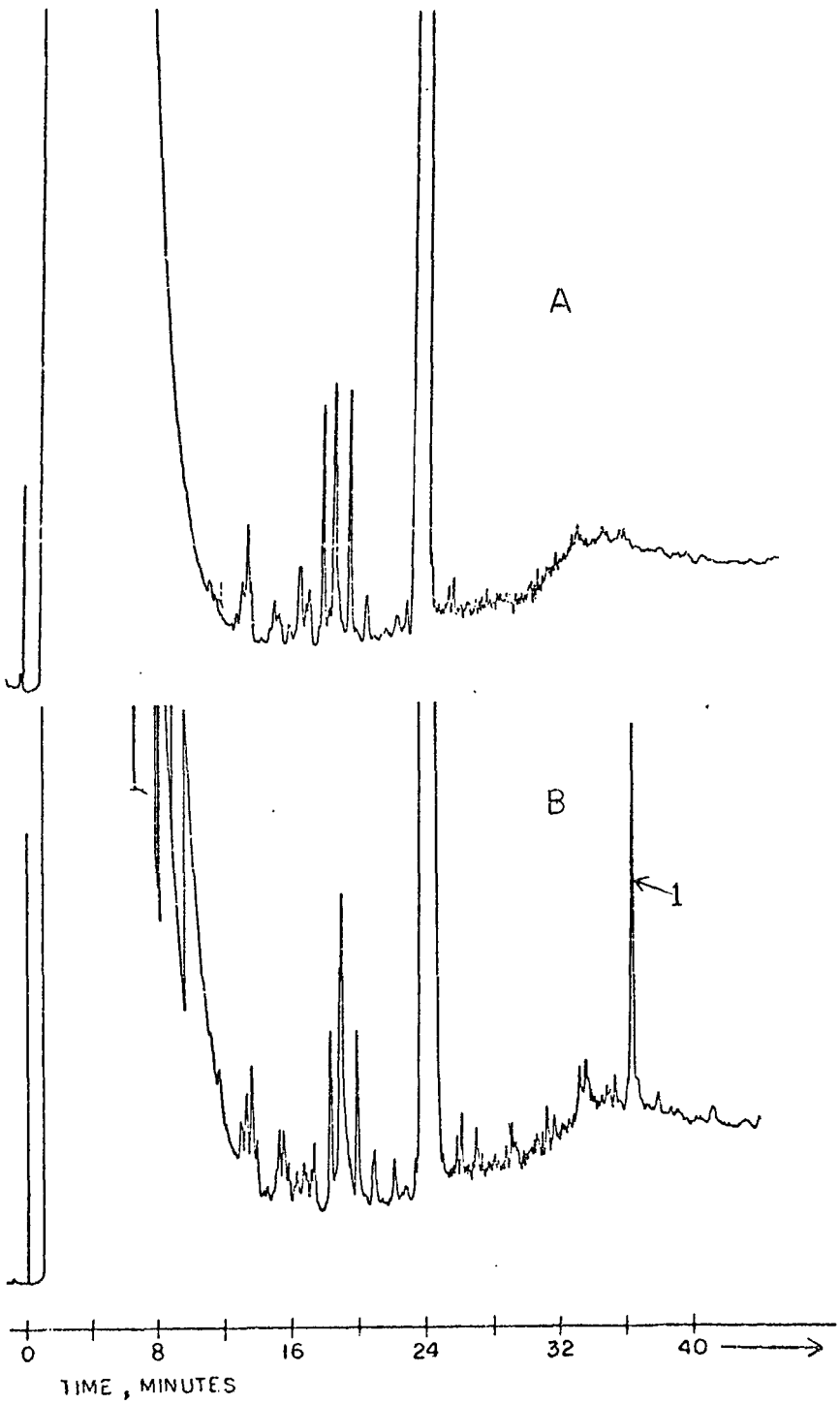

0
8
16
24
32
40
TIME , MINUTES

FIGURE 16. COMPARISON OF

A) Transformed cells vs.

B) Normal cells


A
B
1
0
8
16
24
32
40
TIME , MINUTES

the reproducibility of the technique considerably. With the conditions given, fairly reproducible results have been found. This is illustrated in Figure 17 for a sample of washed schizosaccharomyces pombe (ATCC 24970). Several aliquots of the same top phase were injected on either the same or different days and the results compared.

Samples of this same lyophilized yeast strain were derivatized several times and chromatographed. The reproducibility of the results is illustrated in Figure 18.

A system blank was also a primary consideration due to the multiple steps involved in the determination. Figure 19 shows a chromatogram of a distilled water sample. 500 µl of it were freeze dried, derivatized and run under similar conditions. The blank is very clean.

In spite of attempts to keep the elution conditions completely reproducible the solvent was completely evaporated in some cases but only partially eluted in others. In most cases this did not affect the results as shown in Figure 20. In a few cases however the ratio of peak 1 to peak 2 was inverted as can be seen in Figure 21.

The samples were also repeated biologically at least six times. The samples were grown, harvested and handled independently and the reproducibility is evident in Figure 22 in which schizosaccharomyces pombe (ATCC 24970) is shown. The samples show slight differences but the pattern remains very similar.

The splitless injection can only be used with a double phase system. If the single phase method is used the elution of the solvent

