DETERMINATION OF ACETONE IN NORMAL AND DIABETIC SALIVA BY GAS CHROMATOGRAPHY

by

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ABSTRACT

DETERMINATION OF ACETONE
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BY GAS CHROMATOGRAPHY

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Master of Science
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An analytical method for the determination of acetone in human saliva is described. The method employs head-space gas chromatography with flame ionization detection. Rapid and simple quantitation is accomplished via the internal standard method. The normal limits of acetone in human saliva are established and are examined for correlation with age and length of fast. The relationship between diabetes and increased saliva acetone over normal levels is discussed. Computer assisted literature searches and data reduction analyses are utilized.

ACKNOWLEDGEMENTS

My appreciation is extended to Dr. Francis Smith for his suggestion of this problem and guidance throughout this research. I also thank Dr. Robert Malinowski for his technical advice. Further thanks are extended to my experimental subjects who unselfishly donated saliva specimens.

My utmost gratitude is extended to my wife Kathleen for her unending encouragement and support. Finally, this research is dedicated to the glory of God.

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LIST OF SYMBOLS

SYMBOL OR ABBREVIATION	DEFINITION
A.R.	Analytical reagent
β	Beta
CAS	Chemical Abstracts Service
cm	Centimeter, 1×10^{-2} meter
C.V.	Coefficient of variation
°C	Degrees Celsius
G.C.	Gas chromatography
hr	Hour
ISTD	Internal standard
mL	Milliliter, 1×10^{-3} liter
µg	Microgram, 1×10^{-6} gram
µL	Microliter, 1×10^{-6} liter
min	Minute
mg	Milligram, 1×10^{-3} gram
%	Percent
±	Plus or minus
RT	Retention time of a peak from time of injection to apex of peak, in minutes

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CHAPTER I

INTRODUCTION

In recent years, saliva has been under investigation for use as a biological indicator of health or disease. Saliva is of interest because it is easily obtained in adequate quantities for analysis via a non-invasive technique. There is little work in the literature either on the detection of acetone in human saliva or the relationship of saliva acetone levels to diabetes. This investigation addresses these problems by developing an analytical method for the determination of saliva acetone and by correlating healthy subjects' saliva acetone levels to those of diabetics.

Saliva

The normal fluid of the oral cavity is whole saliva, which is a mixture of secretions from the parotid, submandibular and sublingual glands and from the many minor mucous glands present in the oral mucosa. The total volume of whole saliva produced by a healthy individual during a 24-hour period ranges from 1,000 to 1,500 mL. Although there are individual

variations, the different glands contribute to the total volume of whole saliva as follows: submandibular, 65%; parotid, 23%; minor mucous 8%; and sublingual, 4%.[1] Whole saliva may also contain shed epithelial cells, food debris, bacteria, white blood cells and other particulate matter found in the oral cavity.

Whole saliva may be obtained by either stimulated or unstimulated techniques. Stimulated whole saliva is obtained by allowing the subject to chew on an inert material, i.e. paraffin wax or rubber bands. Other substances such as salt, citric acid or hard candies also stimulate the flow of saliva but could be a source of contamination.

unstimulated saliva is secreted when a waking person is at rest and unaffected by external stimuli.[2] There are three primary methods for the collection of unstimulated whole saliva: (1) draining, (2) suction, and (3) spitting. In the draining method, the subject inclines his head forward so the saliva that collects on the floor of the mouth flows over the lower lip and into a collection container. The suction method employs a vacuum collection device that aspirates the saliva collected after the subject has remained quiet for a fixed period of time. The spitting method allows the subject to actively participate in the

collection process by spitting at regular intervals into a container.

Unstimulated whole saliva collected by the spitting method was used in all determinations. Whole saliva was chosen because of a lack of information in the literature on any possible preferential concentration of acetone in one or more of the salivary glands. Whole saliva was also chosen so that the subjects participating in this investigation could perform the sample collection unaided. The unstimulated technique was used to eliminate any possible cross contamination from the soluble or non-soluble stimulant. The spitting method also allowed the subjects to collect their saliva unaided and eliminated the need for a suction device. Overall, this sample and collection method was employed due to its simplicity, ease of use in instruction and representative sampling.

Metabolism, Diabetes and Ketosis

Cellular mitochondria can produce energy by oxidizing long chain fatty acids in a repetitive series of reactions that shorten the acid chain two carbon atoms at a time. This process, termed β-oxidation, converts a C16 molecule to eight molecules of an intermediate known as acetyl coenzyme A (Acetyl CoA). Oxaloacetate, derived from carbohydrate metabolism,

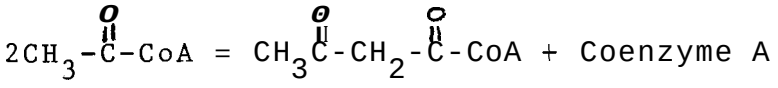
enzymatically condenses with acetyl CoA and prevents it from accumulating in the cell. This condensation reaction produces citrate which is a major component in the Rrebs or tricarboxylic acid cycle. Nearly all food material whether protein, fat, or carbohydrate, undergoes a final oxidation in the Krebs cycle. Therefore, efficient metabolism depends on having sufficient oxaloacetate available to react with acetyl CoA.

In acute starvation or in a state of impaired carbohydrate metabolism, as found in diabetes mellitus, acetyl CoA supplies are greater than those of oxaloacetate. This is due to an excessive release of fatty acids from adipose cells which result in an increased breakdown of these fatty acids by β-oxidation in liver cells. Therefore, the increased supply of acetyl CoA is metabolized via an alternate pathway, one that results in the formation of increased amounts of acetoacetic acid, β-hydroxybutyric acid and acetone. This condition is known as ketosis and the three metabolites as ketone bodies. Accumulation of excessive amounts of acetoacetic and β-hydroxybutyric acids may cause metabolic acidosis.

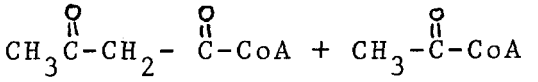
Ketosis develops from an increased production of acetyl CoA as the body attempts to obtain energy by metabolizing stored fat in the absence of an adequate supply of carbohydrates. In order to overcome the

deficiency of glucose, the body liberates large quantities of free fatty acids. As fat is released from storage and the resulting long chain fatty acids are received by functional cells, including those in the liver, they are prepared for degradation by conversion into CoA derivatives. These long chain CoA derivatives inhibit enzymatic production of oxaloacetic acid from glucose or glycogen. Ketogenesis is therefore increased in any condition that promotes the excessive release of fatty acids from adipose cells.

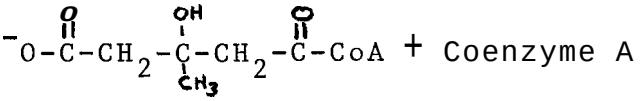
Much of this excess fatty acid production is removed from the blood by liver cells. These cells are largely responsible for the conversion of fatty acids to acetoacetate. Liver cells, unlike muscle cells, are unable to metabolize the resulting acetoacetate. In normal metabolism, acetyl CoA molecules couple with oxaloacetate. Ketogenesis occurs when two acetyl CoA molecules join according to the following reactions.[3] The first reaction forms acetoacetyl CoA which then condenses with a third acetyl CoA molecule in the second reaction to form β-hydroxy-β-methylglutaryl CoA.



