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The application of gas chromatography and mass spectrometry to the study of human diseases: the identification and quantitation of the urinary volatiles associated with a number of genetic defects

Daniel Gerard Burke
University of Wollongong

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THE APPLICATION OF GAS CHROMATOGRAPHY AND MASS SPECTROMETRY
TO THE STUDY OF HUMAN DISEASES. THE IDENTIFICATION
AND QUANTITATION OF THE URINARY VOLATILES ASSOCIATED
WITH A NUMBER OF GENETIC DEFECTS

A THESIS SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
FROM
THE UNIVERSITY OF WOLLONGONG

BY
DANIEL GERARD THOMAS BURKE, B.Sc.

CHEMISTRY
1981

This work is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at Wollongong University. No part of this work has been submitted for a degree at any other University or such institution.

(D.G. Burke)

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Abstract

The past 20 years have seen a rapid growth in studies of inborn errors of metabolism. One of the main aids to this growth has been the concomitant development in organic analytical techniques, especially those based on gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC-MS).

The identification of the organic acids present in body fluids using GC-MS techniques has been remarkably successful in discovering new and diagnosing known inborn errors of metabolism. This type of analysis requires the separation of the organic acid fraction from the body fluid, followed by derivatisation of the acids to increase their volatility thus enabling characterization of the acids by GC and GC-MS. The organic acid profile so produced is now used routinely in some laboratories to screen patients suspected of suffering from a metabolic disorder. The success of this technique has led to the further development of the concept of metabolic profiles of specific classes of metabolites in body fluids.

Workers investigating perfumes and food aromas and flavours have developed specialized techniques for identifying odorous volatile organic compounds. These methods are based on headspace extraction and concentration of volatile organics followed by analysis of the underivatized extract by capillary GC and capillary GC-MS. These methods have been applied to the analysis of volatile metabolites in human body fluids. The profile of volatile metabolites in normal human urine is well characterized.

This thesis describes the application of volatile metabolite profiling to a number of inborn errors of metabolism which are characterized by peculiar body odours. It is shown that the urinary volatiles profile for patients with Phenylketonuria, Maple Syrup Urine Disease, Isovaleric Acidemia and Trimethylaminuria (fishy odour syndrome) is in each case surprisingly different to the normal urinary volatiles profile.

In the Maple Syrup Urine Disease case we found metabolites which occur distal to the metabolic block. Since we had not observed these compounds in the organic acids profile further investigation was needed to ensure that they were not artifacts introduced by the analytical technique. This led to our development of an improved method for analysing volatile short chain fatty acids.

We found a new major metabolite in Isovaleric Acidemia. This compound had not been previously observed because its volatility resulted in excessive losses during sample work up.

We developed a quantitative method for estimating trimethylamine in urine. The technique was based on isotope dilution methodology and used headspace trapping for the extraction of trimethylamine from urine. We used this determination to study some proposed therapies for patients with the fishy odour syndrome.

We concluded that because the volatiles profiles of patients with inborn errors of metabolism mentioned above were strikingly abnormal these profiles could be used as an aid to the diagnosis of the disorders. In addition, by using this technique new and unexpected metabolites which may help clarify the basis of the metabolic distress can be found. Moreover, by adjusting the headspace conditions specific analytes of interest in these cases can be quantified.

INTRODUCTION

Metabolism

All living organisms are committed to a constant chemical change in adaptation to their environment. During the life of a human adult about 60,000 kg of solid material and 40,000 litres of water are processed by his body in response to its needs for energy and form. The basic bodily unit which is constantly engaged in this change is the cell and the processes inside the cell which accomplish these vast feats of chemical engineering are collectively termed metabolism.

Each cell contains a normal complement of approximately 400 molecules of each of about 2,500 different proteins, 10 to 300 million molecules of each of about 1,000 kinds of smaller organic compounds and a variety of nucleic acids. All chemical reactions within the cell are catalysed by enzymes (proteins) and in order for the cell to function harmoniously considerable control must be exerted over the multitudinous metabolic processes to produce an ordered response to alterations in its environment.

The fact that an organism can maintain a normal, constant internal state despite the numerous complex metabolic reactions which it performs and the continuous changes in its environment is due to the sensitivity of specific regulatory mechanisms. The failure of one or more of these mechanisms leads to the metabolic aberrations underlying various disease states. Considerable attention, therefore, has been given to elucidating the many metabolic processes occurring within the human cell.

To understand metabolism we require knowledge of the chemistry of the participating molecules (metabolites), the reactions to which they are subjected, the enzymes participating in these reactions and the regulatory mechanisms which determine the rates of sequential reactions by which any given metabolite is converted to a second then a third and so on. Such a series of steps comprises a metabolic pathway and the integrative operation of the many different pathways constitute metabolism.

For example, one of the best known metabolic pathways is the Krebs or Tricarboxylic Acid Cycle which is the major reaction sequence for providing electrons to the transport system that accomplishes reduction of oxygen to generate chemical energy. Figure 1 presents the major intermediate compounds formed by enzymatic condensation of the starting materials, Acetyl CoA and Oxaloacetic acid. Each step in this sequence represents the enzymatic conversion of a substrate to a product and the product is then used as the substrate for the next enzyme. The operation of the pathway requires the concerted action of all the enzymic activities in that sequence and the regulatory mechanisms are such that if any activity was low or absent then the increase in the concentration of that enzyme's substrate would cause the entire pathway to halt.

Study of Metabolism

Many techniques have been developed for the observation of the chemical reactions of metabolism in living systems and the main questions these are designed to answer are:

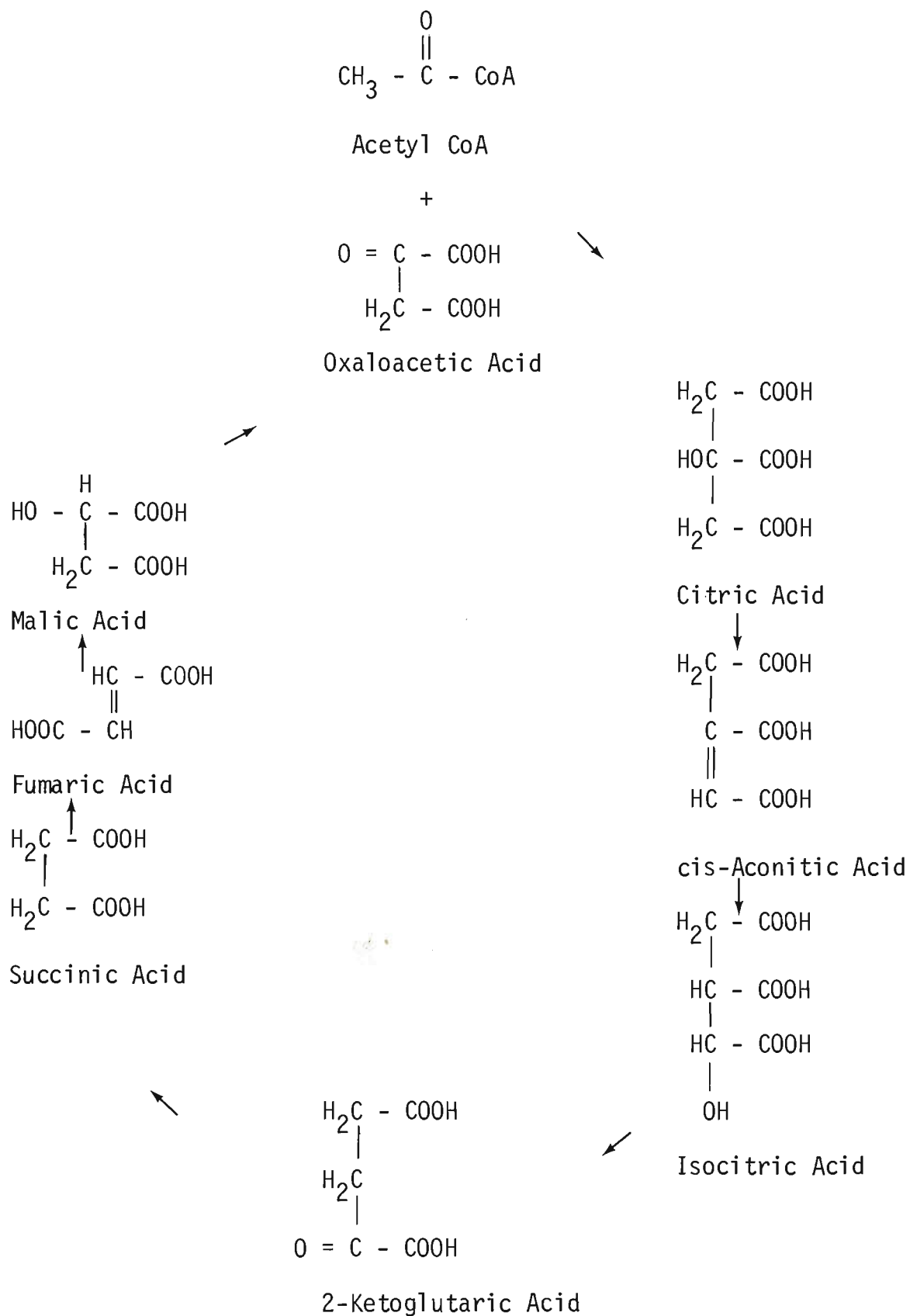


Figure 1: The Tricarboxylic Acid Cycle. Each arrow represents an enzymatically catalysed reaction step giving rise to a different metabolite.

1. What reactions are occurring?
- and 2. How are they controlled?

After observations have been made hypotheses are constructed and tested by experimentally modifying the test organism. Experimental alterations may range from the addition of a minute amount of an isotopically labelled nutrient in the diet of an intact animal to the sacrifice of an animal followed by removal of an organ, isolation of an enzyme from the organ and study of the kinetics of the reaction catalysed by that enzyme. Much metabolic information has been acquired by studies on bacteria and some higher animals but specific information about the human organism is needed. Obviously one cannot justify the sacrifice of any person for this purpose so much attention has been given to the study of individuals with inborn metabolic alterations.

Inborn Errors of Metabolism

Inborn errors of metabolism are disorders in which the organism is structurally intact but has developed a mutation which prevents the normal course of metabolic reactions from occurring. In most cases the error is localised to a single metabolic step and it is generally believed that the cause is by the mutation of only a single gene from among the complete hereditary information. Because only a single metabolic reaction is altered this step is highlighted over all the other metabolic reactions which normally occur. The inactivity of the specific enzyme causes an accumulation of its substrate in the tissues and body fluids and thus it becomes easier to detect and identify this biological intermediate which would normally be present in only minute concentrations.

Once the enzyme substrate is known it is possible to develop an assay for the specific enzymatic activity which normally occurs at that metabolic step and then use the assay to purify the protein responsible for that particular catalytic activity in healthy individuals. When the enzyme has been sufficiently purified its activators, repressors and catalytic rates of reaction can be determined, thus providing some of the answers to the basic questions about metabolism.

The concept of metabolic disorders is, historically speaking, fairly recent. It began with Sir Archibald Garrod who in 1908 suggested that four disorders, namely albinism, alkaptonuria, cystinuria and pentosuria, had certain features in common (1). Firstly, in all four conditions the onset of the peculiar abnormality could be dated to the first days or weeks of life. A second characteristic was their familial occurrence; a third that the conditions were relatively benign and compatible with a normal life expectancy and lastly that there was a high rate of occurrence among the offspring of consanguineous marriages. These disorders he termed "inborn errors of metabolism" and his explanation of this phenomenon bears quoting:

"It may well be that the intermediate products formed at the several stages (of a metabolic pathway) have only momentary existence as such, being subjected to further changes as soon as they are formed and that the course of metabolism along any particular path should be pictured as in continuous movement rather than as a series of distinct steps. If any one step in the process fails, the intermediate product in being at this point of arrest will escape further change, just as when the film of a biograph is brought to a standstill, the moving figures are left foot in air. All that is known of catabolism tends to show that in such circumstances, the intermediate product is being wont to be excreted as such rather than that it is further dealt with along abnormal lines. Indeed it is an arguable question whether, under abnormal conditions, the metabolic processes are ever

thrown out of their ordinary lines into entirely fresh paths with the result that products are formed which have no place in the normal body chemistry. It is commonly assumed that this happens, but if the concept of metabolism in compartments, under the influence of enzymes, be a correct one, it is unlikely a process, that alternative paths are provided which may be formed when for any reason the normal paths are blocked. It is far easier to suppose in such circumstances, normal intermediate products are excreted without further changes."

Now, seventy years later, rapid progress in the biological sciences has validated what was by necessity, supposition in Garrod's time. The original four inborn errors of metabolism have been increased to about two hundred (2-4) and we know more about the chromosomal processes which may give rise to genetically based disorders and the biochemical consequences of these changes.

Most abnormalities of metabolism are caused by changes in the specific rates of one or more metabolic reactions and may produce changes in body composition. The concentrations of most metabolites vary within only a very narrow range for a given healthy individual. A complex series of regulatory mechanisms achieves this consistency by balancing the rate of production of the metabolite against the rate of its utilization. If any of the mechanisms are disturbed then the balance will inevitably change. Regulation of enzymatic activity is considered to occur at two distinct levels; (a) through regulation of the amount of enzyme (control of enzyme synthesis); and (b) through regulation of the activity of a given quantity of enzyme (control of enzyme activity). One example of the latter type of regulatory mechanism is the control of the activity of certain "rate-limiting" enzymes so called because once the substrate is converted to product the biochemical pathway is committed and further steps in the pathway will inevitably occur. Usually these enzymes can be activated, i.e. caused to increase the rate of conversion of substrate to

product, or repressed, i.e. caused to lower the rate of conversion of substrate to product, in response to changes in their environment.

Inborn errors of metabolism prevent the type "a" control mechanism from occurring. This is caused by the protein either not being produced at all, or the production of a defective protein. In these cases type "b" control mechanisms become redundant.

So if an organism is functioning properly the concentrations of the various metabolites within the cellular medium must reflect the balance between their rate of production and removal and any disfunction of the organism would be reflected by a change in the concentration of the metabolite or metabolites associated with the cause of that aberration (5).

Metabolic Profiles

The concept that individuals might have a "metabolic pattern" of concentrations of metabolites in their biological fluids was first developed and tested by Williams and his associates (6) during the late 1940's and early 1950's. Data from over 200,000 paper chromatographic analyses led to the conclusion that there are significant variations in the composition of biological fluids between individuals but that the profiles from the same individual change very little over a substantial period of time. Using these methods he also examined samples from schizophrenics, alcoholics and residents of mental hospitals and produced evidence that there are characteristic metabolic patterns associated with each of these groups.

Recently several workers have also observed that the metabolite pattern of an individual changes very little over lengthy periods of time and that diet seems to have only a slight effect, but there appears to be great

variation between individuals resulting in alterations of the absolute levels at which the various type "b" control mechanisms (e.g. rate-limiting enzymes) will operate.

Chalmers *et al.* (11) have shown that the variations in organic acid excretion depend mainly on individual metabolic differences rather than on dietary factors, and they were able to group the urinary organic acid profile of 420 normal subjects into four categories: (i) unimodal distributions with detectable values in almost all subjects; (ii) apparently unimodal distributions with a number of values below the level of detectability; (iii) bimodal distributions, a unimodal subgroup plus a block of undetectable values; and (iv) irregular distributions with a majority of undetectable values (9).

The assignment of clinical significance to small changes in the excretion of metabolites may only be possible when a relevant mean excretion value can be obtained (e.g. category "i" above) and the correlation of subtle changes in excretion levels to disease states may be extremely difficult.

Inborn errors of metabolism, however, lead to gross changes in the concentration of at least one metabolite - the substrate for the absent enzymatic activity. Usually increases are observed in a small group of metabolites which consists of the substrate from the primary phenotypic lesion as well as some intermediates in the detoxification pathway for that compound.

For example, patients with Maple Syrup Urine Disease (MSUD) are known to excrete large amounts of the 2-keto acids derived from deamination of leucine, isoleucine and valine. However clinical attacks not only result

in the increased excretion of these acids but an accumulation of the corresponding 2-hydroxy acids and all of the metabolites associated with ketoacidosis and lactic acidosis are also observed. Altogether the concentrations of fifteen metabolites (12) in the urine of MSUD patients are observed to increase during attacks.

Detection of Disease States by Metabolite Profiles

The use of metabolic profiling to detect metabolic disorders relies on the ability of this approach to detect large increases in concentrations of any of the group of compounds being analysed. The data analysis therefore usually need not involve statistics and indeed sometimes the clinical significance may be easy to discern. This is in contrast to the approach of Gates *et al.* (13, 14) and Robinson *et al.* (15) who analyse the metabolic profiles of large populations in order to define a "normal" pattern. Once a normal pattern has been shown to exist then sophisticated statistical techniques can be used to decide whether a given sample conforms to the normal pattern or not. Another example of this approach is given by McConnell *et al.* (16) who were able to distinguish profiles of individuals with diabetes mellitus from those of normal individuals by profiling the volatile constituents of the urine. The basic difference between this type of approach and the use of metabolic profiling to detect metabolic disorders lies in the aim of the analysis. The former seeks to ascertain a pattern of metabolic profiles while the latter seeks only to produce a profile of a particular class of metabolites from a selected individual. Of all the data that is available when profiling a class of metabolites the analyst who searches for metabolic disorders is only interested in that data which is obviously grossly different to what could reasonably be classed as normal. So although the physical techniques used

may be the same as for metabolic profiling, the evaluation of the resultant data proceeds along different lines.

One of the most successful methods of metabolic profiling makes use of GC and GC-MS to analyse the organic acid fraction of human physiological fluids (such as blood, urine or cerebrospinal fluid). Large increases in the amounts of metabolites present in these fluids can readily be shown to be clinically important and the use of this technique to detect the increased levels has resulted in the discovery of 23 new metabolic disorders. In addition about 60 previously described inborn errors of metabolism can also be diagnosed in this way (2). The beginnings of this technique go back to 1971 when the Hornings (17,18) introduced the term "metabolic profile" which they defined as, "multicomponent gas chromatographic analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites".

They stated that the object of profile analysis was to correlate a particular metabolite pattern in a chromatogram with the clinical state of the individual from which the sample was taken. Horning's analysis is particularly well suited to studying metabolic disorders as it offers the advantages of:

- (a) detection and identification of a large number of metabolites simultaneously so that a more complete picture of any biochemical change may be given,
- (b) finding new and sometimes unexpected metabolites which would give a clearer indication of the nature of the anomaly and its metabolic effects,

and (c) determining changes in the relative amounts of different constituents compared to the "normal level" when therapy is attempted.

It thus has the potential to yield valuable biochemical information about metabolism as well as reveal the cause of the metabolic distress and sometimes suggest a method of treatment.

The metabolites or biochemical compounds are usually present in only very low concentrations in the biological fluids of healthy individuals. The production of a normal profile thus requires special analytical methods for the extraction, concentration and analysis of the trace components. Thermostable GC liquid phases, lightly packed columns and high sensitivity detectors (19, 20) are required to separate and detect the various organic compounds by GC. The successful combination of a gas chromatograph with a mass spectrometer (GC-MS) by means of molecular separators (21, 22) in 1964 made the identification of each component in a chromatogram by MS possible, and rapid qualitative analysis of complex mixtures is now routine.

Other techniques that have been used for metabolic profiling are paper, thin layer, ion exchange and high pressure liquid chromatography. The detection systems for these types of chromatography are based on derivatising the analytes to yield a product which is monitored by visible or UV photometry. The non-specificity of these detection techniques causes some difficulties in the identification of components, however early work (23-25) showed that it was possible to obtain useful information by such profiling.

Young (26) showed that considerable differences in the carbohydrate profile are apparent for several disease states while Hamilton (27) showed that about 95 ninhydrin positive components of urine could be resolved by HPLC. Later, a group at the Oak Ridge National Laboratory developed a liquid chromatographic system which used a mini-computer to deconvolute overlapping peaks and store the chromatographic data. As many as 150 ultraviolet absorbing substances were found in body fluids (28-31), however GC-MS was needed to identify many of the UV absorbing compounds as aromatic organic and amino acids, emphasising that at present liquid chromatography probably has only a complimentary role to play for metabolic profiling. However, high pressure liquid chromatography-mass spectrometry systems are now commercially available and may become an alternative profiling technique for the less volatile compounds.

Profiling of Organic Acids

The analyses of urinary and blood amino acids by paper and ion exchange chromatography have proved over the last 25 years to be of great clinical value. In a similar manner, chromatographic profiling of organic acids has become of diagnostic importance and has led to an improved understanding of many human disorders and has also been the main stimulus for much of the development of this type of chemical analysis.

Organic acids play a wide variety of roles in the metabolism of man. As mentioned earlier the tricarboxylic acid cycle is the major pathway to provide electrons for the generation of biologically usable energy. The dominant mechanism for the biological synthesis of amino acids is by transamination of 2-keto acids, which are also the first intermediates formed after deamination of amino acids during their catabolism. Sugar acids

play a central role in carbohydrate metabolism and the main end products of glucose metabolism are pyruvic and lactic acids. Lastly, lipid metabolism is concerned solely with the storage and later retrieval of long chain fatty acids in the form of esters of glycerol.

Because of the wide distribution of organic acids in human metabolism and since they are readily analysed by GC and GC-MS when derivatised, many workers have chosen to screen selected populations for disturbances in organic acid excretions. Because GC-MS is an expensive and time consuming technique it is not suitable for the routine screening of pediatric populations (this is inherently the population in which genetic disorders are manifested) and thus only samples from patients suspected of having a metabolic disease are usually analysed. Likely candidates are selected on the basis of a number of criteria which include: peculiar odour, metabolic acidoses, mental retardation, failure to thrive, disturbances in pigment development, severe vomiting in early life and involuntary movements (3).

Precise methodology for the extraction, concentration and derivatisation of the organic acids in biological fluids have now been published (2, 32-35). The organic acids must first be separated from their aqueous medium and since they are present at only low levels it is necessary to concentrate the extract before GC analysis.

There are three principal methods currently in use for the separation of the acidic constituents from urine prior to derivatisation and GLC.

The first method requires the acidification of the urine to liberate the acids from their salts before extraction with an organic solvent

The extract is then dried, the solvent is evaporated and the acids derivatised for GC analysis.

The second is based on the Hornings' (17) ion exchange chromatography technique and has been used extensively (32, 33). Firstly inorganic phosphate and sulphate ions which may interfere in the GC analysis are removed by precipitation as their barium salts. The supernatant is then drawn off and keto acids are converted to their oxime derivatives. An anion exchange column is then used to retain the acids while the basic and neutral compounds are removed by washing with water. Finally the acids are eluted, the solution is dried and the sample is derivatised for GC analysis.

A third more specialised method, makes use of either steam or vacuum distillation (36-39, 70-72) to separate the volatile organic acids from biological material.

Comparatively, solvent extraction yields only qualitative information since very polar or highly water soluble compounds (such as the sugar acids) are only poorly extracted. The ion exchange method gives a more quantitative recovery (13, 32, 33) but since some organic acids co-precipitate (8, 113, 114) or decompose (keto-acids) during the barium hydroxide treatment some important acids may be partially lost. Both methods are not suitable for the analysis of volatile acids or lactones of hydroxyacids as these are likely to evaporate during the work up.

Derivatisation of Organic Acids

Since most GC columns are unstable above 270°C many of the extractable organic acids are not volatile enough for GLC analysis

in their acid form. In addition the polar nature of the acids that are volatile causes peak broadening and tailing and the resultant loss of chromatographic resolution may render profiling impossible. Chemical modification has been used to overcome these problems and the most widely used method for derivatisation is esterification using either a trimethylsilyl (TMS) or methyl donor with or without prior oxime formation of keto acids.

Silylating reagents such as N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) react with hydroxyl groups to form either trimethylsilyl ethers or esters; enolizable ketones and oximes also react. There are a wide variety of esterification reagents available to form alkyl esters (40) but the most common procedure involves the use of diazomethane to methylate carboxylic acids and phenolic hydroxyl groups. The resultant derivatives are usually more volatile and have better chromatographic properties than the free acids since the replacement of the acidic hydrogen by a TMS or a methyl group reduces hydrogen bonding between the analytes and the stationary phase.

Although derivatisation is a fairly simple operation, adequate care must be observed when performing this step. When silylating reagents are used strictly anhydrous conditions must be maintained throughout the procedure as TMS esters readily hydrolyse. Multiple derivatives may be formed with keto acids due to limited enolisation. Diazomethane can cause fumaric and aconitic acids to form pyrazolines in the presence of water and a number of important phenolic acids (e.g. p-hydroxyphenyllactic acid) can yield more than one product.

Another important restriction intrinsic to this method is the inability to detect compounds with latent heats of vaporisation similar to that of the solvent, as they may be lost during solvent removal. These types

of compounds are usually more volatile when derivatised so that even if not completely removed by the evaporation step the derivatives may be masked by co-eluting solvent during gas chromatographic analysis (41, 42 43).

Gas Chromatographic-Mass Spectrometric Analysis

Coupling of gas chromatography and mass spectrometry is achieved by the continuous transfer of the gas chromatographic column eluant to the source of a mass spectrometer. Originally this was made possible by the use of molecular separators which reduce the volume of gas entering the source and enrich the sample relative to the carrier gas solvent (21, 22), however recently the trend has been toward direct connection of capillary columns to the MS source and maintenance of high vacuum by using larger capacity vacuum pumps (34, 44-46).

As the column eluant enters the ion source complete mass spectra are taken either manually as each peak elutes or with computer based equipment at frequent intervals (4-8 secs.) and stored for later evaluation. The technique known as specific ion monitoring (SIM), where the MS is focussed on one or more ions of special interest and only these abundances stored, may also be used for increased sensitivity.

Using this technique Maume *et al.* (47, 48) and Summons *et al.* (49) successfully analysed biological fluids for steroids, catecholamines and amino acids by monitoring ions common to whole classes of these compounds. Although this technique has been shown to be useful it has not become generally popular because some specificity is lost. Its advantages of enabling detection of low concentrations of metabolites which would otherwise

be masked by unresolved components have been largely superseded by advances in instrumental sensitivity and GC resolution.

The technique called "mass fragmentography" or "mass chromatography" first described by Hites and Biemann in 1970 (50) accomplishes the same task as selected ion monitoring without loss of any potentially useful information. Complete mass spectra are taken at frequent intervals (4-8 secs.) and all the spectra for the entire gas chromatogram are stored by a computer for later off-line analysis when the data file may be searched for the abundances of a specific ion which is then plotted against time. Any ion of interest in the entire mass range can be plotted in this way and so the resultant information is equivalent to that given by selected ion monitoring except that the scanning rate may be lower which could result in decreased sensitivity. However, with mixtures containing many unidentified components repetitive scanning of the full mass range, followed by later off-line analysis, provides more useful information but with a loss of some sensitivity.

The main reason for the application of sophisticated computer technology for metabolic profiling has been due to the difficulty in obtaining quantitative data on some of the components of complex biological mixtures. This problem is caused by the low separating efficiency of packed GC columns and thus many of the minor components are poorly resolved. Packed glass or metal chromatographic columns are also reactive towards certain classes of organic compounds. It is necessary to emphasise that even relatively "non-destructible" and low molecular weight compounds suffer some break down when in contact with hot metallic or metal containing systems. The amount of sample loss depends on the structure of the compounds being analysed and this becomes more critical at low concentrations.

The inertness of glass capillary columns had been stressed some time ago (51, 52) and their high resolving power has been known for some twenty years (53). In 1972 Novotny (54) advocated the use of glass capillary columns for the analysis of complex biological mixtures, but it is only in recent years that stable small diameter glass columns have become commercially available. Using such a column Jellum *et al.* (34) discovered six new metabolites associated with an already known organic aciduria (MSUD) which until then had been overlooked. Jakobs *et al.* (12), using the same type of equipment showed that both the L- and D-forms of 2-keto-3-methylvaleric acid were excreted by people with this disorder. This had been previously overlooked as the Di-TMS derivatives of 2-ketoisocaproic and D-2-keto-3-methylvaleric acids could not be separated on packed columns. More recently Spiteller and Spiteller (55, 56) have used the high resolving power of glass capillaries to separate the acidic components of urine. The acids were first separated into eight fractions by TLC before analysis by combined glass capillary GC-MS. About 500 compounds were detected of which only about 200 could be characterised by their mass spectra thus many of the detected compounds are still unknown.

Capillary columns are essential for separating underivatised volatile fractions of biological fluids and almost all the workers in this field employ their greater resolution (15, 41, 42, 45, 57-60). A detailed discussion on this topic follows later.

The use of capillary columns requires some specific modifications to the gas chromatograph and mass spectrometer system. Usually an inlet splitter is required for the use of wall coated open tubular columns (WCOT) but this may not be needed for support coated open tubular columns (SCOT). A direct connection of the column to the mass spectrometer is preferable to the use of molecular separators (44, 45, 46).

The chromatograms produced are often very complex which may make pattern recognition difficult and analysis time for metabolic profiling with capillary columns may be quite large (1-2 hours). Most workers in this field use automated equipment for data processing and statistical analysis (15, 59).

Qualitative Analysis Using Mass Spectrometry

The most common technique for qualitative analysis of biological fluids is the comparison of the mass spectrum of each component to a published spectrum obtained from mass spectral libraries. Since the spectra recorded during a GC-MS run may be contaminated by contributions from liquid phase bleed and unresolved neighbouring peaks it is often necessary to remove the contaminating peaks to obtain a more accurate representation of the spectrum of any component in the mixture. With computer based MS operating systems this can be accomplished rapidly by choosing a spectrum between eluting peaks as a background spectrum and subtracting the abundance at each mass value in that spectrum from that at each mass value in a spectrum from a peak.

In comparison to this simple approach some sophisticated computer programmes have been developed to deconvolute unresolved peaks and automatically subtract background contributions (61, 62). Dromey's programme uses tabular peak models derived directly from the raw data to remove column bleed background from the spectra and to correct for interference from neighbouring elutants and peak saturation (62). Individual components are detected in the data by means of a pair of histograms which statistically characterise the positions of mass fragmentogram peak modes. Components that elute within less than two spectral scan times of each other can be

detected and their mass spectra well resolved.

Comparison of experimental spectra to library spectra can be done manually or with the aid of a computer data base. The computer searching procedures can be much faster than manual operation, but one must be well aware of the search algorithm used in order to interpret the results of the search programme. There are two main categories of computer based library searching; (a) forward library searches and (b) reverse search methods. The forward library search consists of searching the library for a spectrum that matches one spectrum from the GC-MS run. Jellum *et al.* (5) have successfully identified three new metabolic disorders using this type of library searching. The major difficulty with this approach is that mixed spectra from unresolved peaks will not match a spectrum in the library.

The reverse library search techniques examine the spectra from the library for a match to the spectrum from the GC-MS run. One of the most useful variations of this searching technique was proposed by McLafferty *et al.* (63) and is known as "probability based" matching of mass spectra. This procedure involves the assignment of a uniqueness value to each ion in the spectrum based on the frequency of its appearance in all the library spectra and then matching of the most diagnostic ions (least frequent) in the spectrum. Several factors such as relative abundances and number of matched ions are then examined and a "confidence index" is computed. The main advantages of this system are its speed and ability to give a match even with mixed spectra.

Recently a method using direct chemical ionisation mass spectrometry for the qualitative screening of carboxylic acids in urine has been proposed (110). The carboxylic acids are separated from the urine samples by ion exchange chromatography and the underivatized sample is introduced into the

MS by direct probe. The spectra recorded under CI (isobutane) conditions show an interpretable profile and profiles from patients with inborn errors of metabolism can be recognised. The disadvantage of interference inherent in this method may be overcome with the development of MS/MS technology (111).

Quantitative Analysis Using Mass Spectrometry

Most workers in the field of metabolic profiling have reported semi-quantitative results using GC-MS systems with quantitation by GC peak areas and identifications, where necessary, by mass spectrometry. Thus Witten *et al.* (64) reported the means and standard deviations of the excretion rates of 18 acidic compounds from the urinary metabolic profiles of 21 healthy adults who were maintained on a controlled diet. Fourteen of the peaks were identified by MS and at least two peaks contained multiple components. In an excellent study, Thompson and Markey described a similar system (32) and used it for comparing three extraction methods of organic acids. About 60 substances were quantitated in a scale of 0 to +++. Many of the substances were grouped together because they were unresolved by the GC. Later Thompson *et al.* (65) measured peak areas of 24 peaks in urines of 92 "healthy" children and four adults and compared excretion values among the six age groups represented. Bjorkman *et al.* (66) published data from a similar system for 28 substances about half of which were data for unresolved groups of two or more organic acids. Lindstedt *et al.* (67) also quantitated several dicarboxylic acids in the urine of an infant with congenital lactic acidosis. Chalmers *et al.* (7, 8, 11, 43, 68) have made a major contribution with a similar system and have reported quantitative data for more than 20 large peaks in the gas chromatogram some of which were peak clusters containing several components. The results were quantitated by reference to n-tetracosane, the internal standard, in terms of nanograms per

microgram of creatinine. In this way quantitative ranges and frequency distribution patterns of metabolites were reported as a basis for comparison of results from patients whose clinical condition suggests that their excretion values may be abnormal.

Although these workers have used MS in their analyses for identification it was not used for quantitating any of the metabolites. In contrast mass fragmentographic methods have been developed which resolve and quantify components by comparison of peak areas of specific fragment ions (69-71). Gates *et al.* (72) have reported that more than 100 components in a typical mixture of organic acids from urine were automatically identified and quantitated by using retention indices and off-line reverse library searching of selected mass chromatograms from the GC-MS data. Using the same methods they later produced quantitative information on 134 acidic substances and determined the mean absolute concentration of 20 organic acids in adult urines (73). In this type of analysis the peak area of a single ion for each compound is compared to the area of a single ion of the internal standard so the identity of the compounds to be quantitated need not be known. Indeed quantitative information was also produced for 76 "unknown" substances which were defined by four specific ions and a retention index (14). However for these substances absolute quantities could not be determined since the relative response factors and relative recoveries were unknown.

When only one or a small number of known compounds are to be quantified stable isotope labelled analogues of these compounds may be used as internal standards. An intense ion from the mass spectrum of the known compound is chosen and the area of this ion is compared to the area of the corresponding ion of the internal standard. Specific ion monitoring may also be used to improve sensitivity. Interference from fragment ions produced by

the labelled compound may be avoided by using chemical ionisation (CI) mass spectrometry and monitoring the protonated molecular ion species.

The many advantages of using stable isotope analogues as internal standards arise because their physical properties may be so similar to the unlabelled compound that standardisation of all variables and knowledge of recoveries is unnecessary (74). Also if a large amount of internal standard is used, relatively less of the unlabelled compound may be lost through system reactivity since a proportional amount of the internal standard reacts at active sites; detection limits can thus be significantly lowered (75). The cost of preparing the stable isotope analogues coupled with the requirement of a separate internal standard for each compound has largely restricted the use of this technique to areas other than metabolic profiling. Some recently published proceedings provide comprehensive reviews of those areas (76, 77). The principles of this method are equally applicable to direct insertion probe mass spectrometry (DIP-MS) and Truscott *et al.* (78) have quantified anticonvulsant drugs extracted from a 50 µl serum sample in this way. Mee *et al.* (79) using a similar system, were able to determine all the common amino acids in dried blood spots.

Profiling of Volatile Compounds

Since this thesis is concerned with the applications of metabolic profiling of the volatile constituents in urine I have focused attention on the published literature in this field.

Pauling *et al.* (60) were among the first to apply headspace analysis, a technique which had been used successfully by food flavour chemists (80) to analyse volatiles from fruit, to metabolic profiling. The method involves

sweeping the surface of an aqueous solution which contains the volatiles with an inert gas in a closed glass container. The volatile organic compounds are in a dynamic equilibrium between the solution and the gas in the space above it (headspace) and are thus extracted into the inert gas stream which then passes through a trap; the sample is often heated to enhance this extraction. Pauling *et al.* (60) and later Robinson *et al.* (15) used liquid nitrogen cooling of a special pre-column to trap the volatile organics from urine; Teranishi *et al.* (81) analysed volatiles from breath as well as urine in this manner.

However, as mentioned by Politzer *et al.* (82) this trapping method was probably an evolutionary stage in the development of headspace techniques as the condensation of water in the trap becomes a major problem (81). Most workers now adsorb the volatiles onto a hydrophobic porous polymer which is heat stable (58, 45, 83) or onto glass-wool/beads (41, 84, 85, 86). The collected material is then transferred to a gas chromatographic column by heat desorption. Using these techniques Matsumoto (87) was successful in identifying forty-three volatile compounds present in human urine, twenty of which were shown to be ketones and seven to be aldehydes.

Zlatkis *et al.* (58) used trapping of headspace volatiles onto a porous polymer of 2, 6-diphenyl-p-phenylene oxide (Tenax GC) to identify fifty-one compounds in human urine. The same technique was used later to show that the concentrations of ethanol, n-propanol, isobutanol, n-butanol and isopentanol were increased in the urine of diabetic patients as compared to the urine from normal persons (45, 88).

Zlatkis and Andrawes (86) then developed a solvent extraction method followed by adsorption of the organic layer onto glass wool and stripping of the excess solvent. A tenfold excess of diethyl ether was mixed thoroughly

with a blood serum sample and the organic, protein and aqueous layers were separated by centrifugation. A part of the organic layer was injected into a glass tube packed with glass wool, and the solvent was evaporated by passing a stream of helium through the tube. The trapped material was then heat desorbed and swept onto a precolumn. The authors showed that small volumes (100 μ l) of serum and tissue biopsies were sufficient to produce a profile of organic volatiles but no attempt was made to identify any of the compounds.

McConnell and Novotny (59) described a fully automated system for the collection and analysis of volatiles from biological samples which was aimed at acquiring reproducible profiles of large populations. The method was based on adsorption of volatile organics onto a porous polymer trap, using a stream of inert gas with the urine sample heated to 100⁰C. In this case the trap consisted of a small platinum gauze "microbasket" holding Tenax GC. After 30 minutes the microbasket was removed from the sampling unit and sealed in an aluminium capsule by cold welding. The capsule was then placed in an autosampler, which utilised a capsule-type solid injection system, for subsequent fully automated GLC analysis on non-polar glass capillary columns. This system was shown to yield highly reproducible chromatographic profiles of the volatile constituents from different aliquots of a single 24 hour urine sample.

Zlatkis and Kim (84) improving on their previous method (86) described a solvent elution method for the isolation and concentration of volatiles. A small glass column containing porous silica beads (Porasil E) is attached to a novel sampling device called a "transevaporator" and a trapping tube packed with glass wool is connected to the outlet. After adsorption of the sample on the Porasil E column an organic solvent is evaporated through the traps by a stream of helium. High molecular weight substances, solids and water are retained by the column adsorbent (Porasil E) while the volatile

and soluble constituents are eluted from the column by the solvent and flowed to the glass wool trap, from which excess solvent is stripped with helium. The trapped compounds are then heat desorbed from the glass wool in a modified injection port as described above for Zlatkis and Andrawes (86). Complex profiles are obtained with compounds in high yields from as little as 25 μ l of serum although no peaks were identified. No experiments in which urine was used are described.

Stafford *et al.* (85) using techniques described by Zlatkis (84) profiled plasma, urine, breast milk and amniotic fluid. Several compounds were identified and large amounts of benzyl alcohol and benzaldehyde were noted in the plasma of a mother-infant pair.

By slightly modifying the method of Zlatkis and Kim (84), Lee, Nurok and Zlatkis (41) showed that the transelevator technique can be used for both headspace trapping and solvent extraction of volatiles from blood serum and breast milk. The sampling is performed in two steps, the first being the collection of low molecular weight volatiles on a Tenax tube, the second being the solvent extraction of volatiles coupled with collection on glass beads. In the first step the sample is introduced to the Porasil E tube and Tenax GC instead of glass wool is used as a trap for volatiles. A stream of helium gas is passed through the apparatus to transfer the volatile compounds from the porasil tube to the Tenax tube which is then removed from the apparatus for later heat desorption of volatiles as described above. Again very small samples, as little as 25 μ l of breast milk, were used to obtain complex profiles but no compounds were identified and it was stated that this technique is not applicable to urine as this contains too low a concentration of volatiles.

Odours Associated With Disease States

Although it is clear that much attention has been paid to profiling volatile compounds in blood and urine this technique has not been applied for analysing volatile compounds from the fluids of patients presenting with peculiar odours. This is surprising as body odour may be a useful indicator of the diseased state.

The first phenylketonuric patients were recognised because the children's mother complained of a musty or mousey odour (89), now known to be due to the presence of phenylacetic acid in the urine (90, 91). It is clear that knowledge of the identity of the odoriferous compounds would have assisted the clinician.

Later Menkes *et al.* (92) described a syndrome characterised by vomiting and muscular hypertonicity and which was associated with a maple-syrup odour of the urine. Since then knowledge about the so-called "maple syrup urine disease" has increased rapidly, but according to Dancis and Levitz (93) the distinctive clinical feature of the disease is the odour, which has been compared to burned or caramelised sugar. This odour may be caused by the keto-acids which accumulate in the blood and urine of patients with this disorder. Unlike phenylketonuria in which the odour may not be detected until about three weeks after birth, the odour associated with MSUD has been detected in the urine as early as the fifth day and is one of the most important aids in diagnosis (93).

Another disorder of amino acid metabolism which is characterised by a peculiar smell is isovaleric acidemia. Diagnosis is suggested by the distinctive odour described as "cheesy" or "like sweaty feet" (94) and is caused by accumulation of isovaleric acid in the blood and urine of people

with this disorder. As shown in Figure 13, the accumulation arises from a block in the catabolic pathway of leucine after its transamination and oxidative decarboxylation.

In another case the odour, which had also been described as resembling sweaty feet, was found to be due to large quantities of butyric and hexanoic acids (95) in the body fluids.

An odour described as resembling that of rancid butter, or rotten cabbage, has been noted in the urine, sweat and breath of hypermethionemia (96) cases. The smell in the urine was thought to be due to 2-keto-3-methylthiobutyric acid or a derivative of this compound, although the exact metabolic pathway for the formation for this compound was unclear.