FIGURE 17. SCHIZOSACCHAROMYCES POMBE (ATCC 24970)
CHROMATOGRAPHIC REPRODUCIBILITY

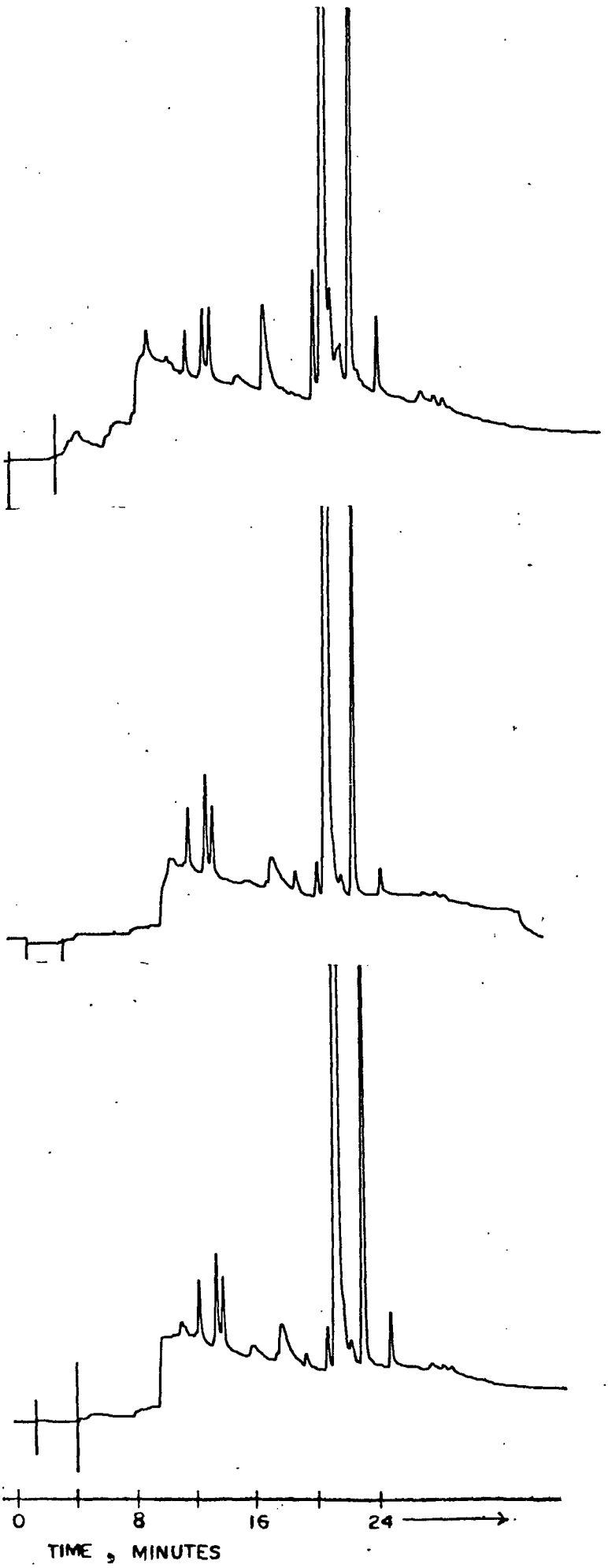

0
8
16
24
TIME , MINUTES

FIGURE 18. SCHIZOSACCHAROMYCES POMBE (ATCC 24970)
SEPARATELY DERIVATIZED SAMPLES

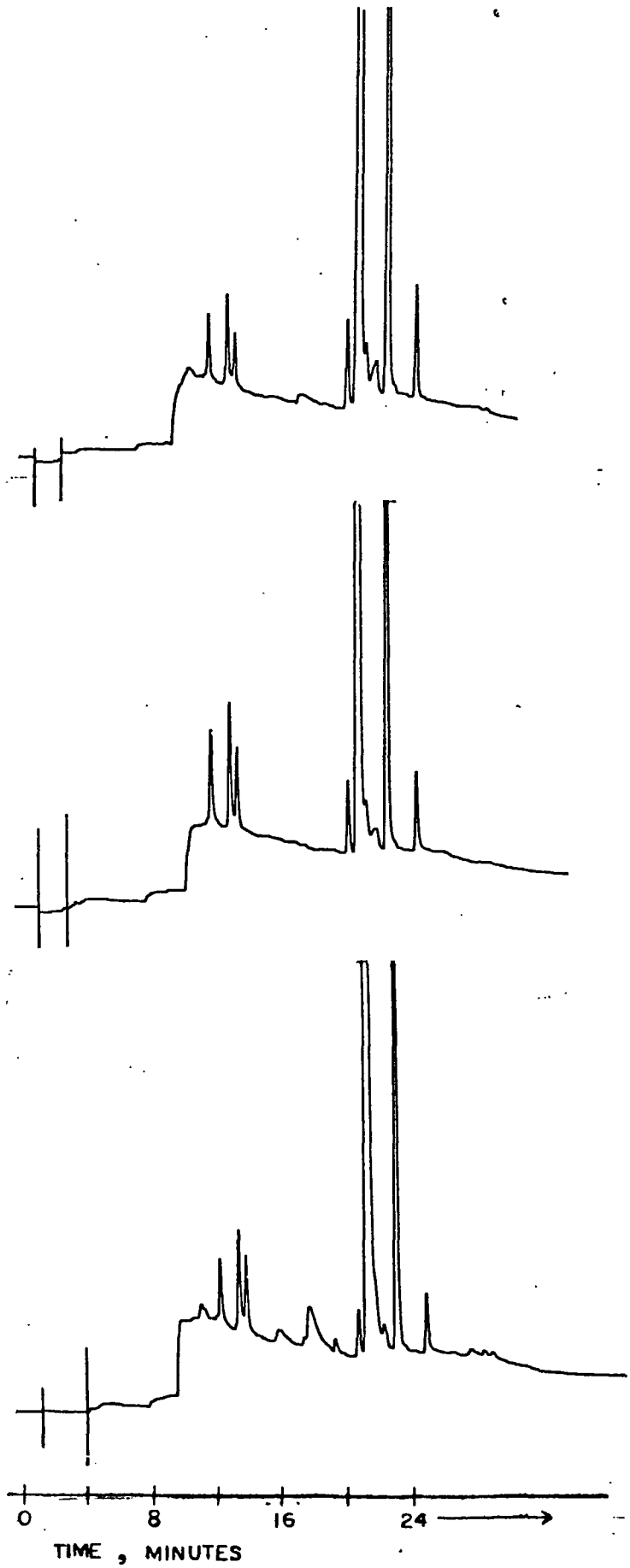

0
8
16
24
TIME , MINUTES

FIGURE 19. BLANK

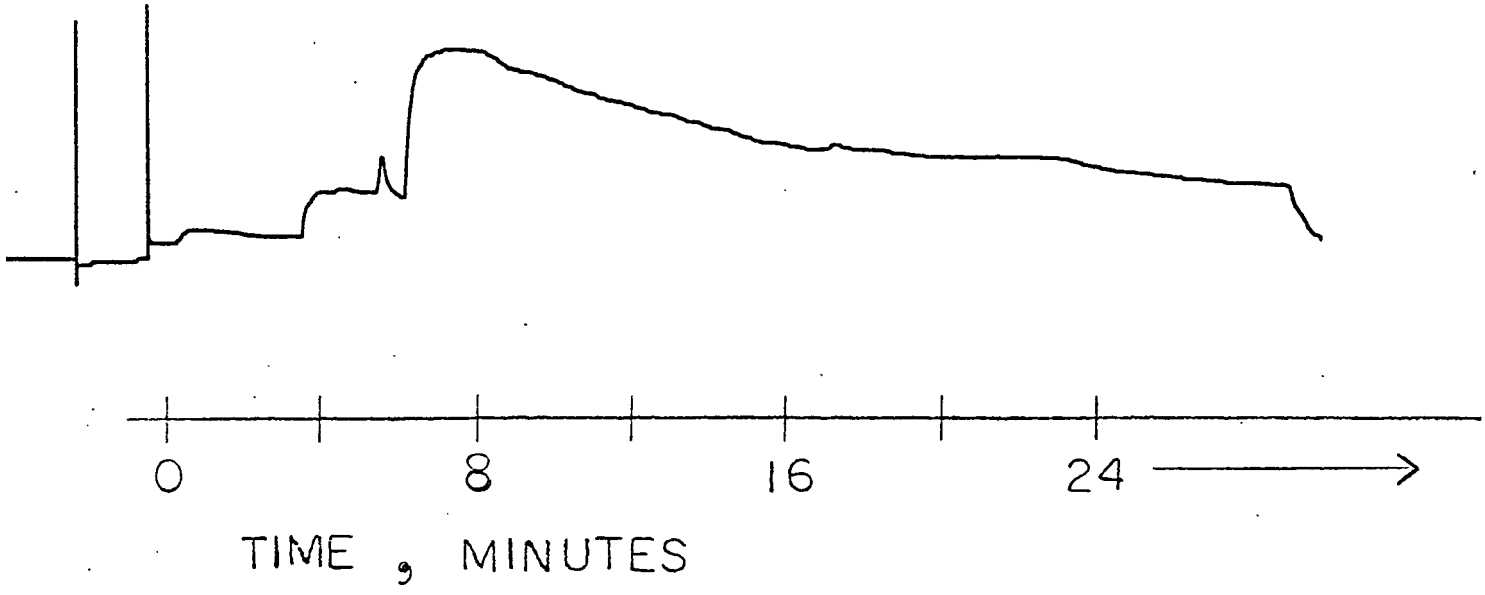

0
8
16
24
TIME , MINUTES

FIGURE 20. SOLVENT ELUTION. NO EFFECT.