Acetyl CoA Acetoacetyl CoA

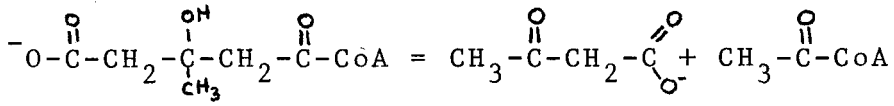


Acetoacetyl CoA Acetyl CoA



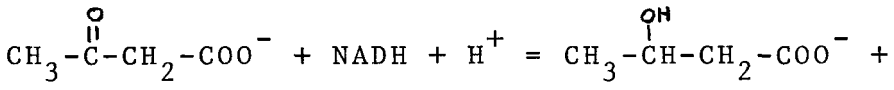
β-Hydroxy-β-Methylglutaryl CoA

This product is cleaved enzymatically to yield acetyl CoA and acetoacetate.



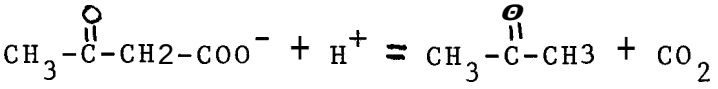
β-Hydroxy-β-Methyl-glutaryl CoA Acetoacetate Acetyl CoA

The resulting acetoacetate is usually reduced to β-Hydroxybutyrate.



Acetoacetate β-Hydroxybutyrate

Free acetoacetate is unstable and some decomposes to form acetone and carbon dioxide.



Acetoacetate Acetone

This decarboxylation reaction accounts for the presence of the elevated levels of acetone found in untreated diabetes. Ketone bodies are utilized by muscle, heart,

and brain cells because these tissues are able to resynthesize the CoA derivatives of the acids involved and then oxidize them and produce energy.

Ketosis is reversed by restoring carbohydrate metabolism to an adequate level in the body. In diabetes mellitus this can entail administration of insulin which permits blood glucose uptake by the cells. This uptake apparently cannot proceed without insulin. Normal metabolism is restored when oxaloacetate is again available and the release of fatty acids from adipose cells slows and reverses.

Chromatography

Recently, a sensitive head-space gas chromatographic technique was utilized by Tomita et al. for the determination of ethanol and acetone in human saliva.[4] This procedure was carried out by applying the analytical method for blood alcohol developed by Wilkinson et al.[5]

In this investigation, the sensitivity of the above determination was increased by standardizing the method as follows: (1) using an appropriate volume (1.0 mL) of saliva sample, (2) injecting 3 mL of head-space gas, (3) maintaining the equilibration time at 5 min., (4) maintaining the equilibration temperature at 60° C, and (5) using an appropriate volume of

internal standard (100 μL). Disposable plastic syringes were used to simplify the method and provide for a clean, uncontaminated injection of all samples and standards.

The chromatograph is a keyboard input instrument that contains a multi-function digital processor. Using the values entered via the keyboard on a separate terminal unit, the processor establishes control over all heated zones (injection ports, column oven, and detectors), carrier gas flows, and recording sensitivities. The processor also analyzes integration data by basing its calculations on the method entered before a run is started. During the run, the terminal traces the chromatogram on heat-sensitive Z-fold paper and prints peak retention times near each peak apex. At the end of the run, the terminal automatically prints the associated internal standard method report under control of the processor.[6]

In the internal standard method, to each sample is added a specified amount of a substance which is known not to be present in the saliva itself. The concentration of one component in the resulting chromatogram is therefore known and the other concentrations can be determined by simple proportion.

$$\text{Amount of Y} = \frac{\text{Area}_Y \times \text{Response}_Y}{\text{Area}_{ISTD} \times \text{Response}_{ISTD}} \times (\text{ISTD Amt}) \times (\text{XF})$$

The calculation is designed to give an absolute amount of Y in the final combined volume of sample and internal standard when the absolute amount of internal standard added is specified. The internal standard method has the following advantages: increased accuracy, calculation of absolute amounts, correction for differences in detector behavior, correction of sample size variations, and only the peaks of interest (including the internal standard) need to be calibrated and eluted from the column.

There are a number of quantitative and semiquantitative methods for the determination of ketone bodies in serum or urine. The generally accepted methods are the semiquantitative Acetest and Ketostix reagents (Ames Co., Div. Miles Laboratories, Elkhart, IN.). Acetest tablets contain a mixture of glycine, sodium nitroprusside, disodium phosphate and lactose. Acetoacetate or acetone forms a lavender-purple colored complex with nitroprusside in the presence of glycine. Lactose increases color development and disodium phosphate provides an optimum pH. This semiquantitative test gives a range of 1+ (purple-lavender color) to 4+ (dark lavender color) reaction. These correspond to 5-10 and 40-50 mg of ketone bodies per 100 mL.

The Ketostix is a modified nitroprusside test using a reagent strip in place of a tablet. This test

gives a positive reaction in 15 seconds with a specimen containing 5 to 10 mg of acetoacetate per 100 mL. As in the Acetest, color blocks represent increasingly more positive reactions. Acetone also reacts, but to a considerably lesser extent.

The internal standard headspace gas chromatographic method has the following advantages over either semiquantitative test. First, the G.C. method reports quantitative amounts. Second, it has an approximately 100 fold greater sensitivity. Third, the G.C. method is specific for acetone and does not suffer interference problems as do the other tests. Fourth, the internal standard adjusts for operator or instrument malfunction.

Computer

A novel approach was used for the literature search accompanying this research. A Tandy TRS-80 Model I microcomputer was used to access an online mainframe computer information service via a modem (modulator-demodulator) and telephone lines. The Dialog Information Retrieval Service (part of the Lockheed Missile and Space Co., Palo Alto, CA) offers online interactive access to literally millions of references. Dialog has some 50 billion bytes of information available online in over 130 individual data bases.[7]

One of these data bases is CA Search produced by Chemical Abstracts Service. This database combines the condensed version of Chemical Abstracts with the controlled vocabulary CA general subject headings and CAS registry numbers, each with its own modifying phrase. Also included is related general subject terminology from the CA index guide. CAS registry numbers are used throughout to represent the corresponding chemical substance. CA Search includes the following sources: journal articles, patents, reviews, technical reports, monographs, conference and symposium proceedings, dissertations and books. CA Search contains all information from the 8th through the 11th collective index from the years 1967 to 1982 inclusive and consists of approximately 5.5 million records. (Table 1)

Dialog is usually accessed through either Telenet or Tymnet, two national data communications networks that have local telephone numbers in many cities. There are various levels at which the Dialog system can be used. Although there are many commands available in the Dialog search language, a relatively small number are used for the majority of search strategies. They are: "Explain", an online help file that provides a detailed description of any specified command. "Select", which sets aside index terms or groups of terms specified into numbered sets

(up to 98). More than one term can be combined into a single select statement by inserting Boolean operators between terms, i.e. Select saliva and acetone. "Expand", displays a listing of index terms that are alphabetically close to the term entered. "Type", displays records online from the sets retrieved from the data base. Various formats can be displayed, i.e. the Dialog reference number, title only or full record. "End/Save", ends a search session and saves the search strategy (individual steps) used in a data base. The strategy then can be used in other data bases by using the "Execute" command. This eliminates the time and expense of entering individual steps every time a different data base is selected.[8]

Table 1

Chemical Abstracts collections available for computer searching via Dialog Information Retrieval Service.