A child with "Oasthouse syndrome" described by Jepson *et al.* (97) had a distinctive unpleasant pungent smell, reminiscent of a brewery or oasthouse. In this condition there is a defect in the transport system which largely affects methionine, and some of the unabsorbed methionine is converted by colonic bacteria to 2-hydroxybutyric acid which is the cause of the characteristic smell. The urine of people suffering from 3-methyl crotonyl glycinuria (98, 99) has an odour of "cat's urine". This odour is believed to be due to the presence of 3-hydroxy isovaleric acid which is formed in the body by β -oxidation of isovaleric acid which accumulates because of a defective enzyme 3-methyl-crotonyl-CoA carboxylase in the catabolic pathway of leucine.

Often the first clue to a diagnosis of the presence of a diabetic ketosis is the fruity aroma of decomposing apples which is due to the presence of ketones in the patient's breath (100).

Smith and Sims (101) showed that schizophrenic and non-schizophrenic sweat could be distinguished by odour. Trans-3-methyl-2-hexenoic acid was first thought to be the compound responsible for the odour (102) but later workers were either unable to find this compound in schizophrenic patients (103) or found it in healthy individuals as well as schizophrenics (104). Earlier, Stein and Wise (105) had proposed an elaborate pathway for the formation of trans-3-methyl-2-hexenoic acid from 6-hydroxydopamine.

Odour has also been used for the diagnosis of some cancers. Lidell describes a patient whose only reason for seeking medical advice was on account of the offensive smell from a leg ulcer which had undergone malignant change (106). This re-inforces other reports by Levine and Fong (107) and Dawson and McIntosh (108) who describe a foul smell from varicose ulcers which had become malignant. Lidell and White (109) report a man whose presenting complaint for a serious squamous cell carcinoma was his offensive body odour which caused his colleagues to refuse to work with him.

MATERIALS AND METHODS

Materials and Methods

Headspace Sampling

The fundamental component for the collection of volatile organic compounds from the enclosed space (headspace) above the aqueous solutions was a trap (Fig. 1a) which consisted of a stainless steel tube with 1/8 inch outside diameter (o.d.) packed with a 100 mg bed of a porous polymer, chromosorb 105 (Johns Manville, Denver, Colo., U.S.A.). Rolled stainless steel gauze plugs held the polymer beads in the tube.

High purity nitrogen (Commonwealth Industrial Gases, Port Kembla), regulated by pressure and flow controlling valves as used by Murray (122), was passed (40 ml/min) into the conical headspace flask via an inlet in the teflon plug (Fig. 2). The plug incorporated a rubber O ring which sealed the flask. Two traps were connected to parallel outlets in the plug and were placed approximately 5 mm from the surface of the aqueous sample. This forced the carrier gas to sweep close to the surface of the solution before passing through the traps to atmosphere. There was no temperature difference between the sample and the trap so condensation of excess water in the trap from the saturated gas stream was avoided. Another trap was placed in the carrier gas inlet line to remove any contaminating organic volatiles.

Heavy lead sinkers kept the flask submerged in a water bath (40°C) for the duration of the headspace sampling (10-20 hours). The sampling procedure left a small amount of trapped water in the tubes which was purged by placing them in a dry flask while continuing the carrier gas flow (10 mins). Finally the tubes were removed from the apparatus, capped with tightly fitting teflon plugs and stored in a desiccator at -15°C.

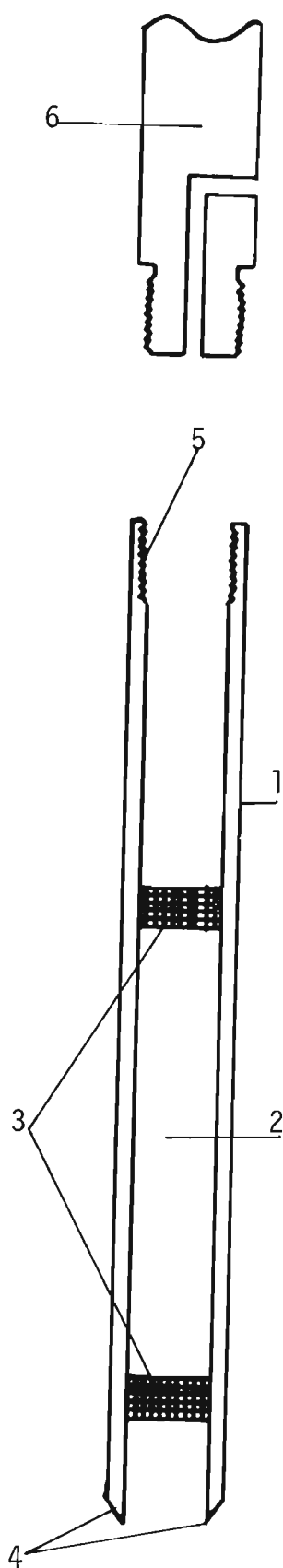


FIGURE 1a: Headspace Trap

- | | |
|--------------------------|---------------------------|
| 1. Stainless Steel Tube | 2. Porous Polymer bed |
| 3. Stainless Steel gauze | 4. 60° Chamfer |
| 5. Internal 6BA thread | 6. Threaded Insertion rod |

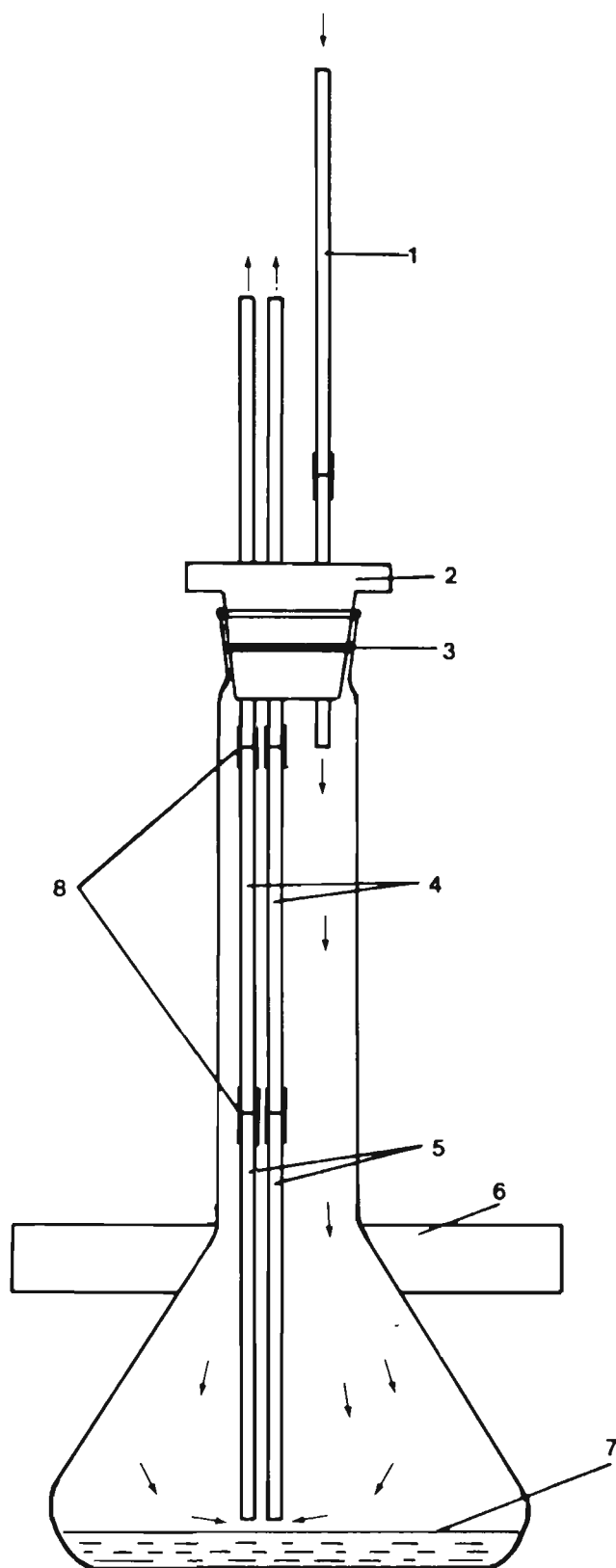


FIGURE 2: Headspace Flask Assembled

1. Carrier gass purifying trap
2. Teflon plug
3. 'O' ring
4. Outlet trapping tubes
5. Empty tubes
6. Lead sinker
7. Urine sample
8. Teflon connectors

Each end of the trap was specially designed for its incorporation into the sample transfer process. One end was threaded for connection to a rod which was used to push the trap into the modified GC injection port. A valve on the GC injection port allowed insertion of the trap without disturbance to the carrier gas flow. The other end of the trap was chamfered to a 60° angle and formed a butt seal when the tube was pushed against a seat with the same angled face in the injection port. The carrier gas (He, 6 ml/min) was thus diverted through the trap in a backflushing direction. The absorbed material was desorbed as the trap was heated to the injector block temperature (150°C) and was collected in a narrow frozen zone of glass lined stainless steel tube (GLT, 1.6 mm o.d. x 0.4 mm i.d.) which connected the injection block to the separating column. This short section of GLT was cooled by a hollow copper probe (Fig. 3a) which was filled with liquid nitrogen and inserted through the GC column-oven lid some minutes earlier. For routine operation, ten min. in the injection port was adequate for complete volatiles recovery, after which the trap was withdrawn.

The condensed volatiles were injected onto the capillary column by replacing the cold probe by a similarly shaped brass probe (Fig. 3b) pre-heated to GC oven temperature or above.

Gas Chromatograph

Most of the work was performed on a Varian model 1800 dual column gas chromatograph. The instrument was fitted with dual flame ionisation detectors (FIDs) and a multi-linear temperature programmer. The carrier gas plumbing on both the inlet and outlet sides of the columns was extensively modified to enable use of capillary columns. For desorption of volatiles from the special traps a modified micro-dipper valve (Packard Instruments, Sydney) was

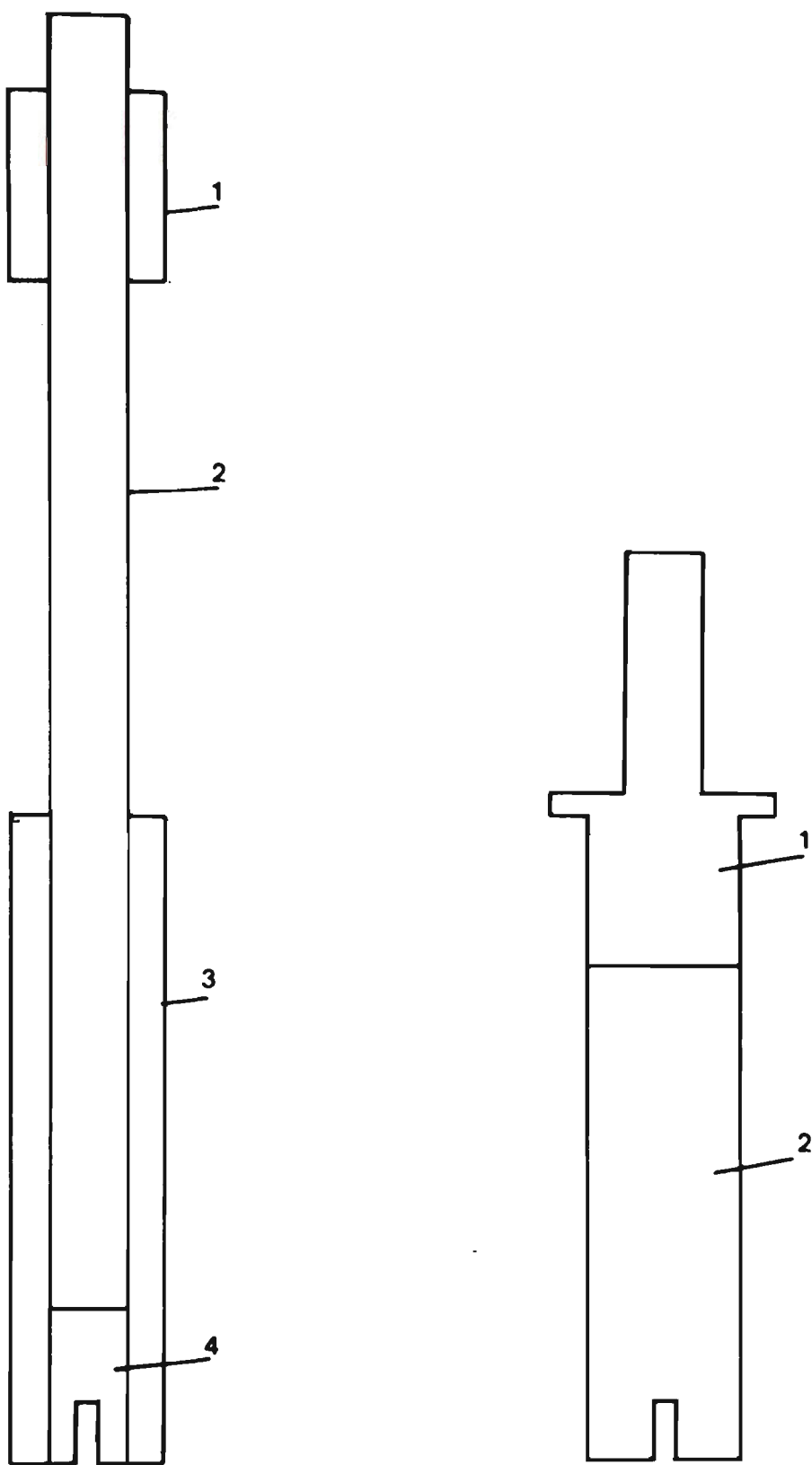


FIGURE 3:

(a) Cold Probe

(b) Hot Probe

- 1. PTFE Insulation
- 2. Copper Tube
- 3. PTFE Insulation
- 4. Channelled brass plug

- 1. Asbestos handle
- 2. Channelled brass rod

connected to the injection port and a stainless steel injection port liner ($\frac{1}{4}$ " o.d. x $\frac{9}{64}$ " i.d.) was fitted. The 60° angled face needed to seal the volatiles trap was incorporated by sleeving the front of the GLT connecting the injection port to the column with a short length (4.5 cm) of stainless steel tube ($\frac{1}{8}$ " o.d.) and chamfering the front of this tube to 60° .

Some work was also performed on a Packard 419 dual column GC. This instrument was fitted with two FIDs and a linear column oven temperature programmer. The electrometer output was set to 2.5 mV full scale and carrier gas plumbing modifications were also needed.

Details of GC modifications for both machines are given in appendix A and are shown schematically in figures 4, 5 and 6. On both GCs the column eluant was divided between the FID and a second external vent by placing a "T" in the column outlet line after the make-up gas had been added and connecting GLT of different internal diameters (i.d.) to the "T" outlets. Both lines were open to atmosphere but since the line with the largest i.d. had the least resistance to flow the carrier gas preferentially took this path. By placing a micro-needle valve (SGE, Melbourne) in the larger ID line the split could be varied by closing the valve thus forcing a greater proportion of carrier gas to flow through the second narrower outlet.

Gas Chromatographic Columns

Support coated open tubular (SCOT) columns (S.G.E., Melbourne) coated with carbowax 20 M liquid phase with 1.0 mm o.d. and 0.5 mm i.d. were used in all profiling work. A 60 metre SCOT OV17 column (S.G.E., Melbourne) was used for some work with butyl esters of short chain fatty acids.

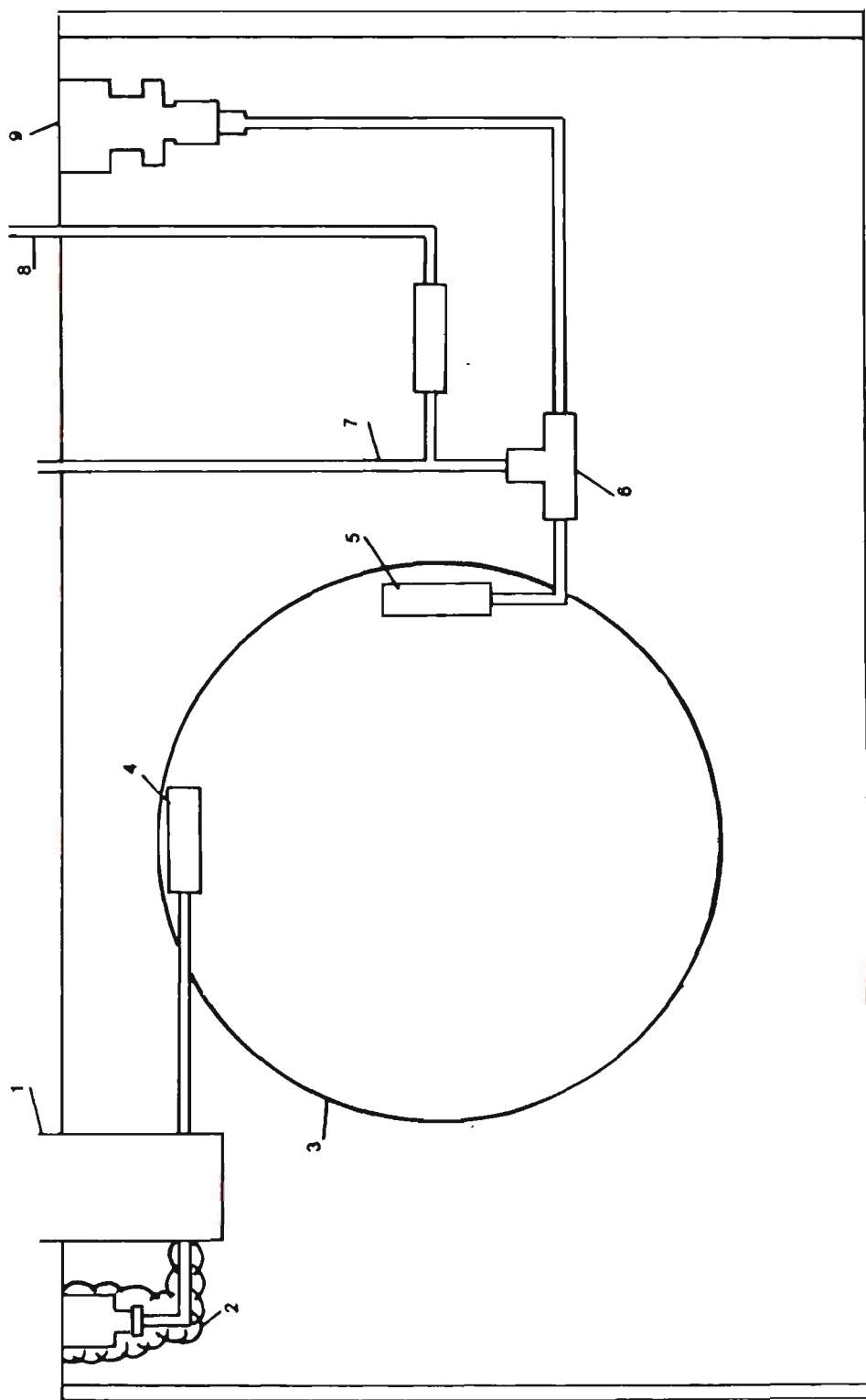


FIGURE 4: Column Oven. Packard 419

- | | |
|---|--------------------------------|
| 1. Cold trap | 6. Low hold up tee |
| 2. Insulation on GLT column inlet | 7. Micro needle valve |
| 3. Capillary column support cage | 8. External vent to atmosphere |
| 4. Low hold up union (connection to capillary column) | 9. FID |
| 5. Make up gas tee (column outlet) | |

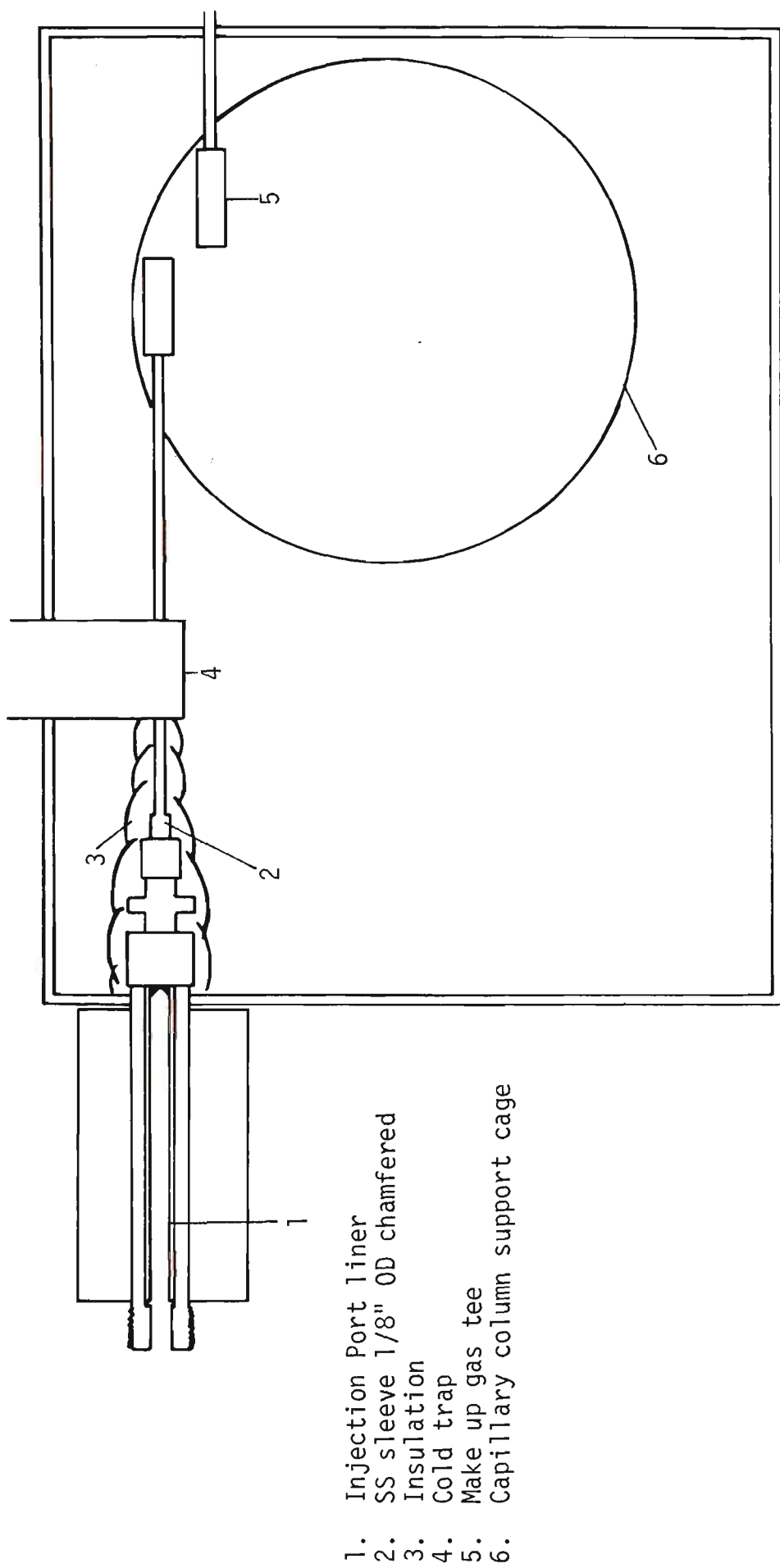


FIGURE 5: Injector block and column oven. Varian 1800

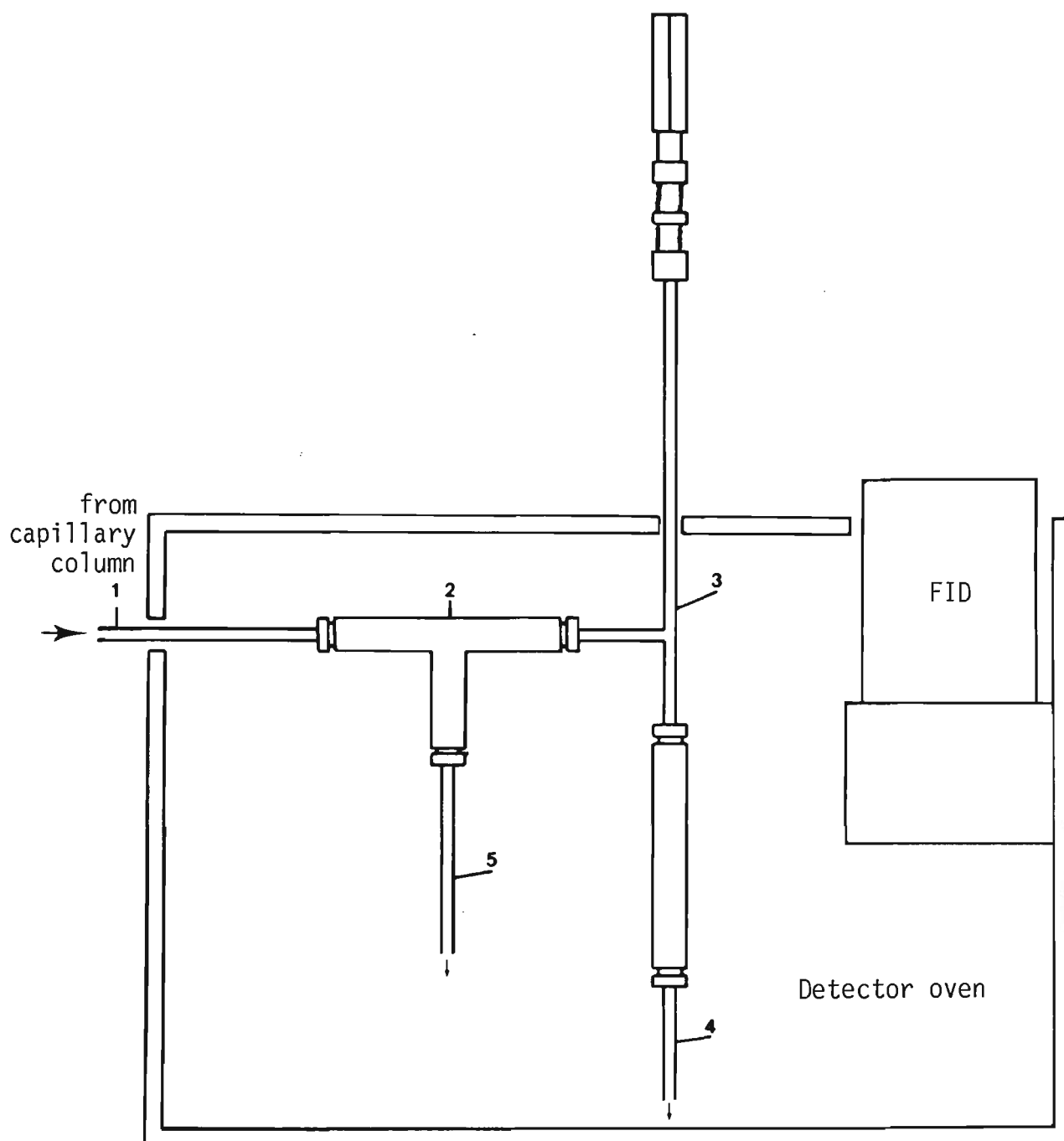


FIGURE 6: Varian 1800 Outlet Plumbing

- | | |
|--|--|
| 1. GC column outlet | 2. Zero dead volume tee |
| 3. Micro needle valve | 4. * LHS = line to FID
RHS = line to MS |
| 5. * LHS = line to vent
RHS = line to FID | |

* The GC was dual column. LHS refers to the column plumbing for the left hand side; RHS refers to the column plumbing for the right hand side.

The efficiencies of these columns calculated by the manufacturer using the formula

$$N_{\text{eff}} = 5.54 \left[(t_r - t_a) / W_{1/2} \right]^2$$

where N_{eff} = number of theoretical plates

t_r = retention time of defined peak

t_a = retention time of air peak

$W_{1/2}$ = width at half height of defined peak and where the defined peak was n-butylbenzene

are shown in Table 1.

Organoleptic Detection and Peak Collection Vent

To determine the odour of a compound as it emerged from the GC column the second arm of the column outlet "T" led outside the GC and was connected to a heated GLT transfer line (1/16" o.d. x 0.6 mm i.d.). This terminated in a position which was convenient for the operator to observe the FID trace and monitor the odour emanating from the vent.

The vent could also be used for preparative work by replacing the heated transfer line with an adopter union (1/8" to 1/16", SGE, Melbourne) incorporating a shortened blue ceramic-teflon front ferrule (1/8", Varian, Sydney). When a peak was due to appear on the FID trace a trapping tube packed with either chromosorb 105 or 5% SF 96 on chromosorb A (60-80 mesh, Applied Science) was pushed into the union. After the peak had eluted the tube was removed, capped and stored in a desiccator at -15°C .

Eluting peaks could also be collected using a "cold-trap" technique by replacing the union adaptor with a low hold up union (1/6", SGE, Melbourne). A capillary tube was connected to the union with a graphite ferrule and a cold probe similar to that described earlier was held over the capillary tube by a

TABLE 1

Efficiencies of capillary columns used for volatiles profiling

Number of theoretical plates (N_{eff})	length (metres)	Manufacturer's serial number
43,100	63	340/113
35,700	40	427/67
31,900	40	524/143
36,800	51	585/156
* 46,700	60	566/85

* This was the OV17 column and the defined peak was
n-tetracosane

bracket connected to the side of the GC. The cold probe was filled with liquid nitrogen and the eluting peak was frozen in a narrow zone (1 cm) of the capillary. When the peak had eluted the capillary was removed and either sealed immediately or flushed with a solvent or derivatising reagent which was drained into a sample vial (1 ml) and sealed.

Mass Spectrometer

The second line of one of the column outlet "T"s from the Varian 1800 GC was connected to an Electronics Associates Incorporated (EAI) Quadrupole 300D mass spectrometer via a single stage jet separator (SGE, Melbourne) and GLT. Details of the GC-MS interface are given in Appendix A. The MS could be operated manually or by a Data General Nova 1220 minicomputer through an EAI 555 Interface (Digital Electronics, Sydney).

The mass analyser was controlled by the computer through a 12 bit digital to analogue converter (DAC). For the TMA analysis the MS was switched to 1-100 mass range so the resolution possible was about 0.02 amu/least significant bit (LSB). However the instrument was detuned so that the mass peaks were approximately 1 amu wide at the base.

The oxygen peak (m/z 32) and the m/z 69 peak in the spectrum of perfluorotributylamine were used to calculate the slope and intercept of the curve relating DAC control number to mass. The values for masses 59 and 60 calculated from the equation thus derived were checked manually for accuracy prior to a batch of analyses.

The programme used for quantitative scanning compensated for electronic drift by stepping the DAC above and below the value calculated for a

given mass by a variable number of LSBs; specifically, the value calculated for mass 59 was 1883 and the relevant variable (samples/peak) was set to 5, so the numbers from 1881-1885 inclusive were used to set the output voltage of the DAC which controlled the mass analyser. For each value an intensity measurement was obtained but only the maximum of the 5 values was reported.

The primary detection system of the Quad 300D was a 12 stage electron multiplier whose output was fed to a high impedance current amplifier. The output from the current amplifier was linked to an integrator which was basically a capacitor charging circuit. When the analyser focused the nominated mass onto the electron multiplier the signal produced by the current amp was integrated for a variable period of time by the analogue integrating circuit and then the accumulated charge was converted to a digital number by an analogue to digital converter (ADC). This number was stored by the computer as the abundance measurement.

Preparation of Tetrabutylammonium Hydroxide

Tetrabutylammonium bromide (4.5 g, 14 mmol) was dissolved in 50 ml of dry methanol (distilled from MgOEt) then silver oxide (8.8 g, 38 mmol) was added and the mixture was stirred for 30 minutes after which dry methanol (50 ml) and ethanol (30 ml) were added to the mixture.

This solution was filtered to remove the silver iodide/silver oxide slurry and centrifuged at 800 *g* to remove remaining suspended material. The resulting alcoholic mixture was stored in plastic bottles and was analysed by TLC for unreacted tetrabutylammonium bromide.

Analysis of Tetrabutylammonium Hydroxide

A pre-coated silica gel TLC plate (Merk, layer thickness 0.25 mm) was spotted with the tetrabutylammonium hydroxide prepared as above. The solvent was evaporated and the plate transferred to a tank containing the solvent mixture ethanol/water/ammonia (10:2:1). After 3½ hours the plate was removed from the tank, dried and developed with iodine vapour.

Preparation of Oximes of Keto Acids

The aqueous sample (2 ml) containing the keto acids was adjusted to a pH of 12 by addition of NaOH (6M, 150 µl); hydroxylamine hydrochloride (25 mg) was added and the mixture was heated at 60°C for 30 minutes.

Solvent Extraction of Organic Acids from Aqueous Solutions

The urine or standard sample (2 ml) was placed in a stoppered centrifuge tube and HCl (6M) was added till the pH of the solution was 1. NaCl (600 mg) was added to saturate the solution. Diethyl ether (2 ml) was then added to the sample and the two phases were mixed thoroughly for two minutes. If a suspension had formed the tube was centrifuged at 800 *g* for 2-5 minutes to separate the aqueous and organic phases before the organic phase was transferred to a test tube. The extraction was repeated twice more and the pooled organic extract was dried by addition of MgSO₄ (1 g) and then filtered into a culture tube (10 ml capacity).

Butylation of Organic Acids

The filtered and dried acid extract was adjusted to a pH of 10-12 by addition of tetrabutylammonium hydroxide (TBA-OH) solution. The solvent was then evaporated under a stream of dry nitrogen while the culture tubes were heated to 50°C. Dimethylacetamide (DMA, 500 µl) and a volume of n-butyl bromide containing ten times the number of moles of TBA-OH used was added to the culture tube and the mixture was heated at 100°C for 60 minutes.

The mixture was cooled and then extracted three times with pentane (1 ml) and the pooled pentane extract was washed with water (½ ml) then transferred to a small sample vial (1 ml capacity). Acetone (50 µl) was added to the vial and the pentane was evaporated under a stream of nitrogen. An aliquot (0.05 µl) of the solution was then injected into the GC.

Silylation of Organic Acids

The solvent was evaporated from the dried and filtered acid extract by using a stream of dry nitrogen. Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, 100 µl) was added to the residue and the solution was transferred to a sample vial (1 ml) and heated at 60°C for 30 minutes. An aliquot (1-2 µl) of this solution was injected into the GC.

Methylation of Organic Acids

Using a stream of dry nitrogen the solvent was evaporated from the dried and filtered organic acid extract. Ethereal diazomethane (200 µl) was

added to the residue and the solution transferred to a sample vial (1 ml). Aliquots (2-5 μ l) of this solution were injected into the GC.

TRIMETHYLAMINE ANALYSIS

Preparation of Standard Solution of Trimethylamine Hydrochloride

A stock solution of trimethylamine (TMA) was prepared by transferring the contents of a 250 ml ampoule of A.R. grade TMA (BDH) into 2 litres of water to give a TMA concentration of approximately 2M. The TMA was then converted to its hydrochloride by titrating a 200 ml aliquot of the stock solution with 6M HCl to pH of one. The salt solution was then extracted twice with chloroform (200 ml) and the extracts were pooled and the solvent evaporated under vacuum. The resulting white crystalline product was redissolved in chloroform, filtered and the solvent evaporated under vacuum. This procedure removed dimethylamine hydrochloride, methylammonium chloride and ammonium chloride which are insoluble in chloroform.

Since the hydrochloride salt of TMA is hygroscopic it was thoroughly dried before weighing. After drying the TMA.HCl for 4 hours in an air oven, a small sample was transferred to a drying pistol and the TMA HCl was further dried under reduced pressure over P_2O_5 . After 24 hours, the vial was removed, capped and the sample was immediately weighed. The sample was then dissolved in water and the solution quantitatively transferred to a volumetric flask.

Internal Standard

A solution of $(CH_3)_3^{15}NHCl$ (Merk, Sharp and Dohme, Canada) was

prepared by dissolving a small aliquot (803 mg) of the solid in water (50 ml). Thorough drying was not attempted. The concentration of this solution was about 8.4mM.

Calibration of Internal Standard

Eight different concentrations of TMA HCl were prepared by pipetting 10, 20, 30, 40, 50, 60, 70, 80 microlitre aliquots of the stock solution into 50 ml Erlenmeyer flasks. Each flask was made up to a volume of 1 ml by addition of water and to each was added 60 μ l of the internal standard solution. The solutions were then transferred to headspace flasks and 5M NaOH (5M, 500 μ l) was added prior to the headspace collection.

Urine Samples

An aliquot of urine (500 μ l) was pipetted into a 50 ml Erlenmeyer flask containing distilled water (500 μ l) and the internal standard solution (60 μ l). The urine solutions were then transferred to a headspace flask and sufficient NaOH (5M) was added to raise the pH to about 14 before the headspace collection was started. In samples where the TMA values were greater than the range covered by the standard curve, the experiment was repeated using a smaller sample volume.

Headspace Collection

The TMA was collected on two traps connected in parallel. Two empty tubes were placed in series to the traps closest to the surface of the

solution to avoid fouling of the porous polymer traps. The flasks containing the TMA solution were immersed in a water bath at 40⁰C for 10 minutes and a carrier gas flow of between 30 and 40 ml/min was used. Then the traps were transferred to an empty flask which was immersed in the water bath for a further fifteen minutes while flushed with dry nitrogen to purge them of adsorbed water. The traps were then removed, capped with teflon end caps, labelled and placed in a desiccator which was stored at -15⁰C.

Gas Chromatograph Conditions

The TMA containing trap was allowed to reach ambient temperature and was then inserted into the GC injection port until the 60⁰ angled faces on the tube and in the injection port were in contact. The trap was removed from the injection port after 60 seconds.

The optimum GC conditions for separation of the TMA from remaining traces of water were obtained with SCOT OV17 column at 100⁰C using a flow rate of 5-6 ml/min. An eluant split to the M.S. of about 90% was used and no make up gas was added to the carrier flow.

Mass Spectrometer Conditions

An EAI Quad 300D controlled by a Nova 1220 computer, was used to repetitively scan the two m/z values 59 and 60 for the duration of the experiment. The instrumental conditions used were: ion energy 8V; extractor 12V; focus 2V; emission 50 uA; electron energy 20V; electron multiplier 1800V; source pressure 1×10^{-5} torr; resolution 29; mass range low.

Data Acquisition

The Wollongong Quad-Magnova Operating System Mark 6 was used in the quantitative selected ion mode. The two m/z values (59, 60) were specified and an integration time of 20 milliseconds with a low integrator gain and within peak scanning gave highly precise results. The coefficient of variation of the TMA samples bled into the source from a sample chamber was usually less than 1% while that of the tail section of the TMA peak from a GC-MS run was usually less than 2%.

The operating system was modified as shown in Appendix B to eliminate the section which calculated and subtracted an arbitrary "noise" value from each intensity and which disregarded any intensity which was less than this value.

A copy of the responses to prompts issued by the operating system is also given in Appendix B. The responses were entered before the TMA containing trap was inserted into the injection port and the computer acquisition of data was commenced after the air peak had passed into the mass spectrometer, i.e. 2-2.5 minutes after the sample was injected.

Then the computer system acquired the abundance data of the two ions for 100 consecutive passes and stored them on a floppy diskette for later off-line data processing.

Data Processing

The data acquired and stored by the operating system was retrieved and processed by a programme called Isotope. This programme searched a nominated

data file for the abundances of the two m/z values specified and computed their ratio if they were above a nominated threshold value. The ratios were then averaged and the standard deviation and coefficient of variation were given. A full description and listing of this programme can be obtained on application to Wollongong University Chemistry Department.

The average ratio so obtained was used as the R_0 term to calculate R_a using the equation:

$$R_a = (R_0 - Q)/(1 - R_0 S)$$

The values for Q , the ratio of m/z 60 to m/z 59 of unlabelled TMA, and S , the ratio of m/z 59 to m/z 60 of labelled TMA, were determined in separate experiments.

Drugs Used for Trimethylaminuria Studies

Becholine: 1.8M choline chloride in a flavoured sorbitol base.

Neomycin: neomycin tablets, Takeda Chemical Industries, Osaka.

Synerlac: Each 50 g contains selected living antibiotic resistant lactic ferments of *B. Bifidus*, *Acidopilus*, *Bulgaricus*, *Thermophilus*, *Streptococcus lactis* (each 3.5 g), aluminium hydroxide (19 g), aluminium silicate (6.25 g) and lactose (6.25 g).

Duphalac: 4-D-Galactopyranosyl-4-D-Fructofuranose (50% w/w/), galactose (8% w/w) and lactose (5% w/w).

Choline Loading Regime for Patients M.H., A.H., S.H., J.R. and N.H.

Subject ingested 8-10 ml Becholine (Medical Research, Sydney)

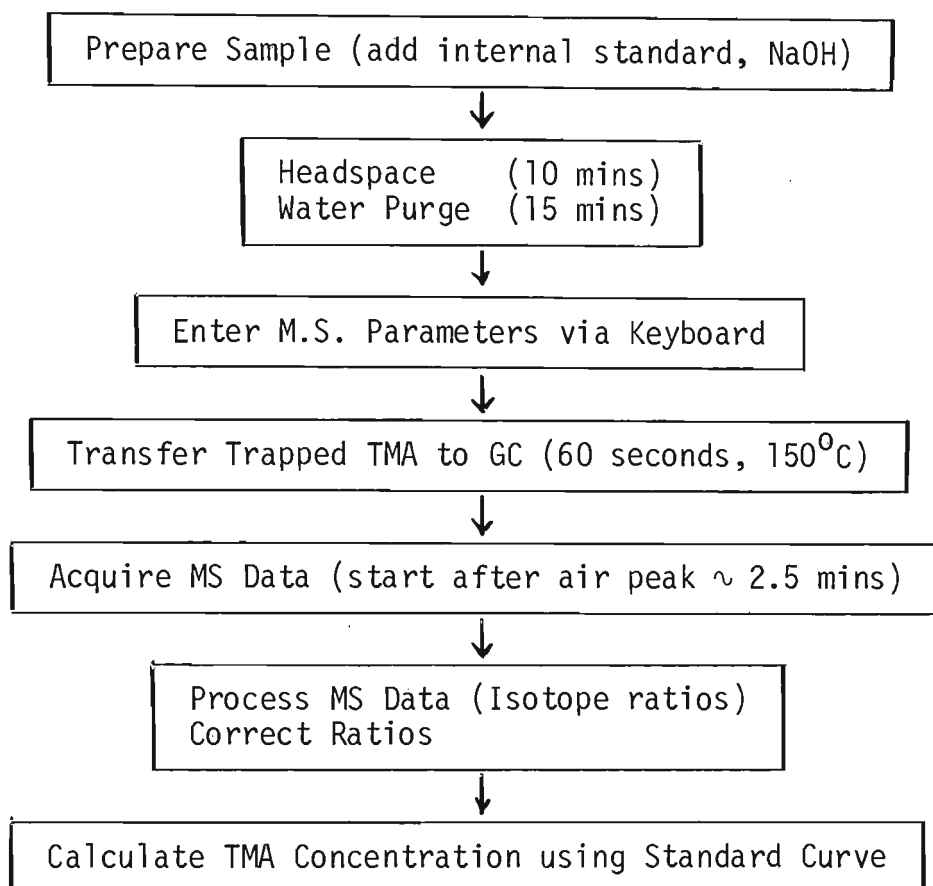


FIGURE 7: Trimethylamine Analysis Flowchart

Each box is described above in detail.

four times daily. One urine specimen was collected within 12 hours after the last dose.