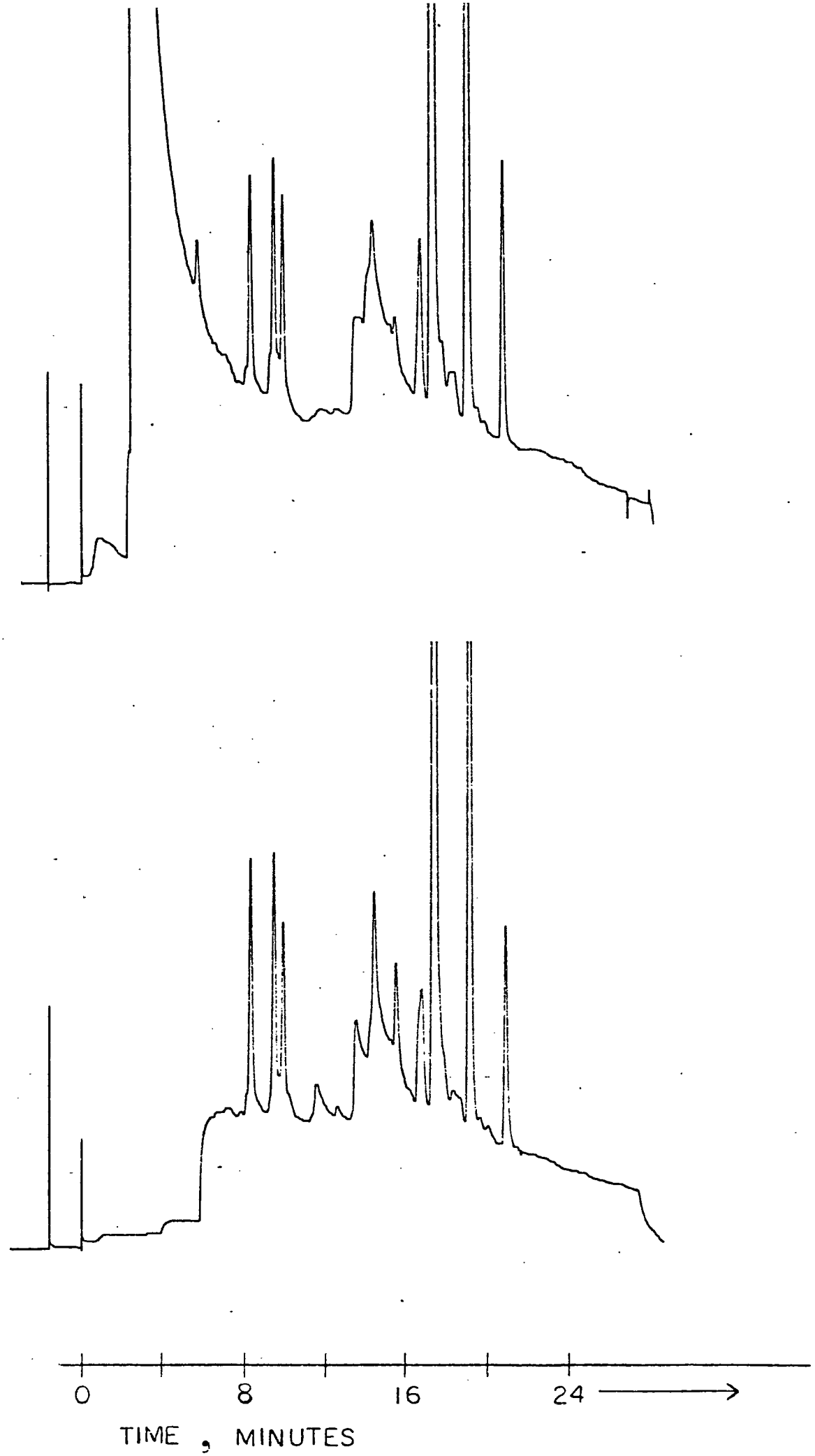

0
8
16
24
TIME , MINUTES

FIGURE 21. SOLVENT ELUTION. PEAK RATIO INVERSION

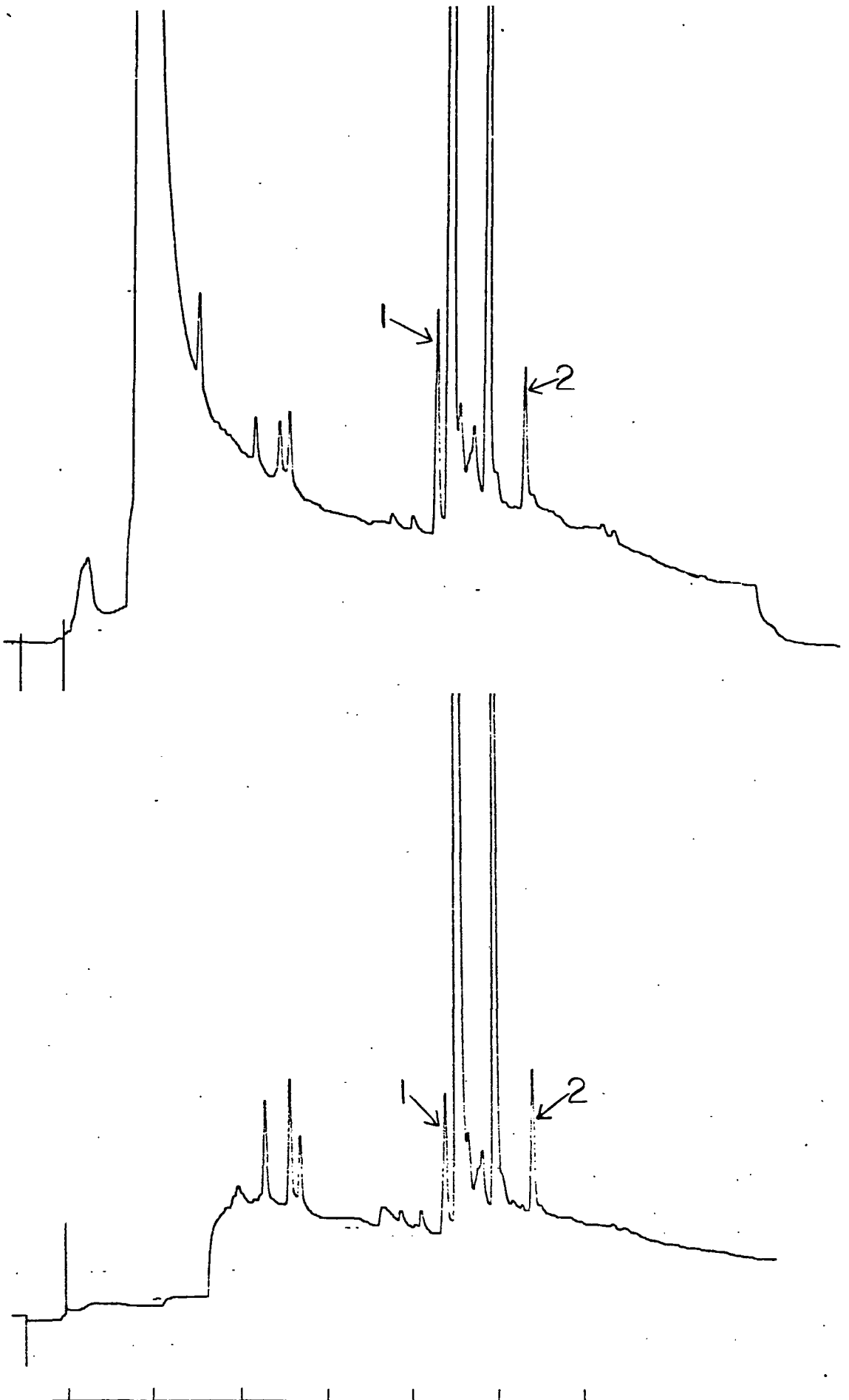
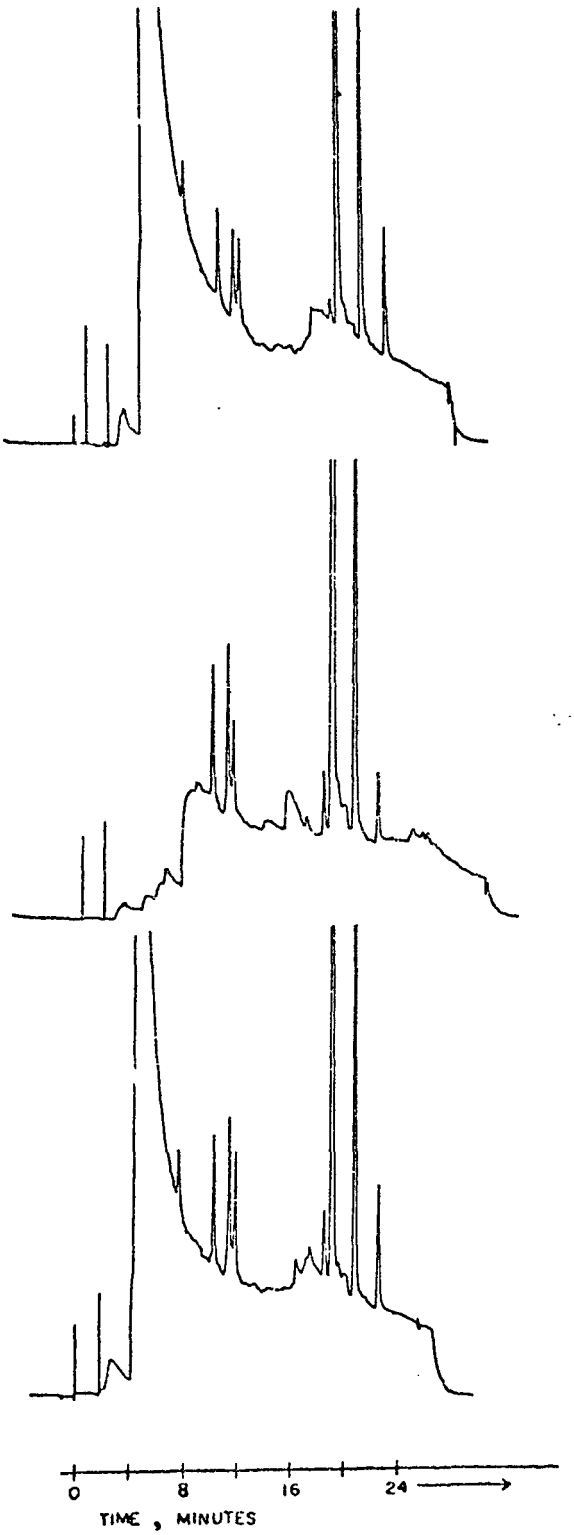

1
2
1
2
0
8
16
24
TIME , MINUTES

FIGURE 22. SCHIZOSACCHAROMYCES POMBE (ATCC 24970)
BIOLOGICAL REPLICATES


0
8
16
24
TIME , MINUTES

is not possible due to the low volatility of the reagent. Even 0.3 µl of the single phase system can not be evaporated under the conditions shown here.

S. Pombe (ATCC 24970) was washed several times to determine the effect of washing. The results are illustrated in Figure 23. (A) is the unwashed yeast, (B) the same yeast washed two times and (C) the yeast washed 5 times. All the washing was done with normal saline solution. There was a slight change in some of the peaks but the effect was not uniform. For all the other strains only unwashed and twice washed cells were compared. There were some differences between washed and unwashed cells but comparison of the four strains either washed or unwashed gave basically the same results.

The comparison of the unwashed cells of the four strains can be seen in Figure 24 and the washed ones in Figure 25. The similarities between the two schizosaccharomyces pombe (A and B) strains are very obvious in both figures. The difference between these two strains and the Candida (C) and Saccharomyces (D) species are also very evident.