File	Collective Index Period	Inclusive Dates	Accession Numbers	Update Frequency	File Size
308	8th	1967-1971	66000001-75157995	Closed file	1,314,655 records
309	9th	1972-1976	76000001-85201798	Closed file	1,772,194 records
320	10th	1977-1979	86000001-91222077	Closed file	1,275,366 records
310	10th	1980-1981	92000001-95231484	Closed file	926,314 records
311	11th	1982-	96000001-	Biweekly (approximately 34,000 records per month)	173,647 records as of June 1, 1982

The literature search for this research included all of the CA search files and was effective in finding several articles that might not have been located by a manual search. A typical search strategy is shown in Figures 1 and 2. This computer-aided literature search dramatically reduced the time required for locating pertinent articles and increased the search specificity through the use of Boolean operators.

In this research, the microcomputer was also used to analyze data generated in the experimental runs for accuracy and precision testing. This included the mean, standard deviation, correlation coefficient, coefficient of variation, standard curve and linear regression plots. All mathematical functions and modeling were accomplished using commercially available software written for this microcomputer.

```
File 310: CA Search - 1980-1981 (See 308, 309, 320, 311)
(Copr. Am. Chem. Soc.)

     Set Items Description
     --- ----- -----------

? SS Saliva? and Gas(W) Chromatography:
        1  1450 Saliva?
        2  3200 Gas(W) Chromatograph?
        3    12 1 and 2
? D 3/6/1-12
3/6/1
95181774      CA: 95(21)181774K Journal
  Gas Chromatographic Determination of Indoles in Human
Mouth Saliva Using a Flameless Alkali Sensitized Detector
(Nitrogen/Phosphorus-Specific Detector, NPD)
  Journal: J. Chromatogr. Sci.     Date: 1981 Volume: 19
Pages: 444-7
```

Fig. 1. Output from a typical Dialog computer search as used in this research. Search of Chemical Abstracts (1980-81) for the key words saliva and gas chromatography, listing twelve articles that have both keywords and displaying one of the articles bibliographic reference.

```
File 320: CA Search - 1977-1979 (See 308, 309, 310, 31-1)
(Copr. Am. Chem. Soc.)

        Set Items Description

? SS Saliva? and Acetone:
        4  1782 Saliva?
        5  3861 Acetone

        6  1  4 and 5
? D 6/6/1
6/6/1
89141322  CA08917141322F Journal: KOKU EISEI GAKKAI ZASSHI
Publ: 78 Series: 28 Issue: 1 Pages: 35-42 Language: Japan
Volatile Matters in the Oral Cavity 1. Determination of
Alcohol and Acetone in Saliva
```

Fig. 2. Output of Dialog computer search of Chemical Abstracts (1977-79) for the keywords saliva and acetone, listing one article having both keywords and displaying the articles bibliographic reference.

CHAPTER II

MATERIALS AND APPARATUS

The following is a list of chemicals used in this research.

NAME	GRADE	MANUFACTURER
Acetone	A.R.	Fisher Scientific Co.
1-Propanol	A.R.	Fisher Scientific Co.
Ethanol	A.R.	Mallinckrodt
Methanol	Pesticide	Fisher Scientific Co.
Acetaldehyde	A.R.	Matheson, Coleman, Bell

Apparatus

All sample analyses were performed using a Hewlett-Packard Model 5830A gas chromatograph with a H.P. Model 18850A GC terminal (Hewlett-Packard, Inc. Avondale, Pa. 19311). The Hewlett-Packard gas chromatograph was equipped with dual flame ionization detectors, with one side connected to a six-foot by 2 mm I.D. coiled glass column packed with Chromosorb 101, 80/100 mesh (Supelco, Inc. Bellefonte, Pa. 16823). A six-foot by 2mm I.D. coiled glass column packed with 3% OV-17 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, Pa. 16823) was connected to the other flame ionization detector.

Additional Apparatus

Septum sealed bottles, 10 mL	Supelco, Inc., Bellefonte, PA 16823
Seal crimper	Pierce Chemical Co., Rockford, IL 61105
Oven	Blue M. Electric Co., Blue Island, IL 60406
Disposable syringe, 5 mL	Becton, Dickinson and Co., Rutherford, NJ 07070
Micropipettor, digital	Scientific Manufacturing Ind., Emeryville, CA 94608
Micropipettor, fixed volume	Drummond Scientific Co., Broomall, PA 19008
Vacutainer tubes, 10 mL	Becton, Dickinson and Co., Rutherford, NJ 07070
Syringe, 10 µL	Hamilton Co., Reno, NV 89510
Timer	Fisher Scientific Co., Cleveland, OH 44123
Microcomputer, TRS-80 I	Tandy Corp., Fort Worth, TX 76102
Hot Plate/Stirrer	Corning Laboratory Products, Corning, NY 14830
Vortex mixer	Scientific Industries, Inc., Bohemia, NY 11716

CHAPTER III

EXPERIMENTAL

Reagents

Preparation of the reagents for G.C. analysis is described below.

Stock acetone solution, 100 μg/mL: Dilute 12.626 μL of acetone to 100 mL with distilled water.

Standard acetone solutions, 20.0, 10.0, 5.0, 1.0, 0.5 μg/mL: prepared by serial dilution of the 100 μg/mL stock solution.

1-Propanol internal standard, 200 μg/mL: Dilute 0.248 mL of 1-Propanol to 100 mL with distilled water.

Sample Collection

Unstimulated whole saliva was collected by actively spitting into a 10 mL Vacutainer tube over a period of approximately 15 minutes. The samples not immediately analyzed were stored in the collection tube in a freezer as it has been demonstrated by this research and others [9] that they are stable for up to one week under these conditions.

Headspace Technique

One mL of saliva or standard and 0.1 mL of 1-Propanol internal standard were pipetted into a 10 mL bottle. The bottle was then sealed with an aluminum tear-away cap lined with a teflon-faced rubber septum. All samples were then vortexed for 30 sec. and incubated in a 60° C water bath for 5 min. A 5 mL disposable plastic syringe fitted with a 25 gauge needle was heated to 70° C in an oven, then used to extract 3 mL of headspace gas from the sealed bottle. This volume was then injected into the G.C. without delay.

G.C. Analysis

Optimum operating conditions for the Chromosorb 101 column were determined to be as follows: column temperature isothermal at 150° C, injection port temperature 250° C, flame ionization detector temperature 300° C, chart speed 1.0 cm/min, attenuation 2↑5 (X32) slope sensitivity 0.20, carrier gas (nitrogen) flow 30 mL/min. Hydrogen and air were used as flame ionization detector gases with flow rates of 40 mL/min and 220 mL/min respectively.

CHAPTER IV

RESULTS

Figure 3 shows a sample chromatogram obtained from a standard solution containing 10 μg/mL of acetone (RT=1.88 min) and 200 μg/mL of 1-propanol internal standard (RT=2.77 min) using flame ionization detection and the Chromosorb 101 column. These peaks were in general agreement with those obtained by Tomita[10] on the GC-1 chromatography system. The identity of acetone and 1-Propanol were also confirmed by injection of standards and unknowns on a second chromatography system: 6 foot by 2 mm I.D. coiled glass column containing 3% OV-17 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA 16823) (Fig. 4). The peak at 0.61 min. is present in all Chromosorb 101 column runs including blanks and appears to be normal detector response to a 3 mL head-space injection into the system.