Intestinal Sterilisation Regime for Patients M.H. and R.G.

Two urine samples (50 ml each) were collected from the subjects on the first day of the experiment and 5 ml of HCl (6M) was added to each sample. Then followed a course of neomycin (500 mg/day) for one week. On day 7 one urine sample (100 ml) was taken and acidified with HCl (10 ml, 6M).

Regimes for Choline Load Responses

(a) Neomycin

- Day 1. Becholine (1 ml) taken orally 4 times (10 a.m., 2 p.m., 6 p.m. 10 p.m.). Urine collected for next 12 hours.
- Day 2. Urine collected till 10 a.m. Neomycin (500 mg) taken before evening meal.
- Day 3. Neomycin (500 mg) taken 3 times.
- Day 4. Neomycin (500 mg) taken 3 times.
- Day 5. Neomycin (500 mg) taken 3 times.
- Day 6. Neomycin taken 3 times. Becholine (1 ml) taken orally 4 times (10 a.m., 2 p.m., 6 p.m., 10 p.m.). Urine collected for next 12 hours after 10 p.m.
- Day 7. Urine collected till 10 a.m. Neomycin taken before breakfast.

(b) Synerlac

- Day 1. Becholine (1 ml) taken orally 4 times (10 a.m., 2 p.m., 6 p.m., 10 p.m.). Urine collected for next 12 hours.
- Day 2. Urine collected till 10 a.m. Synerlac (2.5 g) taken orally before evening meal.
- Day 3. Synerlac (2.5 g) taken orally twice.
- Day 4. Synerlac (2.5 g) taken orally twice. Becholine (1 ml) taken orally 4 times (10 a.m., 2 p.m., 6 p.m., 10 p.m.). Urine voided during 12 hours after 10 p.m. collected.
- Day 5. Urine collected till 10 a.m.

(c) Duphalac

- Day 1. Becholine (1 ml) taken orally 4 times (10 a.m., 2 p.m., 6 p.m., 10 p.m.). Urine collected for next 12 hours.
- Day 2. Urine collected till 10 a.m. Duphalac (5 ml) taken orally before evening meal.
- Day 3. Duphalac (5 ml) taken orally 3 times. Becholine (1 ml) taken orally 4 times (10 a.m., 2 p.m., 6 p.m., 10 p.m.). Urine collected for 12 hours after 10 p.m.
- Day 4. Urine collected till 10 a.m.

RESULTS AND DISCUSSION

Results and Discussion

Although clinicians frequently associate peculiar body odours with disease states (115, 116) to our knowledge no attempt has been made to identify the odorous constituents of urine as an aid to the diagnosis of these diseases. A number of workers have shown that low molecular weight, volatile metabolites are present in urine and these include ketones, alcohols, furans, pyrroles and sulphur compounds (15, 45, 58, 59, 60, 81, 83, 87, 88, 217). The gas chromatographic profile of these compounds has been extensively investigated by Robinson *et al.* (15) who developed automated equipment which enabled analysis of samples from large populations. Computer matching procedures were needed to process the chromatographic data since the volatiles profiles sometimes contain over 200 constituents. Their aim is to use this data for orthomolecular diagnosis, that is the quantitative assessment of the state of molecular health of the human body. They have demonstrated that there are diagnostically strong patterns present in most of the disease groups (e.g. breast cancer, Huntington's disease) they studied (117).

McConnell *et al.* have recently reported similar equipment in which the data collection procedures are sufficiently standardised to enable computer based pattern recognition techniques to be applied for profile characterisation. Using this system they were able to distinguish volatiles profiles of individuals with diabetes mellitus from those of normal individuals. Earlier work by Liebich and Al-Babbili (118) shows that the urinary concentrations of some aliphatic alcohols are abnormally increased in these patients.

Several problems have limited the extraction of useful information from complex volatiles profiling data. Profiles vary considerably from individual to individual due to such factors as diet and genetic make up.

In some cases these variations are of the same magnitude as those which might be expected to occur due to pathological changes. A diagnosis of such a condition this requires comparison of large sets of normal and pathological profiles so that "normal" variations can be eliminated from the pathological profiles.

None of these workers has applied the technique as an aid in the diagnosis of specific metabolic disorders which are associated with peculiar odours. This laboratory is associated with the genetics research unit at the Royal Children's Hospital, Melbourne, and routinely examines suspect organic acid profiles by GC-MS. Because of the success of metabolic profiling of organic acids and amino acids in the diagnosis and study of inherited metabolic disorders the logical extension of using a volatiles profile for the diagnosis of inborn errors of metabolism was apparent.

Although a variety of techniques for volatiles profiling have been reported in the literature (15, 16, 19) we selected the procedure described by Murray (112). The reasons for our selection were: (a) the technique had been highly developed and thoroughly tested by applications associated with food flavours and aromas (120); (b) the additional equipment requirements for the modification of the gas chromatograph were minimal; (c) the geographical proximity to Dr. Murray's laboratory enabled consultation and the author's training in headspace chromatography.

With this technique the volatiles are extracted by passing a stream of inert carrier gas over the urine sample contained in a sealed glass flask which is kept at a constant temperature (40°C). A small stainless steel column packed with a porous organic polymer and fixed to the outlet of the flask traps the volatiles extracted from the urine by the purging gas. The trap is then placed in the GC injection port where the volatiles are heat

desorbed at 150°C and flushed onto the GC column by the carrier gas.

For the purposes of this work volatiles can be defined as those organic compounds which can be trapped by Murray's headspace technique and chromatographed without derivatisation.

After our gas chromatograph had been modified as described in the experimental section the equipment was used to obtain the volatiles profiles of a number of healthy individuals.

The volatiles profile from the author's urine is shown in Figure 8. This profile was highly reproducible since some 20 control runs on different urine samples taken from the author over a period of 3 years showed only minimal variation. Since our aim was to investigate profile differences between normals and individuals with inborn errors of metabolism, it was not essential to establish the identity of each GC peak. Some large peaks in the normal profile, however, were readily identified by matching library spectra (121) and are listed in Figure 8. For the sake of completeness we have listed the mass spectra of the 6 unidentified GC peaks in appendix B. We have compared our profile results with those published by other workers who used similar headspace chromatographic techniques and capillary GC-MS (Table 2). It can be seen that our procedure gives comparable results to theirs with normal subjects.

As did Robinson and Pauling (117), we also found that the volatiles profile varies greatly between individuals, but that the profile from the same individual was remarkably reproducible. This observation is pertinent to those workers who seek to find small but significant changes in the metabolic profile of a selected population compared to a normal one. We were not unduly perturbed by the variability of the volatiles profile between

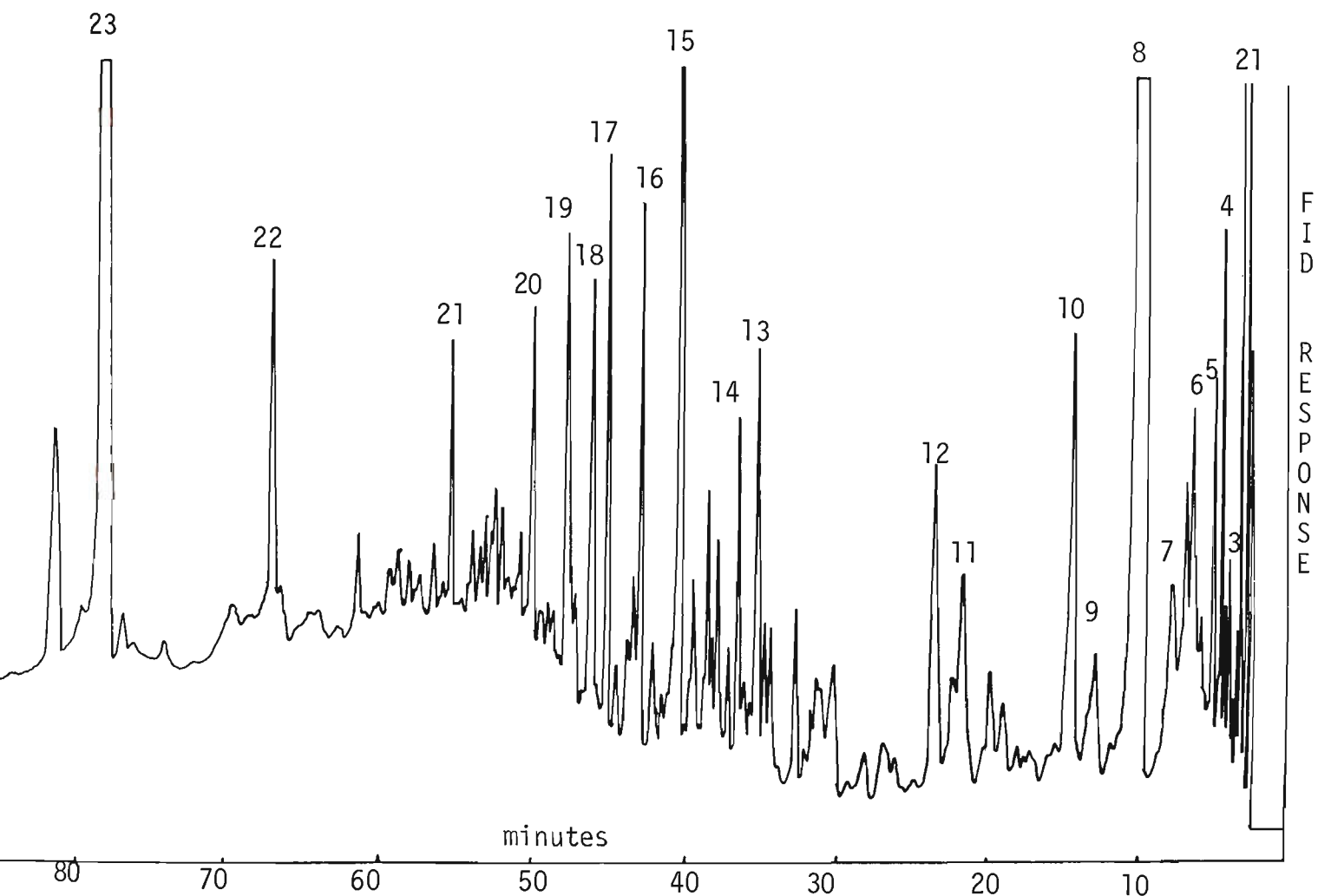


FIGURE 8: Chromatogram of volatiles from the author's urine (50 ml, pH 6)

Headspace volume: 24.56 l, (HS630)

Column: SCOT CW20M 50 m

Temperature programme: 80°C 10 mins. then 3°C/min. to 185°C,
and 185°C for 30 mins.

1 = ethanol, 2 = acetone, 3 = 2-butanone, 4 = hexane,
5 = 2-pentanone, 6 = 2-hexanone, 7 = 3-heptanone,
8 = 4-heptanone, 9 = xylene, 10 = UN1, 11 = UN2,
12 = 3-methyl-2-heptanone, 13 = benzaldehyde,
14 = 2,5-dimethylpyrazine, 15 = butyrolactone,
16 = furfural, 17 = UN3, 18 = UN4, 19 = naphthalene,
20 = UN5, 21 = dimethyldisulphide, 22 = p-cresol,
23 = UN6.

TABLE 2

Volatile compounds found in human urine vapour. Compilation from references 83, 16, 122

HYDROCARBONS

hexane
B-pinene
benzene, toluene,
p-methylpropylbenzene

ALCOHOLS

ethanol
n-propanol
n-butanol, isobutanol
isopentanol
p-cresol

KETONES

2-propanone
2-butanone; 2,3-butanedione
2-pentanone; 2,3-pentanedione
3-methyl-2-butanone
3-penten-2-one
3-pentanone, 3 methylcyclopentanone
4-methyl-2-pentanone
3-methyl-2-pentanone
4-methyl-3-penten-2-one
3-hexanone, 2-hexanone
5-methyl-3-hexanone
4-heptanone
3-heptanone
2-heptanone
4-octanone
3-octanone
2-nonanone
cyclohexanone
carrone
piperitone
thiolan-2-one

ALDEHYDES

butanal
propionaldehyde
3-methylbutanal
2-methylbutanal
benzaldehyde

HETEROCYCLICS

(a) Furans

2-methylfuran
2,3-dimethylfuran
2,4-dimethylfuran
2,5-dimethylfuran
methylethylfuran
2-ethylfuran
2,3,5-trimethylfuran
C₄-furan
C₄-pentylfuran
acetylfuran, furfural

(b) Pyrroles

pyrrole, 1-butylpyrrole
1-methylpyrrole
2-methylpyrrole
dimethylpyrrole

(c) Thiophene

(d) Pyrazines

methylpyrazine
2,3-dimethylpyrazine
2,3,5-trimethylpyrazine
2-methyl-6-ethylpyrazine
vinylpyrazine
2-methyl-6-vinylpyrazine

SULPHUR COMPOUNDS

dimethyl disulphide
propylene sulphide
2,3-dithiabutane
allylisothiocyanate
butenylisothiocyanate

ALKYL HALIDES

chloroform

individuals because our experience with inborn errors of metabolism suggested that major profile distortions would occur in such cases.

To establish a volatiles profile for a particular disorder, samples from more than one individual suffering from this disorder must be analysed. However in some cases this was not possible as most of the metabolic disorders studied are so rare that few cases were available. Maple Syrup Urine Disease, for example, has an incidence of only 1 in 200,000 live births and only 1 diagnosed case of this disease was available to us. In such cases we do not claim that our volatiles profile is necessarily characteristic of all sufferers of the disorder, merely that for the sample we analysed the profile was distinct enough to enable its classification as abnormal.

This thesis describes and characterises the components present in the volatiles profiles from patients with phenylketonuria, maple syrup urine disease, isovaleric acidemia, propionic acidemia, Zellweger's Syndrome and trimethylaminuria. Urine samples from patients with these disorders were shown to give volatiles profiles which were surprisingly different to normals.

Phenylketonuria

The infant with phenylketonuria (PKU) is not clinically abnormal at birth and the transformation of an apparently normal baby into a severely defective one during the first year of life is the most striking clinical characteristic of the disease. In the main there are no consistent neonatal abnormalities for the first month or two, after which the usual developmental milestones are delayed with the result that 35 percent of patients cannot walk and 63 percent cannot talk at the age that they could normally be expected to do so (90). Deficient pigmentation with blond hair and blue eyes is characteristic. Convulsive seizures occur in about 26 percent of cases beginning between 6 and 18 months of age and stopping spontaneously before adulthood.

In 1934 Folling (123) identified phenylpyruvic acid in the urine of patients suffering from this condition and 3 years later Penrose and Quastel (124) suggested the name "phenylketonuria" for this defect, and this name has remained in common usage to date. Jervis (125) showed that the liver of such patients is deficient in the ability to convert phenylalanine to tyrosine. A dietary therapy was reported by Bickel *et al.* (126) 23 years after Folling's discovery and nowadays a diet restricting intake of phenylalanine and tyrosine can prevent the inevitable mental retardation of an untreated individual (90). The next major milestone in the history of the disease was the development of a simple screening method for blood phenylalanine concentration by Guthrie (127), which is based on the prevention of growth inhibition of *Bacillus subtilis*. Application of mass screening for phenylketonuria has disclosed a spectrum of conditions in which phenylalanine is elevated with gradations of defective phenylalanine hydroxylase activity as well as other enzyme defects which result secondarily in hyperphenylalanemia.

Hyperphenylalanemia is no longer considered synonymous with phenylketonuria and an accurate diagnosis is required for proper clinical management (128). Much effort has been devoted to the study of the organic acid excretion of some of these patients in the hope of assisting in the clinical diagnosis of PKU and Hyperphenylalanemia relationships and to get a better biochemical understanding of the defects and the various variants.

The failure to convert phenylalanine to tyrosine causes the accumulation of phenylalanine in the body fluids. In addition, there are also increased concentrations of several phenylalanine metabolites in the urine mainly phenylpyruvic- phenyllactic- and 2-hydroxy phenylacetic- acids. The organic acid profile of PKU patients has been well characterised (129, 130, 131, 132) and is reproduced in Figure 9.

The biochemical pathway of phenylalanine metabolism is shown in Figure 10.

Quantitative studies on the organic acids excretion in PKU have shown that the milder variants of PKU excrete the same metabolites as the classical patients but at lower levels (133-136).

Urinary Volatiles

Urine samples from untreated phenylketonurics were obtained from patients (aged 16-25 years) in an institution for the mentally retarded. The disorders were detected during a screen of such institutions for inborn errors of metabolism and the diagnosis was based on elevated levels of phenylalanine in blood.

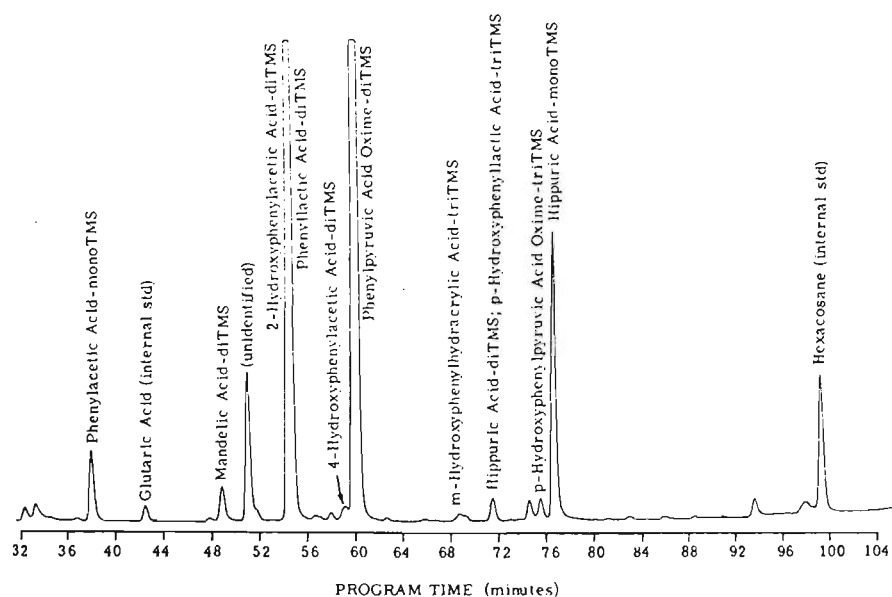


FIGURE 9: The gas chromatographic profile of the oxime-TMS derivatives of the extracted urinary organic acids from an untreated phenylketonuric patient. Reproduced from Thompson *et al.* (129):

Column: 9 feet x 2 mm ID glass, 10% OV17 on
gas-chrom Q (80/100 mesh)
Temperature programme: 60°C, then 2°C/min. to 270°C.

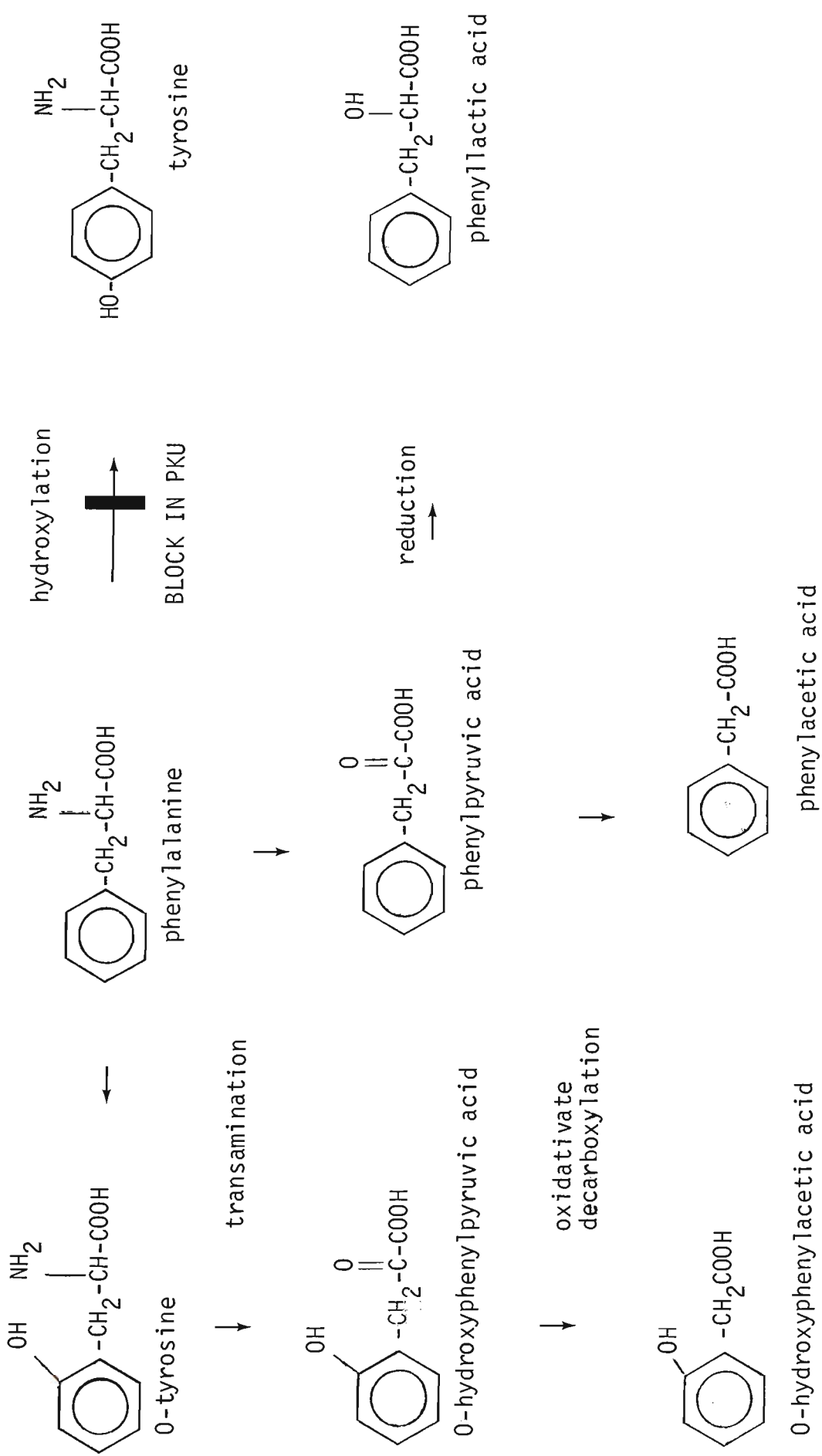


FIGURE 10: A portion of the metabolism of phenylalanine in man. The solid bar denotes the position of the metabolic block in phenylketonuria. An increase in the concentration of all the metabolites shown (except tyrosine) is observed in the body fluids of phenylketonurics.

The chromatogram of the urinary volatiles (Figure 11) of the first patient showed only one major peak which had the same mass spectrum and retention time as an authentic sample of benzaldehyde.

Subsequent headspace extractions were performed at acid pH and Figure 12 is typical of the chromatograms obtained from three other PKU patients under these conditions. There were only four major peaks: 4 heptanone, benzaldehyde, p-cresol and phenol with a minor peak for benzoic acid.

Benzoic acid has been reported in the urine of patients with high serum and urine levels of phenylalanine (137, 138) and it was suggested that its main source is from bacterial degradation of absorbed phenylalanine in the intestinal lumen. Hansen *et al.* (139) have shown that urinary benzoic acid can also arise from the degradation of hippuric acid by urinary bacteria.

Benzaldehyde has not been reported as a urinary metabolite in PKU but is a normal constituent of the urinary volatiles (58, 45). Quantitation was not attempted, however the amounts detected in these cases (in the order of 10^{-7} moles/ml) were at least an order of magnitude greater than the amounts detected for normal individuals. Although several workers have observed benzaldehyde in urinary volatiles profiles (83, 87, 140) its origin has not been discussed. Its occurrence at abnormally high levels in PKU patients strongly implicates phenylalanine as its precursor. Benzoyl CoA is an intermediate in the enzymatic formation of hippuric acid from benzoic acid and glycine. It is possible that free benzaldehyde arises from benzoyl CoA and that the increased level of benzoic acid in the body fluids of phenylketonurics results in the increased level of urinary benzaldehyde which we observed.

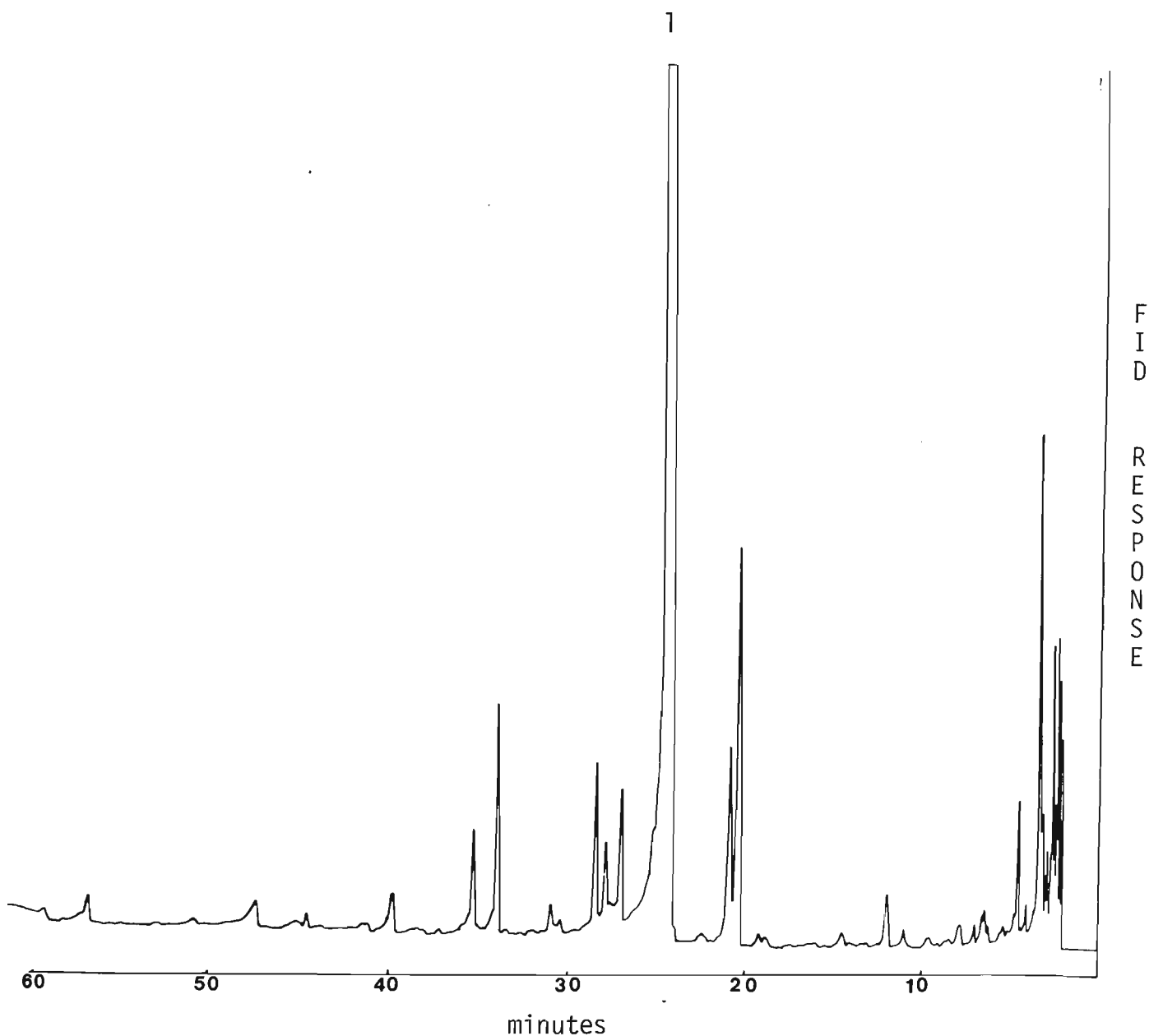


FIGURE 11: Urinary Volatiles Profile of an Untreated Phenylketonuric Patient.

The volatiles were extracted from the urine (40 ml), pH=5) of a phenylketonuric (R2015) by a 27 litre headspace (HS 712).

Column: SCOT CW20M 50 metres

Temperature programme: 70°C 4 min. then programmed at 3°C/min to 226°C, isothermal at 226°C for 8 minutes

Peak No. 1 = benzaldehyde

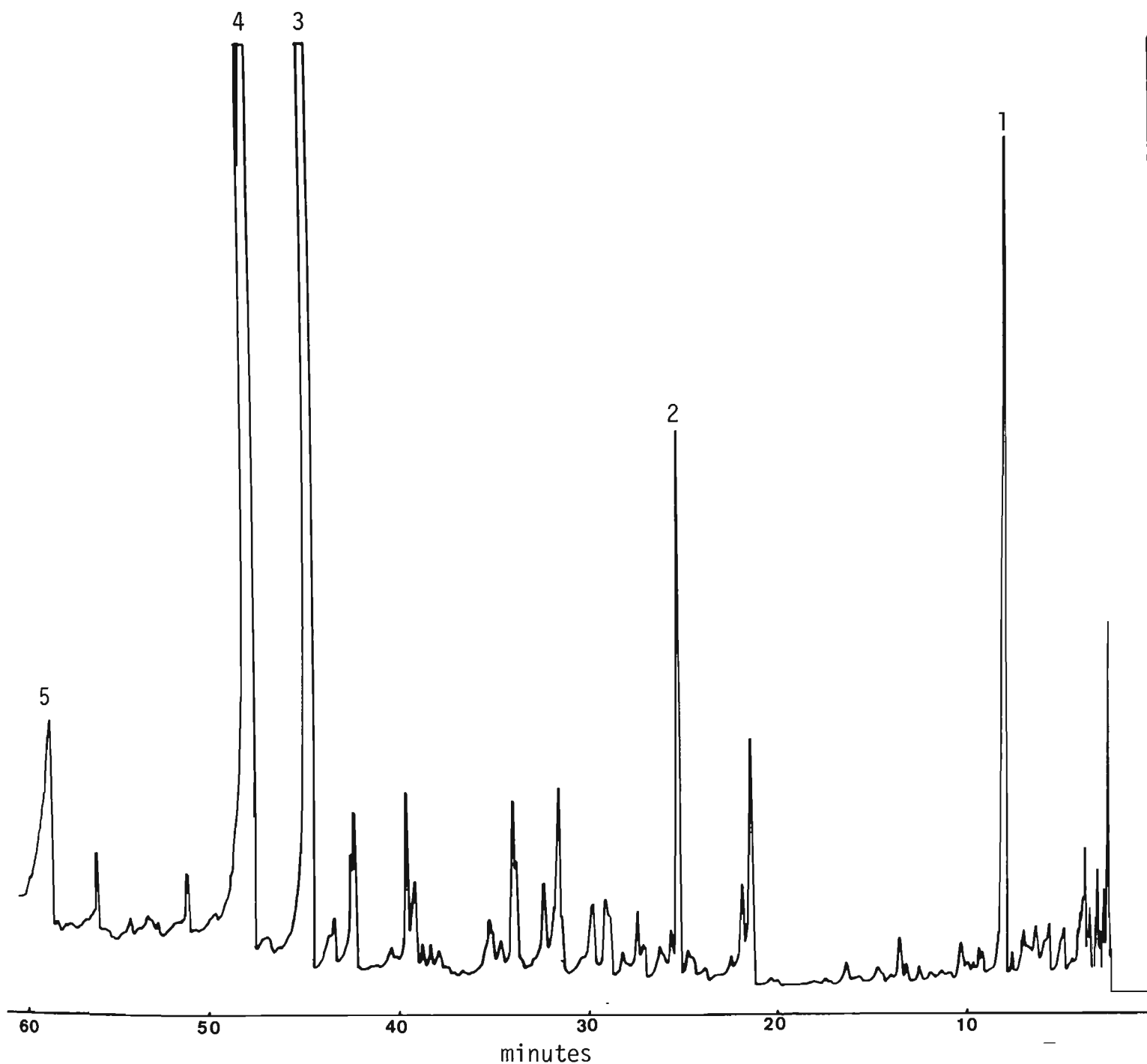


FIGURE 12: Acidic Urinary Volatiles Profile of an Untreated Phenylketonuric Patient.

The volatiles were extracted from urine (36 ml, pH=1) of a phenylketonuric (R2019) by a 31.8 litre headspace (HS 729).

Column: SCOT CW20M 50m

Temperature programme: 70°C 4 min, then programmed at 3°C/min to 226°C, isothermal at 226°C for 8 minutes.

Peak identities: 1 = 4-heptanone, 2 = benzaldehyde,
3 = p-cresol, 4 = phenol, 5 = benzoic acid.

Urinary phenol and p-cresol have been reported as bacterial degradation products of unabsorbed tyrosine (141-143) and are normally excreted at a combined rate of about 50-100 mg/day (2, 144) so their occurrence in the urine of PKU patients is to be expected since the amount of unabsorbed tyrosine in the intestine of these patients would be comparable to that in normals. It is a common practice in hospital laboratories to add phenol or cresol to urine samples as a preservative, however for these samples neither phenol nor p-cresol was added.

Liebich and Huesgen (57) have shown that 4-heptanone arises from a precursor, possibly by decarboxylation of a 3-ketoacid, during headspace collection. It is a normal constituent of urinary volatiles being excreted at a rate of between 10 and 30 μg per 24 hours, but the amount detected increases when the urine is heated during volatiles extraction. The amounts I detected in PKU patients were not abnormal.

The commonly observed urinary metabolites of PKU patients (phenylpyruvic and phenyllactic acids) were not detected by our headspace analysis. This may be due to their polarity and consequent high boiling points. These properties may have resulted in (a) a low yield in the headspace extraction or (b) retention by the polar liquid phase of the GC column or a combination of both these phenomena. The last peak observed in the PKU volatiles profile from acidic urine was benzoic acid and both phenyllactic and phenylpyruvic acids have higher boiling points and greater water solubilities than this compound.

The distinctive feature of the volatiles profiles of phenylketonuric patients was the predominance of the large peak for benzaldehyde when neutral urine (pH-5) was used for headspace extraction. This contrasts sharply with the large number of smaller peaks in the volatiles profiles of normal urines.

Maple Syrup Urine Disease

Maple Syrup Urine Disease (MSUD), so named because of the maple syrup like odour of the urine of affected patients, was first described by Menkes *et al.* (92) in 1954. To date some 50 cases have been reported (93) and it is probable that many cases have either not been reported or have not been correctly diagnosed (122).

MSUD leads to progressive acidosis, seizures, coma and usually death in the first few days or weeks of life unless it is recognised early enough so it can be treated properly (93). The few untreated children who have survived the neonatal period have exhibited severe irreversible brain damage, have episodes of hypoglycemia (low levels of blood glucose) and usually die within the first two years of life.

The metabolic defect is due to the inability of the patient to further catabolise the keto acids derived from the branched chain amino acids: leucine, isoleucine and valine and results in the accumulation of these acids in the body fluids. Figure 13 shows the catabolic pathways for the branched chain amino acids and the position of the block in MSUD. The metabolite in the urine which is responsible for the maple syrup-like odour is usually ascribed to the keto acid derived from isoleucine (145).

Because of the complexity of the oxidative decarboxylase enzyme which metabolises the branched chain amino acids a number of variants of this disorder are known (147, 156) and Dancis (146) has classified these variants into 3 major groups on the basis of the patients ability to tolerate protein.

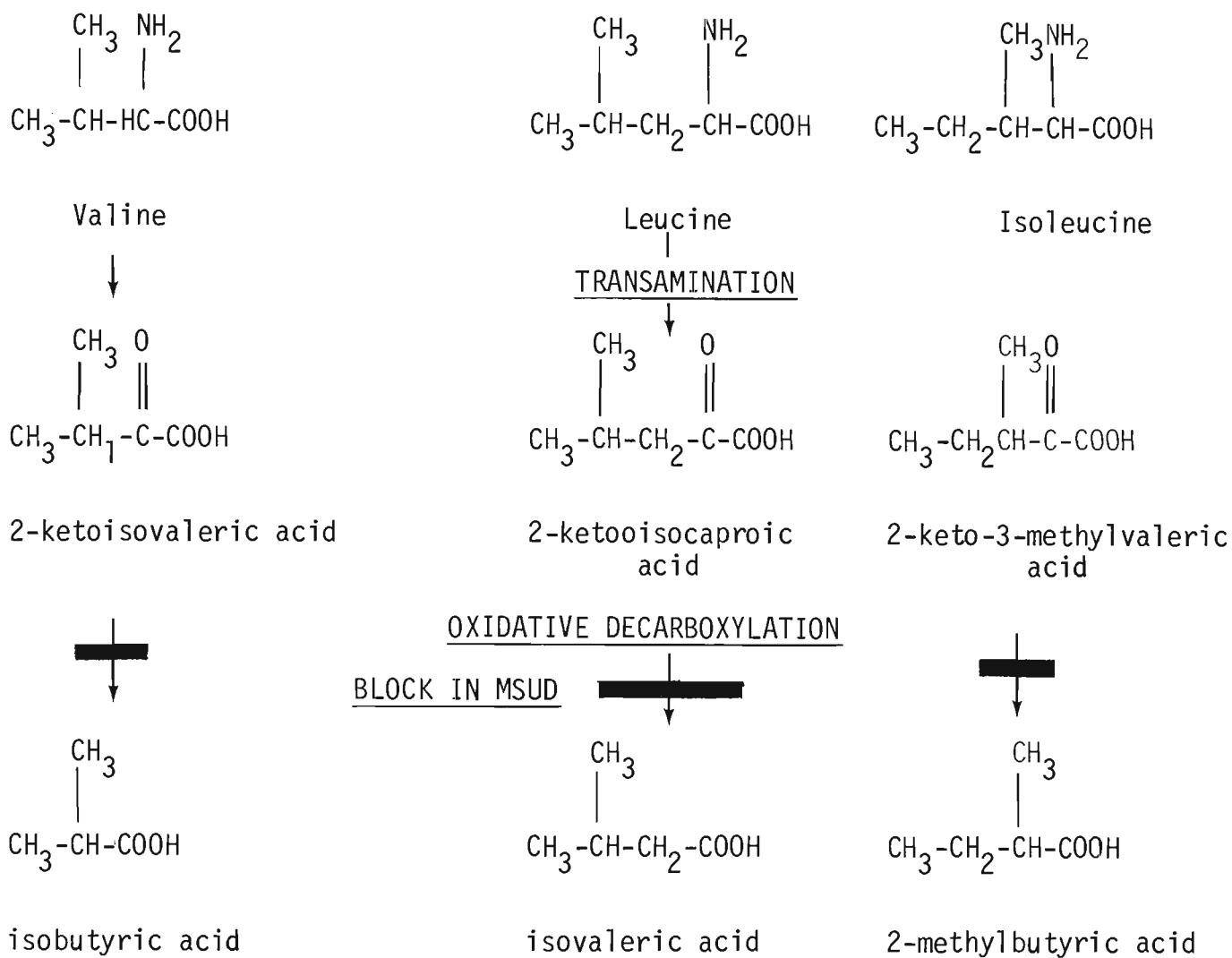


FIGURE 13: The first 2 enzymatic reactions in the catabolism of the branched chain amino acids. The solid bar denotes the position of the block in MSUD.

Case History

S.W. was the first child of unrelated Australian parents and weighed 3.7 kg at birth. She remained well for the first week, but on day 8 developed writhing movements and refused feeds. She was acidotic and rapidly became comatose, but no unusual odour was reported.

Urinary and plasma levels of leucine (1553 μM) and valine (298 μM) were greatly elevated. No data is available for isoleucine levels. Urinary organic acids GC profile showed elevated levels of 2-hydroxyisovaleric acid, 3-hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-keto-3-methylvaleric acid. These data led to the diagnosis of Maple Syrup Urine Disease by the medical staff of the Oliver Latham Laboratory, Sydney, Australia.

Subsequently, cultured fibroblasts from this patient were shown to produce $^{14}\text{CO}_2$ from ^{14}C -leucine at only 0.9% of the normal rate.

Treatment was by administration of a diet devoid of branched chain amino acids. The child responded well and after one week branched chain amino acids (630 mg/day) were re-introduced to her diet.

She was well until aged 15 months when she presented with general failure to thrive and ataxia. Plasma levels of leucine (857 μM) and isoleucine (190 μM) were high but valine was normal. Again the GC profile of organic acids showed elevated levels of 2-hydroxyisovaleric acid (0.86 mmole/mmol creatinine), 3-hydroxyisovaleric acid (0.32 mmole/mmol creatinine) and 2-hydroxyisocaproic acid (0.22 mmole/mmol creatinine). Since this time she has presented irregularly with similar symptoms, however, her

overall development is acceptable with an IQ in the low values of the normal range.

The first urine sample used for headspace analysis (SW1) was obtained from this patient at 19 months of age when she again presented with elevated plasma levels of leucine (1090 μM), isoleucine (381 μM) and valine (484 μM) and increased urinary levels of 2-hydroxyisovaleric (9.1 mmole/mmol creatinine), 3-hydroxyisovaleric (0.8 mmole/mmol creatinine), 2-hydroxyisocaproic (1.2 mmole/mmol creatinine) and 2-keto-3-methylvaleric (1.9 mmole/mmol creatinine) acids.