The feasibility of classifying these particular strains of yeast has been demonstrated. The determination was also carried out as a blind test in which the biologist identified the samples only as A, B, C and D. All the samples were identified correctly.

Work is currently underway to apply this method to 20 species and subspecies of yeast. The results of this survey would indicate whether it is possible to apply this method for taxonomic purposes on a wider basis.

FIGURE 23. SCHIZOSACCHAROMYCES POMBE (ATCC 24970)

EFFECT OF WASHING

A) Unwashed
B) Washed two times
C) Washed five times

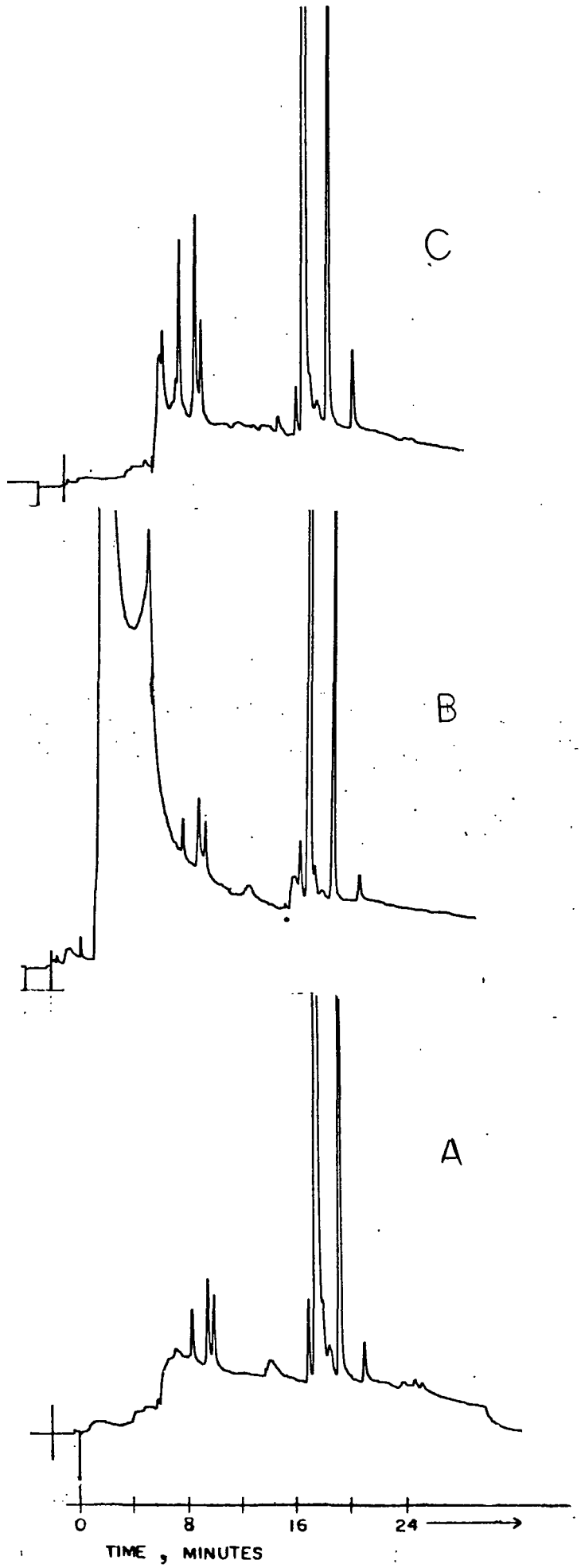

C
B
A
0
8
16
24
TIME , MINUTES

FIGURE 24. COMPARISON OF FOUR YEAST STRAINS UNWASHED

A) Schizosaccharomyces pombe (ATCC 24970)

B) Schizosaccharomyces pombe (972 h^-)

C) Candida albicans 1

D) Saccharomyces cereviseae (ATCC 8824)