Figures 5 and 6 show representative chromatograms of a healthy subject's saliva and a diabetic patient's saliva, respectively. The quantitation is calculated and displayed in the "AMT" column. These amounts are concentrations in μg/mL.

Figure 7 shows the standard curve and Table 2 shows the regression statistics for detector response

linearity. As shown, the detector response is linear over the 0.5 to 20.0 μg/mL range with a detection limit of approximately 0.05 μg/mL of acetone.

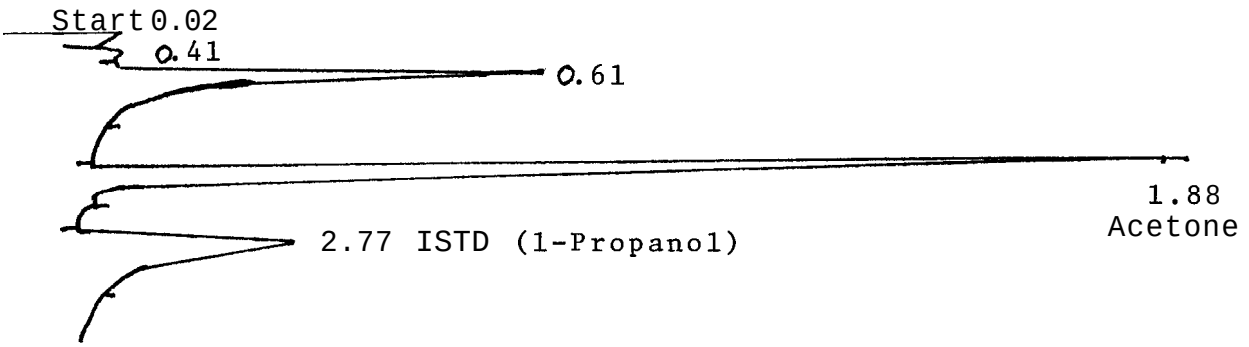


Fig. 3. Chromatogram of a standard acetone solution (10 μg/mL) with internal standard. Conditions: Chromosorb 101 column isothermal at 150° C; injection volume, 3 mL headspace; injection port temperature, 250° C; flame ionization detector temperature, 300" C; nitrogen carrier gas flow of 30 mL/min; flame ionization detector gases, hydrogen at 40 mL/min and air at 220 mL/min.

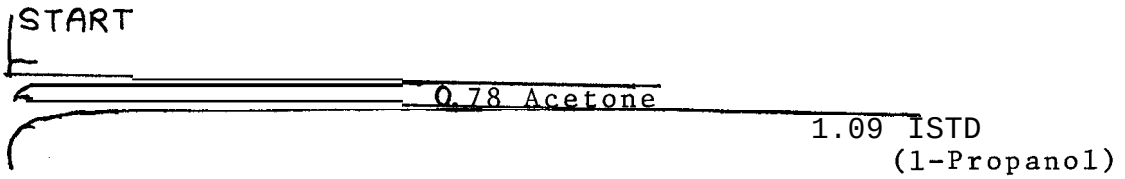
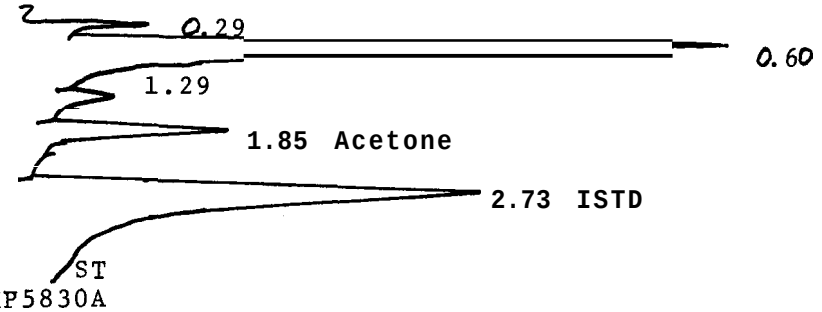


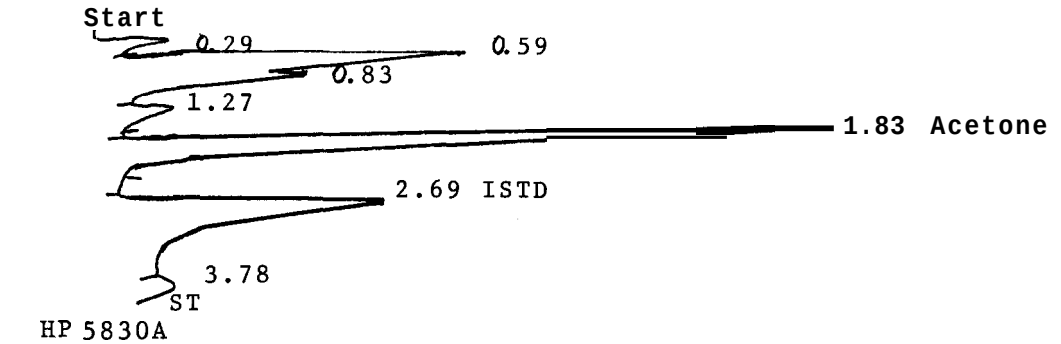
Fig. 4. Chromatogram of a standard acetone solution (100 μg/mL) with internal standard. Conditions: OV-17 column isothermal at 30° C; injection volume 1μL; injection port temperature, 250° C; flame ionization detector temperature, 300° C; nitrogen carrier gas flow of 30 mL/min; flame ionization detector gases, hydrogen 40 mL/min, air 220 mL/min.



RT	EXP RT	AREA		CAL#	AMT
1.85	1.84	3735		2	0.972
2.73	2.75	15600	R	1	

XF: 5.0000 E-1 ISTD AMT: 4.0000 E+1

Fig. 5. Chromatogram of a healthy subject's saliva with internal standard. Conditions: same as Fig. 3.



HP 5830A

ISTD

RT	EXP RT	AREA		CAL #	AMT
1.83	1.82	28540		2	6.432
2.69	2.70	18920	R	1	

XF: 5.0000 E-1 ISTD AMT: 4.0000 E+l

Fig. 6. Chromatogram of a diabetic patient's saliva with internal standard. Conditions: same as Fig. 3.