Urinary Volatiles

Figure 14 shows the chromatogram of the urinary volatile compounds obtained by us by headspace chromatography from a sodium chloride saturated urine sample (SW1). The difference between this chromatogram and that from a normal control of the same age is striking. The normal "volatiles finger print" is characterised by a large number of smaller peaks whilst this trace was dominated by a few large peaks which eluted at the higher temperatures of the chromatogram.

We were unable to ascertain the identity of the largest peak (peak 5, fig. 14) from the e.i. spectrum obtained by capillary GC-MS (Figure 15) although it was believed to be due to a mixture. The peak was collected into a liquid nitrogen cooled capillary tube via a column eluant splitter and a chemical ionisation (c.i.) mass spectrum was obtained by direct insertion probe (Figure 16). From the e.i. and c.i. data a tentative identification of isovaleric acid was made. The preparative chromatography was repeated and the collected material was esterified with BSTFA (Figure 17).

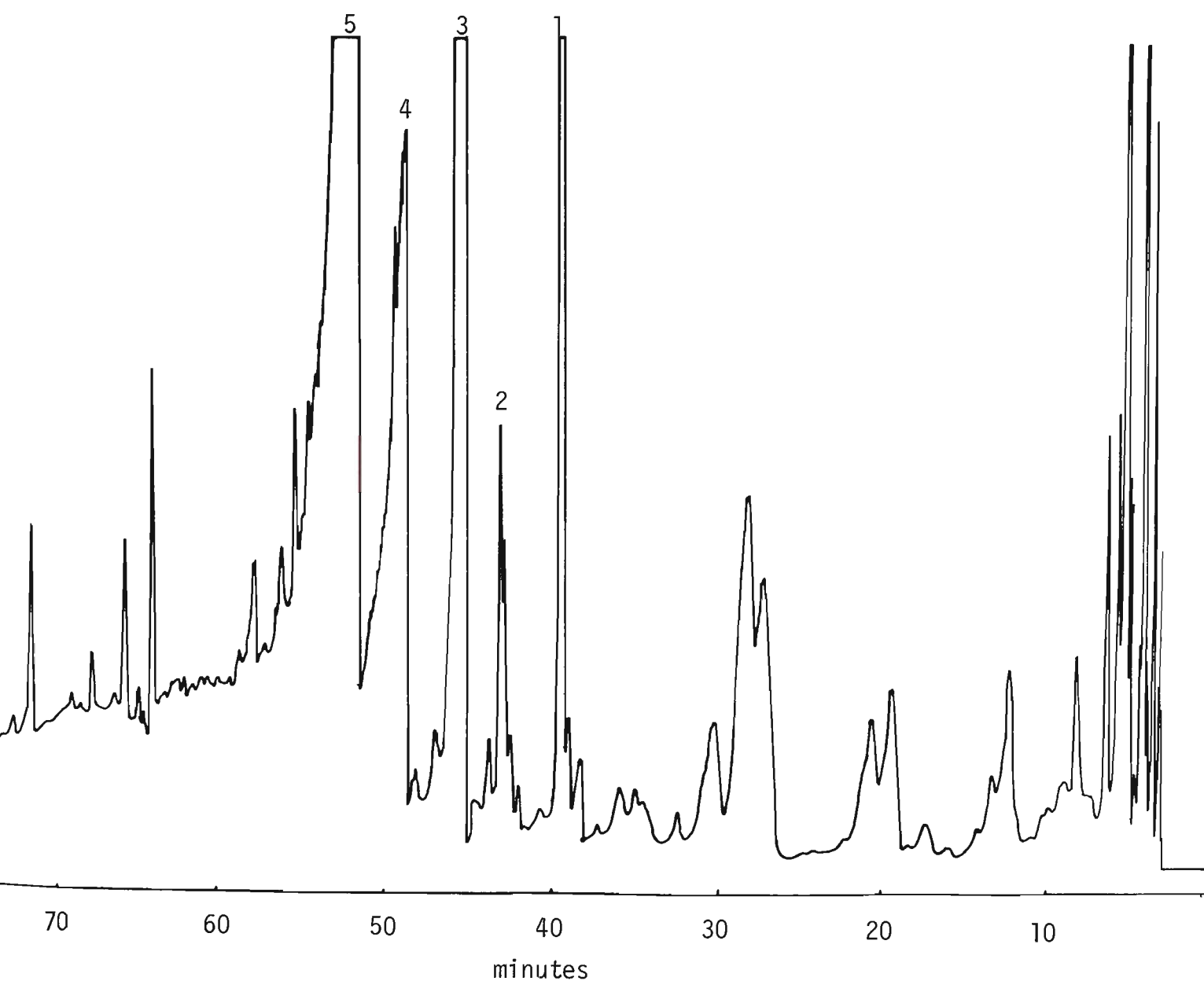


FIGURE 14: Urinary Volatiles Profile of a Patient with Maple Syrup Urine Disease.

The volatiles were extracted from a 50 ml urine sample (SW1) from patient S.W. by a 33 litre headspace (HS 634).

Column: SCOT CW20M 50 m

Temperature programme: 70°C 8 mins, then programmed at 2.5°C/min to 185°C, isothermal at 185°C for 26 minutes

Peak identifications: 1 = furfural, 2 = benzaldehyde, 3 = unknown, 4 = acetophenone, 5 = mixture of isovaleric and 2-methylbutyric acids.

The spectrum for peak 3 is given in Appendix D.

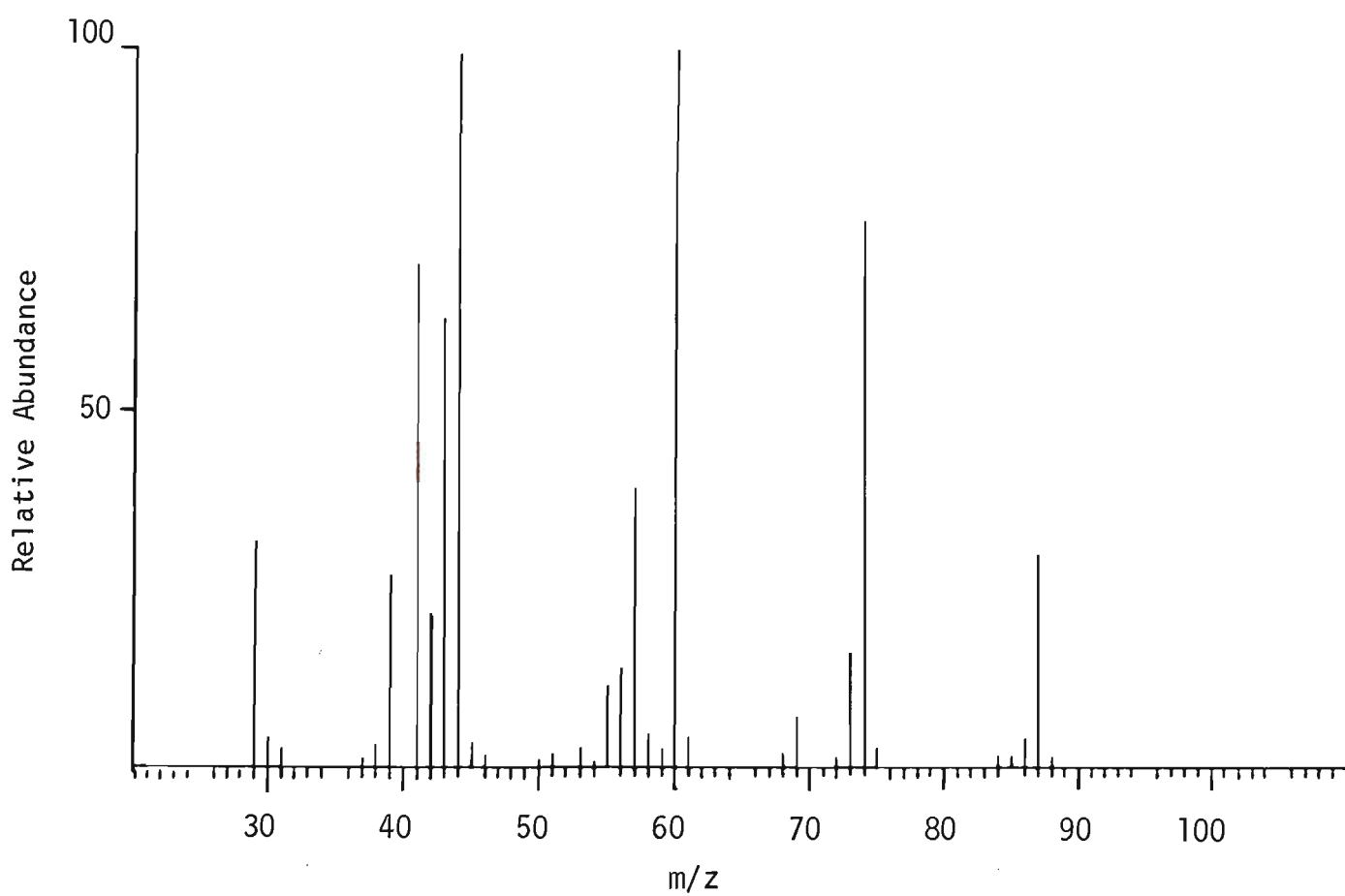


FIGURE 15: Mass Spectrum (e.i.) of peak 5 from Figure 14.

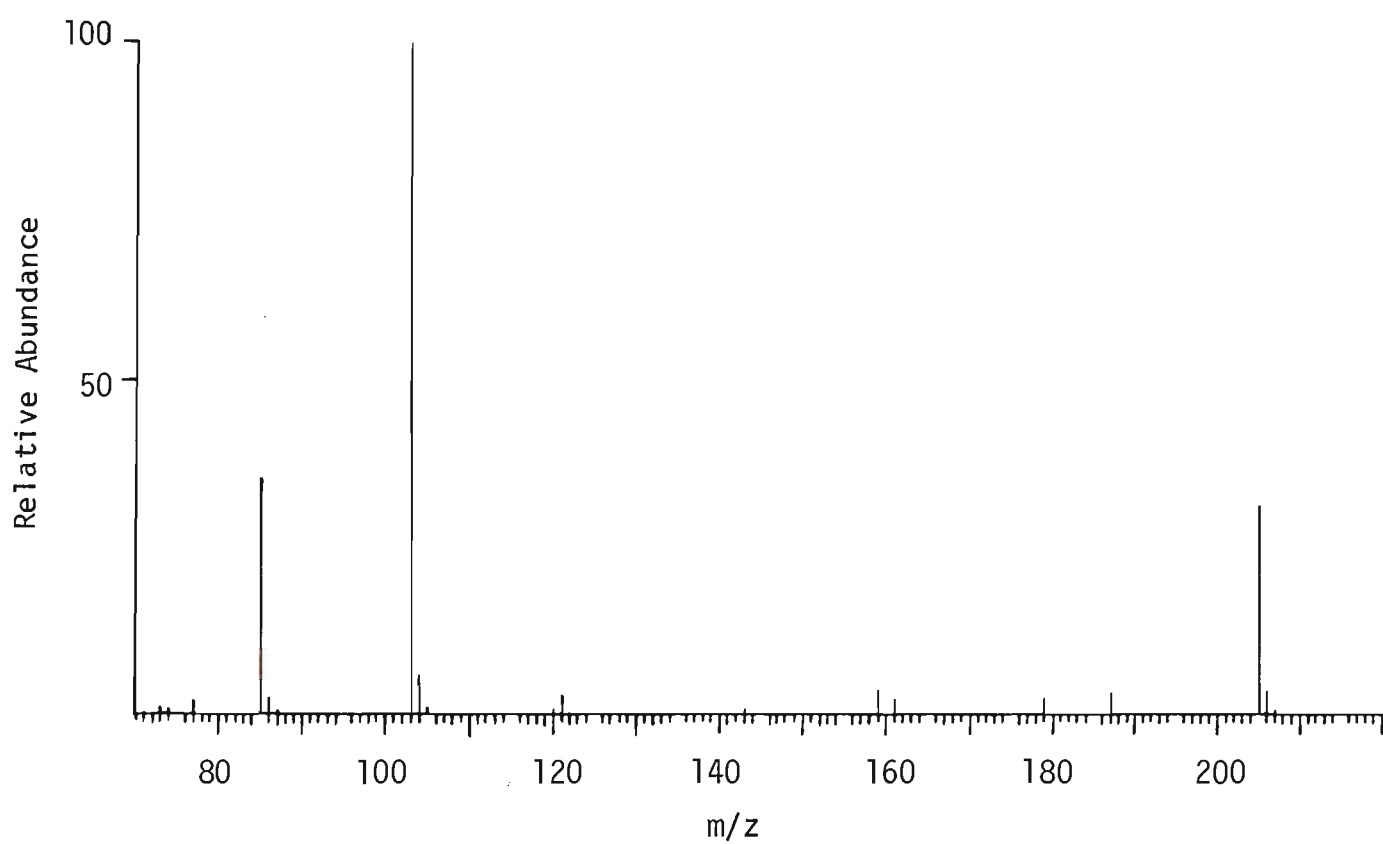


FIGURE 16: Mass Spectrum (c.i., isobutane) of peak 5 from Figure 14.

The compound has a molecular weight of 102 ($[M+H]^+ = 103$). The ion at 85 is due to loss of H_2O from $[M+H]^+$ and the ion at 205 is due to the dimerisation of the compound in the ion source ($2 \times 102 = 204$, $[M+H]^+ = 205$). Both these phenomena are characteristic of carboxylic acids.

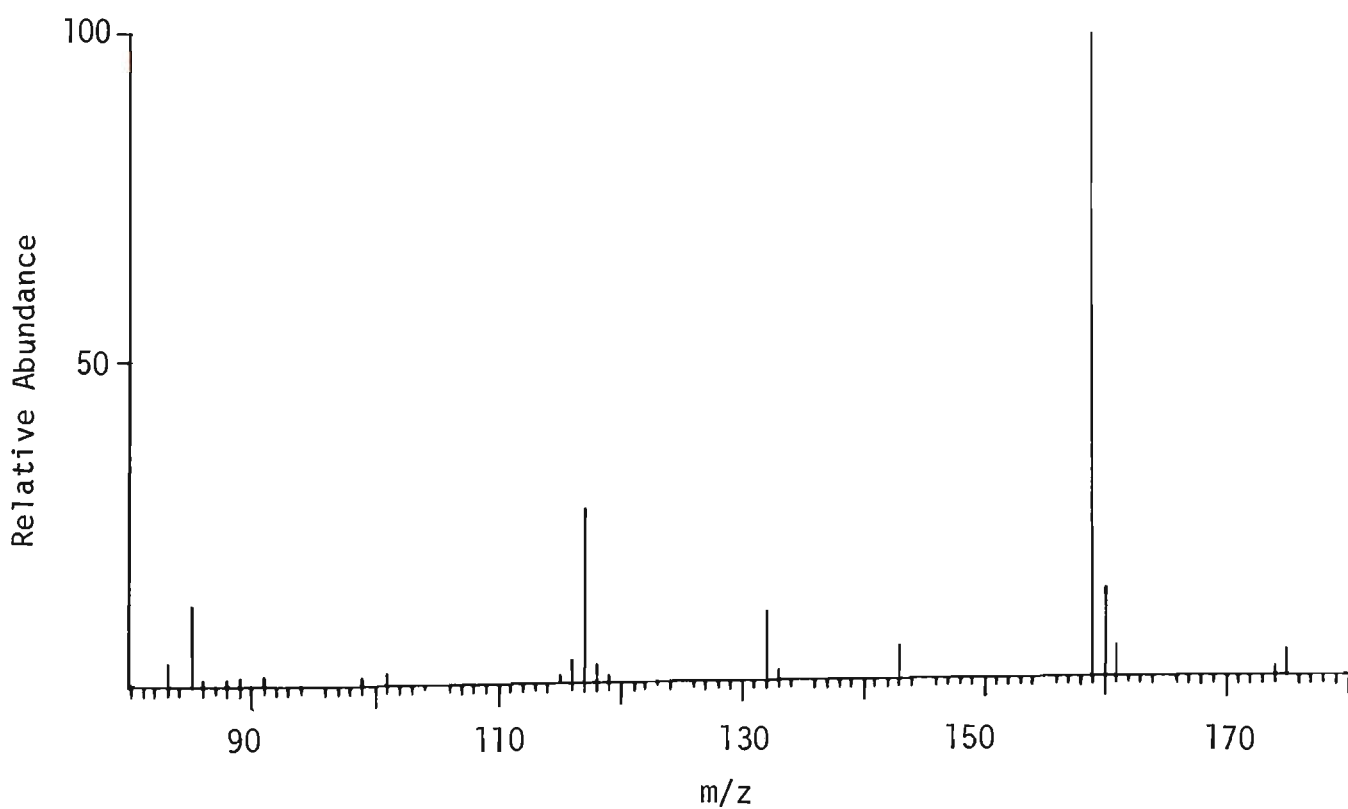
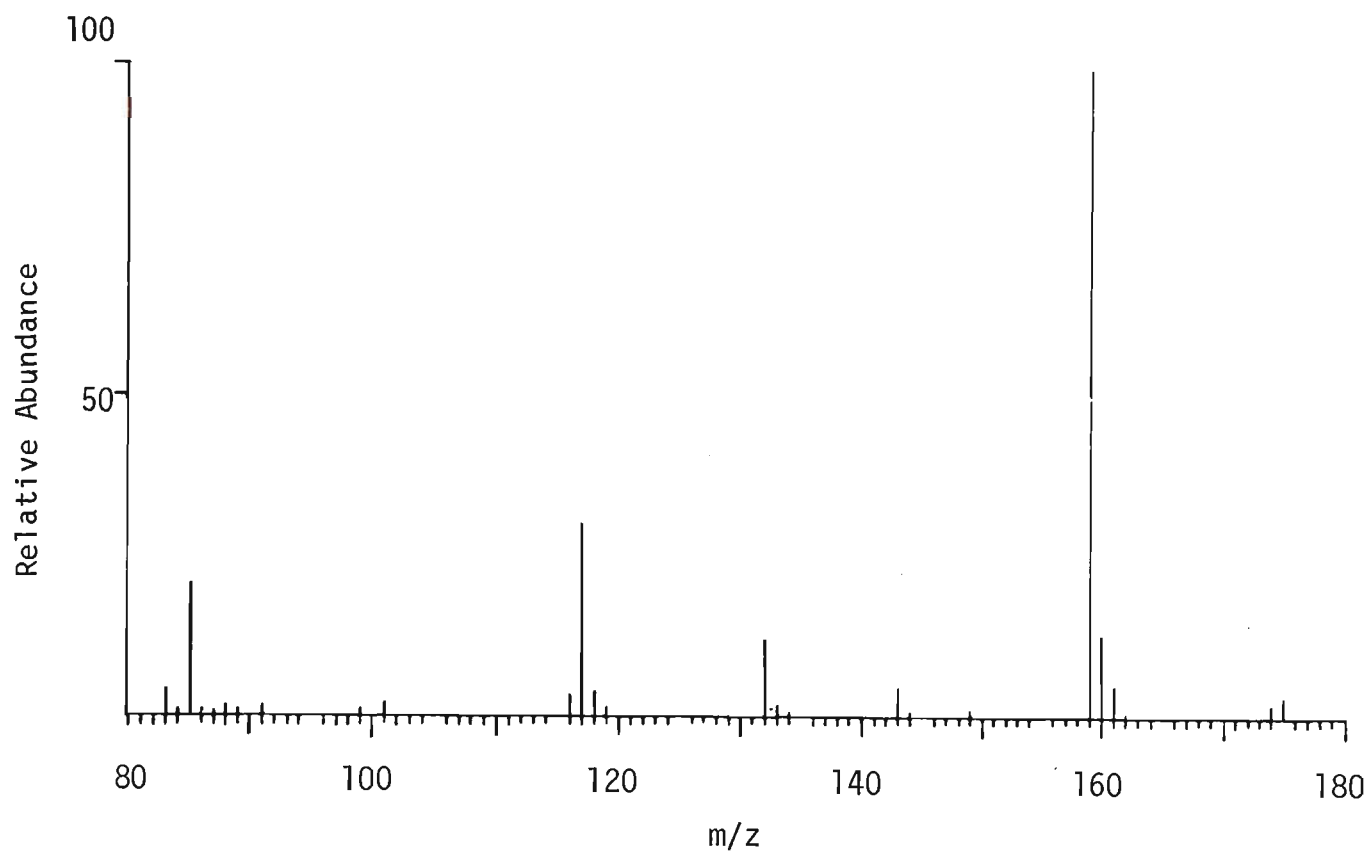


FIGURE 17: Mass spectra of (bottom) the second peak in the chromatogram obtained by isolating peak 5, Figure 14 using preparative capillary GC and derivatising the condensate with BSTFA and (top) the TMS derivative of an authentic sample of isovaleric acid.

The chromatograms for both derivatives contained two peaks which had the same molecular weight; one peak had the same retention time as an authentic sample of isovaleric acid. The mass spectrum of this compound had a McLafferty ion at m/z 132 for the TMS derivative and at m/z 74 for the methyl ester which indicate that there is no branching at the alpha carbon. The other GC peak from the collected and derivatised material had corresponding ions at m/z 146 and m/z 88 which indicate a methyl group on the alpha carbon. Also the capillary GC-MS e.i. spectrum had ions at both m/z 60 and m/z 74 which correspond to the McLafferty ions for a carboxylic acid unbranched and branched at the alpha carbon respectively. Based on this data the identity of the other compound in peak 5, Figure 14 was 2-methylbutyric acid. Peak 5, Figure 14 thus consisted of a mixture of isovaleric and 2-methylbutyric acids. The identities of the other peaks in Figure 3 were assigned by matching with library spectra.

Since the urine sample was several months old at the time of analysis it is possible that the large amounts of isovaleric and 2 methyl butyric acids present in the sample could have been formed by degradation of the amino and keto acids present in the sample. Figure 18 shows the chromatogram of the volatiles from a fresh sample (SW2) of urine from patient S.W. and is even more striking than the first. Apart from the presence of isovaleric and 2-methyl butyric acids other large GC peaks were also detected. The identity of peak 4 was confirmed as 2 keto-3-methylvaleric acid by preparative capillary GC as used for the previous sample. Peak 2 (Fig. 18) gave spectrum characteristic of a hydroxy acid but it could not be conclusively identified (Appendix D).

Another sample (SW3) was obtained from this patient after she had been treated for a urinary tract infection. It was possible that bacterial degradation of the amino acids or keto acids in the bladder may have been the

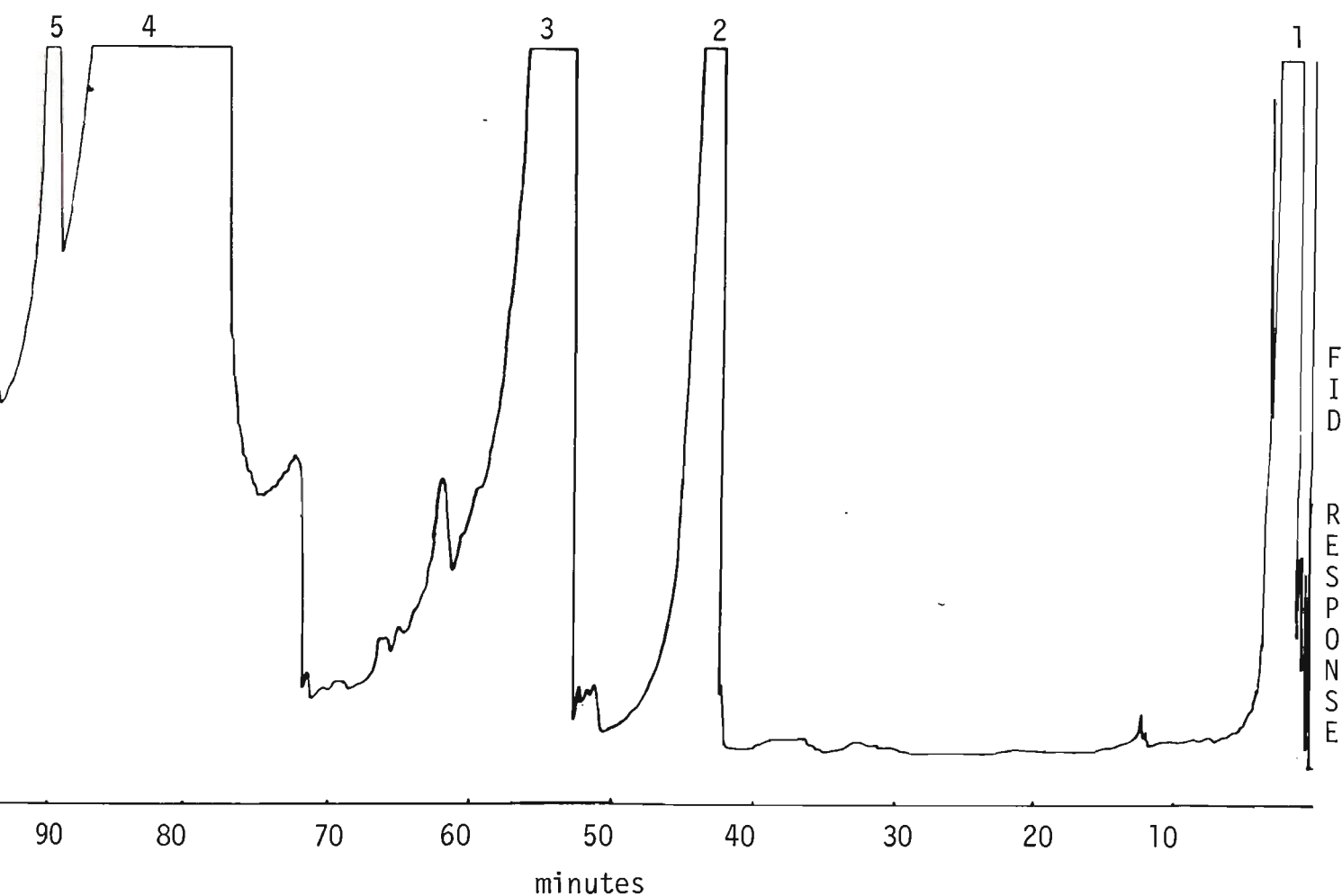


FIGURE 18: Urinary Volatiles Profile of Patient S.W. (sample SW2)

The acidic volatiles were extracted from a 25 ml urine sample (SW2) from patient SW by a 24 litre headspace (HS 666).

Column: SCOT CW20M 50m

Temperature programme: 70°C for 8 mins, then 1.5°C/min to 179.5°C, isothermal at 179.5°C for 24 minutes

Peak identifications: 1 = breakthrough of isovaleric and 2-methyl-butyric acids, 2 = unknown, 3 = mixture of isovaleric and 2-methyl-butyric acids, 4 = 2-keto-3-methylvaleric acid, 5 = p-cresol.

The spectrum for the TMS derivative of peak 2 is given in Appendix D.

origin of the short chain acids in the urine. The volatiles chromatogram obtained for this specimen (Figure 19) was similar to that obtained for the previous sample (Figure 18).

To check that our observation of the short chain acids was not due to their production during headspace collection from 2-ketoacids control experiments were performed in which an acidic solution of 2-ketoisocaproic acid was headspaced under the same experimental conditions as the urines from patients SW. No isovaleric acid peak was found. This also showed that the keto acid did not decarboxylate during chromatography. Conversely a separate headspace collection was performed on a sample of patient SW's urine which had been reacted with NH_2OH prior to analysis to protect the keto function. Isovaleric acid was still found.

Previous analyses of the urinary organic acids for this patient had not shown the presence of any short chain fatty acids. Those analyses consisted of solvent extraction of the acidified urine followed by concentration of the pooled organic extract and derivatisation with BSTFA. This analysis was repeated, but instead of completely evaporating the solvent from the pooled extract its volume was reduced to about 100 μl . Isovaleric acid was detected only when the solvent was not completely evaporated. The GC temperature programme was also altered by starting with the column at ambient temperature (40°C). Thus it is likely that previously the short chain fatty acids were either evaporated during sample concentration or co-chromatographed with the solvent peak.

Urine samples SW2 and SW3 both had a strong, unpleasant odour similar to yeast extract and by organoleptic monitoring of the column eluant via a splitter the compound responsible for this odour was found to be isovaleric acid. Although this was the only smelly compound detected

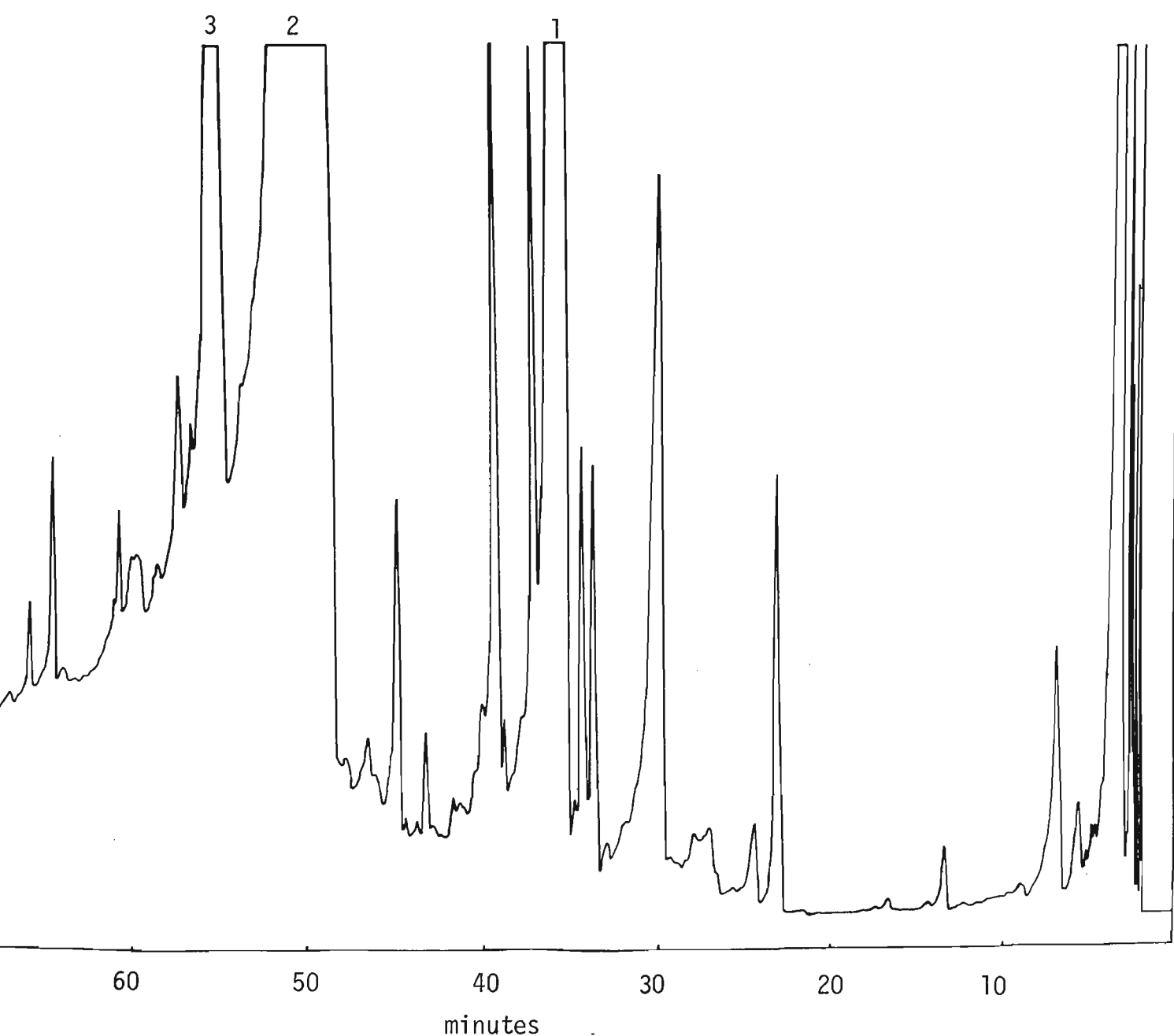


FIGURE 19: Urinary Volatiles Profile of Patient S.W. (sample SW3)

The acidic volatiles were extracted from a 50 ml urine sample (SW3) from patient SW by a 28.8 litre headspace (HS 700).

Column: SCOT CW20M 50 m

Temperature programme: 70°C for 6 mins, then 3°C/min to 200°C.

Peak identifications: 1 = mixture of isovaleric and 2-methyl-butyric acids, 2 = 2-keto-3-methylvaleric acid, 3 = p-cresol.

it was not certain whether it is the compound responsible for the classically described odour of "maple syrup" or "burning sugar".

There may be no simple explanation for the observation of isovaleric and 2-methylbutyric acids in the urine of this MSUD patient. When Tenaka *et al.* discovered isovaleric acid in the biological fluids of two patients it was not accompanied by the other metabolites associated with MSUD or ketoacidosis and this led to the diagnosis of a new inborn error of metabolism which was named isovaleric acidemia (148, 149). Recently, however, elevated levels of both isovaleric and 2-methylbutyric acids as well as the three short chain fatty acids propionic, isobutyric and butyric have been reported in the urine of a patient with periodic attacks of dyspnea followed by ketoacidosis (150). This patient also excreted large amounts of 3-hydroxyisobutyric, acetoacetic, adipic and suberic acids, and mono-unsaturated suberic and sebacic acids. More pertinent is the report of a new variety of MSUD by Harkness *et al.* (151) in which the plasma levels of isobutyric and isovaleric acids were elevated. This patient also had high levels of the amino acids leucine, valine, isoleucine as well as the keto acids (2-ketoisovaleric, 2-ketoisocaproic, 2-keto-3-methylvaleric). Leucocytes from peripheral blood from this patient showed normal rates of leucine decarboxylation.

These observations may point to an area of branched chain amino acid metabolism which has not yet been fully elucidated. There is still vigorous discussion concerning the metabolic block responsible for this disorder and many workers concede that our current knowledge of branched chain amino acid metabolism is not complete and thus the results may be subject to more than one interpretation (152). However a number of points have been clarified. It was originally thought that the three keto acids shared one decarboxylase but subsequent work has since shown that more than one decarboxylase is

present (153, 154, 155). For example Goedde and Keller (157) have shown that there are three different enzyme complexes for the oxidative decarboxylation of the three branched chain 2-ketoacids. Exact knowledge of the reaction mechanism, the coenzyme utilization or the affinity to enzymes or parts of enzyme complexes is not available but the requirement for many cofactors and discrete sub-activities (decarboxylase, lipoamide oxidoreductase etc.) has been documented. Thus the heterogeneity of the reported MSUD cases is not surprising. Not only is the biochemistry associated with these pathways exceedingly intricate but the analytical methods required to detect, identify and quantify these metabolites are still under development.

Despite the variety of case reports in the literature only our case and the one reported by Harkness *et al.* (151) have confirmed the presence of short chain fatty acids in the urine. A probable mechanism for their formation could be through bacterial action in the gut on the elevated levels of the branched chain amino acids and keto acids. The production of volatile fatty acids by colonic bacterial species is well known (144) and the degradation of tyrosine to p-cresol by intestinal bacteria is also well documented (141, 144, 143). Since p-cresol was found in these urine samples it is highly probable that intestinal flora could also be responsible for the degradation of unabsorbed branched chain amino acids to the volatile fatty acids observed. It is interesting to note that the MSU "odour" appears in affected people after about the first week of life (158) - which is about the time when infection of the gut occurs. The exact mechanism by which the central nervous system damage is produced is not understood (126) but isovaleric acid, like other short chain fatty acids, is neurotoxic (159) and it is possible that these acids, produced in response to the elevated levels of amino acids, cause the neurologic damage as well as the characteristic odour described in this disorder. The

difficulty in detecting the volatile acids has been responsible for a mis-diagnosis before (160) and could account for their not having been observed in other earlier cases.

If the isovaleric acid does arise from endogenous metabolism then its appearance in this case as well as the expected MSUD metabolites could be explained by a common factor inhibiting both the oxidative decarboxylase complexes and the acyl dehydrogenases resulting in the reduction of flux in these pathways. However, speculation cannot result in a satisfactory resolution of these questions, and confirmation will depend on the aquisition of pertinent enzymological data.

Despite the complicated questions arising from the identification of the major peaks in the volatiles profile from this patient the difference between that profile and the volatiles profile from a healthy individual is immediately apparent. Unfortunately due to the rarity of this disorder we were only able to study a single case, however if the origin of the short chain fatty acids is from bacterial action on the elevated levels of branched chain amino and keto acids in the body fluids then inevitably this process will occur in all cases who survive the first week of life and workers may now be able to check this possibility.

Determination of Isovaleric Acid as its Butyl Ester Derivative

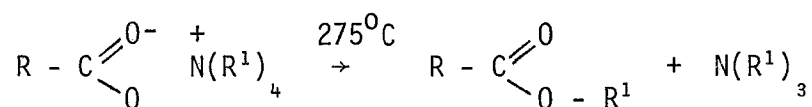
Although we have proved the presence of isovaleric and 2-methylbutyric acids in the urine of this MSUD patient by headspace chromatography their presence is difficult to explain since in the catabolic pathways of leucine and isoleucine they occur distal to the block. The question of whether these short chain acids were artifacts of the headspace technique was resolved by developing a method to determine isovaleric acid without using headspace chromatography.

These acids present some analytical problems if GC is used not only because of their volatility but also due to their polar nature and the possibility of their interaction with the GC column. Esterification with either diazomethane or BSTFA decreases their polarity and thus improves GC characteristics but also increases the volatility of the components. This causes some acids to co-chromatograph with the solvent and results in excessive losses during work up. In addition the mass spectra of the acid derivatives do not contain many characteristic ions and this makes unequivocal identification of "unknowns" difficult. When a solvent extraction technique is used to isolate the acids from urine large losses can occur during the removal of the solvent. This step also concentrates residual water which may be present in the extract and this can interfere in subsequent derivatisation procedures. To minimize these problems, one should ensure complete removal of H_2O prior to concentration and that samples are not evaporated to dryness.

A potentially useful method was developed which overcomes most of these problems. The acids were extracted with an organic solvent

from acidic urine and the pooled extract was titrated to pH = 12 with a quaternary alkylammonium hydroxide. The extracted acids were thus converted to involatile quaternary ammonium salts so evaporation of the extract to dryness, without loss of volatile acids was possible. It was found that drying of the extract by azeotroping residual water with dichloromethane was preferable to using anhydrous MgSO_4 .

A solution of the quaternary alkylammonium salt of the acid could then be injected directly into the GC where the alkylammonium salt is converted to the alkyl ester and the tertiary alkylamine by-product in the injection port (161).



A portion of the chromatogram obtained using this method with urine sample SW3 is shown in Figure 20. Peaks 1 and 2 were identified as butyl-2-methylbutyrate and butyl isovalerate respectively and their quantities were estimated to be in the order of 10^{-6} moles/ml which is approximately an order of magnitude lower than the amounts of keto-acids detected in urine samples from this patient. The mass spectra of these two peaks are shown in Figures 21 and 22.

Although the alkyl ester can be produced in the GC injection port it was found that this procedure rapidly degraded the GC column and the tertiary alkylamine by-product interfered in the GC analysis. We also observed that hexamethylcyclotrisiloxane and octamethylcyclotrisiloxane were liberated when the reaction mixture containing TMAH salts was injected onto the GC column suggesting that a reaction between any excess quaternary alkylammonium hydroxide and the glass column or silylated solid support material occurred. This reaction also seemed to occur during storage of

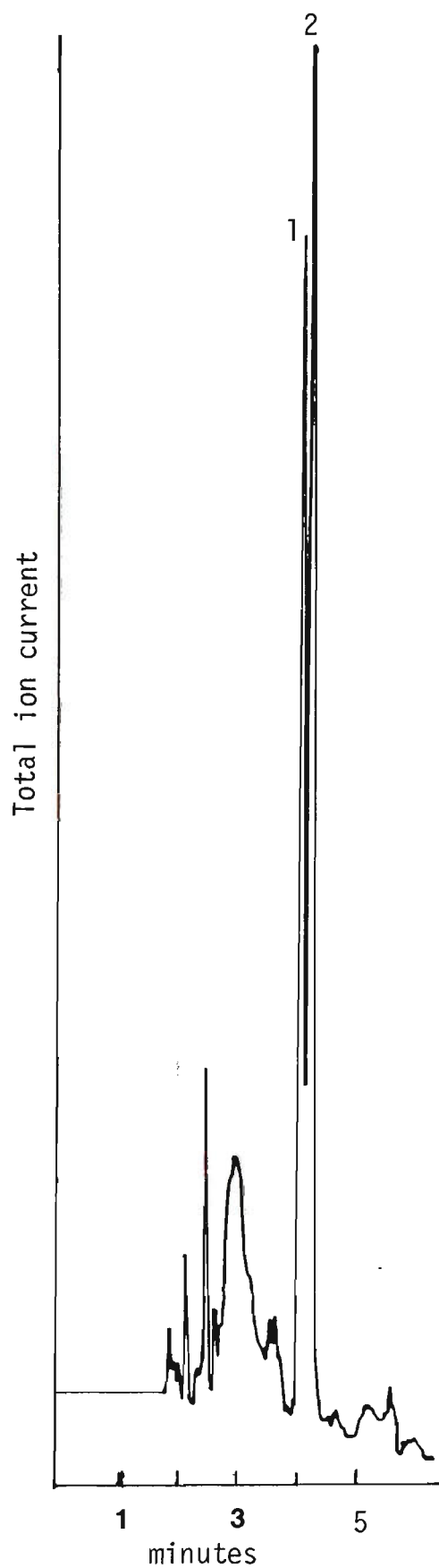


FIGURE 20: A portion of the chromatogram of butylated urinary organic acids from patient S.W. (sample SW3).

1 = butyl 2-methylbutyrate, 2 = butyl isovalerate.

Column: 20 m SE54, Jaeggi, Switzerland
Temperature programme: 60°C, 4°C/min to 250°C
Instrument: Varian, Mat 44.
Carrier gas: Helium, 2 ml/min.