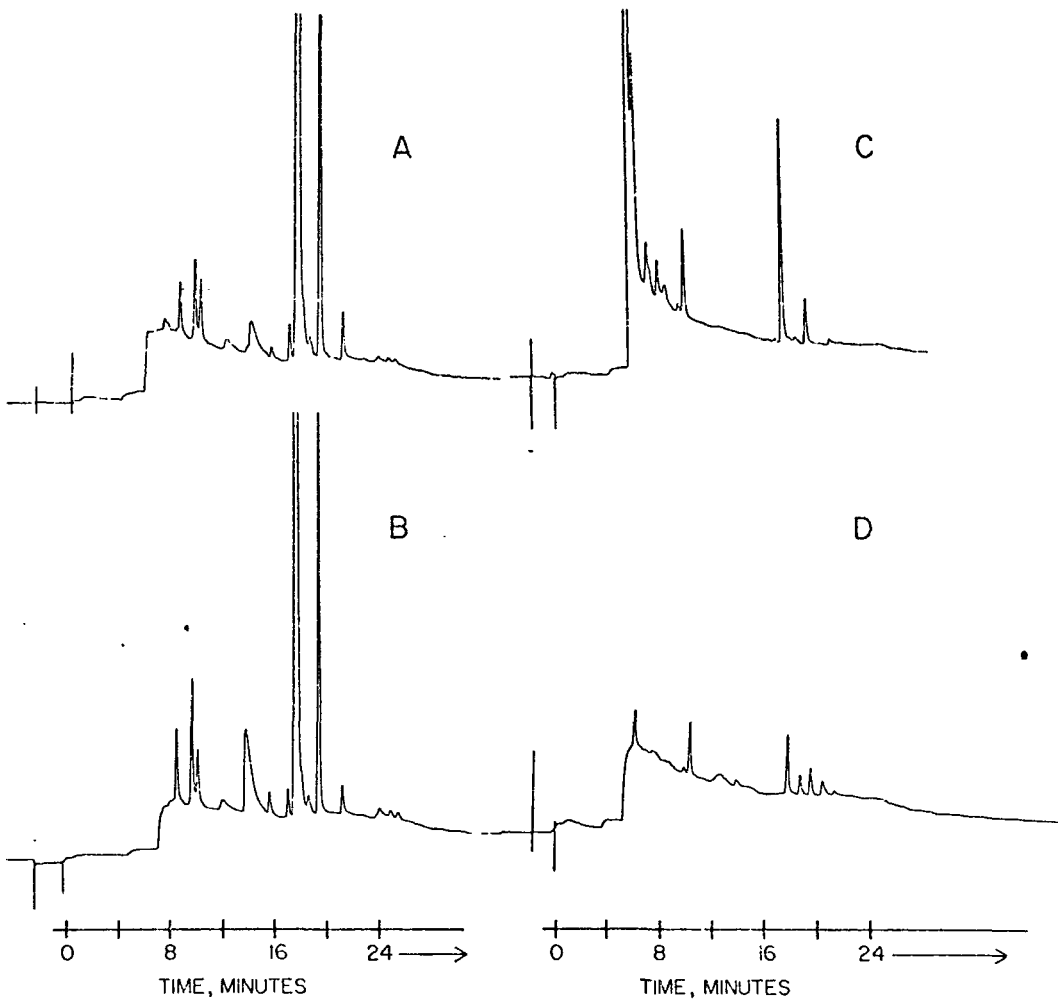

A
C
B
D
0
8
16
24
TIME, MINUTES
0
8
16
24
TIME, MINUTES

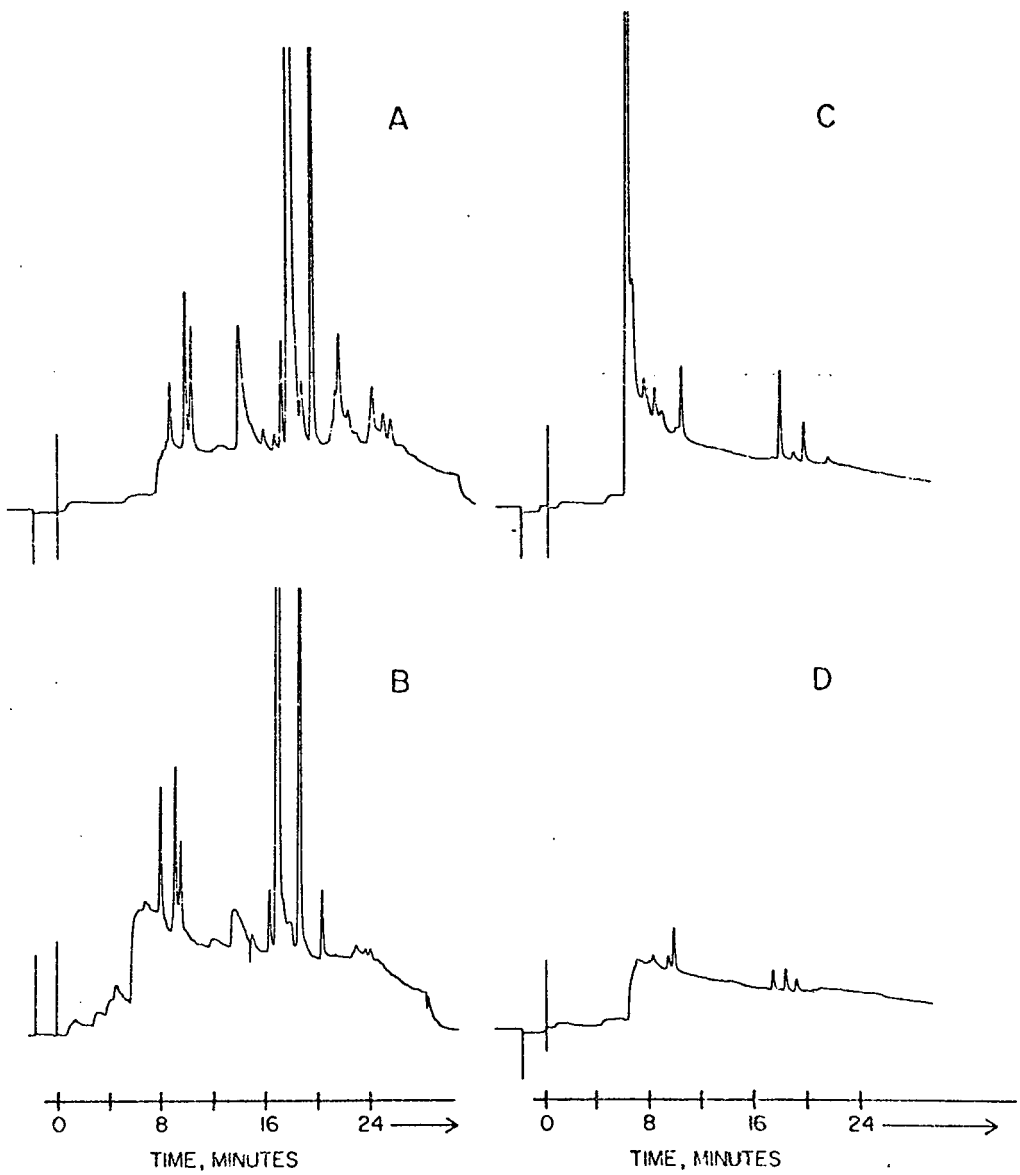
FIGURE 25. COMPARISON OF FOUR YEAST STRAINS WASHED

A) Schizosaccharomyces pombe (ATCC 24970)

B) Schizosaccharomyces pombe (972 h^-)

C) Candida albicans 1

D) Saccharomyces cereviseae (ATCC 18824)


A
C
B
D
0
8
16
24
TIME, MINUTES
0
8
16
24
TIME, MINUTES

As it was mentioned before the spent medium was also compared because it was thought that the metabolic products would be different. That was found to be the case. In Figure 26 the chromatogram of new medium is shown. In Figure 27 a comparison of the spent medium of the four strains is made. The similarities and differences are again very obvious.

It should also be noticed that the time at which the sample is harvested is very important. Care should be exercised to harvest the cells after the growth curve has reached its maximum. This was determined by sampling at different intervals during the growth stage. The best results were found during the plateau attained after maximum growth had been reached.

The use of yeast grown directly on agar plates was also considered. This offered several advantages. The sample could be harvested faster because the adaptation period required for liquid broth could be eliminated and growth curves would not be necessary. The results for the four strains are illustrated in Figure 28. The two saccharomyces pombe show remarkable similarity and the other two species are again different. Unfortunately the peak ratios vary.

In spite of the variation different samples were still different enough to allow their identification. This variation might be due to differences in the growth pattern that depends on the amount of sample inoculated, a factor that is very difficult to control. Another observation is that the number of peaks present is less than that observed in the yeasts grown on liquid broth. Saccharomyces

FIGURE 26. PYG MEDIUM

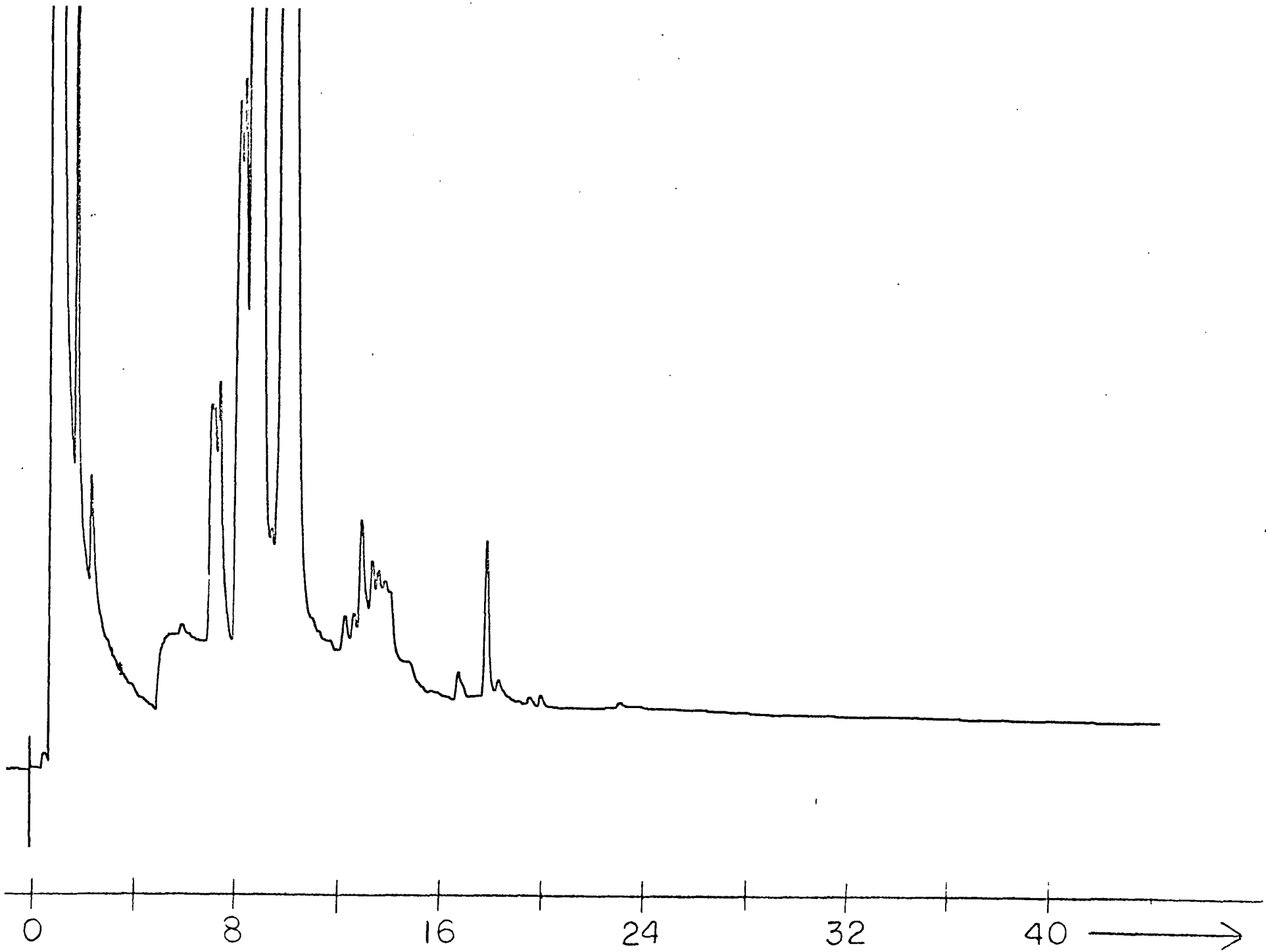

0
8
16
24
32
40
TIME . MINUTES

FIGURE 27. SPENT PYG MEDIUM FROM

A) S. Pombe (ATCC 24970)

B) S. Pombe 972 h^-

C) C. Albicans 1

D) S. Cereviseae (ATCC 18824)