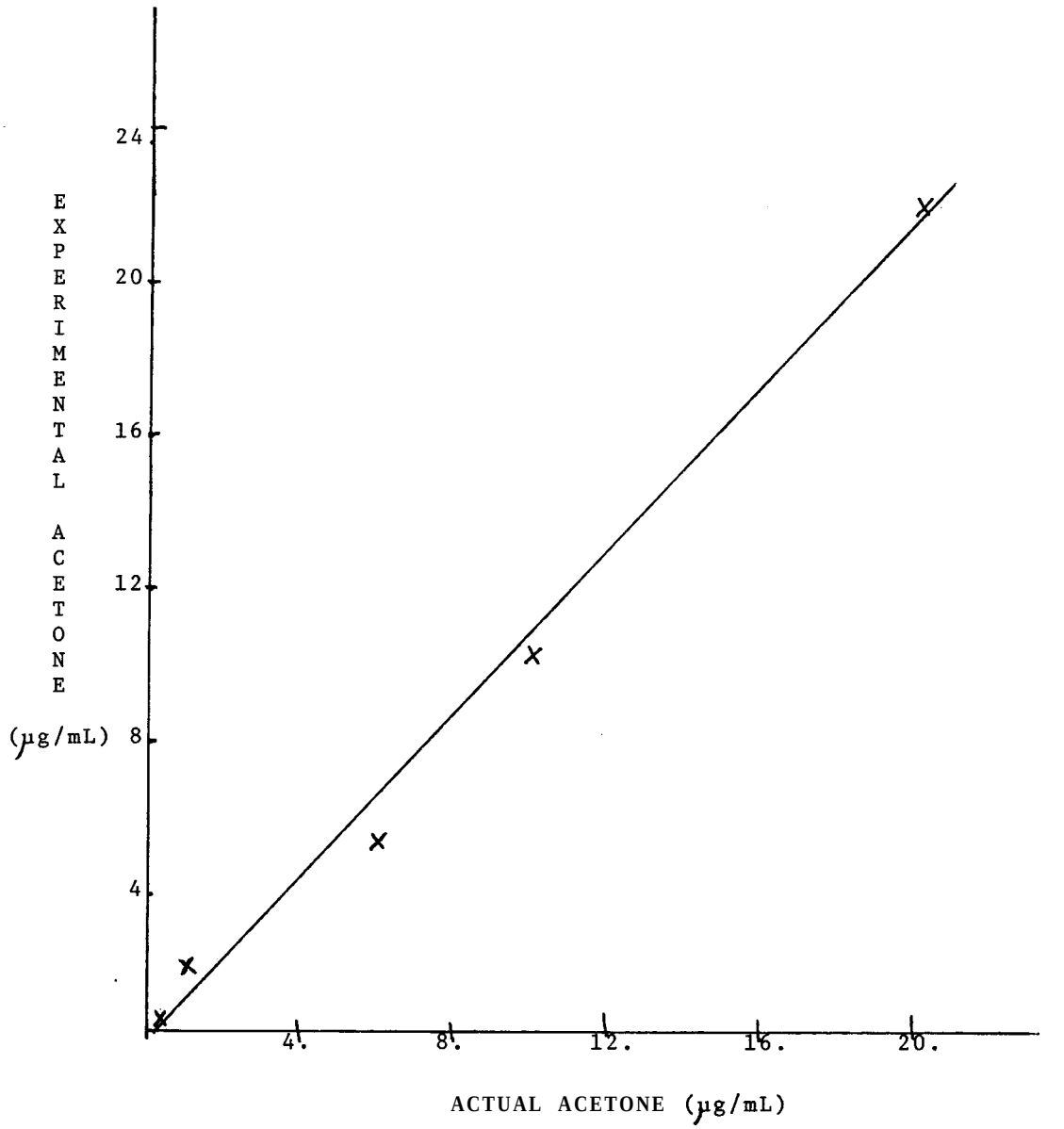


Fig. 7. Plot of standard curve for experimentally determined acetone concentrations (µg/mL) vs the actual acetone concentrations (µg/mL) showing detector response linearity.

Table 2

Regression statistics for the standard curve (Fig. 7) of experimentally determined acetone concentrations vs. actual acetone concentrations.

Variable X: Actual Acetone	Variable Y: Experimental Acetone
Mean of X = 7.300	Mean of Y = 7.890
Standard Deviation of X = 7.208	Standard Deviation of Y = 8.048

Number of Pairs (N) = 5

Correlation Coefficient (R) = .998

Degrees of Freedom (DF) = 3

Slope (M) of Regression Line = 1.114

Y Intercept (B) for the Line = -.244

Table 3 is an analysis of variance for precision data obtained on both healthy subjects' saliva and spiked water samples. The coefficients of variation are 4.65, 3.52, and 2.79% which compare favorably to those obtained by both Tomita[11] and Wilkinson[12].

Figure 8 depicts a representative plot of the accuracy data and corresponding regression statistics are found in Table 4. Data are from both spiked water

samples and spiked healthy subjects' saliva samples.

Table 5 lists descriptive statistics for analytical results on saliva obtained from healthy subjects. Included are the mean, range, minimum, maximum, variance, and standard deviation.

Table 6 shows the distribution of analytical results on saliva obtained from healthy subjects over a ±3 standard deviation range. The data show 93.9% of all healthy subjects' saliva acetone values fall within a ±2 standard deviation range. The histogram in Figure 9 shows this distribution in a graphical format.

Figure 10 shows the plot and Table 7 lists the regression statistics for a comparison of healthy subjects' saliva acetone concentrations (µg/mL) vs the length of the subjects' fast (hrs). This plot shows a large scatter of points represented by a slightly positive sloping regression line.

Figure 11 shows the plot and Table 8 lists the regression statistics for a comparison of healthy subjects mean saliva acetone concentrations (µg/mL) vs the age of the subjects (years). The plot again shows a relatively large scatter of points represented by a very slightly positive sloped regression line.

Table 9 is a listing of the statistics for the analytical results on saliva obtained from diabetic subjects.

Table 3

Analysis of variance for precision data. Shown are the sample subject, mean, standard deviation and coefficient of variation for the gas chromatographic method used in this research.

Sample	Number of Samples	Mean	Standard Deviation	Coefficient of Variation (%)
Subject Saliva (JMS)	4	.367	.017	4.650
Subject Saliva (VF)	3	.365	.012	3.520
Spiked Water	5	.259	7.231 E-03	2.785