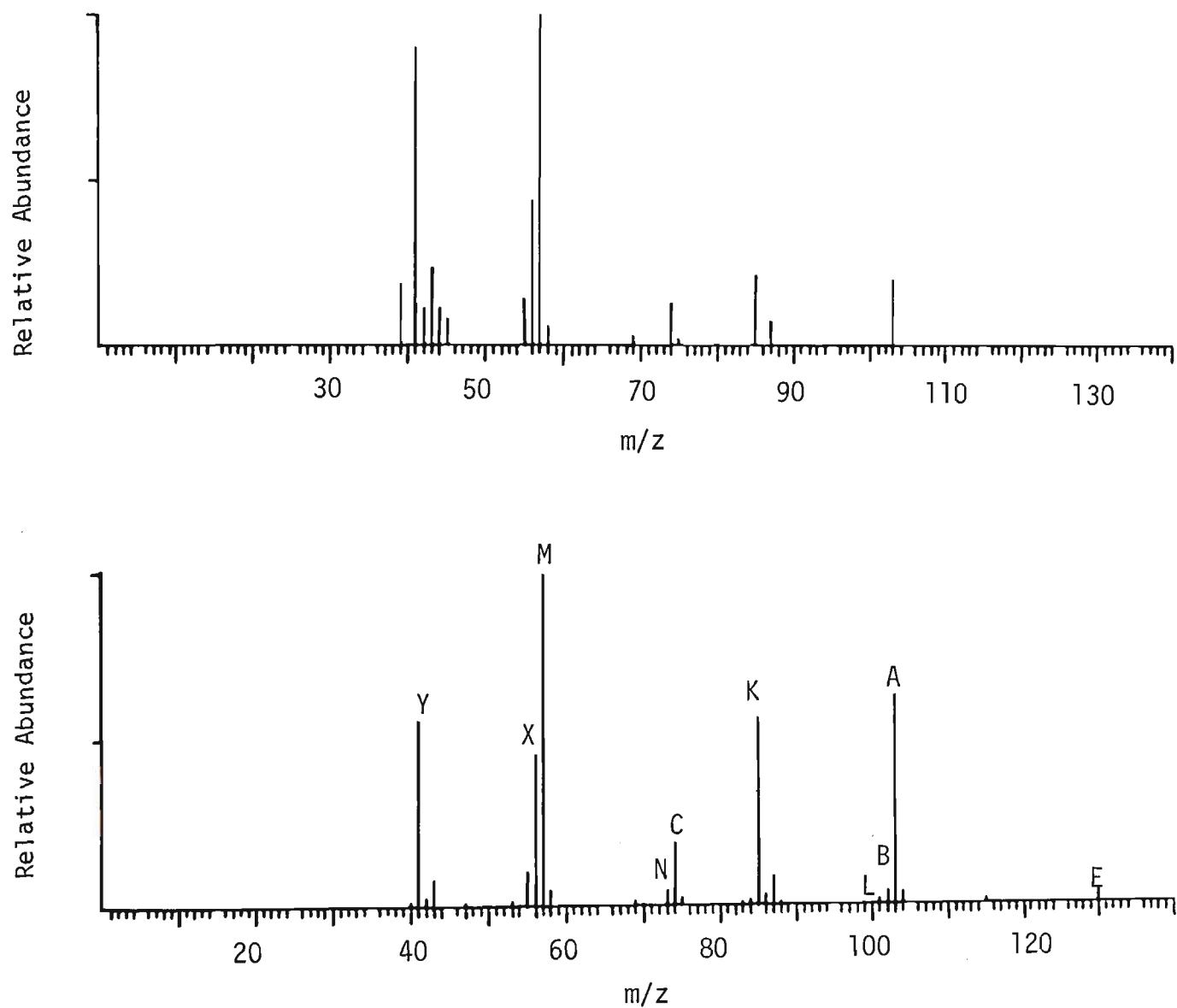


FIGURE 21: Mass spectra of (top) authentic butyl 2-methylbutyrate and (bottom) peak 2, Figure 20. The letters are used to designate ion structures in the following discussion. These spectra were obtained on different instruments - the instrument used for the authentic compound was less sensitive at higher masses hence the ion E was not observed.

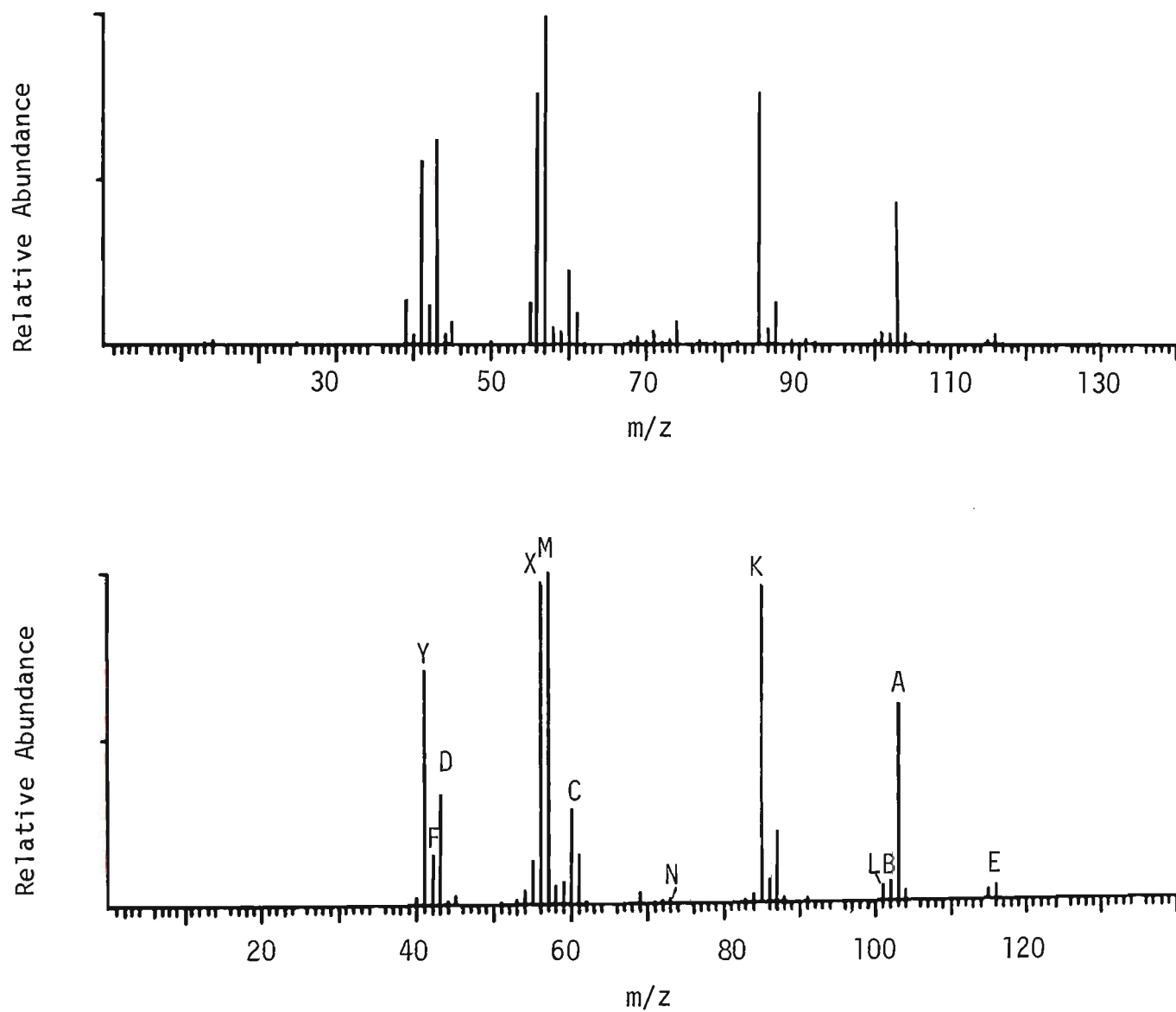
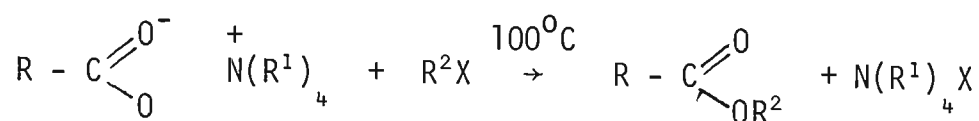


FIGURE 22: Mass spectra of (bottom) peak 1, Figure 20 and (top) butyl isovalerate (library). The letters are used in the following discussion to designate ion structures.

the alcoholic quaternary alkylammonium hydroxide in a glass container.

To overcome these problems the quaternary salts were converted to esters prior to the GC analysis by heating in dimethylacetamide (DMA) with butyl bromide.



The resulting esters were then extracted from this solution with pentane and concentrated by removing the excess pentane with dry nitrogen. It was desirable to wash the pentane extract with water to remove traces of DMA before evaporation.

Butylbromide was chosen because the volatility of the resulting butyl esters allowed ready separation from the solvent on low polarity GC columns (packed or capillary). In addition the mass spectra of these compounds contained diagnostic re-arrangement ions involving the butyl side chain thus allowing improved mass spectral identification.

The efficiency of this method for extracting and butylating short chain fatty acids was tested by extracting an aqueous solution of isobutyric acid (500 μl , 4.6 mM) with diethyl ether and adding an equimolar amount of butyric acid (500 μl , 4.6 mM) to the extract. The acids were converted to their butyl esters by heating their dried tetrabutylammonium salts with butylbromide in DMA. An equimolar amount of chromatographically pure butyl 2-methylbutyrate (50 μl , 46 mM) was added to the final solution of butyl esters. The relative amounts of butyl isobutyrate and butyl butyrate recovered thus gave a measurement of the efficiency of the extraction procedure while the relative amounts of butyl butyrate and butyl 2-methylbutyrate gave a measurement of the efficiency of the conversion

of butyric acid to butyl butyrate. The esters were separated by GLC and the relative peak heights were used as an estimate of the relative amounts of each ester since their peak widths were equivalent. The average of five determinations for both ratios gave a butyl isobutyrate/butyl butyrate ratio of 96.3%; a butyl butyrate/butyl 2-methylbutyrate ratio of 96.9%; and a butyl isobutyrate/butyl 2-methylbutyrate ratio of 93.1%. Thus the solvent extraction of the acids was 96.3% efficient, the butylation was 96.9% efficient and the overall procedure resulted in a 93.1% yield.

Figure 23 shows the GC profile obtained by extraction and butylation as described above of a normal urine to which was added the following at a concentration of approximately 3 μ M: acetic, propionic, butyric, isobutyric, 2-methylbutyric, isovaleric and n-valeric acids. The e.i. spectra obtained for these peaks and the corresponding library spectra of the same compounds are given in Appendix I.

Mass Spectra of Butyl Esters of Short Chain Fatty Acids

There is an extensive literature on the mass spectra of alkyl esters. For example the mass spectra of some 31 compounds have been listed by Sharkey *et al.* (162) and Beynon *et al.* (163) have studied 27 esters under high resolution mass spectral conditions. Specific re-arrangement ions of butyl acetate, propanoate and hexanoate have been studied with the aid of deuterated analogues by McFadden and his co-workers (164, 165). Willimans, Budzikiewicz and Djerassi (166) performed similar experiments with methyl butyrate. Correlations of spectra with structure are clearly evident among all the esters. Major peaks are formed by homolytic scission of the bonds adjacent to the carbonyl group (α cleavage) to give the ions K and L.

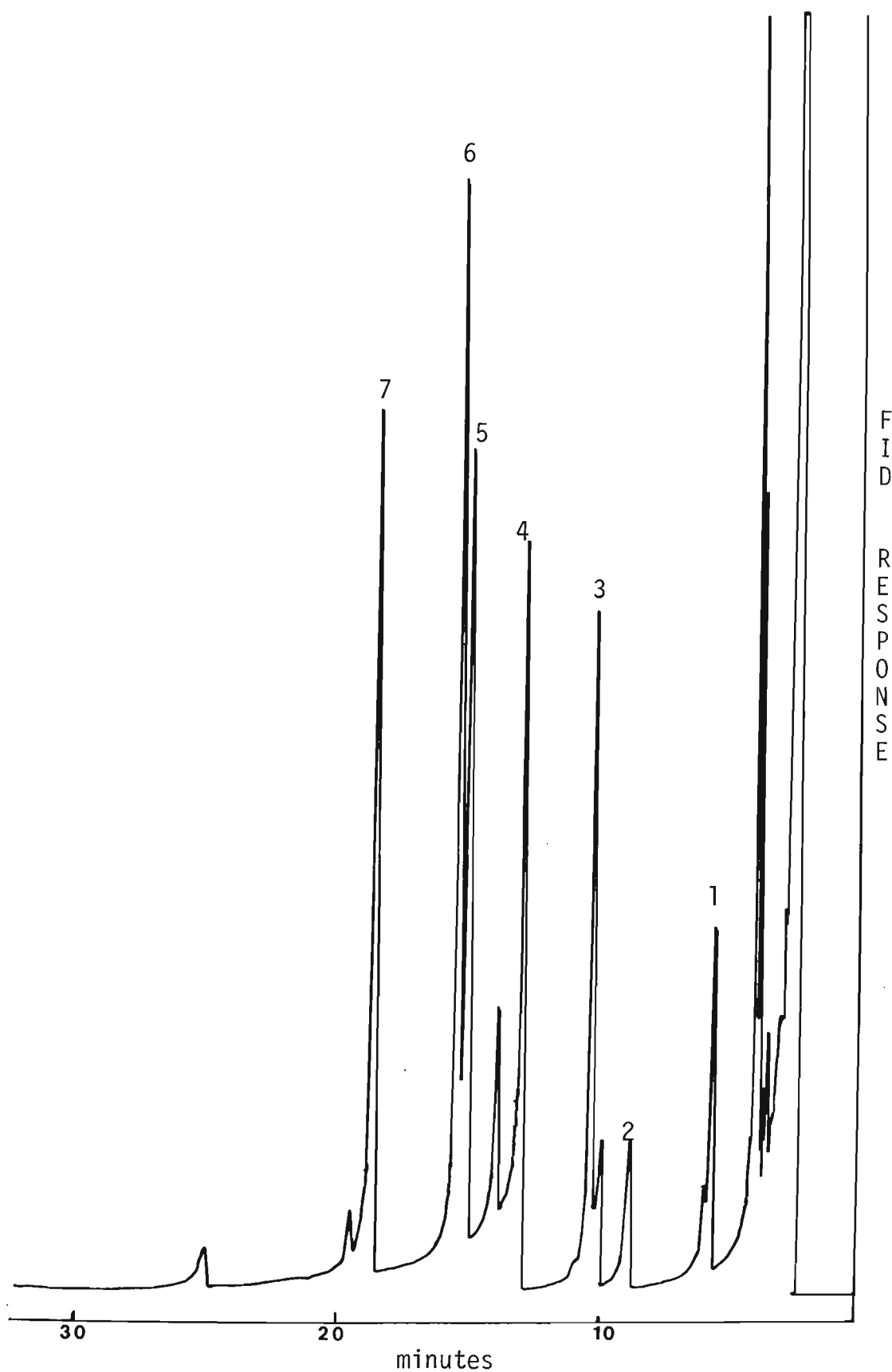


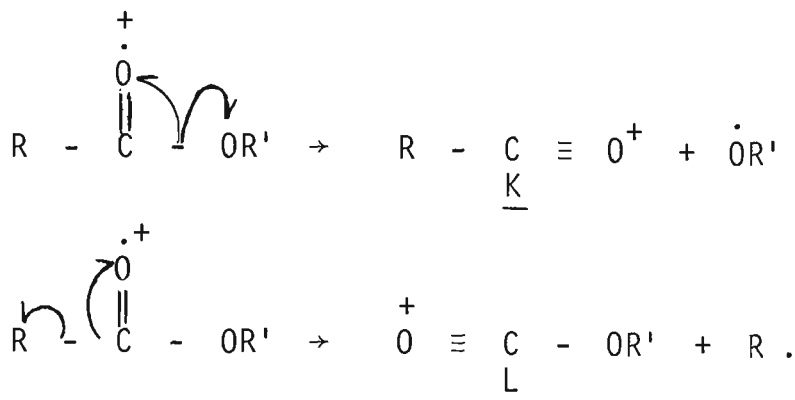
FIGURE 23: Chromatogram of the butyl esters of seven short chain fatty acids.

1 = n-butyl acetate, 2 = n-butyl propionate,
3 = n-butyl isobutyrate, 4 = n-butyl n-butyrate,
5 = n-butyl 2-methylbutyrate, 6 = n-butyl isovalerate,
7 = n-butyl n-valerate.

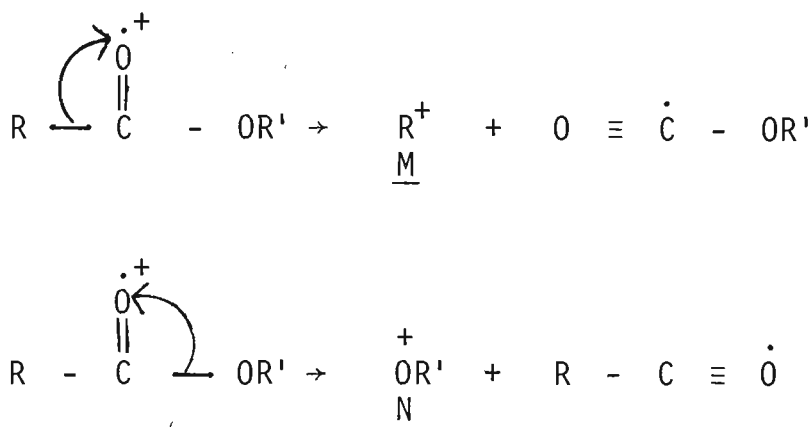
Column: SCOT OV17 60 m (SGE # 566/85)

Carrier gas: Helium, ~5 ml/min.

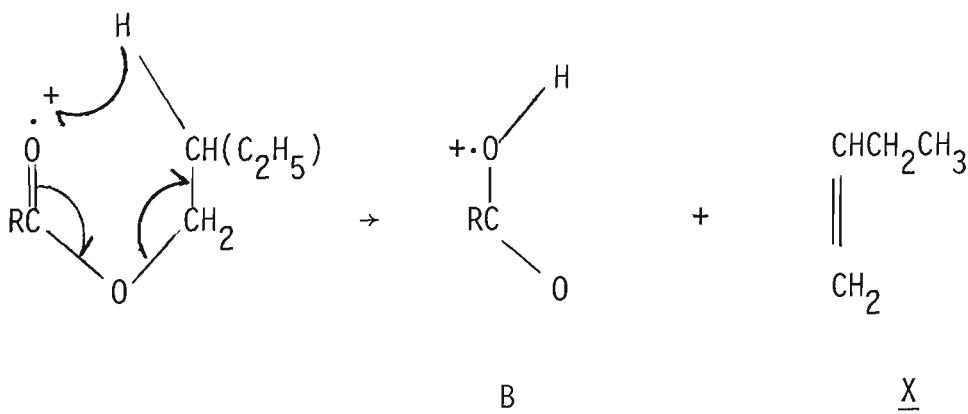
Temperature programme: 70°C 4 mins, then 3°C/min to 214°C.



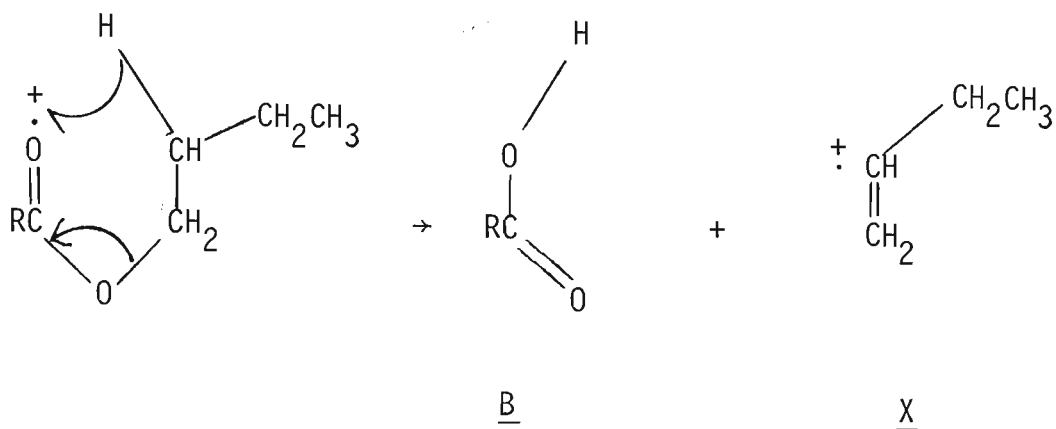
Heterolytic cleavage of this bond results in the ions M and N, but these are usually not as abundant as the ions K and L (167).



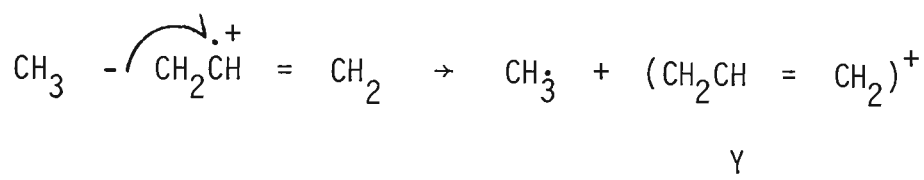
Ions which are more characteristic than either of these four are derived from McLafferty rearrangements as shown below. With butyl esters this rearrangement is always possible (since a hydrogen gamma to the carbonyl group is always available) and yields the ion B. The olefinic fragment X



may also retain the charge due to some heterolytic scissions.

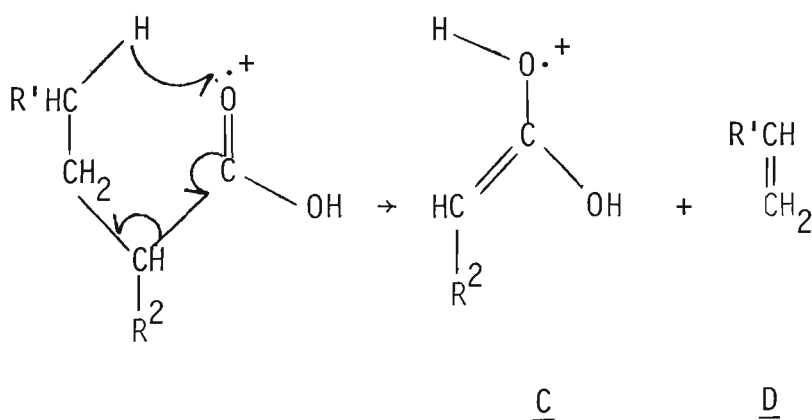


The odd electron ion X may then lose a neutral methyl radical by homolytic scission to give the allylic ion Y.

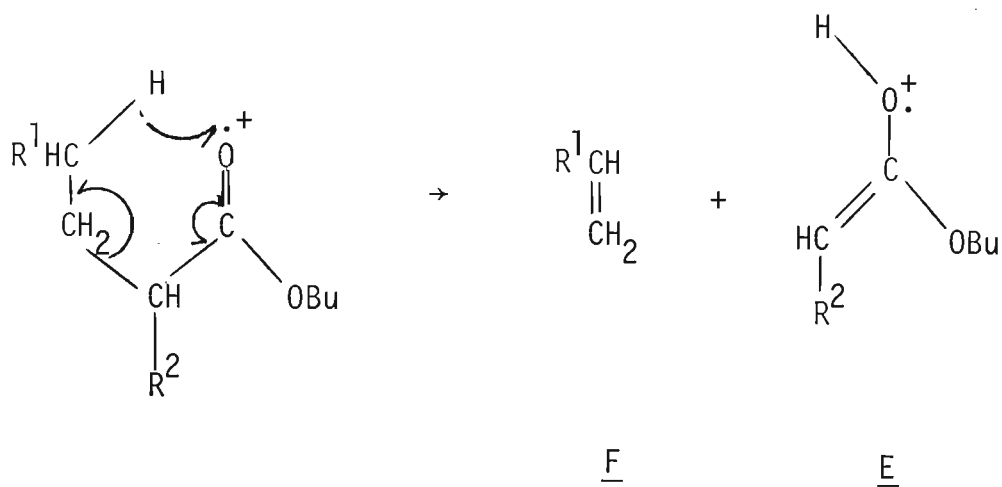


Both ions X and Y are prominent in the spectra of all butyl esters (168).

The ion B has the same formula as the molecular ion of the acid from which the ester is derived. Since ion B is an odd electron ion further fragmentation occurs to produce all the ions seen in the free acid spectrum. Also if the alkyl chain of the acid is 4 carbons or larger a further McLafferty rearrangement occurs to yield the fragments C and D.



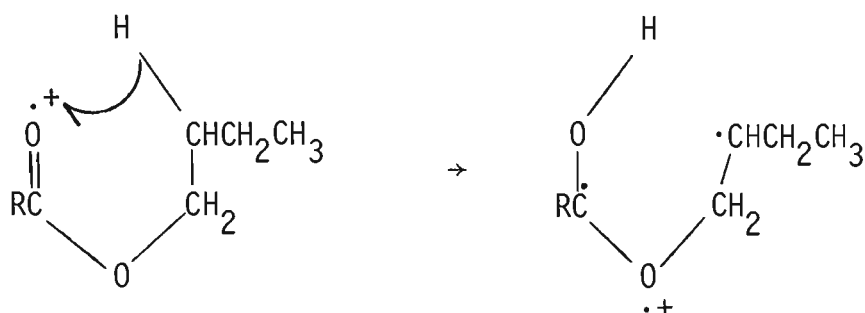
With esters derived from acids of appropriate chain length this type of rearrangement can occur instead of the rearrangement to produce ion B thus yielding fragments E and F (F has the same formula as D).



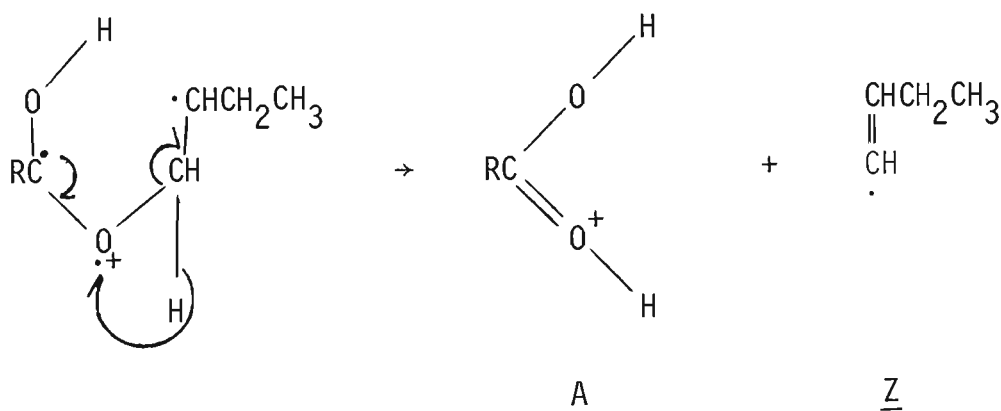
Thus the ion C ($m/z = 116$ when $\text{R}^2 = \text{H}$) formed by this process only appears in the spectrum of butylisovalerate, butyl-n-valerate and butylbutyrate. The corresponding ion for acids branched at the alpha carbon appears at $m/z = 130$ and is diagnostic for such branching as in the case of butyl-2-methylbutyrate.

Another very important diagnostic ion having the formula $(\text{RCOO} + 2\text{H})^+$ and involving the rearrangement of two hydrogen atoms is seen in the spectra of ethyl and high alkyl esters, and usually has a greater abundance than the ion B. The spectra of deuterated secondary butyl acetates (169) have been used to show that in these rearrangement ions the gamma and delta carbon atoms supply 80% of the rearrangement hydrogen atoms and the remainder come from the beta carbon. This occurs even though the beta-hydrogens are in the labile tertiary position. Black and co-workers (164) found that in n-butyl acetate the gamma-carbon atom is the main source of supply for the migrating hydrogen atoms. Djerassi and Fenselau (170) found by studying the spectra of n-butyl propionate and its deuterated analogues that the hydrogens could originate from every possible carbon atom but that those bonded to the beta- and gamma-carbons provide the largest supply. Based on these observations a proposed mechanism for the scission of three bonds to give the "double rearrangement" ion is shown below and consists of two steps. Firstly, the gamma hydrogen is transferred to the carbonyl oxygen to produce an intermediate structure in which the positive charge is

delocalised as in the first step of a McLafferty rearrangement.



Secondly, the beta-hydrogen acts as a nucleophile since there is now an extra bonding electron associated with the beta-gamma carbon bond where a pi bond is also beginning to form due to the second homolytic cleavage of the McLafferty rearrangement. Homolytic cleavage of the carbon-hydrogen bond at the beta-carbon and hydrogen transfer results in the formation of the even electron ion A and the neutral radical Z.



A possible reason for McLafferty's (163) not having observed the beta-hydrogen transfer could be through steric hinderance from the extra gamma methyl group on the beta-carbon of sec-butyl acetate.

Figure 24 summarises the major fragmentation pathways for the butyl esters of the short chain acids as discussed above and Tables 3 and 4 show the mass values of these ions for some acids.

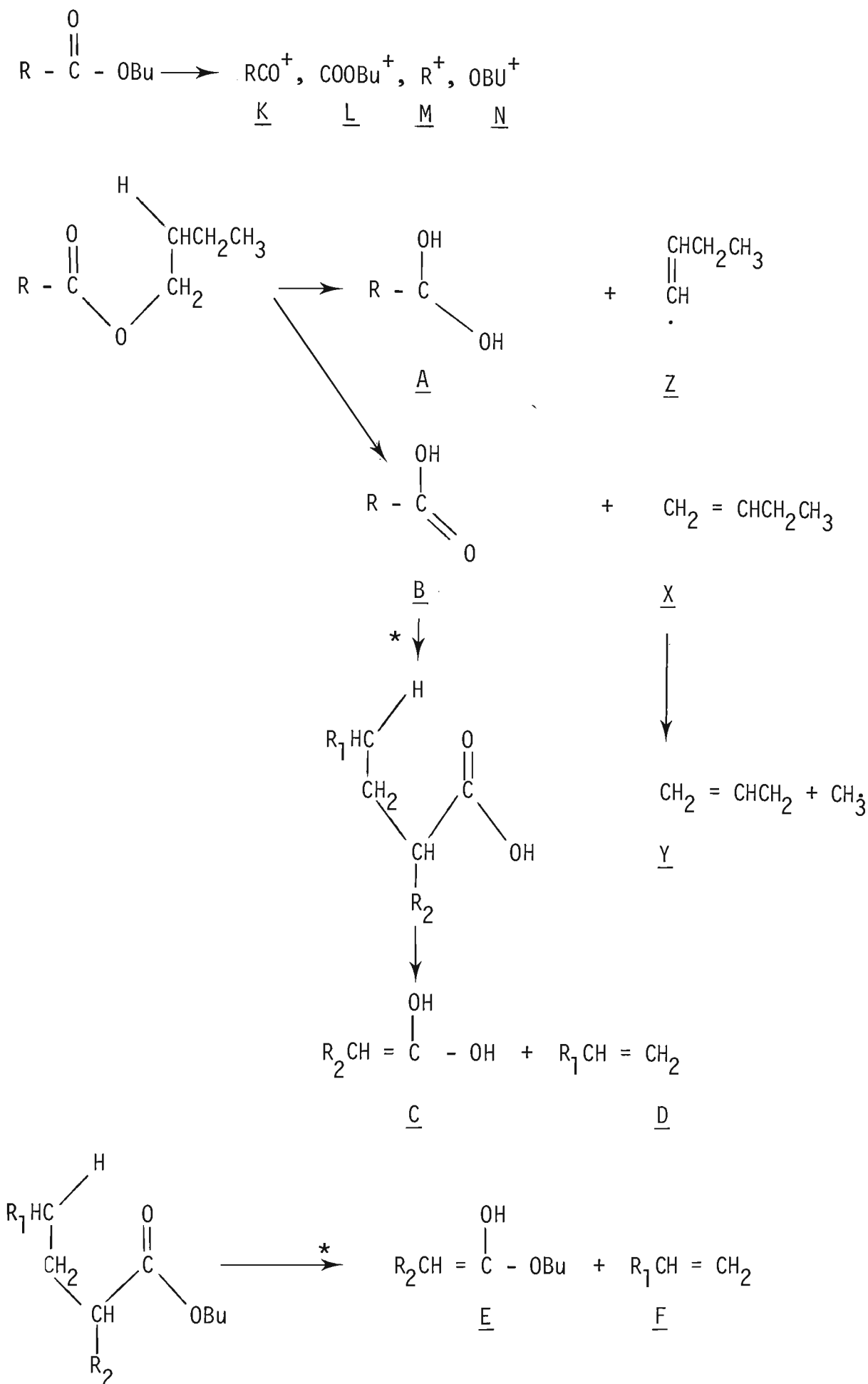


FIGURE 24: Major fragmentation pathways of butyl esters of short chain fatty acids. The letter under the fragment is used to represent it in Tables 3 and 4.

* This fragmentation pathway is only possible when $\text{R} = \text{R}^1 - \text{CH}_2 - \text{CH}_2 - \underset{\text{R}^2}{\underset{|}{\text{CH}}} -$

TABLE 3

Mass values corresponding to the characteristic fragments shown in Figure 24

Name	R-	R ₂	A	B	C	E	D/F	K
Acetic	CH ₃ -	na	61	60	-	-	-	43
propionic	CH ₃ CH ₂ -	na	75	74	-	-	-	57
butyric	CH ₃ (CH ₂) ₂ -	H	89	88	60	116	28	71
isobutyric	(CH ₃) ₂ CH ₂ -	na	89	88	-	-	-	71
isovaleric	(CH ₃) ₂ CH CH ₂ -	H	103	102	60	116	42	85
2-methylbutyric	CH ₃ CH ₂ CH(CH ₃)-	CH ₃	103	102	74	130	28	85
n-valeric	CH ₃ (CH ₂) ₃ -	H	103	102	60	116	42	85
caproic	CH ₃ (CH ₂) ₄ -	H	117	116	60	116	56	99
heptanoic	CH ₃ (CH ₂) ₅ -	H	131	130	60	116	70	113
caprylic	CH ₃ (CH ₂) ₆ -	H	145	144	60	116	84	127

TABLE 4

Mass values of fragment ions common to all butyl esters

Fragment	Formula	m/z value
L	$\text{COOCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$	101
M	$\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$	57
N	$\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$	73
X	$\text{CH}_2 = \text{CHCH}_2\text{CH}_3$	56
Y	$\text{CH}_2 = \text{CHCH}_2$	41

Isovaleric Acidemia

Isovaleric acidemia was first described by Tanaka *et al.* (148) in two patients who were investigated because of a persistent smell in their urine, sweat and breath described as the odour of "sweaty feet". The compound responsible for this odour was subsequently identified by gas chromatography/mass spectrometry (GC-MS) as isovaleric acid. Since leucocytes from children with this disease were shown to have a decreased capacity to degrade isovaleric acid to carbondioxide it was proposed that isovaleric acid accumulates in these patients as a result of a defect in the activity of isovaleryl CoA dehydrogenase, an enzyme in the leucine catabolic pathway (Fig. 13).

Children with this disorder are characterised clinically by episodic attacks of vomiting, lethargy, ketosis and metabolic acidosis and with the presence of an offensive smell. During periods of remission however, behaviour may be normal and the characteristic smell absent.

In an attempt to explain the absence of symptoms and smell during remission, Tanaka and his colleagues examined urine samples from patients with isovaleric acidemia for evidence of alternative pathways of isovaleric acid metabolism. Two abnormal metabolites were discovered. These were identified as isovalerylglycine (171) and 3-hydroxyisovaleric acid (172). Isovalerylglycine was found to be the major abnormal metabolite excreted during periods of remission, and it was suggested that significant quantities of 3-hydroxyisovaleric acid and free isovaleric acid accumulated only when the capacity of the glycine conjugation system was exceeded. This situation could arise if increased amounts of isovaleryl CoA were produced as a result of either increased catabolism due to an infection or due to a higher protein intake.

A fourth metabolite, methylsuccinic acid, was identified by Baerlocher (173) in the urine and liver of a patient with isovaleric acidemia who died at 14 days of age. This compound has since been found in the urines of two other patients with isovaleric acidemia (174).

There appears to be two forms of isovaleric acidemia: a mild, late-onset type, and a severe form manifesting itself in the first week of life (175), however the underlying reason for these different clinical presentations is not known.

Case History

A.D. was admitted to the Royal Children's Hospital on the 8th day because of acidosis, dehydration, depressed conscious state and an abnormal smell. He was the first child of unrelated Australian parents, born at term after a normal pregnancy, weighed 3.7 kg and was breastfed. An abnormal odour had been noted from birth. Jaundice and abnormal twitching movements were noted on day 2, followed by poor feeding, vomiting, hypotonia and cerebral depression. Presumed sepsis was treated with antibiotics from day 2, without benefit. A pseudomonas organism was grown from the blood on admission and again 4 days later.

On admission he looked very ill, was severely dehydrated and acidotic (blood pH 7.19, pCO_2 19 mm Hg; bicarbonate 6.8 mmol/l) and base excess - 20.6 mmol/l, mildly jaundiced, convulsing and hyperreflexic. White blood cell ($4,400/mm^3$) and platelet counts ($60,000/mm^3$) were depressed.

Blood ammonia was 100 $\mu\text{mole/l}$. Urinary carboxylic acid GC profile was grossly abnormal and led to prompt diagnosis of isovaleric aciduria.

Treatment was by intravenous fluids, peritoneal dialysis, and oral glycine (0.5 g 6 hourly) and biotin (25 mg 6 hourly) supplements. Some improvement occurred but neutropenia ($168/\text{mm}^3$) and thrombocytopenia ($10,000/\text{mm}^3$) increased. Despite continuing these measures he deteriorated acutely on day 12 with pseudomonas and candida albicans infection. Ventilator support, change of antibiotics and exchange transfusion (twice) led to marked improvement. Parenteral nutrition was commenced using a low leucine content and improvement continued.

A further collapse occurred on day 18 and led to his death; an event which was not precisely explained at autopsy which revealed widespread patchy damage and patchial haemorrhage in main tissues, including brain and myocardium.

Urinary Volatiles

In contrast to the normal volatiles profile discussed earlier, this patient's profile (Figure 25) was dominated by two large peaks. Peak 2 was shown to correspond to isovaleric acid (Figure 26). Peak 1, the major peak in the chromatogram, could not be immediately identified from its mass spectrum however the fragmentation pattern suggested that it may be due to a methyl substituted butyrolactone. This proposal was confirmed by synthesis of 3-methylbutyrolactone by the method of Owen and Sultanbawa (176). The mass spectrum of this synthetic 3-methylbutyrolactone is shown in Figure 27. The proportion of 4-hydroxisovaleric acid existing in the lactone form in the child's urine is not known as lactone formation increases on acidification.

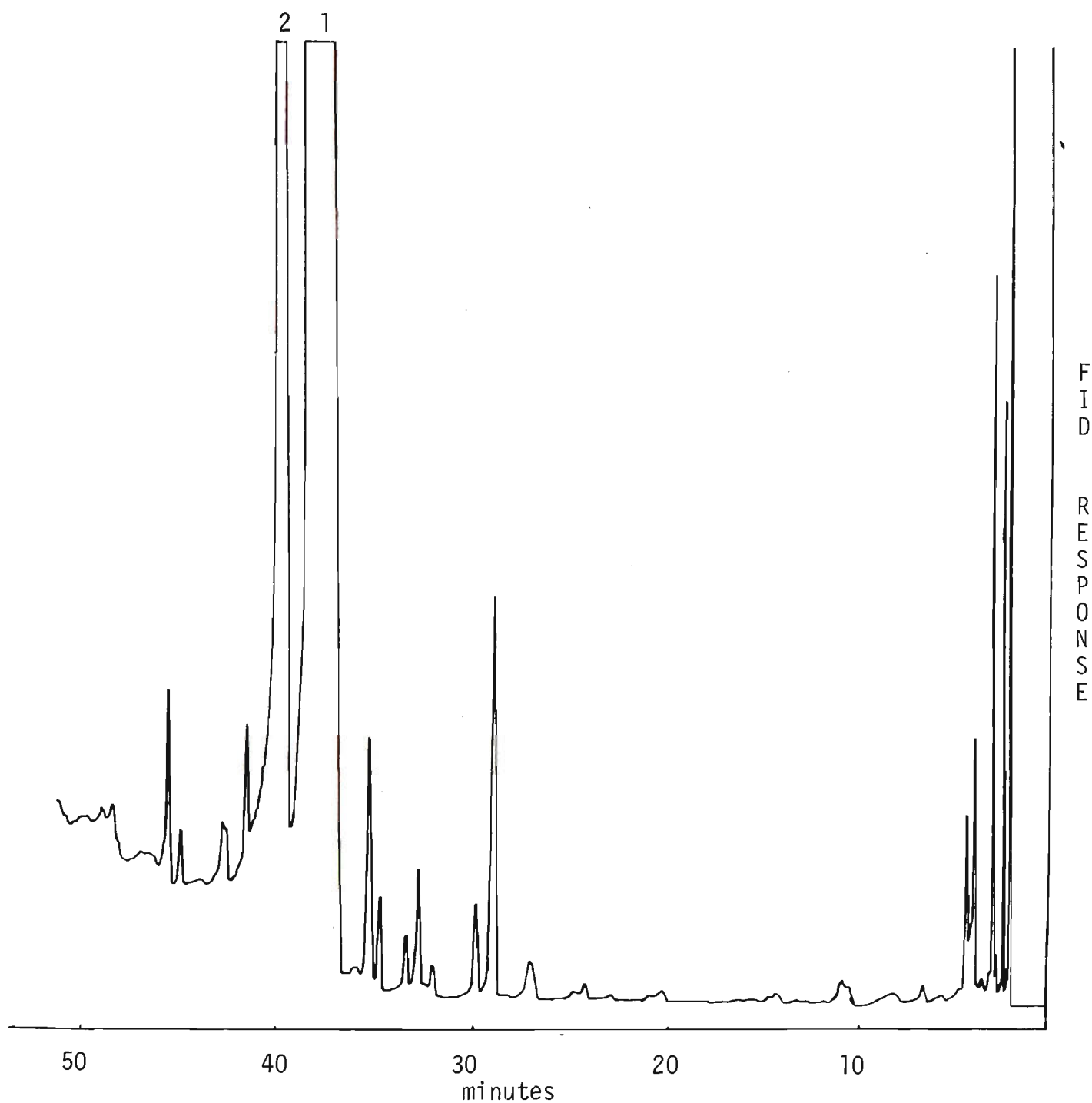


FIGURE 25: Urinary Volatiles Profile of Patient A.D. The volatiles were extracted from a 5 ml urine sample (pH = 1, #14108) by an 83 litre headspace (HS 933).