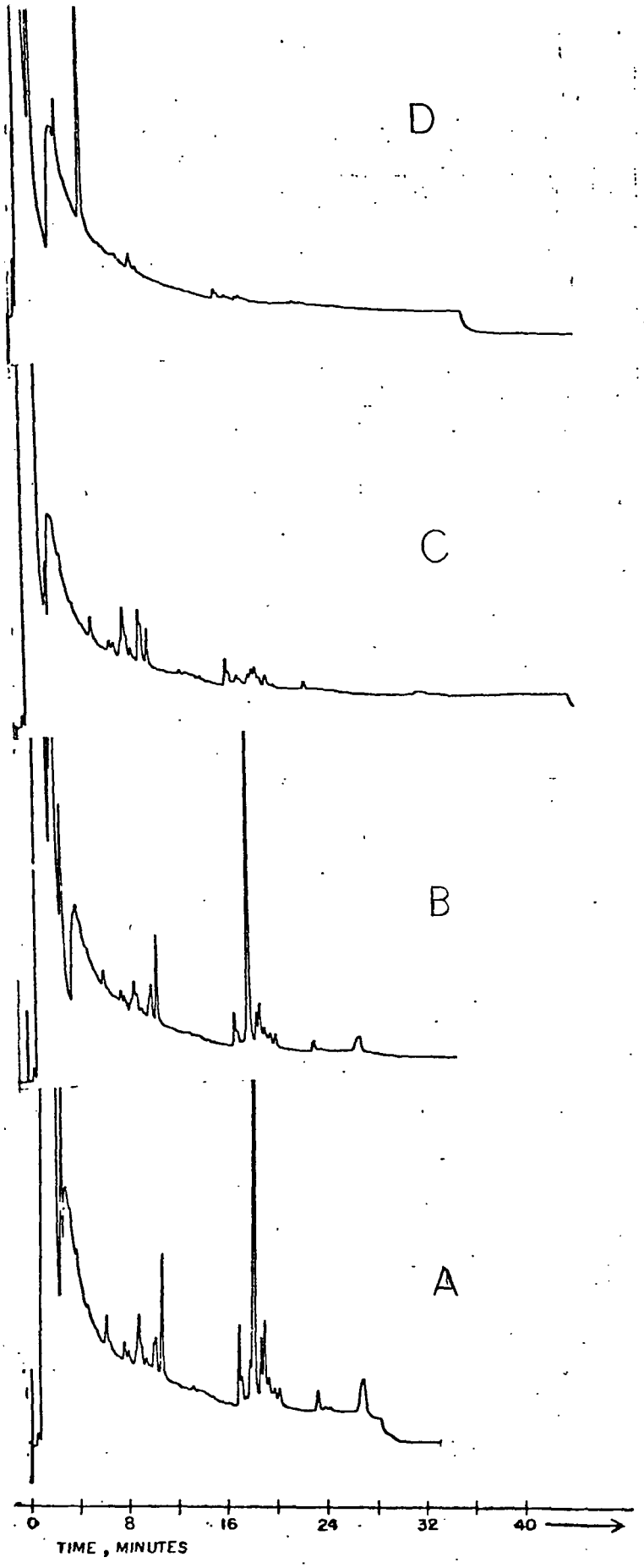

D
C
B
A
0
8
16
24
32
40
TIME , MINUTES

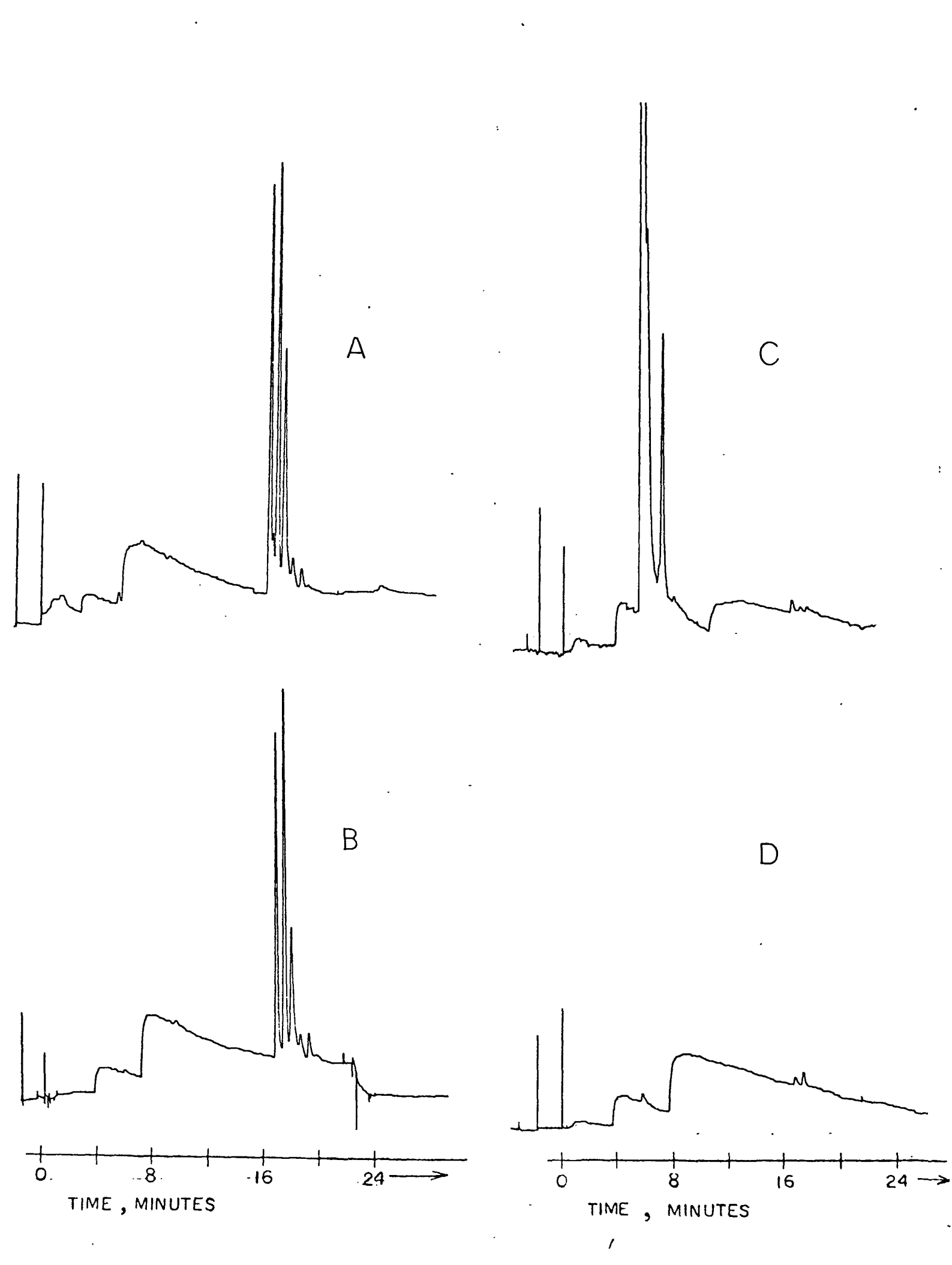
FIGURE 28. COMPARISON OF FOUR YEAST STRAINS GROWN ON PYGA MEDIUM

A) Schizosaccharomyces pombe (ATCC 24970)

B) Schizosaccharomyces pombe (972 h^-)

C) Candida albicans 1

D) Saccharomyces cereviseae


A
B
C
D
0
8
16
24
TIME , MINUTES
0
8
16
24
TIME , MINUTES

cereviceae does not show any while the other 3 strains show only a few each. These fewer peaks would limit the possibility of further using this technique to identify other microorganisms. However this could be a concentration effect and could be solved by freeze drying the yeast as it was done for the ones grown on liquid broth. It has been found that the yeast looses about 25% of its weight after freeze drying. The bigger amount of yeast and the smaller top phase attained this way could give the concentration necessary to see other peaks.

A dry sample would afford the possibility of a smaller top phase and hence a higher concentration of silylated sugars. Work in this area is progressing along this line. This may well result in more peaks appearing in the chromatogram.

D. HEADSPACE. APPLICATION TO YEAST SAMPLES.

The determination of volatiles in yeast samples by headspace analysis offers several problems. The samples were harvested and washed 5 times with normal saline solution. This eliminates the possibility of contaminants coming from the medium. The samples were run immediately after harvesting and washing. The effect of freezing them for storage purposes has not been studied. The length of sampling we found satisfactory was 30 minutes with a flow rate of helium of 30 cc/min. The sampling temperature was a very important factor to consider. Temperatures over 40°C will kill the yeasts and this can,

of course, affect the results. The compromise sampling temperature used was 30°C although it is a temperature difficult to control. The condenser water temperature was room temperature which was cool enough to avoid the water reaching the adsorbent trap. Once the sample is adsorbed in the Tenax trap it can be stored at -10°C for several days. The Tenax trap is placed in 20 x 125 mm culture tubes (Kimax, Kimball Products, Owens, Illinois), fitted with Teflon-lined caps and then stored. The samples were run, however, as soon as possible. In the majority of the cases they were run as soon as the sampling was completed.

Figure 29 shows the results obtained for three independently grown samples of schizosaccharomyces pombe. The chromatograms are not identical but the similarities in the last part of the chromatogram are evident. The differences in the first part of the chromatogram might be due to some contamination or to problems in the control of the sampling temperature.

The blank run also shows some impurities that can not be eliminated. The most likely source of contamination is the distilled water used in the preparation of the saline solution. The blanks were obtained by rinsing the headspace sampling container with the washing solution and then sampling under the same conditions as the sample. The chromatogram is illustrated in Figure 30.