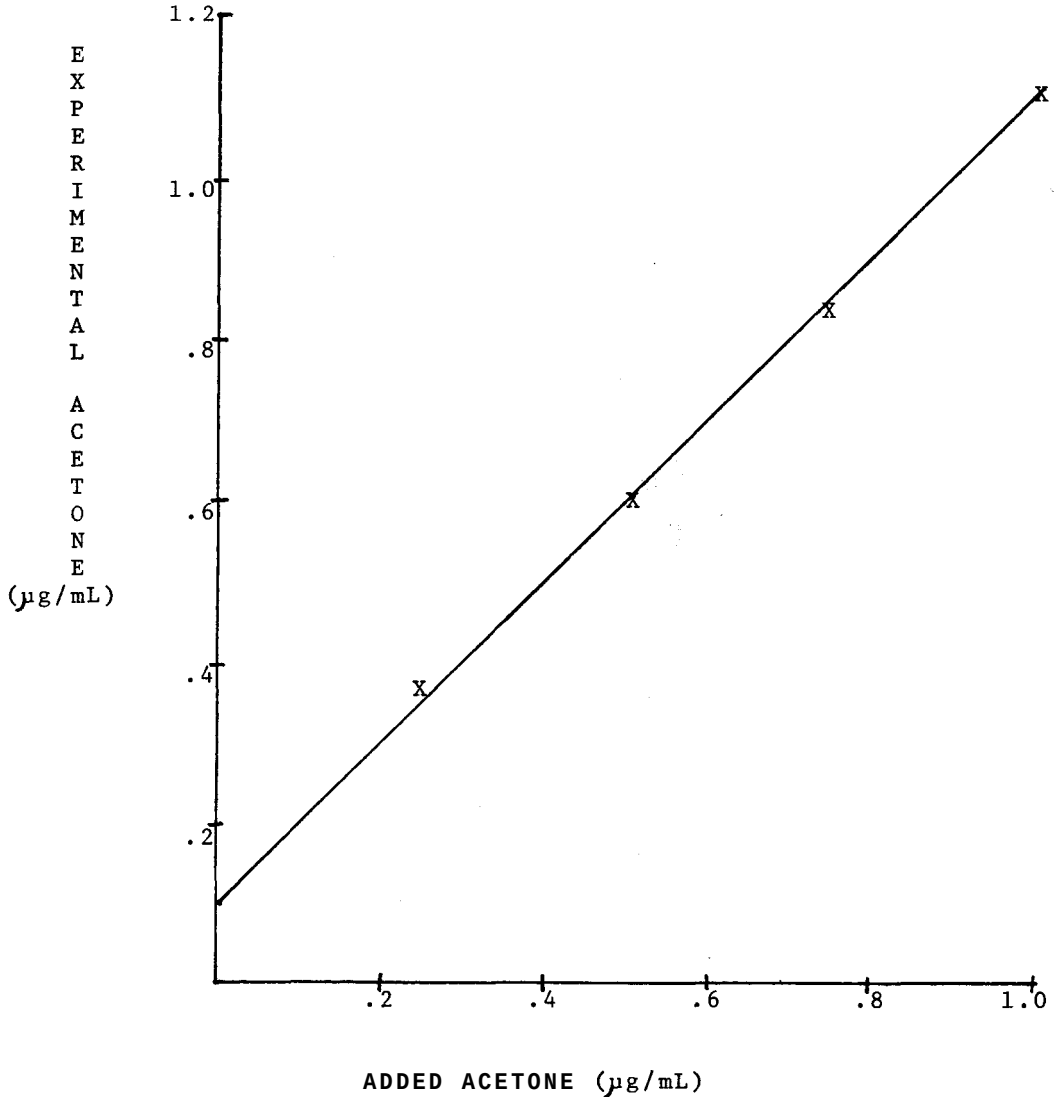


Fig. 8. Plot of accuracy data. Experimentally determined acetone concentrations (µg/mL) vs added acetone concentrations (µg/mL). The added acetone concentration is the actual amount of acetone added to either a saliva or water sample for accuracy testing.

Table 4

Regression statistics for the accuracy data plot (Fig. 8). Experimentally determined acetone concentrations vs added acetone concentrations.

Variable X: Added Acetone	Variable Y: Experimental Acetone
Mean of X = .625	Mean of Y = .734
Standard Deviation of X = .279	Standard Deviation of Y = .275

Number of Pairs (N) = 4

Correlation Coefficient (R) = .998

Degrees of Freedom (DF) = 2

Slope (M) of Regression Line = .983

Y Intercept (B) for the Line = .12

Table 5

Statistics for analytical results on saliva obtained from healthy subjects.

Variable: Healthy Subjects Saliva Acetone (μg/mL) Sample Size (N) = 49

Sample Statistics:

Mean	= .774	Range	= 1.273
Variance	= .098	Minimum	= .277
Standard Deviation	= .314	Maximum	= 1.550

Variable: Healthy Subjects Mean Saliva Acetone Values (μg/mL) Sample Size (N) = 9

Sample Statistics:

Mean	= .732	Range	= .719
Variance	= .041	Minimum	= .305
Standard Deviation	= .202	Maximum	= 1.024

Table 6

Distribution of analytical results on saliva obtained from healthy subjects.

DISTRIBUTION OF VARIABLE:
HEALTHY SUBJECTS' SALIVA ACETONE (µg/mL)

INTERVAL	FREQUENCY	PERCENT	CUMULATIVE PERCENT
0.000 to 0.143 µg/mL	0	0.0	0.0
0.144 to 0.458	6	12.2	12.2
0.459 to 0.773	21	42.9	55.1
0.774 to 1.088	14	28.6	83.7
1.089 to 1.403	5	10.2	93.9
1.404 to 1.719	3	6.1	100.0
TOTAL	49	**100.0**	

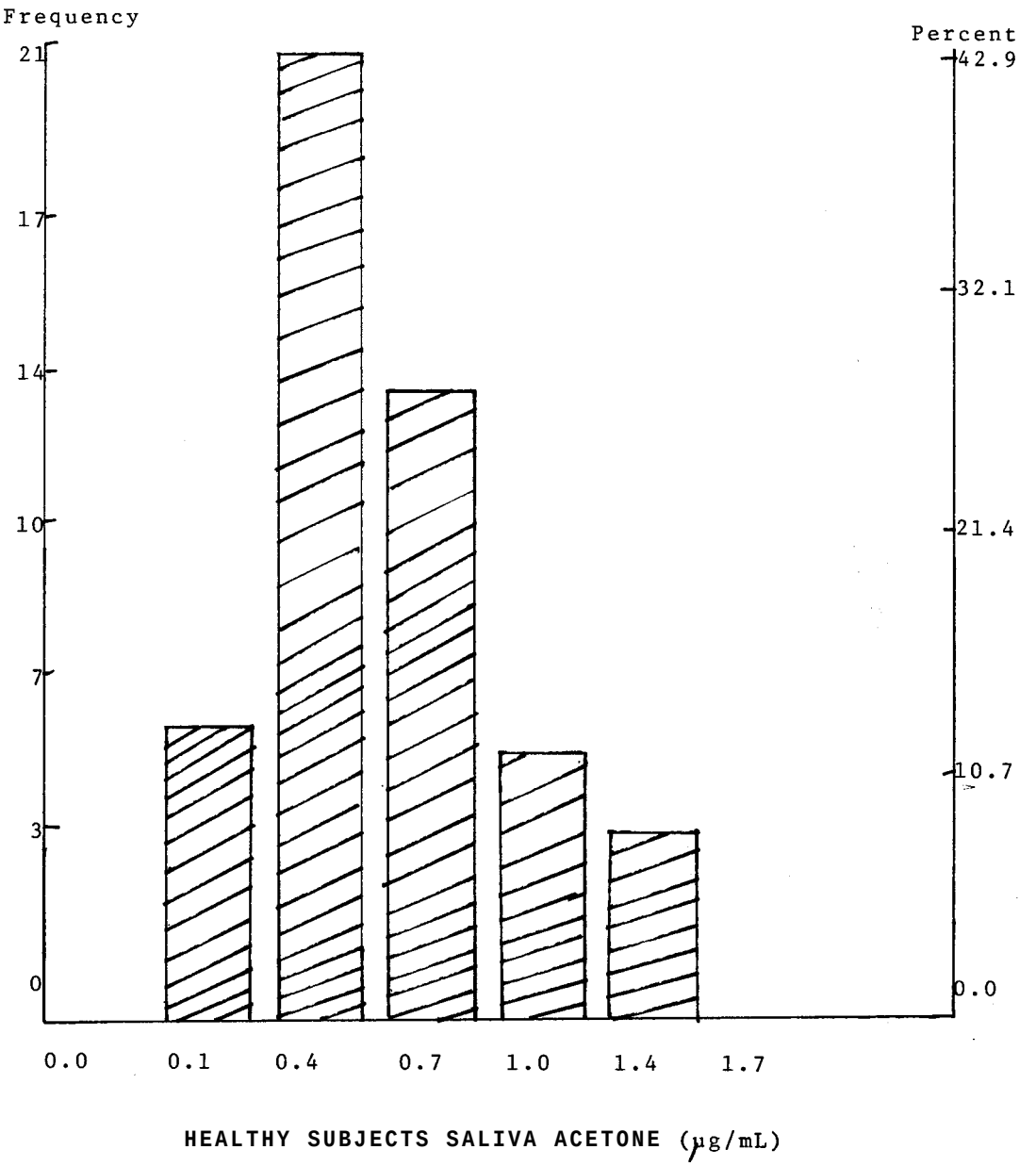


Fig. 9. Histogram of the distribution of analytical results on saliva obtained from healthy subjects.