Column: SCOT CW20M 50m

Temperature programme: 70°C 8 mins, 3°C/min to 200°C.

Carrier gas: Helium, ~5 ml/min.

1 = 3-methylbutyrolactone, 2 = isovaleric acid.

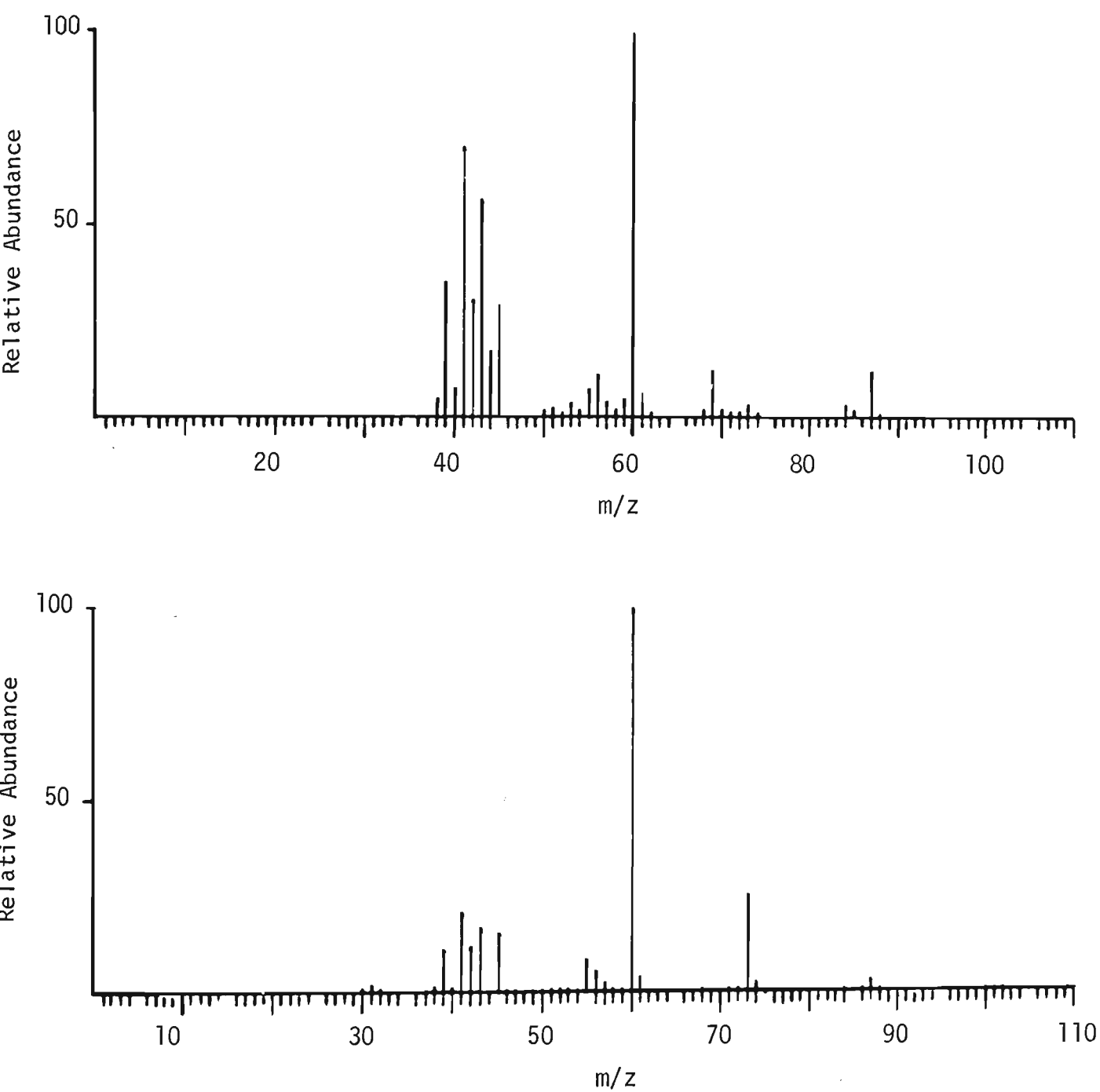


FIGURE 26: Mass spectrum (e.i.) of (top) peak 2, Figure 26, and (bottom) matching library spectrum of isovaleric acid.

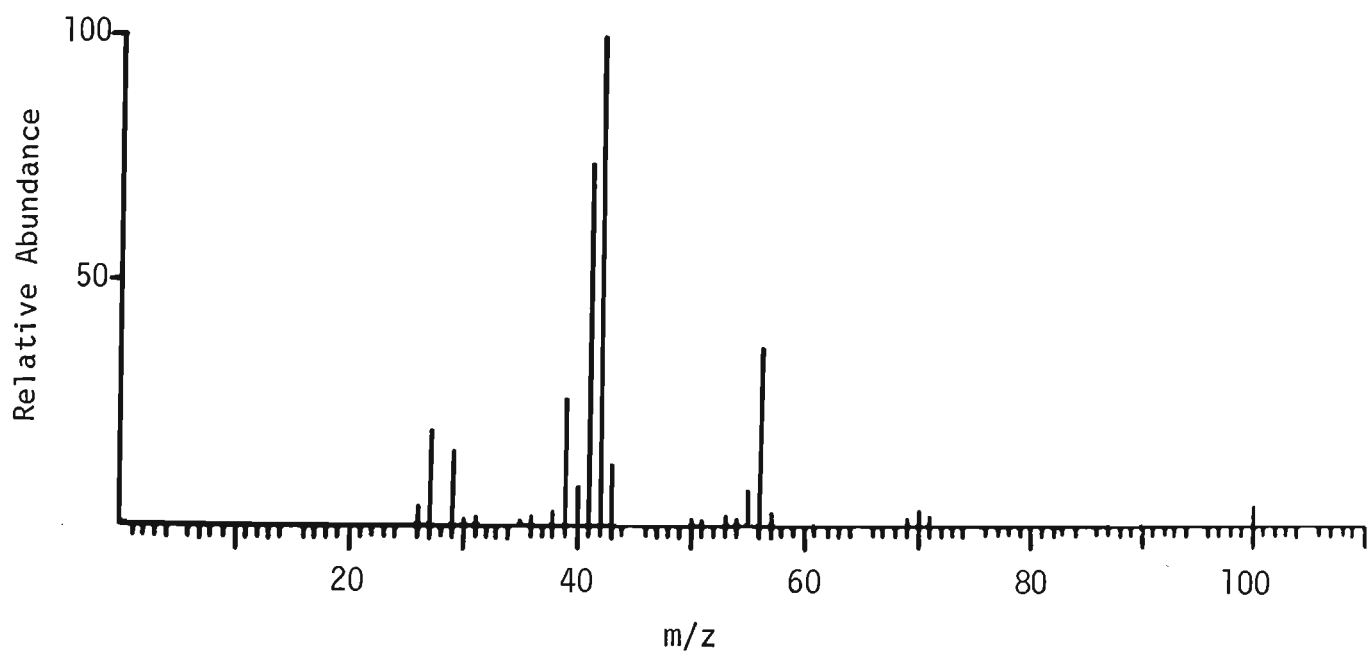
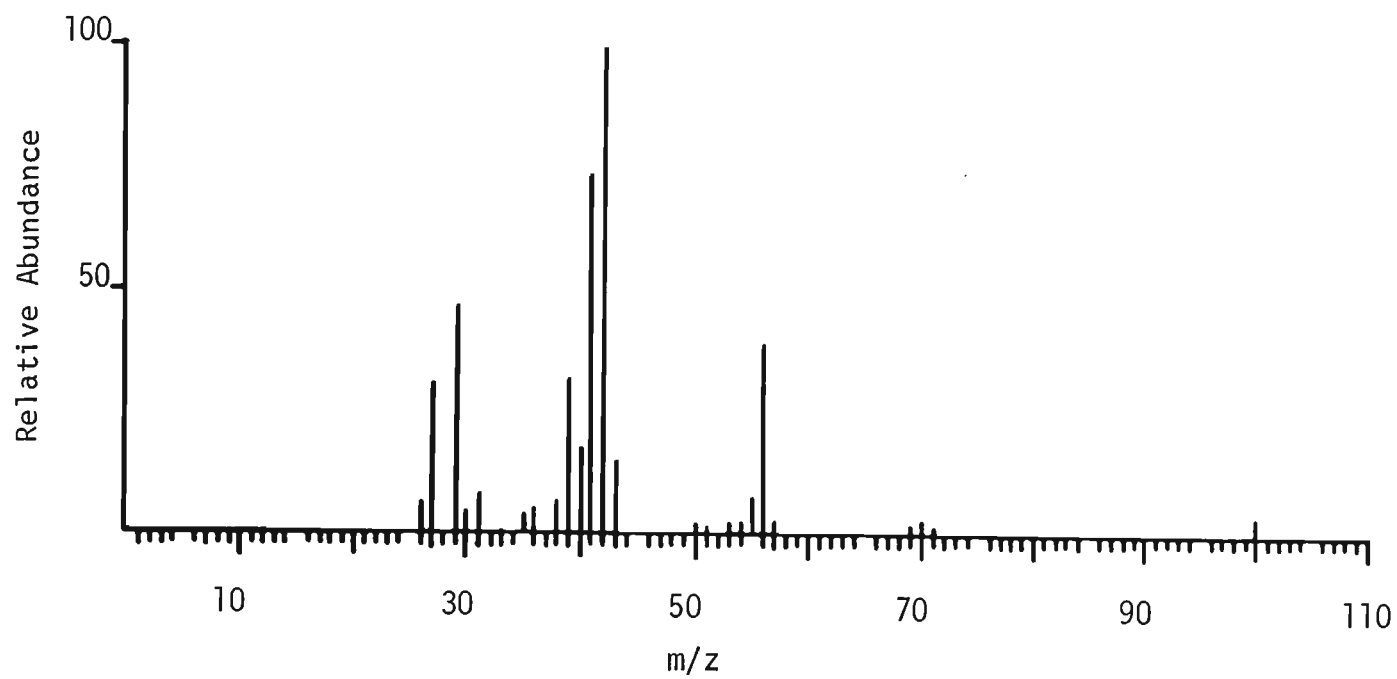


FIGURE 27: Mass Spectra (e.i.) of (bottom) peak 1, Figure 26 and (top) synthetic 3-methylbutyrolactone.

4-Hydroxyisovaleric acid has not been previously described as a metabolite found in excess in Isovaleric Acidemia. It is presumably formed by the ω -hydroxylation of isovaleric acid and as such this metabolite is probably the intermediate in the pathway responsible for the conversion of isovaleric acid to methylsuccinic acid. This pathway is shown in Figure 28. The ω -oxidation pathway has been studied in guinea-pig liver by Wakabayashi and Shimazono (177) who found that oxidation of the terminal or ω -carbon of straight-chain fatty acids took place in two distinct steps. The first step, which occurs in the microsomes, results in the formation of the ω -hydroxy fatty acid. This species is then further converted to an ω -carboxy fatty acid by a dehydrogenase located in the soluble fraction of the cell. The discovery of both 4-hydroxyisovaleric acid and methylsuccinic acid in the urine of our patient parallels the observation by Kuhara and Matsumoto (178) that rats fed the branched chain fatty acid, diproplacetic acid (epilim) excrete both 5-hydroxy-2-propylpentanoic acid and 2-propylglutaric acid in the urine (Figure 29).

Since 4-hydroxyisovaleric acid was a major urinary metabolite in this patient it is pertinent to speculate on the reason why this compound has not been described previously in children with isovaleric acidemia. Most probably this compound has been missed because the majority is converted to 3-methylbutyrolactone under the acid conditions used for the solvent extraction of urinary organic acids. Thus, although 3-methylbutyrolactone is extracted into the organic layer it is probably lost during solvent concentration and the remainder will not be derivatised and so will co-chromatograph on GC with the solvent peak. The general problem of lactonisation and the resultant loss or modification of possibly valuable diagnostic metabolites seems to have been largely ignored by workers when discussing the procedures used, and the problems encountered, in the screening of children for inborn errors of metabolism.

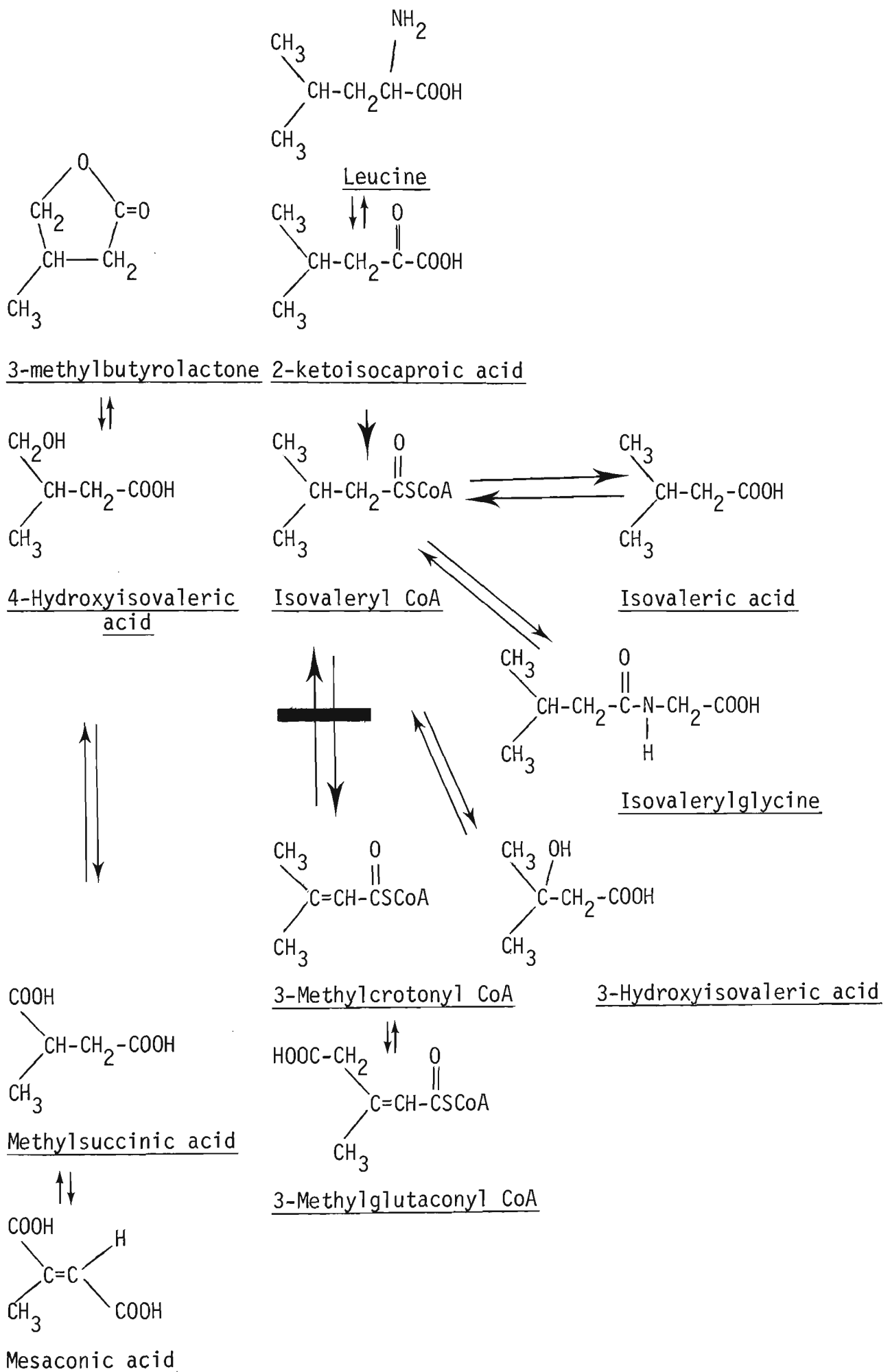
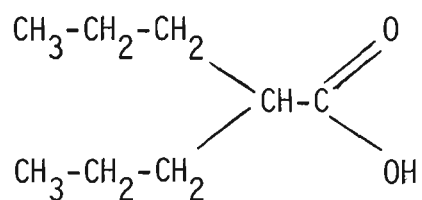
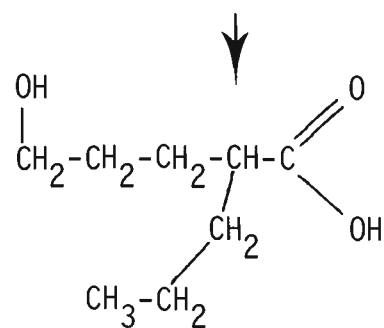


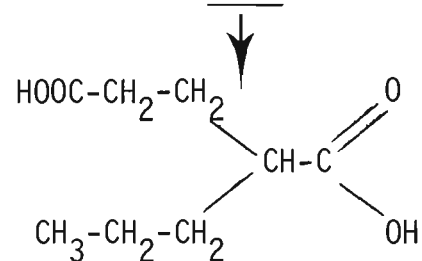
FIGURE 28: Metabolism of Leucine. The position of the metabolic block in isovaleric acidemia is shown by the solid bar. Proposed routes to the formation of methylsuccinic acid and 3-methylbutyrolactone are shown.



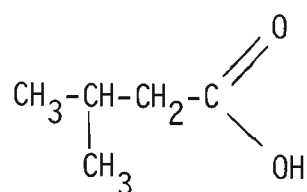
dipropylacetic acid (epilim)



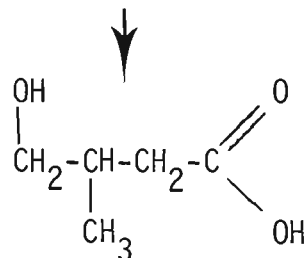
5-hydroxy-2-propylpentanoic acid



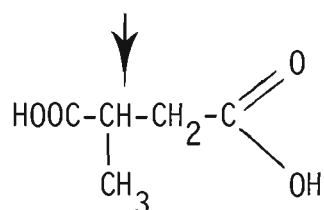
2-propylglutaric acid



isovaleric acid



4-hydroxyisovaleric acid



methylsuccinic acid

FIGURE 29: The formation of Methylsuccinic Acid in man is similar to formation of 2-propylglutaric acid in rats.

Whilst the existence of isovaleric acidemia has now been recognised for more than 12 years and numerous cases have been reported in this period many fundamental questions concerning this disease remain unanswered. Thus, the reason for the apparent existence of two forms of this disease is unknown as is the exact mechanism involved in the formation of two of the major secondary metabolites, 3-hydroxyisovaleric acid and methylsuccinic acid. Although the existence of isovaleric acidemia may imply that isovaleryl CoA dehydrogenase exists as a separate enzyme, and Tanaka and others have obtained supportive evidence for this proposal (179), it has been suggested that isovaleric acidemia could simply be the result of a mutational change in substrate specificity in the enzyme which normally dehydrogenates branched and straight chain fatty acids (134).

Two more cases of Isovaleric Acidemia have since presented at the Royal Children's Hospital, Melbourne, and their urine samples were analysed at Professor Halpern's laboratory. In both cases the same volatiles profile was observed as in the case described above. The profile was dominated by two large peaks, one of 3-methylbutyrolactone and the other of isovaleric acid. In addition, the GC profile of the urinary organic acids of these patients showed a large peak for 4-hydroxyisovaleric acid.

Thus the identification of the large peaks in these volatiles profiles has led to the discovery of a new metabolite in isovaleric acidemia. This has given us a greater understanding of the metabolic pathways involved in leucine catabolism and we can now state that the presence of both isovaleric acid and 4-hydroxyisovaleric acid or its lactone in the urine is diagnostic of isovaleric acidemia. By using headspace chromatography both these diagnostic metabolites were detected

simultaneously and this led to the differences which make this profile characteristic for Isovaleric Acidemia.

Propionic Acidemia

The first reported occurrence of propionic acidemia was in 1961 when Childs *et al.* (180) described a male infant (E.G.) who suffered episodes of metabolic ketosis and protein intolerance. The child was shown to have elevated plasma glycine level. The diagnosis of propionic acidemia was only established at a later date and was based on the description of three other patients; one patient with massive propionate accumulation in blood (181), another with impaired propionate oxidation in leukocytes (182) and from a third with both propionic acidemia and defective carboxylase activity (183). Nearly twenty children with similar clinical and biochemical findings have since been described but some were subsequently shown to suffer from methylmalonic acidemia (184).

The enzymatic defect was established by Hsia *et al.* (182) who demonstrated that leukocytes from a patient produced only negligible quantities of CO_2 from propionate compared to leukocytes from a number of controls. Their patient's cells oxidised methylmalonate and succinate normally. This data showed that the primary metabolic defect in the patient lay in the conversion of propionyl CoA to D-methylmalonyl CoA, a reaction which is catalysed by propionyl CoA carboxylase.

The metabolic pathway involving propionyl CoA is shown in Figure 30. Nearly all the propionate used by man is derived from the catabolism of lipids and proteins. The branched chain amino acids isoleucine and valine as well as methionine threonine and cholesterol and fatty acids with an odd number of carbon atoms are all precursors of propionyl CoA (185). Three enzymatic reactions are responsible for the conversion of propionyl CoA to

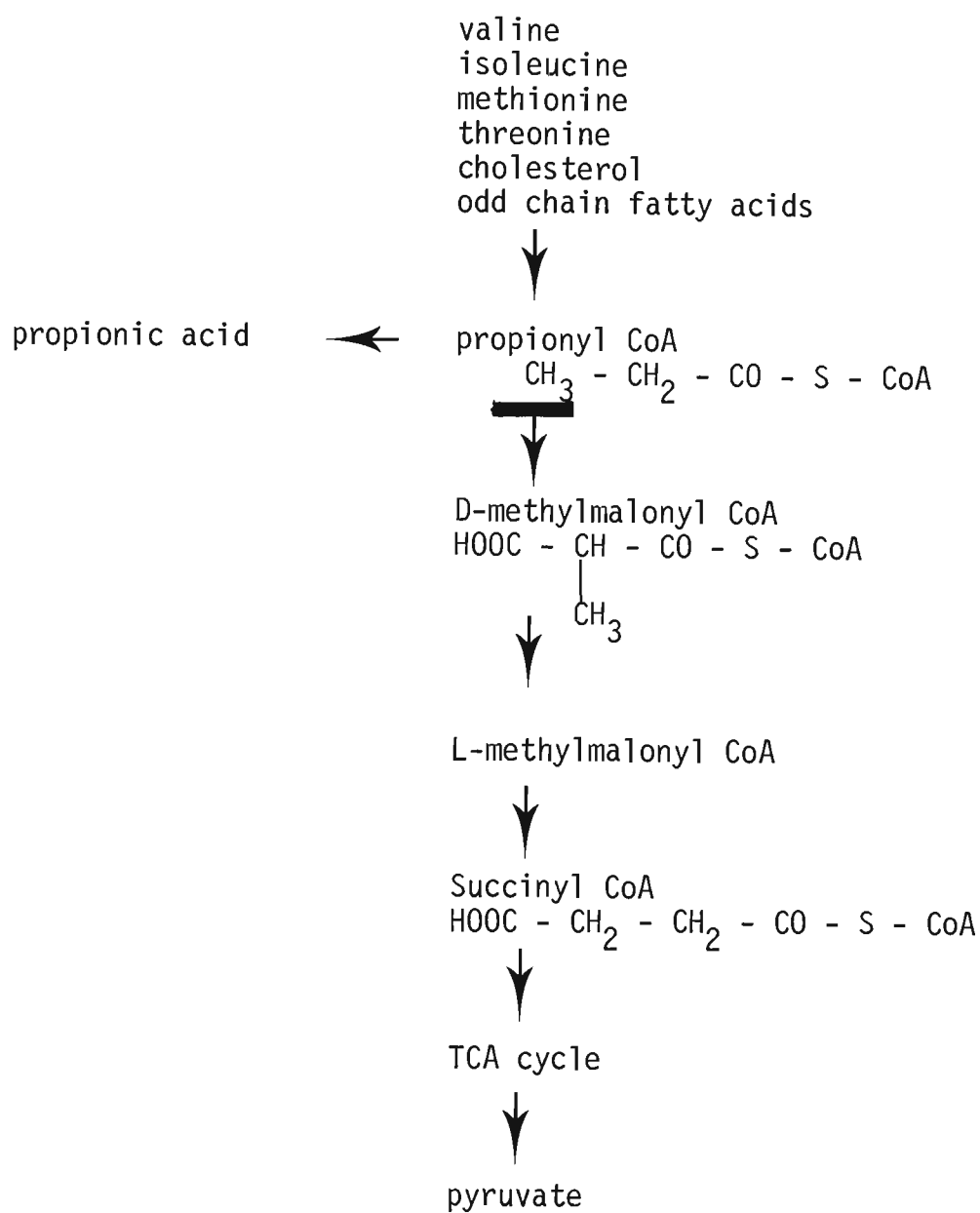


FIGURE 30: A portion of the metabolism of propionate in man. The solid bar denotes the position of the block in patients with propionic acidemia.

succinyl CoA. Succinyl CoA may then enter the tricarboxylic acid cycle to form oxaloacetate then pyruvate which can be converted to glucose via the gluconeogenic pathway.

Case History

Z.K. was born at term after a normal pregnancy, labour and delivery. Birth weight was 2.820 kg. The infant was initially fed a milk formula containing 2.8 g protein per 100 ml but he was noted to be unwell at 48 h, with depressed conscious state and acidotic breathing, and was said to smell ketotic. Mild jaundice was present. Laboratory investigation revealed a marked metabolic acidosis (pH 7.06, bicarbonate 4 mM l^{-1} , pCO_2 14 mmHg and base excess -25) and the nitroprusside test of urine for ketones was strongly positive. Intravenous sodium bicarbonate was given.

Patient Z.K. was admitted to the Royal Children's Hospital at 60 hours of life with metabolic acidosis, hypothermia, lethargy and poor feeding. Blood ammonia was elevated ($600 \text{ } \mu\text{M}$) and GLC examination (SP1200/ H_3PO_4 liquid phase) of serum extracts by the hospital laboratory revealed a large peak with the same retention time as propionic acid. The quantity of propionic acid in blood was estimated at 6 mM. High voltage electrophoresis showed elevated lysine and cystine in urine and elevated lysine and decreased alanine in serum. Thin layer chromatography (TLC) of urinary ketones, as the DNP derivatives, showed increased acetone, butanone and pentanone. The initial full blood count was normal, but later leucopenia ($3.2 \times 10^3 \text{ mm}^{-3}$) and thrombocytopenia ($15 \times 10^3 \text{ mm}^{-3}$) appeared. Treatment consisted of intravenous 10% dextrose, peritoneal dialysis from 65 h of life,

exchange transfusions at 90 and 110 h of life, and megavitamin therapy including biotin 100 mg orally immediately after the second exchange transfusion. Rapid improvement in the infant's clinical condition began around this time and the amount of propionic acid found in his blood was drastically reduced. The decrease in blood propionate was mirrored in the blood ammonia levels which decreased to $93 \mu\text{M}$ on day 4 of life. Dietary protein was introduced and increased to $1.5 \text{ g kg}^{-1} \text{ day}^{-1}$ on the eleventh day of life. Biotin was continued at a dose of 10 mg daily. The impression of biotin responsiveness gained during the initial illness was to have been formally tested later, however a further metabolic crisis developed after two months of relatively good progress on biotin and protein restriction. Hyperammonemia ($1700 \mu\text{M l}^{-1}$) and propionic acidemia proved uncontrollable and he died.

Urinary Volatiles

The method described above for extracting short chain fatty acids from urine followed by derivatization with butyl bromide was used to detect the presence of propionic acid in urine from this patient at 4 days of age. The chromatogram obtained is shown in Figure 31. Peak 1 had the same retention time as mass spectrum as an authentic sample of propionic acid which was butylated in the same way (Figure 32) and the CI (isobutane) molecular ion of this peak was seen at m/z 131.

Nyhan and colleagues have shown that other propionate derivatives (methyl citrate, propionyl glycine, 3-hydroxypropionate and tiglic acid) also accumulate in the urine of affected subjects (186-188),

however none of these compounds were detected by our method. Unfortunately urine from this patient was not available prior to dietary treatment, thus our results might not rule out the possibility of the above metabolites being present during the acute period of the disease. In addition to the experiments discussed above, this patient's volatiles profile was investigated by Professor Halpern's group. It was found that the profile was dominated by a single large peak which was identified as propionic acid.

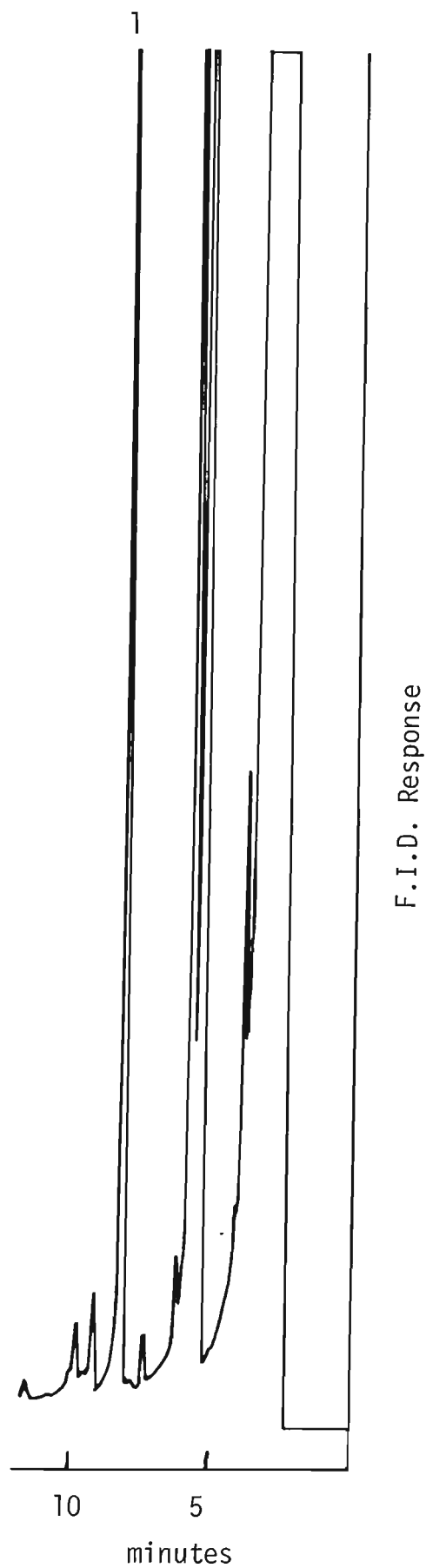


FIGURE 31: Chromatogram at the butylated urinary organic acids from patient Z.K.

Column: SCOT OV17, SGE (566/85)

Carrier gas: Helium, ~ 5 ml/min.

Temperature programme: 70°C 2 mins, $3^{\circ}\text{C}/\text{min}$ to 210°C .

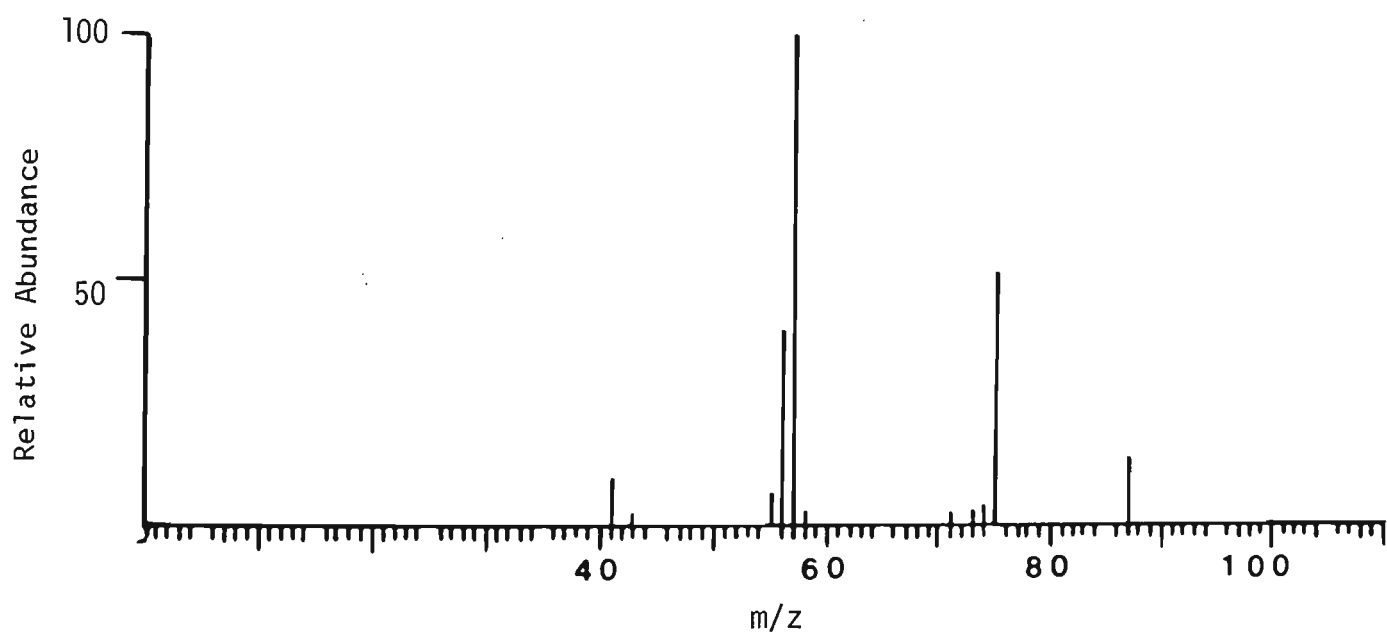
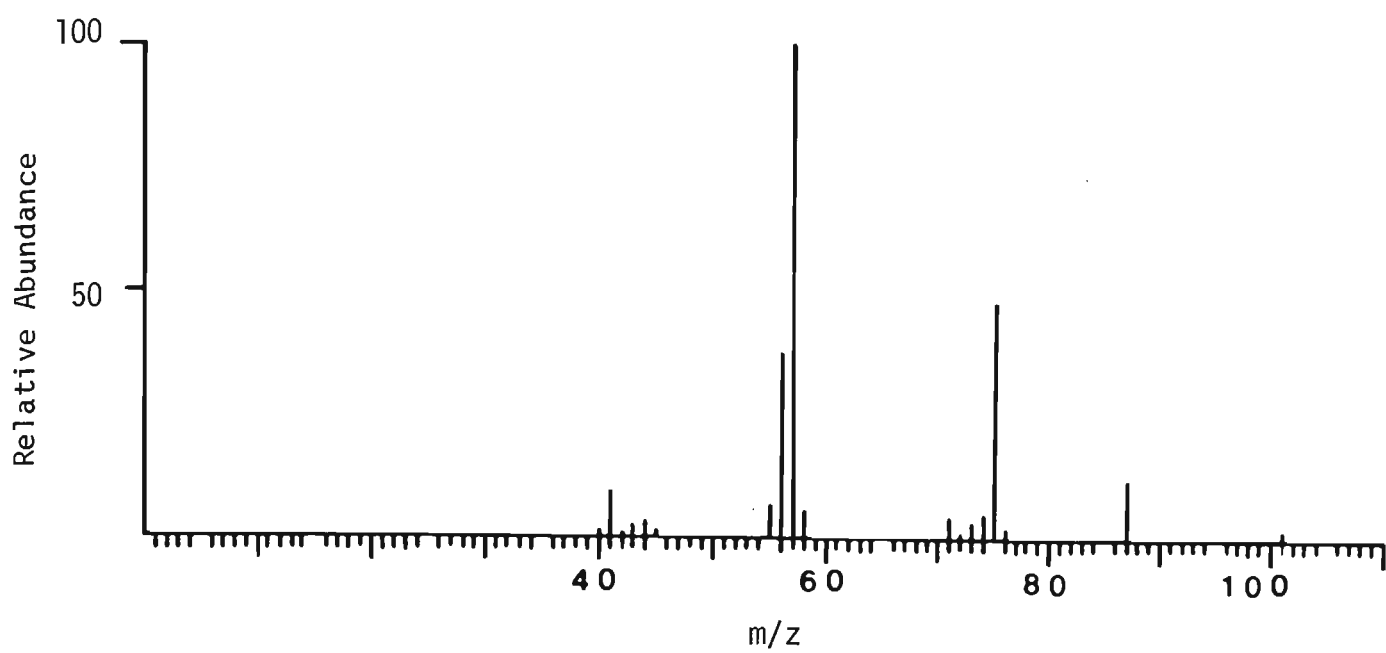


FIGURE 32: Mass Spectra (e.i.) of (bottom) peak 1, Figure 32 and (top) authentic butyl propionate.

Trimethylaminuria

In 1970 Humbert *et al.* (189) described a six year old girl who periodically had a fishy odour associated with increased excretion of trimethylamine (TMA) in her urine. In addition the patient had the clinical stigmata of Turner's syndrome (hematologic abnormalities) and splenomegaly. However, later findings of TMA in the urine of patients without these extra symptoms (190, 191) and Calvert's (192) observation of a patient with the extra symptoms but without the presence of TMA in the urine suggest that the association of all the symptoms in Humbert's patient was fortuitous.

Thus there appear to be no complications with regard to growth and development due to this disease but one of its major effects is the social rejection encountered by sufferers because of their offensive body odour (216).

In man, TMA has a dietary origin (189, 191) and is believed to be a product of intestinal bacterial action. TMA is formed from choline, or its phosphatidyl derivative lecithin. The most important dietary sources of choline are egg yolk, liver and kidney. An additional source of TMA is trimethylamine-N-Oxide (TMA-O) which is a normal constituent of salt water fish (190). In the body the TMA is produced in the gut and it is then absorbed and oxidised in the liver to the odourless N-oxide by a microsomal mixed function oxidase (Higgins, 193). This experimenter also showed that in the patient described by Humbert (189) the TMA oxidase system was defective in a liver biopsy (193). Such a defective enzyme system may cause an accumulation of TMA in the body fluids which is then excreted in the urine and sweat. The proposed block in the detoxification pathway is shown in Figure 33.

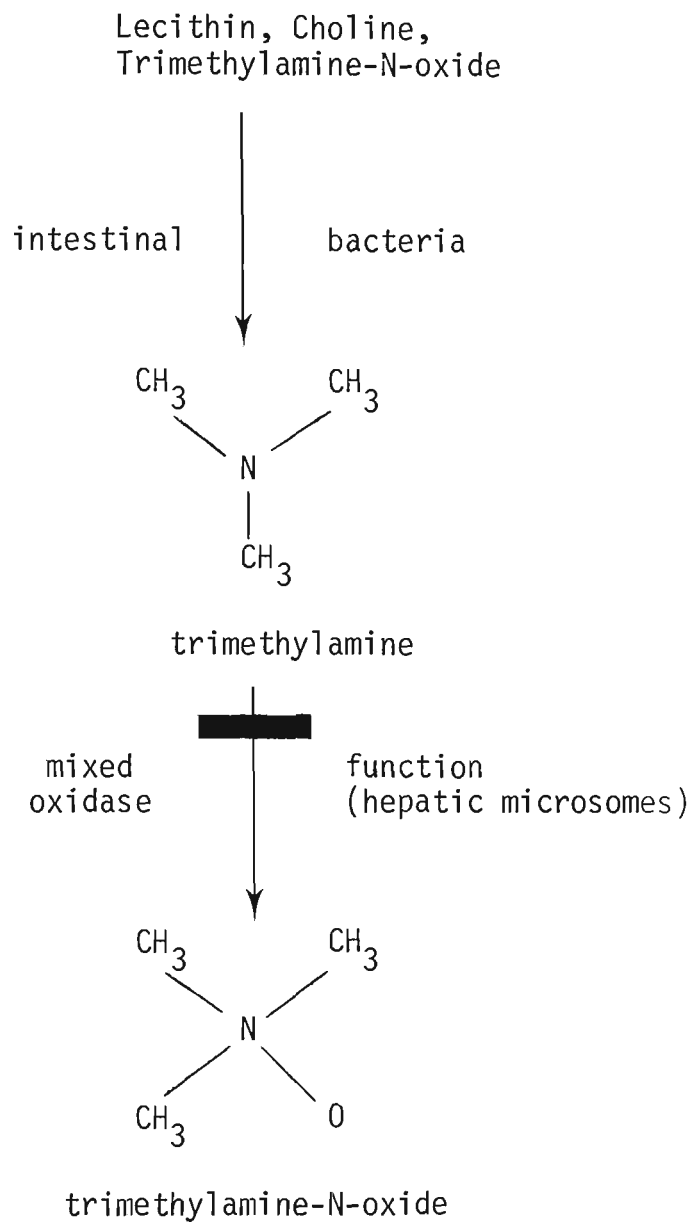


FIGURE 33: The origin and metabolic fate of trimethylamine in man. The solid bar denotes the position of the proposed block in metabolism of trimethylamine in patients with the "fishy odour" syndrome.

Urinary Volatiles Associated with Trimethylaminuria

The volatiles profiles of patients suffering from this disorder were strikingly simple. The profiles were dominated by a single large peak which was usually the first peak to appear on the GC chromatogram. This compound was identified as trimethylamine by GC-MS and was present at such high levels that in the short time required to saturate the collection trap no other compounds were extracted in detectable quantities. Figure 34 is typical of the profiles obtained from a number of such patients.