The results were encouraging enough to try other strains of microorganisms and compare them with each other. In Figure 31, the chromatograms of three yeast strains are illustrated. Figure 31(A) is schizosaccharomyces pombe (972 h^-) and (B) is schizosaccharomyces

FIGURE 29. SCHIZOSACCHAROMYCES POMBE (ATCC 24970)
VOLATILES BIOLOGICAL REPLICATES

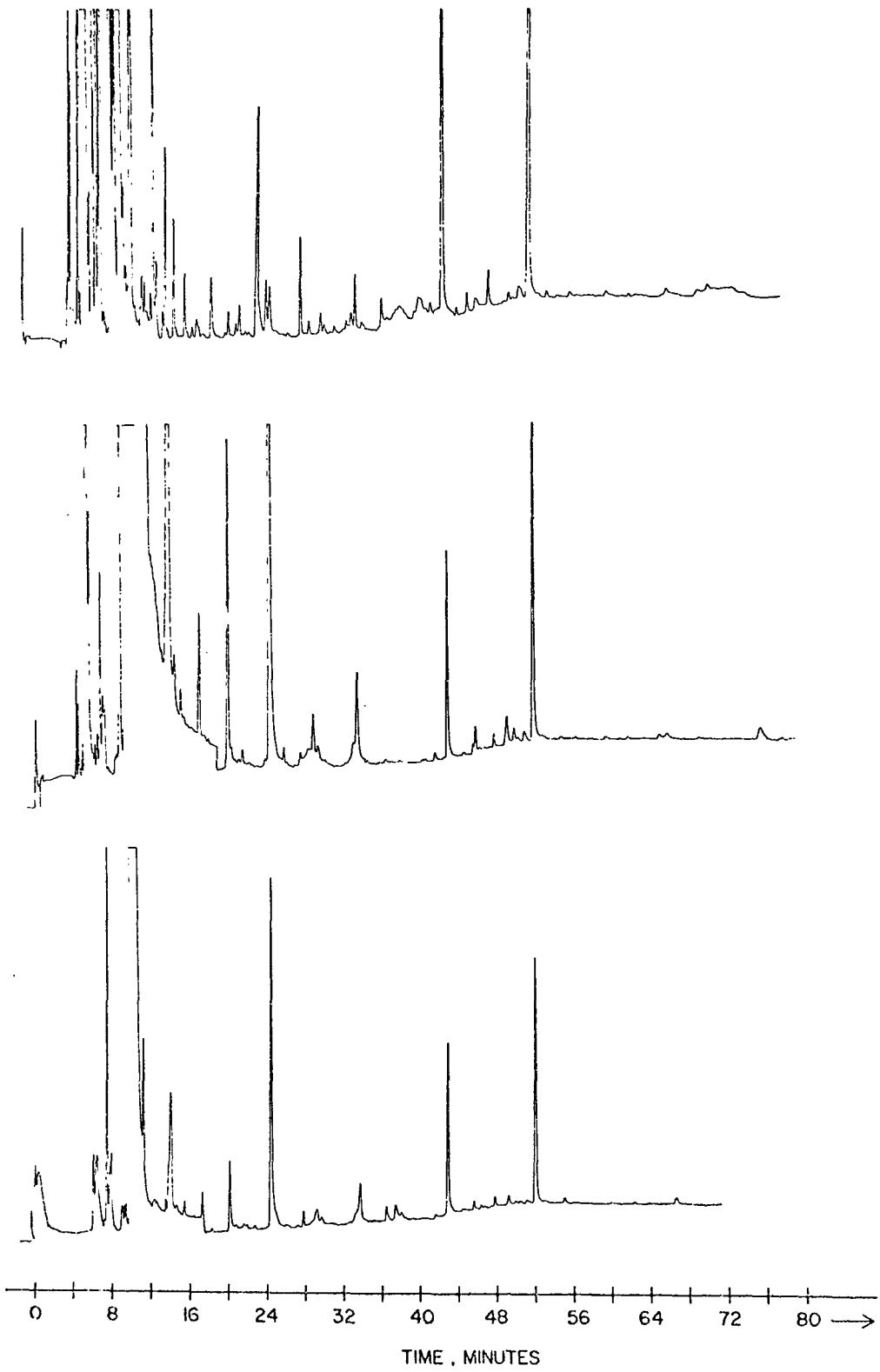

0
8
16
24
32
40
48
56
64
72
80
TIME , MINUTES

FIGURE 30. VOLATILES BLANK

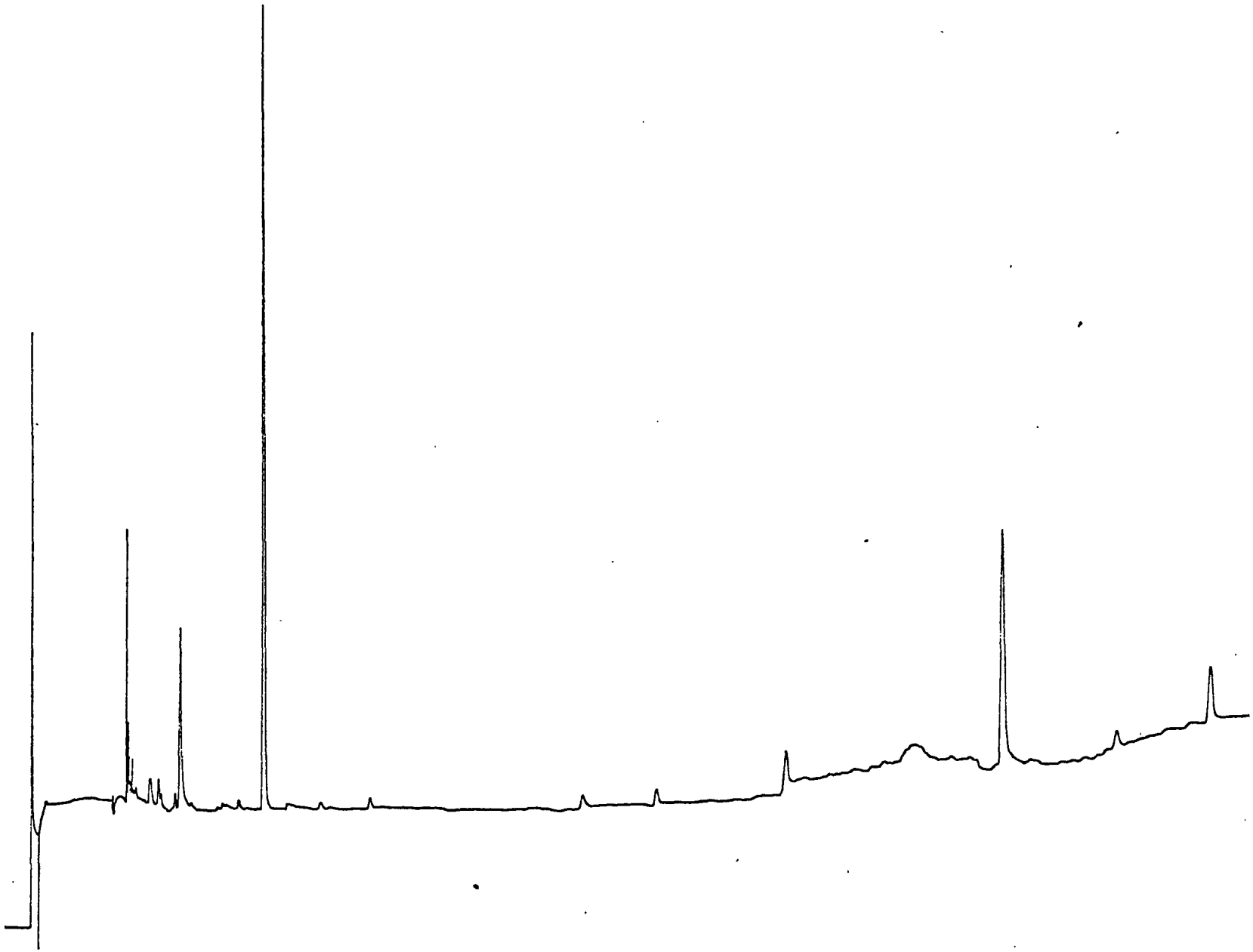
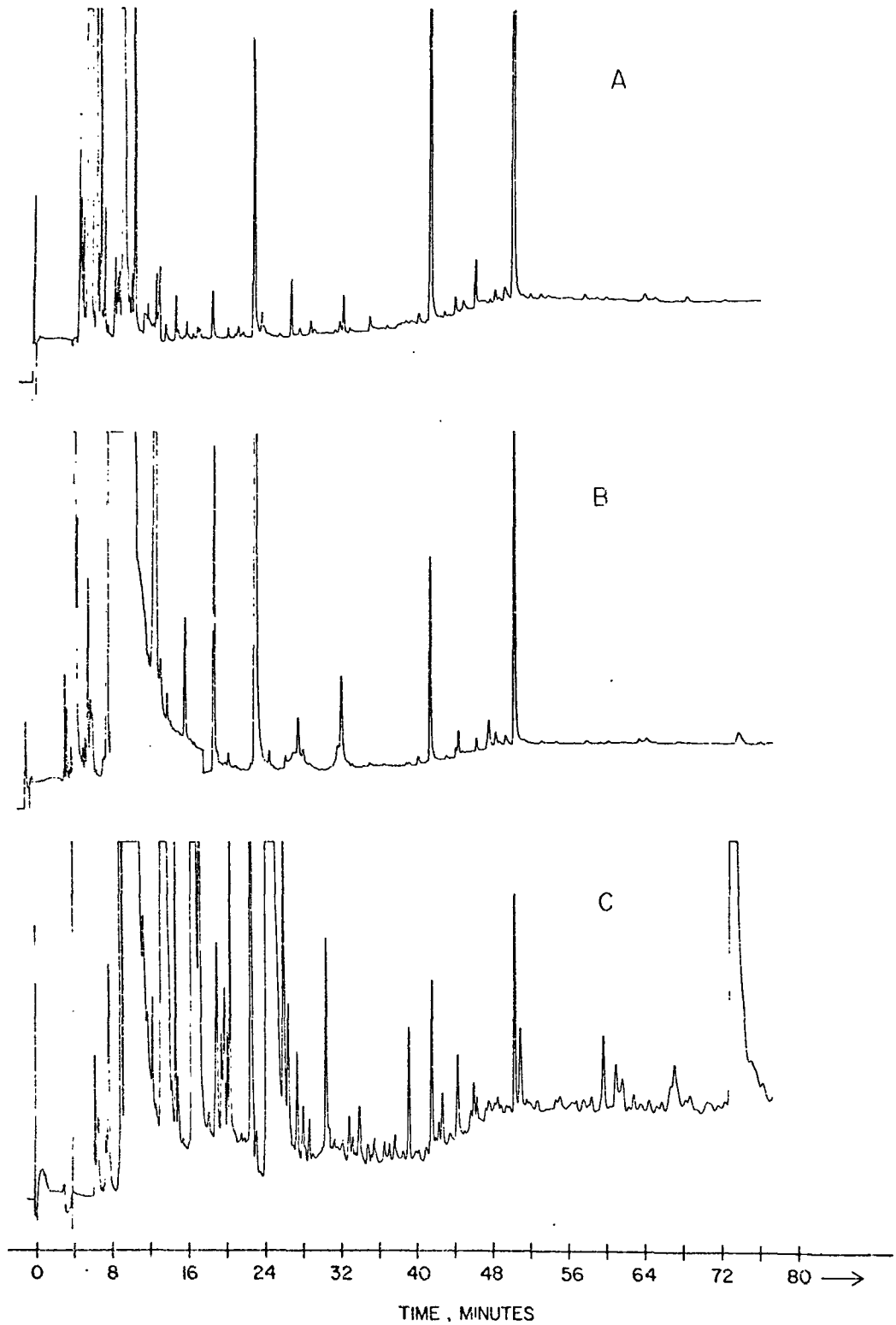