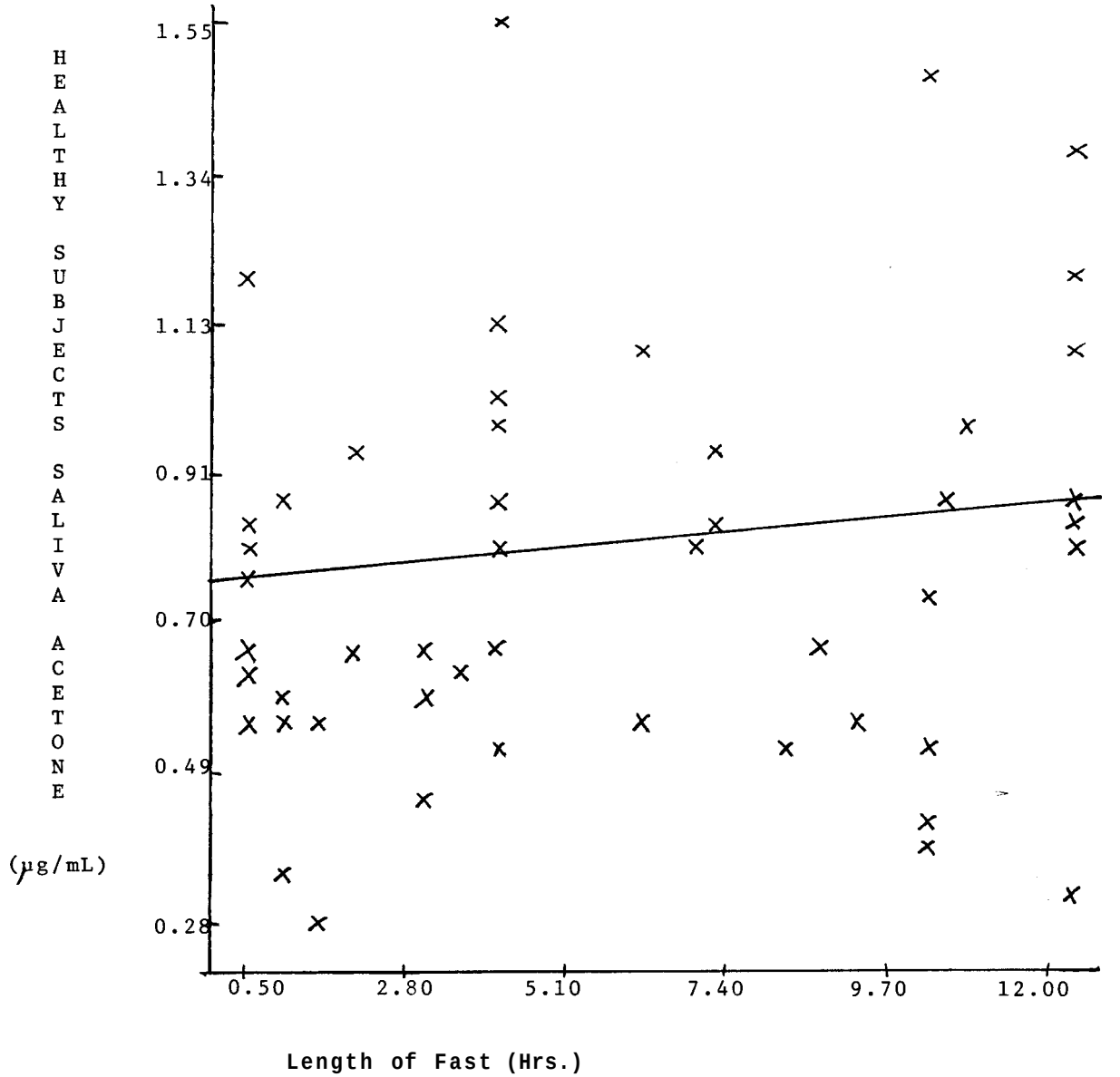


Fig. 10. Plot of healthy subjects' saliva acetone concentrations (μg/mL) vs the length of subjects fast (hours).

Table 7

Regression statistics for the plot (Fig. 10) of healthy subjects' saliva acetone concentrations (μg/mL) vs. the length of subjects' fast (hours).

Variable X: Length of Fast (Hrs.)	Variable Y: Healthy subjects' Saliva Acetone (μg/mL)
Mean of X = 5.061	Mean of Y = .774
Standard Deviation of X = 4.151	Standard Deviation of Y = .314