Since this profile was quite characteristic of this defect we were using this method for the diagnosis of this disease. Contrary to earlier reports (191) we have found trimethylaminuria to be relatively widespread. In this laboratory ten patients have been confirmed as suffering from this disorder over a period of about three years and we are aware that a similar number has been seen in other laboratories (D.M. Danks personal communications).

Higgins *et al.* (193) have suggested that this disorder may be an inherited enzymatic abnormality. To establish whether an inheritance pattern existed for our patients the family members of a sufferer were subjected to an oral load of choline and urine samples taken from them after the load were analysed for the presence of TMA. Both parents and one of three siblings excreted large quantities of TMA in their urines. A hereditary basis for this disease was also supported by our later observation of elevated urinary TMA in two first cousins.

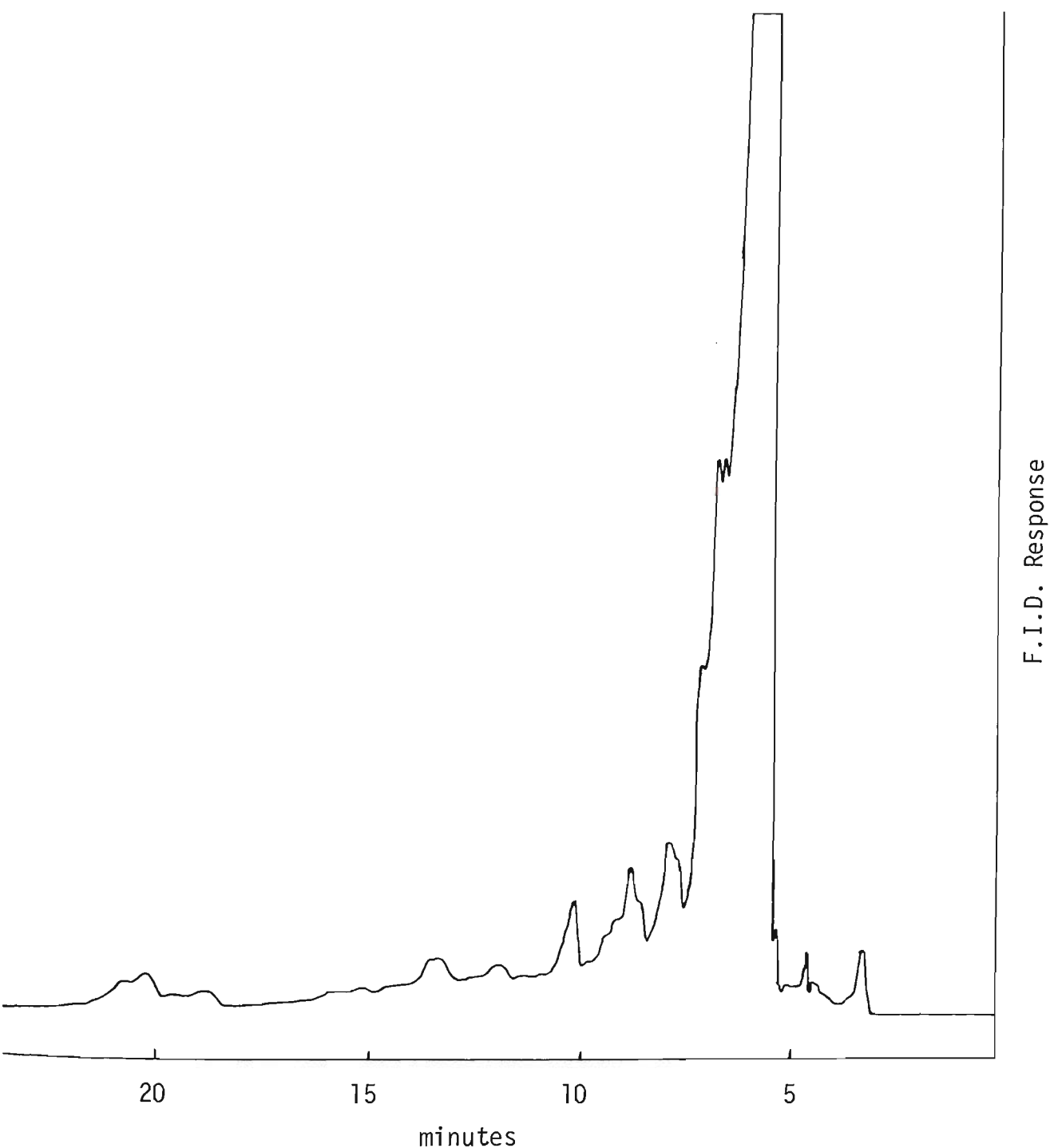


FIGURE 34: Urinary Volatiles Profile of a patient (J.D.) with Trimethylaminuria. The large peak was identified by GC-MS as trimethylamine. The volatiles were extracted from a 2 ml urine sample (pH = 14) by a 36 litre headspace (HS 593)

Column: SCOT CW20M 50m (SGE, Melbourne)
Temperature programme: 70°C 4 mins, 2.5°C/min to 180°C
Carrier gas: Helium, ~3 ml/min.

These studies exposed the need for a quantitative estimate of urinary TMA levels. In addition since dietary manipulation does not always control the fishy body odour of these patients (194) more detailed quantitative studies on the influence of diet on TMA production were planned. The method used to diagnose this disorder was not suitable in its original form for the quantitative work entailed in these further investigations since the headspace was sampled for 20 hours and the whole extract was used for a single GC analysis. Considerable variability in yield was also encountered even when internal standards with similar chemical properties were used. By changing some of the experimental conditions, using TMA labelled with a stable isotope as an internal standard in conjunction with single ion monitoring we were able to use the headspace technique in a quantitative mode.

Many published methods for TMA analysis originate from the fish processing industry and are based on quantitative gas chromatography. The aliphatic amines such as dimethyl-, methyl-, triethyl- and trimethylamine are first extracted from a sample of tissue and converted to their salts by the addition of mineral acid. The aqueous extract may then be concentrated before injection onto a basic GC column where TMA and other bases are released from their salts. Generally porous polymers (195-197) or polar stationary phases mixed with KOH or NaOH (198-204) are used in packed columns to separate the components of the amine mixtures. The advantages of speed and simplicity of these methods are countered however by some important disadvantages intrinsic to this type of analysis. It is frequently found that after several analyses the injection of a water blank will produce a TMA peak. This is due to the presence of "active sites" on the GC column which reversibly bind polar compounds such as TMA, resulting in ghosting and tailing, both of which may interfere with peak area calculations.

The adsorbed water on the column may also change the polarity thus causing variations in retention times and consequent uncertainty of the identity of the GC peaks. These factors have resulted in unusually low precision even when internal standards were used.

In our present work some of these problems were overcome by collecting the TMA onto a porous polymer trap by headspace chromatography. The headspace conditions were adjusted to suit the chemical properties of TMA. The pH of the sample was adjusted to 14 by the addition of concentrated NaOH thus eliminating interference from urinary volatile acids. The duration of the headspace sampling was reduced to ten minutes giving a swept volume of approximately 350 ml which is sufficiently small to trap only compounds with very high vapour pressures at 40°C. The desorbing period of the porous polymer trap in the GC injection port was reduced to sixty seconds and GC separation of TMA from by products was improved by using a polar (CW20M) high resolution support coated open tubular (SCOT) column for subsequent gas chromatography. Under the standard conditions of this analysis TMA gave a reproducible retention time of 3.7 ± 0.3 mins and analyses of urines from healthy subjects consistently failed to show the presence of any interfering GC peaks.

The inclusion of a headspace extraction in the analysis introduced several new variables, however it was possible to overcome most of the difficulties by using a stable isotopically labelled internal standard. N^{15} labelled TMA was most suitable for this purpose as its relevant chemical properties are identical to unlabelled TMA but its difference in molecular weight can be used for quantifying TMA. This was achieved by measuring the abundance of the molecular ion of unlabelled TMA relative to the abundance of the corresponding ion of the labelled internal standard of the TMA peak on the GC. Because the eluting front of the peak caused

too rapid a change in concentration of sample in the MS ion source the relative molecular ion (m/z 59, 60) abundance ratios were used for quantifying TMA instead of the relative molecular ion peak area ratios. The molecular ion abundances were taken from the peak maximum till the lowest ion abundance fell to twice the background level since that part of the GC peak approximated constant sample pressure in the MS source. Figure 35 shows the plot of total ion intensity against spectrum number (time) for a typical TMA peak and Table 5 (Appendix F) presents the computer printout of the abundances and their ratios for the tail section of this peak.

The internal standard used in this assay for TMA was ^{15}N -TMA. The amount of TMA present in the sample was calculated by comparing the molecular ion intensity of the unlabelled species to that of the labelled species and relating this ratio to a calibration curve for the internal standard. Since the intensity ratios were related to the standard curve the amount of labelled standard used need not be known exactly provided that the same amount is used for each assay.

The base peak of the labelled compound is the $[M - 1]^+ = m/z$ 59 ion which has the same mass as the molecular ion of the unlabelled compound. Also the $[M + 1]^+ = (m/z$ 60) peak of unlabelled TMA contributes to the abundance of the molecular ion of labelled TMA. A simple mathematical manipulation can be used to calculate the abundances at m/z 60 due solely to the labelled species and at m/z 59 due to the unlabelled species.

The abundances measured at m/z 59 consists of a finite contribution, a , from the unlabelled TMA added to the contribution b , from the labelled TMA. Likewise the abundance at m/z 60 consists of the contribution, x , from the unlabelled TMA added to " y " from the labelled TMA. Thus the observed ratio of molecular ion abundances, R_o , can be denoted as such:

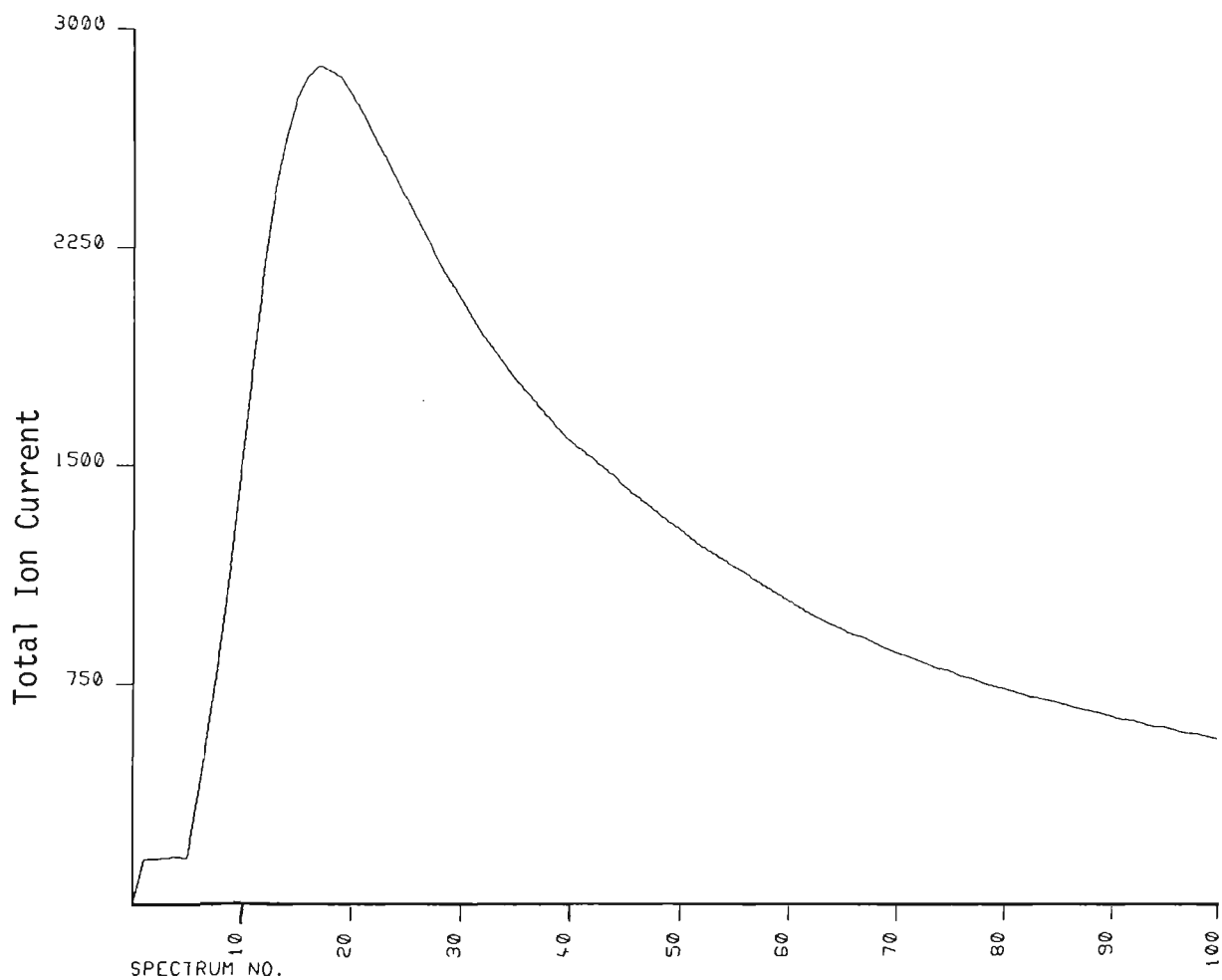


FIGURE 35: Total ion chromatogram of volatiles from headspace No. 840a showing peak tailing. Concentration of TMA in source reached a maximum in 17 scans and then decreased to 2 x background in 80 scans. The time taken for 100 scans was approximately 90 seconds.

$$R_o = \frac{a + b}{x + y}$$

At constant sample pressure the ratios x/a and b/y are constant and can be determined experimentally.

$$\text{Let } \frac{x}{a} = Q \text{ or } x = aQ \quad 2$$

$$\text{and } \frac{b}{y} = S \text{ or } b = yS \quad 3$$

Substituting equations 2 and 3 in equation 1 gives:

$$R_o = \frac{(a + yS)}{(aQ + y)}$$

$$R_o (aQ + y) = a + yS$$

$$aQR_o + R_o y = a + yS$$

$$a - aQR_o = yR_o - yS$$

$$a(1 - QR_o) = y(R_o - S)$$

$$a/y = (R_o - S)/(1 - QR_o) \quad 4$$

Conditions were selected which both minimise fragmentation (so that the ratio S would remain constant) and prevent ion-molecule reactions (keeping Q constant). Instead of the normal 70eV ionisation energy commonly used for electron impact ionisation, an electron energy setting of 20V significantly reduced fragmentation and changed the ratio Q from greater than 2 to approximately 1.3 with a concomitant increase in absolute abundance of the molecular ion species. The ratio Q ($[M + 1]^+/M^+$) of unlabelled and S ($[M - 1]^+/M^+$ of labelled) were determined prior to the sample analysis by admitting the labelled and unlabelled TMA into the MS from a sample reservoir.

The molecular ion abundance ratios obtained from fifty scans were averaged to determine these ratios and approximately forty scans were averaged to obtain the observed ratio, R_o , for a TMA sample or standard.

Accuracy of Ratios Q and S

Under these conditions the ratios Q and S could be determined with very high precision mostly with coefficients of variations of 1% or less. The ratio Q could not be determined with great accuracy since the intensities for 59 and 60 were at the extremes of the dynamic range of the instrument. Five separate determinations of the ratio Q gave a mean value of 0.06 which was used in all calculations. The ratio S was determined over a period of 4 weeks and the mean of the ratios was 1.31 (CV = 3.7%) and was used in all further calculations. Determinations of the ratios Q (Table 6) and S (Table 7) are given in Appendix F.

A batch of samples consisted of four sets of duplicates (i.e. eight tubes) and were run over a period of 90-120 minutes. To ensure that instrumental conditions gave a ratio within the range of experimental error the ratio S was checked at the beginning of the batch, after 4 tubes and after all tubes were run. The results of some of these determinations are shown in Table 8 Appendix F.

Calibration of Internal Standard

Using the above values for Q and S in equation 4 gives

$$R_a = (R_o - 1.31)/(1 - 0.06 R_o) \quad 5$$

where $R_a = a/y$, the true ratio of molecular ions. Figure 36 is a plot of equation 5 showing that linear relationship between R_a and R_o is approximated

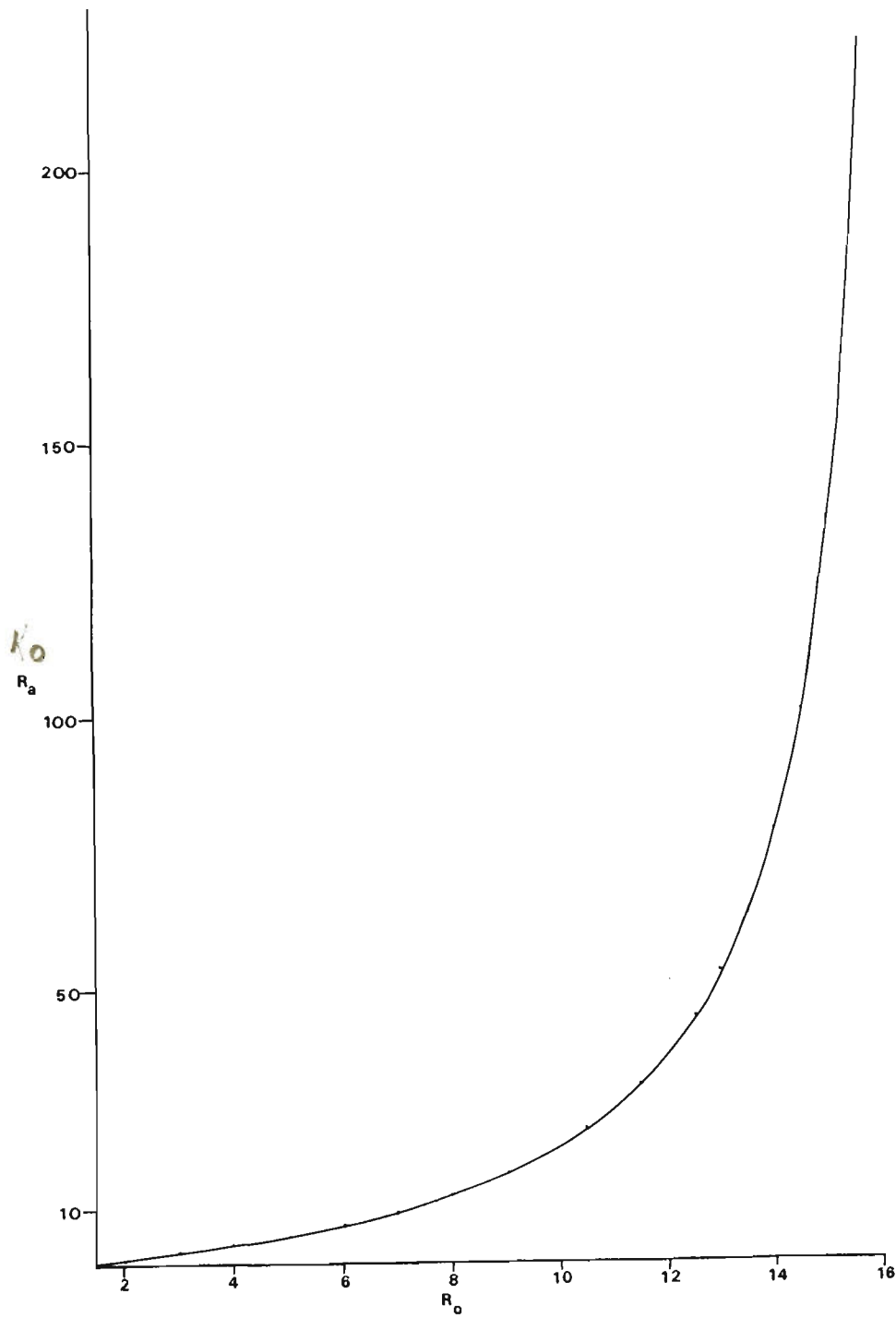


FIGURE 36: Plot of $R_a = \frac{R_o - 1.31}{1 - 0.06R_o}$

Showing approximate linear relationship to R_o of 6.

up to an R_0 value of 6; after this the relationship is exponential. The amounts of labelled and unlabelled compounds used were chosen to fall in the R_0 range of 1.5 - 3.0 since the error involved in determining this ratio would not be amplified when converting to the R_a value.

A calibration curve was prepared with eight discrete points and shows that the relationship between R_a and the quantity of TMA is linear. Table 9 summarises the results obtained for the eight points and also presents the results of the linear regression analysis for the calibration curve. The curve is plotted in Figure 37 and Appendix F lists the formulae used in these calculations.

The standard error of the estimate of the value for TMA, which is a measure of variability of the value about the regression line for a particular ratio of molecular ions averaged over all values, was calculated to be 0.02 and means that the larger values were determined more precisely. This error measurement, since it is based on the final figures given by the analysis gives an indication of the overall accuracy of this assay since at this point all errors have been summed. That is, this assay gives a measurement for the amount of TMA in the sample within the range $0 - 0.80 \pm 0.02 \mu\text{moles}$.

Two more calibration curves were prepared after the mass spectrometer electronics had been extensively overhauled and fresh solutions of the unlabelled TMA were prepared. The results for these two curves are given in Appendix F.

TABLE 9

First Calibration Curve for Trimethylamine (TMA)

Amount of TMA (μ moles)	Peak Height Ratio (R_a)
0.17	0.32, 0.33
0.26	0.60, 0.58, 0.61, 0.61
0.34	0.75, 0.74, 0.72
0.43	0.91, 1.06, 1.04, 0.91, 0.97
0.52	1.20, 1.15
0.60	1.35, 1.35, 1.33
0.69	1.51, 1.55, 1.55
0.78	1.72, 1.67, 1.68

intercept = 0.008
slope = 2.204
correlation coefficient = 0.995

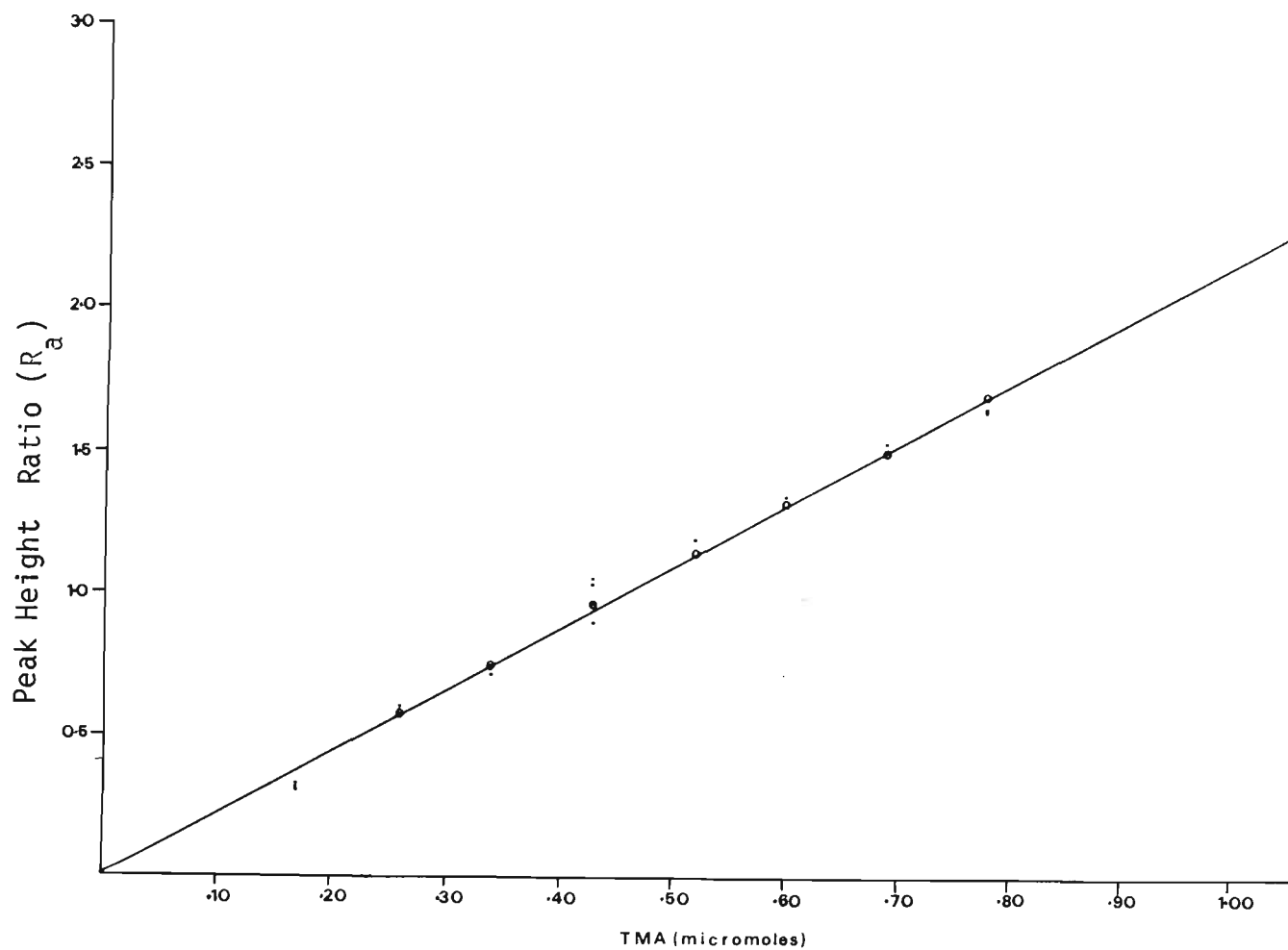


FIGURE 37: Calibration curve for trimethylamine (TMA)
The peak height ratio is the ratio of the abundance of the molecular ion of unlabelled TMA to the abundance of the molecular ion of ^{15}N -TMA internal standard.

Analysis of Samples from Patients suffering from Trimethylaminuria

The results of this analysis for samples of urine from people considered by clinicians to be suffering from the "fishy smell" syndrome are given in Table 10. In each case the absolute level of TMA in the urine was greater than 0.1 $\mu\text{mole/ml}$ while TMA was either not detected or was below the 0.1 $\mu\text{mole/ml}$ level in the urine of healthy subjects given in Table 11.

In order to compensate for the differences in concentration of TMA in urine caused by dilution of the urine by excess ingested water all results were compared to creatinine values of the urine samples. Assuming that individuals excrete a constant daily amount of creatinine this comparison then may provide a more independent estimation of the rate of excretion of TMA in the urine. However, the amount of creatinine excreted is proportional to body mass so this manipulation is more valid for comparisons between the same individual rather than among different individuals as the extra assumption that no significant difference exists in the body masses of the individuals being compared may not be valid. This assumption is not true when comparing children to adults and certainly this limitation must be borne in mind when analysing such results. Caution must also be observed when using results for creatinine concentration obtained from public hospitals as considerable variations were found between hospitals for the same samples.

Subject to these constraints, however, it was found that a useful figure could be obtained by calculating the excretion of TMA relative to creatinine on a molar basis and converting this figure to a percentage. The resulting value was termed relative molar

TABLE 10

Level of TMA in urine of people with "fishy odour" syndrome

Name	(a) TMA (μ moles/ml)	(b) Creatinine (μ moles/ml)	Relative excretion (a/b)%
J.D.	0.89	4.0	22.3
M.H.	0.51	8.8	5.8
R.G.	2.39	14.3	16.7
N.L.	0.46	3.8	12.1
M.H.	0.72	11.6	6.2
M.H.	0.32	4.9	6.5
N.L.	0.44	4.4	10.0
M.H.	0.60	6.9	8.7
C.C.	0.52	4.9	10.6
S.B.	0.26	3.1	8.4
C.K.	1.37	6.0	22.8

TABLE 11

Level of TMA in urine of healthy subjects with no "fishy odour"

Name	(a) TMA (μ moles/ml)	(b) Creatinine (μ moles/ml)	Relative excretion (a/b)%
M.S.	n.d.*	n.a.†	-
D.S.	n.d.	n.a.	-
S.S.	0.09	3.7	2.4
X.R.	0.10	21.4	0.5
M.M.	0.10	4.5	2.2
X.S.	0.16	9.0	1.8
X.S.	0.14	10.4	1.3
X.S.	0.10	11.3	0.9
X.S.	n.d.	n.a.	-
D.B.	n.d.	n.a.	-
J.R.	0.11	7.6	1.4
N.H.	0.02	5.8	0.3

* n.d. = not detected

† n.a. = not applicable

excretion (RME). Thus it was shown that all people considered as suffering from the "fishy odour" syndrome exhibit a relative molar excretion of TMA of greater than 3%, while healthy subjects are usually greatly below this level or in the not detected range (0 - 0.1%).

Patient Therapy Studies

Choline Loading

The accumulation of TMA may be more marked when the affected person ingests a large quantity of a precursor compound. So to test if diet is the origin of TMA and to determine if an inheritance pattern existed for the patient (M.H.) levels of TMA were measured in the urine before and after her ingestion of a large dose of choline chloride. The levels of TMA in her parent's (A.H., S.H.) urine after the same choline dose were also measured. The urinary TMA levels were measured for two control patients (N.H., J.R.) before and after the same choline dose for comparison.

The results of these determinations are presented in Table 12 and show that before the choline load control patients had urinary levels of TMA in the normal range but the value for M.H. abnormal. A dramatic increase in urinary TMA excretion can be seen for M.H. after the load, however only one parent (S.H.) had a level after the load which was above normal. The urinary TMA level of one control patient (J.R.) remained marginally inside the normal range while that of the other (N.H.) increased to just over the normal range.

These observations support both the hypotheses that TMA is formed in the gut by bacterial action and also that dietary choline is a

TABLE 12

Levels of TMA in urine of the parents of a fishy odour syndrome child and controls before and after choline load

Name	Before load		After load	
	TMA $\mu\text{moles/ml}$	RME ^a %	RME ^a %	TMA $\mu\text{moles/ml}$
M.H.	0.51	5.8	2088.5	41.77
S.H.			12.5	0.15
A.H.			0.8	0.13
J.R.	0.11	1.4	2.9	0.14
N.H.	0.02	0.3	5.0	0.81

(a) Relative Molar Excretion (RME) is calculated by dividing the TMA value by the creatine value

precursor compound. However, the view that these patients suffer from an inherited inability to metabolise TMA to TMA-O was not supported since a heterozygous recessive pattern was not observed in patient M.H.'s parents. This agrees with results by Marks *et al.* (191) who were not able to detect TMA in the urine of their patient's family members. The inability to demonstrate a recessive inheritance pattern in these cases together with the more widespread occurrence than was originally thought casts doubt on the assertion that this syndrome is an inborn error of metabolism. An alternative explanation may be that the excessive amount of TMA in these patients' biological fluids is the result of intestinal infection by a bacterial species similar to those responsible for fish spoilage (196, 198, 199). The organism may not be widespread in the community, but if infected the sufferer would expose other family members to subsequent infection. If bacterial infection is the cause of this disorder then isolation and identification of the causative bacterial species from the intestinal tract of "smelly" patients and determination if previously normal subjects could be changed to "smelly" subjects by specialised intestinal infection with these species may resolve the question of the reason for the occurrence of this syndrome.

Intestinal Sterilization

Since TMA is believed to arise from intestinal bacterial action, sterilization of the gut with a broad spectrum antibiotic such as Neomycin should decrease the level of TMA excreted in the urine. Table 13 presents the TMA levels of two patients (R.G. and M.H.) before and after a one week course of Neomycin. After this treatment the levels fell to within the normal range and both patients noted a reduction in odour.

TABLE 13

Level of TMA in urine of trimethylaminuriacs before and after
neomycin treatment

Patient	Before treatment		After treatment	
	TMA $\mu\text{moles/ml}$	RME ^a %	RME ^a %	TMA $\mu\text{moles/ml}$
R.G.	2.39	16.7	1.7	0.21
M.H.	0.32	6.6	0.5	0.05

Intestinal sterilization was studied in another "fishy odour" syndrome patient (N.L.) by examining his response to a choline load before and after the course of antibiotics. The results presented in Table 14 show that after sterilization a choline load did not produce an abnormal TMA level.

Replacement of intestinal flora

Two therapies were examined (Synerlac and Duphalac) which are claimed by the manufacturers to replace proteolytic flora in the intestine with sarcharalytic species thus reducing production of amines in the gut. Synerlac contains living, antibiotic resistant strains of selected bacteria with lactose and causes the change in flora by introducing the new species directly to the intestinal lumen (205). Duphalac contains a synthetic disaccharide (4-D-galactopyranosyl-4-D-fructofuranose) which can be used as a carbon source only by specialised saccharalytic bacteria (205). The replacement of the proteolytic flora occurs by providing enough of this disaccharide to select species which can metabolise it.

Two patients (N.L. and M.H.) were given 2.5 g of Synerlac twice daily for two days and their responses to choline loading were determined before and after this treatment. The results of these determinations are shown in Table 15. Only one patient (M.H.) had a significant reduction (measured by RME) in excretion of TMA to about the normal range after the treatment.

Two different patients (N.L. and R.G.) were given Duphalac (5 ml, three times daily) for one day only and their response to choline loading before and after treatment were determined. Table 16 shows the results of

TABLE 14

Urinary TMA levels of patient N.L. showing his response to a choline load before and after intestinal sterilization

Choline load		Choline load after Neomycin	
TMA μmoles/ml	RME %	RME %	TMA μmoles/ml
2.56	25.9	1.1	0.16

NOTE: TMA level under non-stress conditions is: 12.1% RME

TABLE 15

Urinary TMA levels of two fishy odour syndrome patients showing their responses to a choline load before and after synerlac treatment

Name	Choline load		Choline load after Synerlac	
	TMA μmoles/ml	RME %	RME %	TMA μmoles/ml
N.L.	0.46	12.1	15.0	0.90
M.H.	0.72	6.2	3.6	0.46

TABLE 16

Urinary TMA levels of two fishy odour syndrome patients showing their responses to a choline load before and after Duphalac treatment

Name	Choline load		Choline load after Duphalac	
	TMA $\mu\text{moles/ml}$	RME ^a %	RME ^a %	TMA $\mu\text{moles/ml}$
N.L.	2.99	15.6	7.8	0.93
R.G.	0.49	2.9	3.3	0.38

NOTE: TMA levels under non-stress conditions
are: N.L. 12.1% RME and
R.G. 16.7% RME

these determinations. In one patient (N.L.) TMA excretion was halved but was still above the normal range while the other patient's excretion of TMA was unaltered but was very close to the normal range to start with.

For both these treatments the results are inconclusive. However, the presence of some positive results suggests that there may be some instances when the use of these therapies may be advantageous. It is apparent that the condition of the intestinal flora when therapy is commenced will have an overwhelming effect on the results of the therapy. The flora may be so virulent that the new species introduced by Synerlac therapy do not survive in large numbers. Duphalac may be ineffective unless there are species present which can metabolise the synthetic disaccharide constituent. With this in mind the above results may be interpreted as follows: the therapies were successful only in those patients whose intestinal flora were in a sufficiently receptive state. Therefore it may be useful to first sterilize the gut with neomycin, which we have shown will eradicate the species which produce TMA, followed by either of the above therapies. It may also be useful to determine prior to these tests whether the bacterial species contained in Synerlac produce TMA from choline, as this use of Synerlac was not envisaged by the manufacturers.

Zellwegers Syndrome

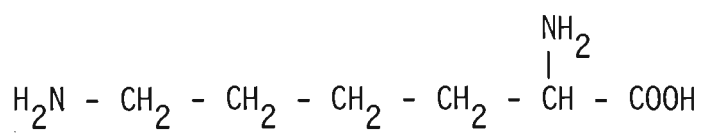
The distinctive cerebro-hepato-renal syndrome of Zellweger was defined by a series of publications in 1964-1968 (206-209). The clinical features and the pathologic changes in the brain and kidney have been very consistent. The syndrome is characterised by severe hypotonia, liver abnormalities, renal cortical cysts, cerebral dysfunction, characteristic facial appearance with hypertelorism and high forehead, eye abnormalities, skeletal malformation, failure to thrive and death in early infancy. Autosomal recessive inheritance is well established but the basic biochemical defect has not been clearly identified and the description and interpretation of the changes in the liver have varied.

An abnormal excretion of pipecolic acid, a metabolite of lysine as shown in Figure 38, was found in four patients from three different families (210). Increased amounts of tri- and dihydroxycoprostanic acid were also found in bile, serum and urine of patients with the Zellweger syndrome (211).

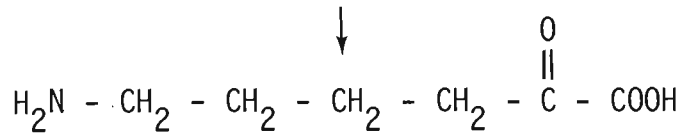
Urinary Volatiles

Patient C.S. was admitted to the Royal Children's Hospital, Melbourne, at one month of age. The Zellweger syndrome diagnosis was based on the characteristic appearance described above and the detection of pipecolic acid in the urine (88 micromoles/mmol creatinine).

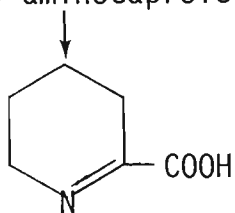
Suspecting a disturbance in the metabolism of lysine, the referring laboratory had analysed the urine by direct injection onto an alkaline FFAP GLC column and had observed a broad peak whose area appeared to increase



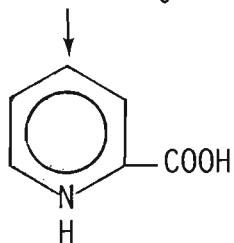
Lysine



2-keto-6-aminocaproic acid



Δ^1 - Piperidine 2-carboxylic acid



Pipecolic acid

FIGURE 38: A portion of the catabolic pathway of Lysine showing the derivation of pipecolic acid.

after the patient had undergone a lysine load. This presumed basic or neutral volatile compound was searched for by headspace chromatography, performed at alkaline pH.

Figure 39 shows the volatiles profile obtained from a urine sample (S13037) after this patient had undergone a lysine load. The identities of the peaks indicated are based on matching library spectra and the number in brackets after the compound identification is the confidence index computed by the probability based matching programme (63). The closer the confidence index approaches to unity the better is the match and it can be seen that all the matching spectra have an index of approximately 0.98. However even though this approach gave a highly probable match the identities of most of the peaks were not verified as standard compounds were not available for a comparison of GC retention times, and the isomeric structures of some of the compounds could not be deduced from their mass spectra. Thus these spectra and the matching library spectra for comparison are given in Appendix G.

With the reservation in mind that these identifications are only tentative until suitable authentic compounds can be analysed it can be stated that the appearance of such a wide variety of alcohols in the urinary volatiles is most unusual.

It is possible that the mixture of these alcohols would have chromatographed as the broad peak seen on the alkaline FFAP column.

The relationship that these compounds have with the syndrome, if any, or with the lysine loading is not clear. Bacterial involvement in producing the alcohols from precursor metabolites is indicated as the previously observed broad peak on the alkaline FFAP column was absent from

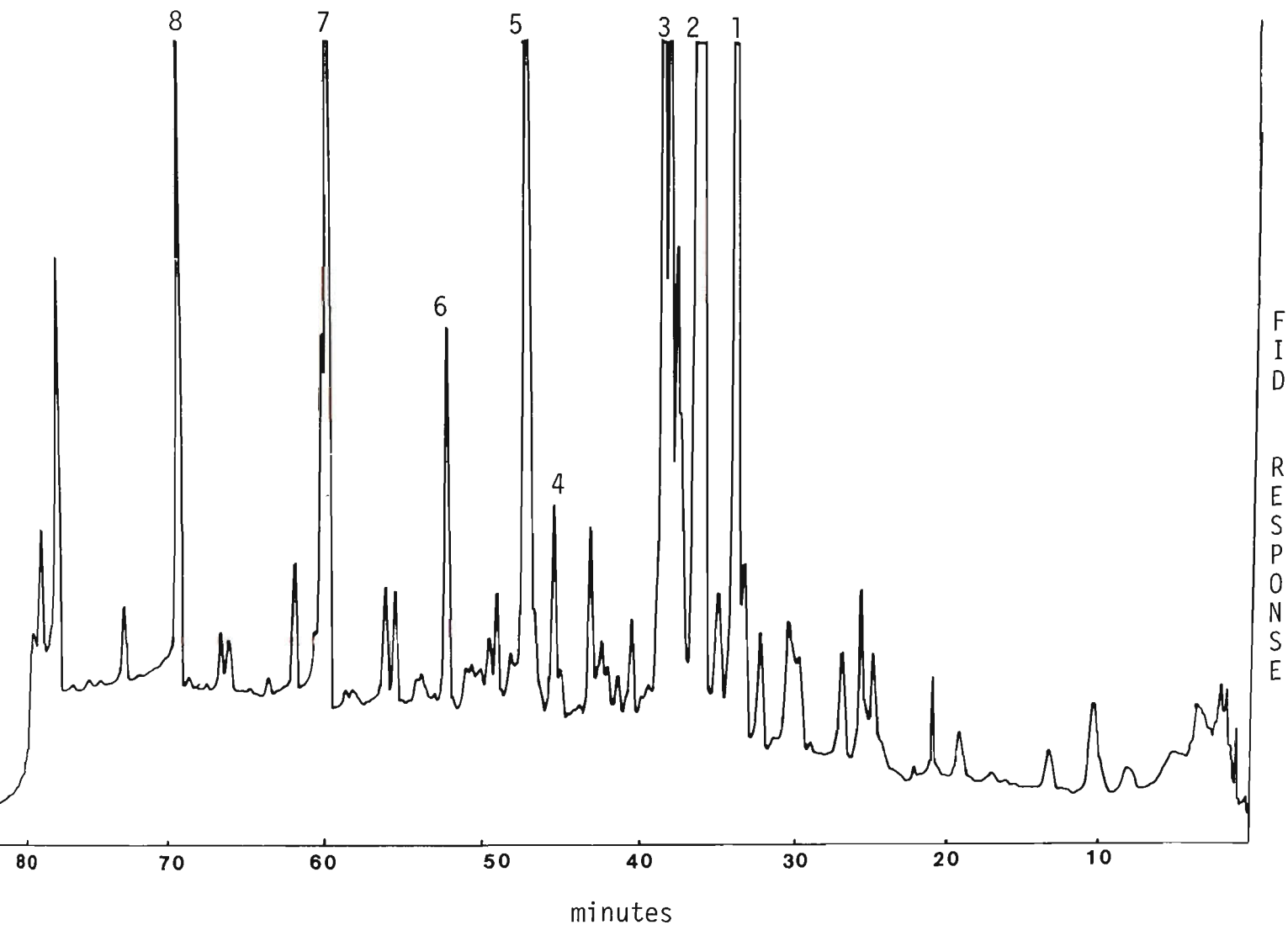


FIGURE 39: Volatiles profile of a patient (C.S.) with Zellweger's Syndrome. The volatiles were extracted from a 21 ml urine sample (pH = 14) by a 78.6 litre headspace (HS 854a)

Column: SCOT CW20M 40m

Temperature programme: 70°C 4 mins, 2°C/min to 198°C,
198°C 12 mins.