FIGURE 31. VOLATILES

A) Schizosaccharomyces pombe (972 h^-)

B) Schizosaccharomyces pombe (ATCC 24970)

C) Saccharomyces cereviseae (ATCC 18824)


A
B
C
0
8
16
24
32
40
48
56
64
72
80
TIME , MINUTES

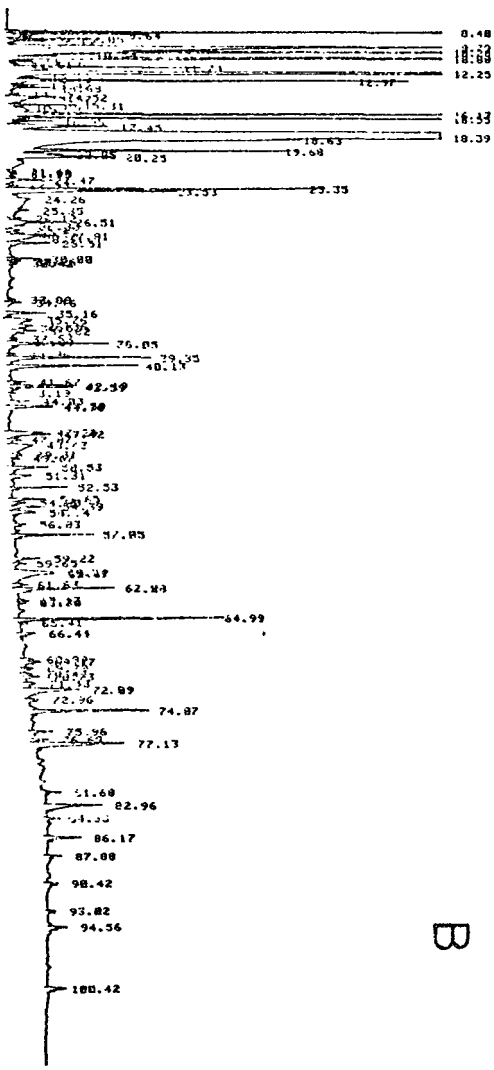
pombe (ATCC 24970). The similarities between these two yeasts are very obvious. Figure 31(C) is the chromatogram of saccharomyces cereviseae (ATCC 18824) and differences in the profile can be observed especially in the first and in the last part of the chromatogram when this sample is compared to the first two. Saccharomyces cereviseae is of great importance in the food and beverage industries.

The application of this technique to diseased or contaminated yeast strains might be very useful since it would be possible to compare the changes, if any, in the profile caused by the disease. The use of selective detectors could also be very useful in this problem since a lot of the aromas are caused by sulfur compounds.

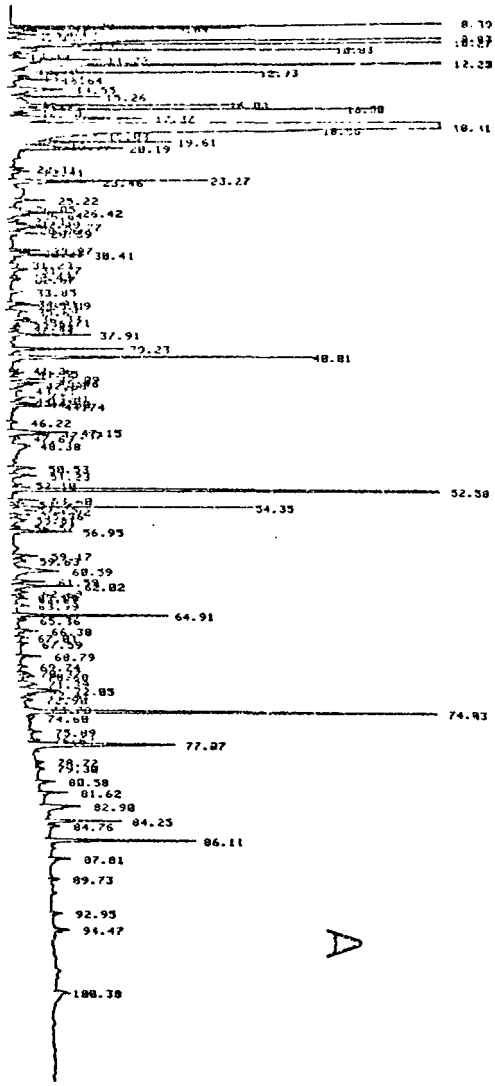
E. TRANSEVAPORATION. APPLICATION TO CELL CULTURES.

The transevaporation technique was applied to both the normal and the transformed cells. 80 µl of each were needed for the determination. The amount of volatiles is very small compared to the ones normally obtained from the serum but this was expected. The chromatograms are shown in Figure 32. 32(A) represents the normal cells and (B) the transformed ones. Some differences were shown especially in some peak ratios and also one of the early peaks was different.

FIGURE 32. TRANSEVAPORATION. COMPARISON OF NORMAL VS. TRANSFORMED CELLS


8.48
12.25
18.39
19.68
20.25
23.35
42.39
62.84
64.99
74.87
77.13
82.96
86.17
87.08
98.42
93.82
94.56
100.42

B


12.23
19.61
23.27
38.41
37.91
40.81
52.58
54.35
64.91
74.93
77.07
81.62
82.98
84.23
86.11
87.81
89.73
92.95
94.47
100.38

A

CONCLUSION

The use of a concentration technique for the derivatization of oligosaccharides was tested. The results were very good when the method was applied to the analysis of oligosaccharides by gas chromatography in samples that included liquids, solids or semisolids. The method was further developed to allow the determination of even smaller amounts of oligosaccharides. A slow injection technique and a splitless injection were developed. A concentration factor of up to 500 can be achieved with this method. These methods were applied to the analysis of mono- and oligosaccharides in cell cultures and yeast. The methods were found to be fast and reproducible for all these different samples. The splitless injection was found to be particularly useful in the taxonomic classification of four strains of yeast.

It is believed that the development of these methods will be of considerable help to life scientists involved in determining structures of complex glycolipids and glycoproteins. It will also be of interest to biologists working on the composition of cell walls.

The determination of volatiles in yeast and cell cultures was also undertaken. The results are encouraging but more effort is necessary to evaluate some of the future applications of these determinations and the best conditions for these determinations.

CONCLUSION

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