Number of Pairs (N) = 49

Correlation Coefficient (R) = .14

Degrees of Freedom (DF) = 47

Slope (M) of Regression Line = .010

Y Intercept (B) for the Line = .720

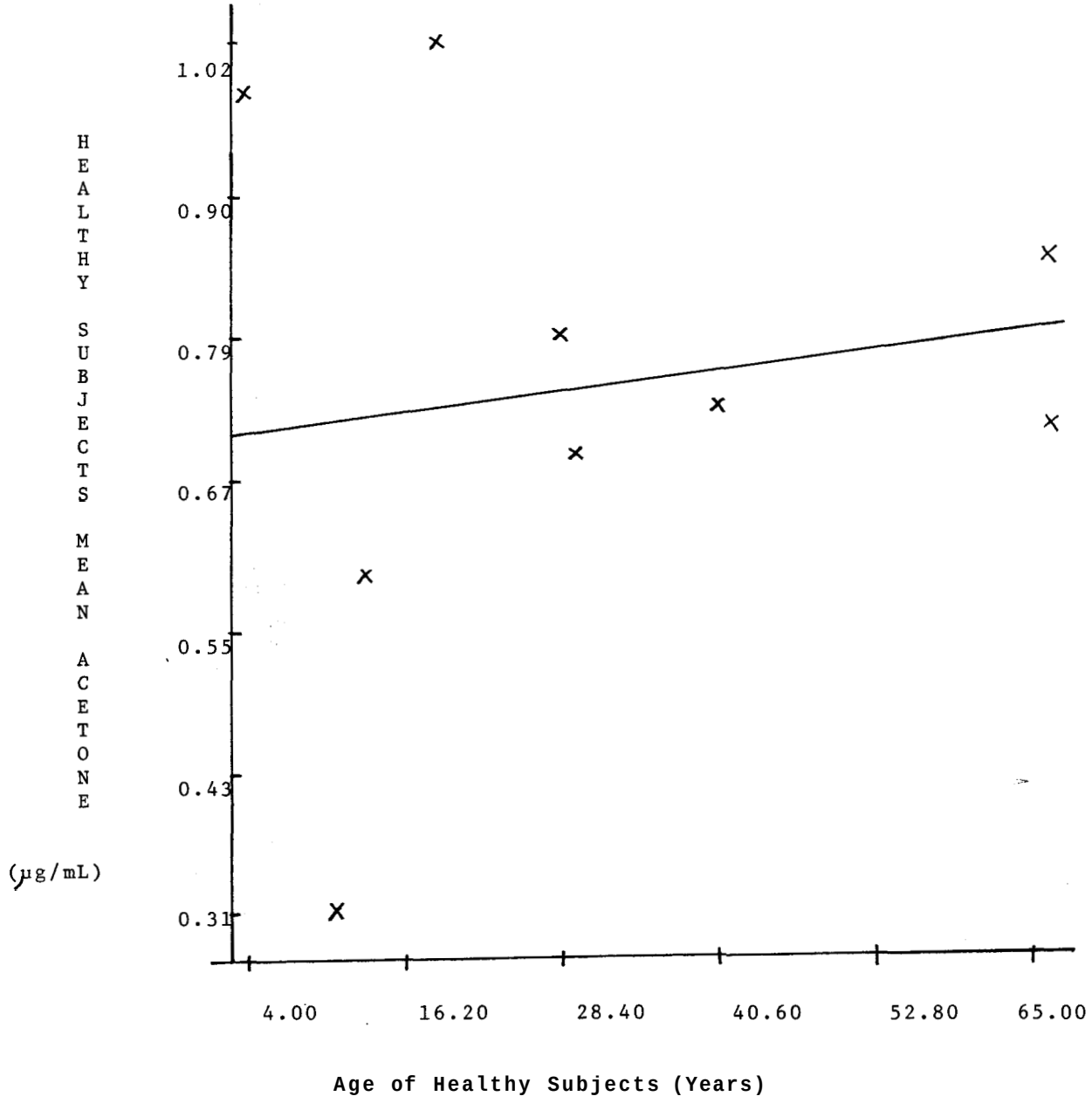


Fig. 11. Plot of healthy subjects' mean acetone concentrations (μg/mL) vs age of healthy subjects (years.)

Table 8

Regression statistics for the plot (Fig. 11) of healthy subjects' mean acetone concentration (μg/mL) vs age of healthy subjects (years).

Variable X:	Age of Healthy Subjects (Years)	Variable Y:	Healthy subjects' Mean Acetone (μg/mL)
Mean of X =	30.555	Mean of Y =	.732
Standard Deviation of X =	20.992	Standard Deviation of Y =	.202

Number of Pairs (N) = 9

Correlation Coefficient (R) = .081

Degrees of Freedom (DF) = 7

Slope (M) of Regression Line = 7.811 E-04

Y Intercept (B) for the Line = .708

Table 9

Statistics for analytical results on saliva obtained from diabetic subjects.

Variable: Diabetic Subject Saliva Acetone (µg/mL) Sample Size (N) = 4

Sample Statistics:

Mean =	4.663	Range =	6.868
Variance =	8.293	Minimum =	1.590
Standard Deviation =	2.879	Maximum =	8.458

CHAPTER V

DISCUSSION

The headspace gas chromatographic technique is capable of effectively separating the acetone and internal standard components in a minimal analysis time of four minutes. The excellent regression statistics and coefficients of variation for the accuracy and precision data validate the general methodology. The standard curve and detector sensitivity data also confirm the effectiveness of this method for determining unknown sample values. The use of the disposable polyethylene syringe for sample injection lends to the simplicity and economy of the method while controlling any cross-contamination between samples. Levels of acetone in both standards and unknown samples remain unchanged after their storage in the freezer for a week without addition of a preservative. This allows the analyst to readily "batch" process a group of samples over this time period.

Other parameters in the method such as incubation temperatures, incubation times, injection volumes and G.C. operating values were easily reproduced both between runs and from day to day. The injection technique was the only part of the method that did require a modicum

of operator experience. This is because of the increased *G.C.* injection port backpressure generated with the injection of the 3 mL sample volume. Care was needed to smoothly inject the volume against this backpressure. This problem could be overcome with the use of an automated injection system which could be added to the *G.C.* at a later date.

Statistics on the healthy subjects' saliva acetone values reveal a mean of 0.774 µg/mL with a minimum of 0.277 and maximum of 1.550 µg/mL for N=49. For purposes of comparison, it may be noted that published serum or plasma acetone values range from 3.0 to 20.0 µg/mL.[13] The distribution of the healthy subjects' saliva acetone values are 93.9% contained in ±2 S.D. of the mean and 100% contained in 23 S.D. which produces the nearly "normal" distribution curve as represented by the histogram in Figure 9.

Another variable examined was that of the healthy subjects' saliva acetone concentrations vs the length of the subjects fast. As shown in Figure 10 there is an overall small increase in saliva acetone levels as the fast continues. This is relatively consistent with the metabolic processes involved as described above. Further study could be done to extend the 12 hour fast time limit to see if indeed there is an absolute correlation between fasting and saliva acetone level.

Probably the most revealing data result from a comparison of healthy and diabetic subject acetone levels. The mean healthy subject level is 0.774 μg/mL as compared to the mean diabetic level of 4.663 μg/mL. This difference is consistent with the impaired metabolic processes involved in diabetes. Although no absolute correlation between diabetes and saliva acetone values can be drawn, it appears from this study that there is a considerable difference between a healthy subject's saliva acetone level and that of a diabetic. Perhaps this information could be used to further develop the clinical applicability of this method in the screening of a population for diabetes.

CHAPTER VI

CONCLUSIONS

From this investigation, the following conclusions can be drawn: (1) a headspace gas chromatographic technique with the required sensitivity, accuracy and precision can be used for the determination of saliva acetone in both healthy and diabetic subjects. (2) There appears to be a distinct correlation between increased saliva acetone levels and diabetes. (3) This rapid non-invasive and relatively economical technique could be adapted for clinical use in those laboratories already using G.C. for other organic substance identification and/or quantitation.

REFERENCES

1. Shaw, J.H., Textbook of Oral Biology, Philadelphia, PA: W.G. Saunders Co., 1978, pp. 593-615.

2. Rankow, R.M. and Polayes, I.M., Diseases of the Salivary Glands, Philadelphia, PA: W.B. Saunders Co., 1976, pp. 32-51.

3. Teitz, N.B., Fundamentals of Clinical Chemistry, Philadelphia, PA: W.G. Saunders Co., 1976, pp. 485-487.

4. Tomita, M., et al., KOKU EISEI GAKKAI ZASSHI 28(1). 42 (1978) (Japanese).

5 Wilkinson, P K. et al. Analytical Chemistry 47(9) 1506, (1975)

6. Hewlett-Packard- Inc., Series 5830A Gas Chromatograph Instrument Manual. (1974) pp. 2-10.

7. Miastkowski, S., BYTE, 6(6), 88, (1981).

8. Dialog Information Service, Guide to Searching, (1982), pp. 6-2.

9. Tomita, et al., p. 40.

10. Ibid., p. 38.

11. Ibid., p. 37.

12. Wilkinson, et al., p. 1509.

13. Teitz, p. 1206.