1 = 6-methyl-1-heptanol (0.979), 2 = 2-ethyl-1-hexanol (0.980),
3 = octylalcohol (0.981), 4 = acetophenone (0.980),
5 = nonylalcohol (0.981), 6 = 4-t-butylcyclohexanol (0.860),
7 = benzyl alcohol (0.981), 8 = unknown.

this patient's urine after intestinal sterilization with neomycin.

Determination of the source of these alcohols then may provide useful information as to the cause of this disorder.

CONCLUSIONS

Some Conclusions about the Value of the Volatiles Profile in Diagnosis

In each of the above cases of inborn errors of metabolism, the pathological profile was dominated by a few major components instead of the large number of minor components usually present in normal profiles. Such a simplified profile was entirely unexpected and made identification and subsequent diagnosis of these metabolic disorders surprisingly simple. Because of these large profile differences this technique is now used routinely in our laboratory for the diagnosis of inborn errors of metabolism. A further 12 cases of other known inborn errors of metabolism have now been examined by Professor Halpern's group and in each case a simple profile has been found. They also confirmed that in their hands this technique leads to highly reproducible profiles which are diagnostic of the disease studied.

The headspace trapping technique we used seemed to enhance the differences between the volatiles profiles of normals and patients with inborn errors of metabolism. This effect could have been due to the nature of the adsorption process of the organic compounds on the porous polymer (chromasorb 105) trap. Simpson (212), using a similar headspace system to ours for wine aroma analysis, has shown that the quantities of many of the compounds retained by the chromasorb 105 trap reaches an upper limit. If the adsorption sites are non-selective, i.e. a number of different types of compounds can be adsorbed by the same site, then the component with the greatest partial pressure in the headspace could saturate the adsorptive sites in the trap thus preventing the adsorption of minor components. Evidence that chromasorb 105 is non-selective has been presented by Murray (112) and the magnitude of the increase in concentration of diagnostic metabolites in the body fluids of patients with inborn errors of metabolism is often a factor of 10 or more (185). These two phenomena,

i.e. (1) non-specificity of adsorbent and (2) headspace dominated by only one or very few major components, may have combined to produce the chromatographic profiles which were so different to the normal.

Initially the time taken for a single headspace extraction was about 20 hours, 50 ml of urine was used and the total extract was used for a single GC analysis. However later results showed that enough material (of the major components at least) may be extracted from a greatly reduced sample volume and using a shorter extraction period. The results from the isovaleric acidemia case indicated that the use of a small volume of urine (200-500 μ l) was preferable to the larger volumes (50-100 ml) originally used.

When small sample volumes were used the water completely evaporated thus all the volatile components were swept into the trap. In some instances the recovery of volatiles was higher (based on GC peak areas) when a smaller volume of urine was used. This indicated that the solubility of some components in the aqueous phase of the headspace system was a limiting factor in the extraction process.

Our observation that three headspace extractions on the same 50 ml volume of a urine sample from the MSUD patient yielded the same profile also suggested that using large sample volumes was not efficient. These findings agree with that of Zlalkis and Kim (84) that complete removal of water from the sample is therefore superior.

There was some doubt that the technique as described was suitable for quantitative analysis, because one cannot assume that all the volatiles are collected on the adsorbent or that all the collected volatiles can be quantitatively desorbed. We thus believe that quantitation required the

addition of an internal standard to the sample prior to headspace chromatography. Since it is difficult to select a standard with the same volatility and adsorption properties as the analyte we think quantitation was best achieved using isotope dilution methodology.

By adjusting the headspace variables and using a labelled internal standard we were able to use this technique for the quantitative estimation of trimethylamine. The method we developed was reliable, used only small urine volumes (0.1-1 ml) and was very accurate. Because of the extreme volatility and polarity of trimethylamine no other method for its estimation has comparable accuracy, reliability or sensitivity.

The principle of this method could be extended to other compounds whose volatility causes problems with reproducibility. In these cases a different stable isotopically labelled internal standard would be needed, preferably not containing deuterium as the reduction in polarity may cause a significant difference in headspace distribution coefficient and GC retention time, and the extraction and sample transfer conditions could be modified to suit the different chemical properties.

Many of the components extracted by headspace chromatography from urine are ketones. Based on their observations of the time and temperature dependence on the yield of 4-heptanone from urine, Liebich and Huesgen (57) have suggested that the ketones may arise by decarboxylation of β -ketoacids. Truscott *et al.* (213) have also shown that the presence of 3 pentanone in the urine of patients with propionic acidemia is due to the decarboxylation of the β -keto acid, 3-keto-2-methylvaleric acid. In 20 hours and at 37°C, half the precursor was converted to the ketone. Even though we performed the headspace extraction at 40°C rather than the more common 100°C the decarboxylation of β -ketoacids could be

expected to increase if the urine sample was acidified and must be suspected when aldehydes or ketones are detected. Apart from decarboxylation of β -ketoacids, there have been no reports in the literature of, and this laboratory has not found any, artifacts introduced by this extraction procedure.

Another reason for cautious interpretation of the information in the volatiles profile was illustrated when we received one urine sample in which a dietary metabolite was mistaken for an organic aciduria. The volatiles profile for this patient (A.G.) is shown in Figure 40 where a very large peak identified as octanoic acid was observed. Later it was revealed that this patient had been placed on a formula food Pregestimil^R, which consists of protein hydrolysate, added amino acids and vitamins and medium chain triglycerides (214). Octanoic acid constitutes 25.8% of the total fatty acids in this formula food, which has also been found to induce excretion of 3-OH-butyric, adipic, suberic and sebacic acids (215).

This again illustrates the point made by Jellum (2) that when analysing biological fluids for diagnostic purposes, drug and special dietary therapy must be known.

Our discovery of the lactone of 4-hydroxyisovaleric acid in the urine of the patient with isovaleric acidemia illustrates an advantage of this technique. As with other profiling methods, previously undiscovered metabolites may be found and these may lead to a more complete understanding of the metabolic pathway under study. Thus headspace chromatography in its own right may have an important role to play in the study of inborn errors of metabolism as well as in the diagnosis of these diseases. This role may also be applicable to other areas of metabolism where conventional methods have failed to reveal the underlying biochemical abnormality.

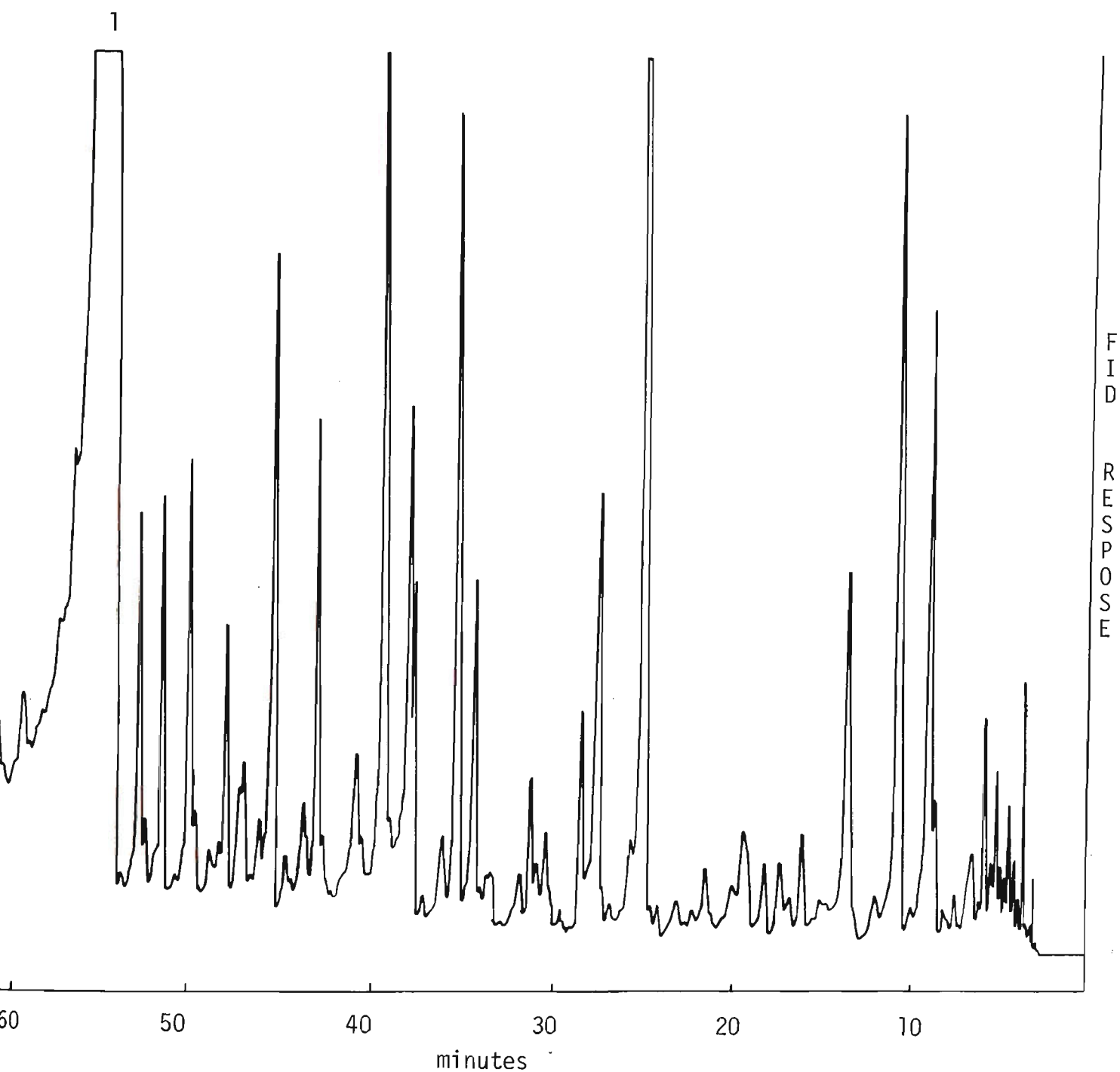


FIGURE 40: Urinary volatiles profile of patient A.G. The volatiles were extracted from a 20 ml urine sample (pH = 1) by a 50 litre headspace (HS 920a')

Column: SCOT CW20M (S.G.E., #586/156)

Temperature programme: 70°C 4 mins, 3°C/min. to 200°C

1 = Octanoic acid.

APPENDIX A

Modifications to gas chromatographs.

Modification of Gas Chromatographs

Materials

Column support cage, jet separator, glass lined stainless steel tubing (GLT), micro-needle valves, low hold up unions, zero-dead volume unions and tees, make up gas tees and capillary columns were supplied by Scientific Glass Engineering (SGE), Melbourne. All stainless steel (SS) tubing was obtained from Tube and Pipe Sales, Sydney. Swagelok and Cajon fittings were supplied by Sydney Valve and Fitting, Sydney; and the Micro-Dipper valves were obtained from Pakcard Instruments, Sydney.

Modification of Packard 419

Micro-dipper Valve

The Micro-Dipper valve was modified by lining the lower shaft with 9/64" ID x 1/4" OD SS tube to a distance of 1.5 cm from the end connected to the GC injection port. The upper shaft was lined with the same tube which continued beyond the top of this section by 3.7 cm. To the top of this tube a Cajon 2UT-A-4 ultra-torr adaptor was silver soldered. See Figure A1.

Carrier Gas Plumbing and Injection Port

The carrier gas inlet line to the injection port was replaced by a hypodermic SS tube (0.81 mm OD x 0.46 mm ID) to increase the column back pressure and thus allow the use of the existing flow control equipment.

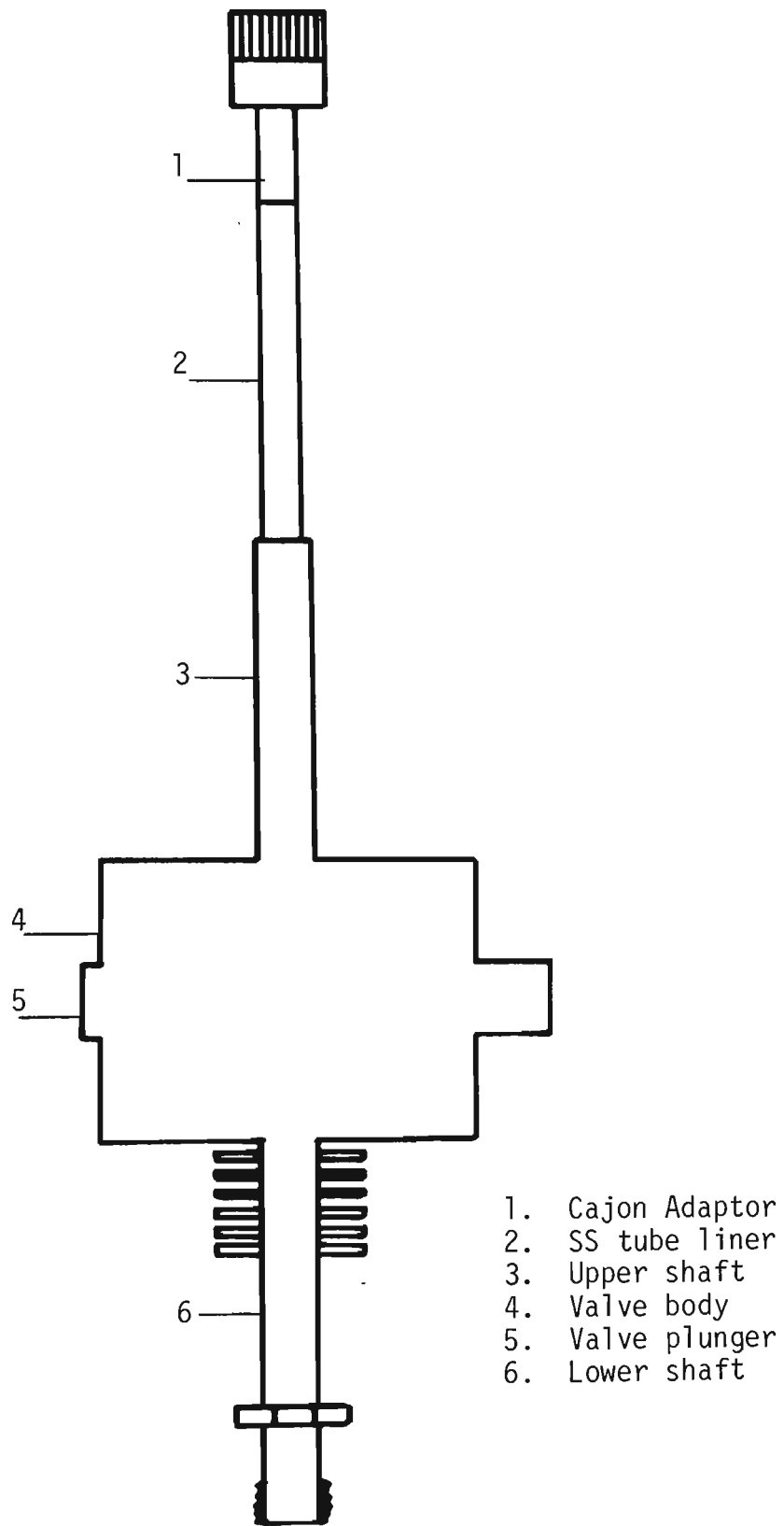


FIGURE A1: Microdipper Valve

The standard 6 mm OD x 3.57 mm ID SS insert was fitted to the injector block and the outlet fitting for this was cut in half and tapped to fit a 1/16" swagelok half union. From this union GLT (1/16" OD x 0.4 mm ID) was continued to the SGE low hold up union on the column support cage. The inlet side of the GC system did not include a stream splitter. See Figure A2.

The part of the inlet plumbing which protruded into the oven up to the inlet cold trap was insulated with glass wool and asbestos lagging. See Figure A3.

Column Outlet Plumbing

From the column outlet "make-up gas" tee piece GLT (1/16" OD x 0.6 mm ID) was taken to a low hold up tee. One side of this tee led through GLT (1/16" OD x 0.4 mm ID) to the FID base which had been fitted with an 1/8" to 1/4" adaptor while the other side was connected to a micro-needle valve whence by GLT (1/16" OD x 0.6 mm ID) through the detector block to vent outside the column oven. See Figure A3.

Column Inlet Cold Trap

A 3.5 cm hole was drilled in the oven lid over the GLT column inlet line and this was lined with a 14 cm length of SS tube (3.2 cm OD x 3.0 cm ID) to the end of which was fastened the GLT column inlet. See Figure A3.

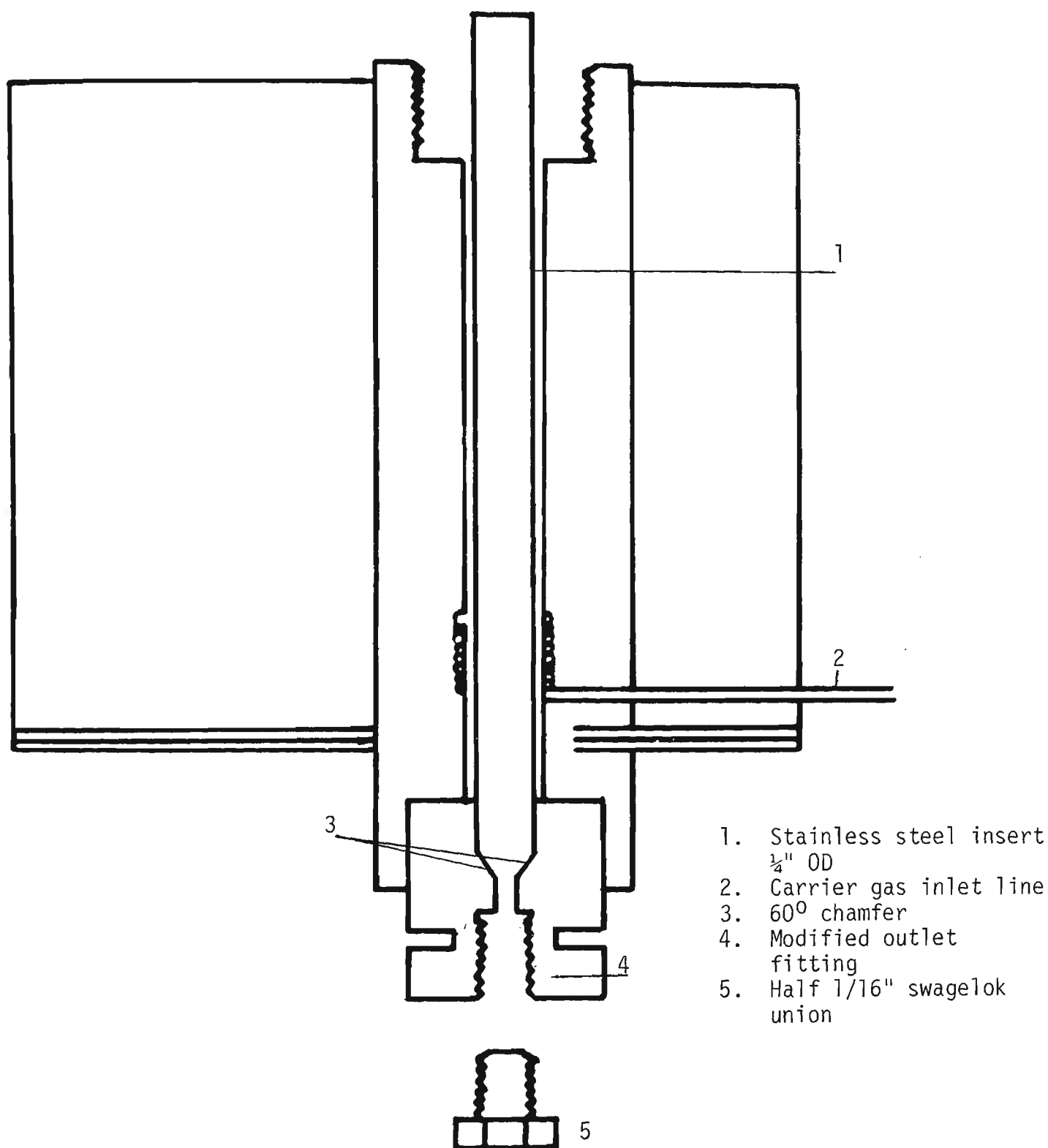


FIGURE A2: Packard 419 Injection Port

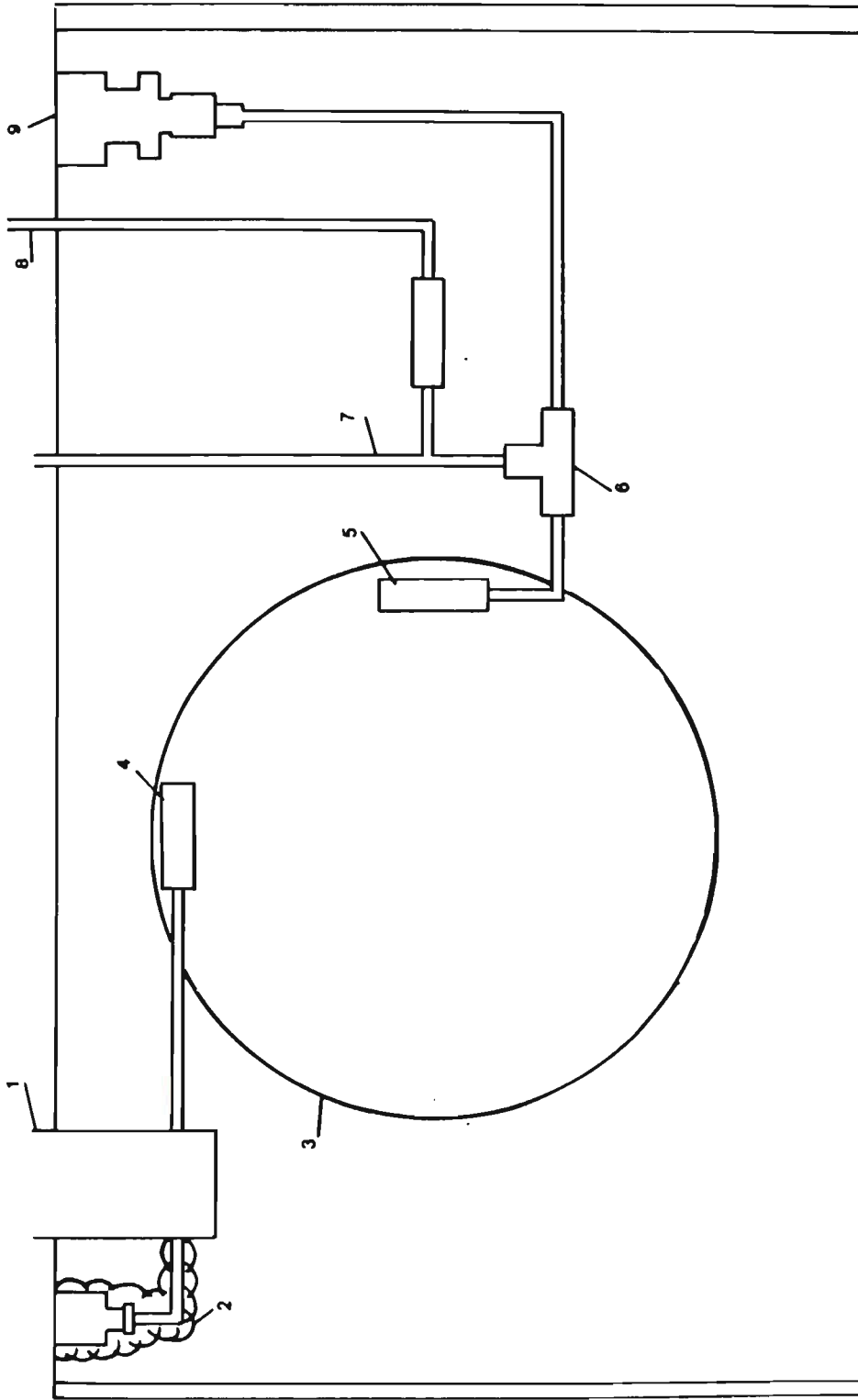


FIGURE A3: Column Oven. Packard 419

- | | |
|---|--------------------------------|
| 1. Cold trap | 6. Low hold up tee |
| 2. Insulation on GLT column inlet | 7. Micro needle valve |
| 3. Capillary column support cage | 8. External vent to atmosphere |
| 4. Low hold up union (connection to capillary column) | 9. FID |
| 5. Make up gas tee (column outlet) | |

Cold Probe

A 26 cm length of 1.2 cm ($\frac{1}{2}$ ") OD copper tube was plugged at one end with a 1.5 cm length of 1.2 cm OD channelled brass rod. The plugged end was insulated by sleeving with a 13 cm length of teflon tube (2.8 cm OD) channelled at the lower end. Liquid nitrogen was poured into the bore of the copper tubing when this probe had been lowered through the lined hole in the column oven lid so that the channel in the brass plug fitted snugly over the GLT column inlet. See Figure A4(a).

Hot Probe

An 8 cm length of 2.7 cm OD brass rod was channelled at one end to fit over the GLT column inlet, and insulated at the other end for handling. This probe was heated to about 150⁰C then inserted in the same manner as the cold probe. See Figure A4(b).

Modification of Varian 1800

Microdipper Valve

The lower shaft of the previously modified Micro-Dipper valve was replaced by a female threaded connector to the Varian GC injector block. See Figure A1.

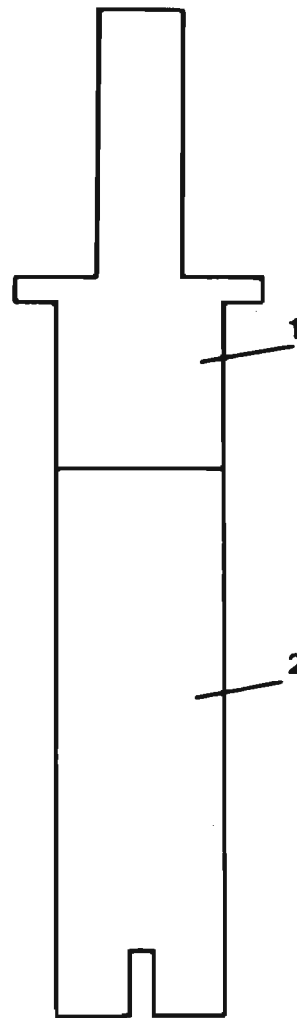
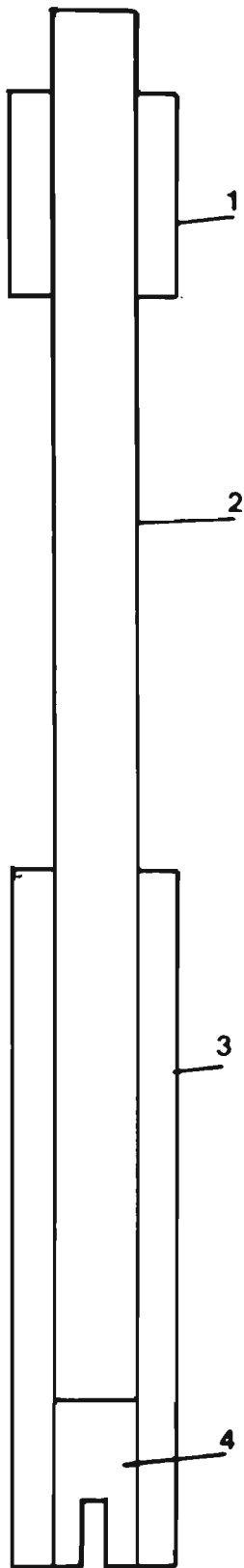


FIGURE A4: (a) Cold Probe

1. PTFE Insulation
2. Copper Tube
3. PTFE Insulation
4. Channelled brass plug

(b) Hot Probe

1. Asbestos handle
2. Channelled brass rod

Carrier Gas Plumbing and Injection Port

A 30 cm coil of SS tube (1/16" OD x 0.007" ID) was connected in series to the carrier gas line to increase the column back pressure and allow the use of the standard flow control system.

The standard Varian 1/4" OD x 9/64" ID SS injection port liner was connected to GLT (1/16" OD x 0.4 mm ID) sleeved at one end with a 4.5 cm length of 1/8" OD SS which protruded past the end of the GLT by 3 mm where it was chamfered to a 60° included angle to accept the collection tubes. Only 3 mm of the sleeved section protruded in to the column oven, the rest being housed in the injection port liner. That length of the column inlet GLT which extended from the column oven wall to the inlet cold trap was insulated by winding with glass wool and lagging with asbestos tape. See Figure A5.

Column Inlet Cold Trap

The column oven lid was replaced by two lateral sections; the rear section being attached to the automatic lid opening device while the front section had two 3.5 cm holes lined with SS tube which was connected to the column inlet GLT. The cooling and heating probes were inserted through these holes for either column. See Figure A5.

Column Outlet Plumbing

The left hand side (LHS) column outlet from the make up gas tee was GLT (1/6" OD x 0.6 mm ID) which led through the column and detector oven

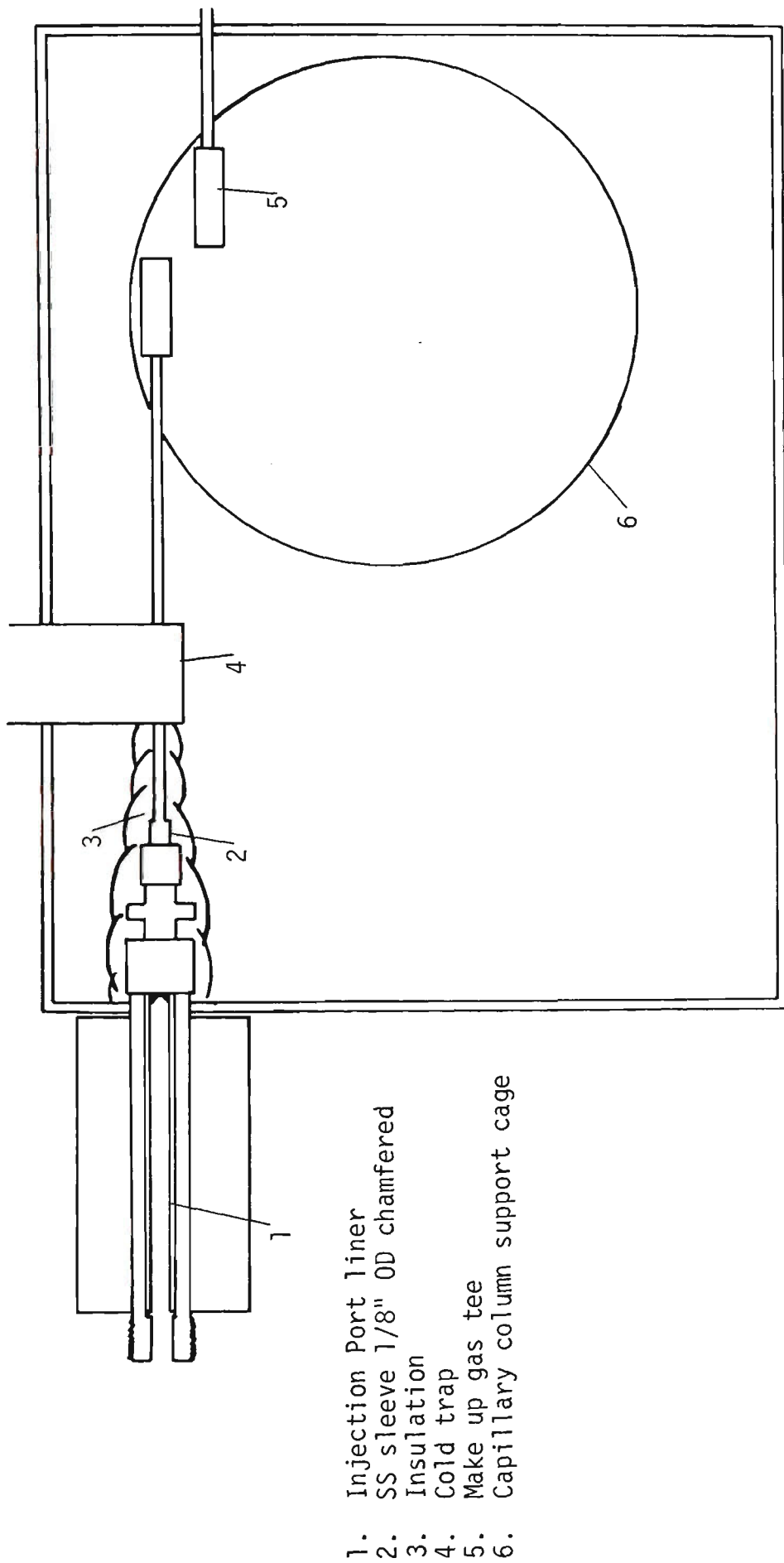


FIGURE A5: Injector block and column oven. Varian 1800

walls to a zero dead volume tee in the detector oven. A Micro-Needle valve was connected to one side of the tee and from this GLT (1/16" OD x 0.4 mm ID) sleeved at one end with 1/4" OD SS tube led to the FID. The other arm of the tee was connected directly to a 40 cm length of GLT (1/16" OD x 0.6 ID) which passed through the detector oven wall and terminated on the outside of the gas chromatograph in a 1/16" to 1/8" adaptor. See Figure A6.

The split was variable from 100% to the FID by blocking the vent outlet, to 50/50 with the needle valve completely open and the vent outlet unblocked and from 50/50 infinitely variable to 100% to vent by adjustment of the micro-needle valve.

The outlet line from the right hand side (RHS) column led through the oven wall to a zero dead volume tee in the detector oven. The arm of the tee which led to the mass spectrometer (MS) had the micro-needle valve in series and was constructed of a wider bore GLT (1/16" OD x 0.6 mm ID) than that which led to the FID (1/16" OD x 0.4 mm ID). Thus the split on this side was infinitely variable from 0-100% to the MS. See Figure A6.

GC-MS Interface

The GC-MS interface consisted of a jet separator which was connected via 1/4" to 1/8" adaptors with graphite on both sides to (1/8" OD x 1.8 mm ID) lines. On the GC side of the jet the GLT outlet of the column was inserted into the bore of the 1/8" GLT and the seal was made by an 1/8" to 1/16" adaptor. The GLT (1/8" OD x 1.8 mm ID) on the MS side extended through the source GC inlet to a distance of approximately 2 mm from the Faraday cage.

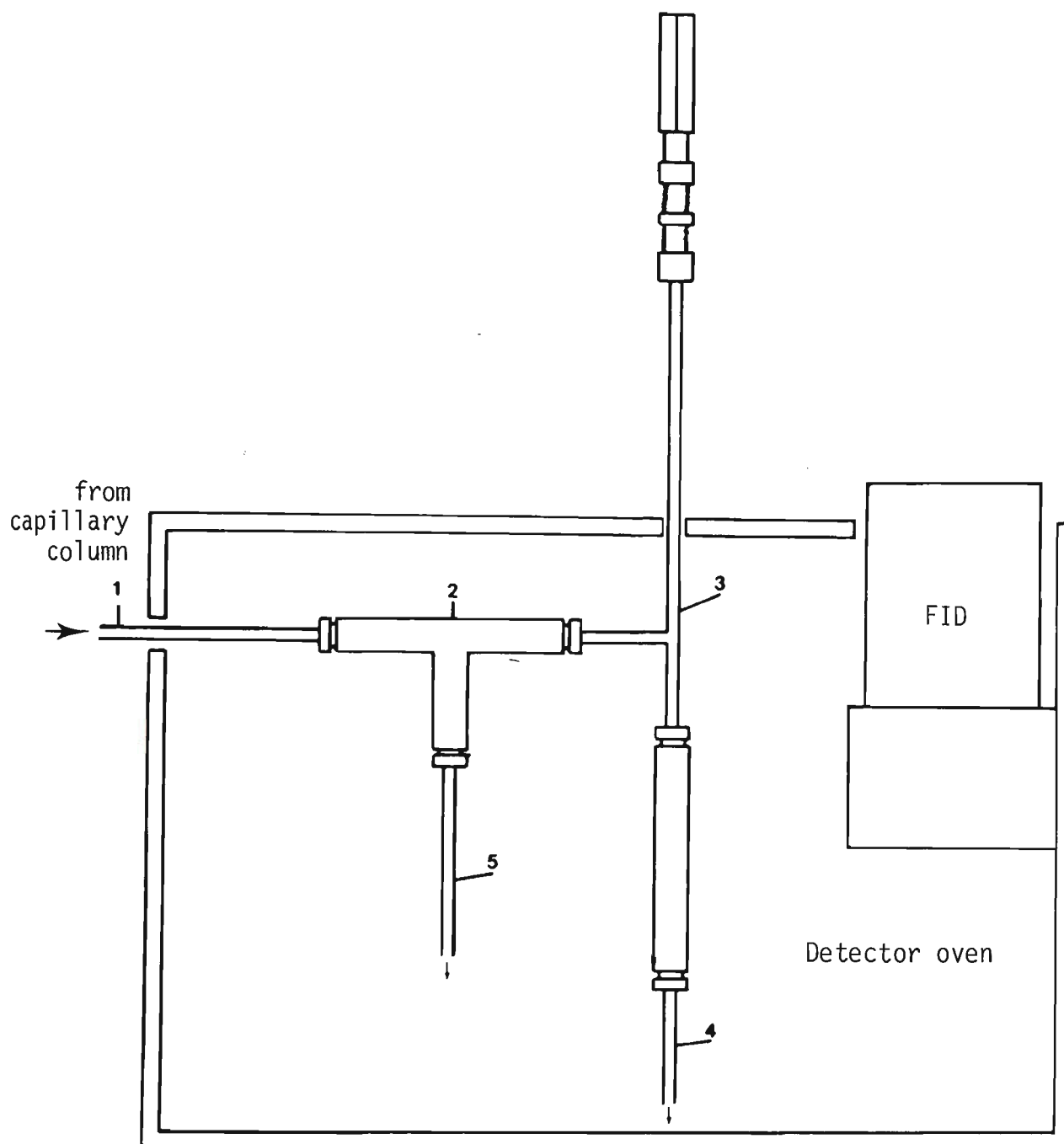


FIGURE A6: Varian 1800 Outlet Plumbing

- | | |
|-------------------------|-------------------------|
| 1. GC column outlet | 2. Zero dead volume tee |
| 3. Micro needle valve | 4. * LHS = line to FID |
| 5. * LHS = line to vent | RHS = line to MS |

* The GC was dual column. LHS refers to the column plumbing for the left hand side; RHS refers to the column plumbing for the right hand side.

An Edwards ED 50 2 stage rotary vacuum pump was connected to the vacuum arm of the jet separator, via a Nupro $\frac{1}{4}$ " on/off valve.

APPENDIX B

Operating system variables for trimethylamine analysis.

APPENDIX B

Operating system changes to eliminate noise correction calculations

<u>LOCATION</u>	<u>CHANGED TO (OCTAL)</u>
NLOOP + 22 ₈	JMP .+2 (402)
NOISE + 2	JMP .+4 (404)
INREAD + 4	JMP .+2 (402)
MSRND + 6	JMP .+7 (407)
MSRND + 25 ₈	JMP .+5 (405)

Responses (underlined) to prompts, issued by the Woolongong Quad-Mag-Nova operating system, for quantitative selected ion monitoring in the trimethylamine analysis.

OPTION: S

LOW MASS RANGE? Y

ENTER FILE NAME

HS XXXX LMR DIS L20 5P EE20V

INTEG GAIN: L

INTEG TIME (MSECS) = 20

COMPLETE SCAN? N

MASS VALUES

ENTER LIST

59, 60

DOUBLE INT TIME AT M = 100

SAMPLES PER PEAK = 5

NUMBER OF SCANS = 100

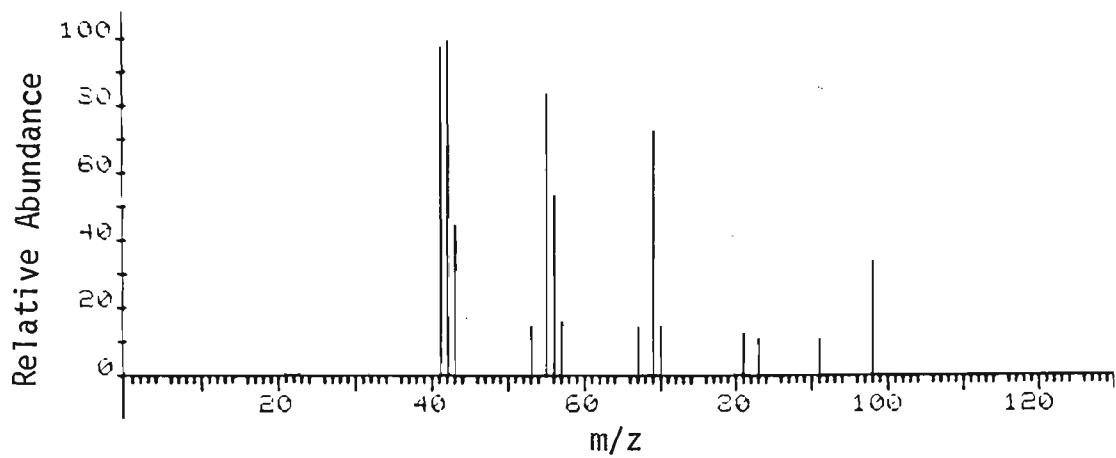
DISPLAY? N

DELAY TIME (SECS) = 0

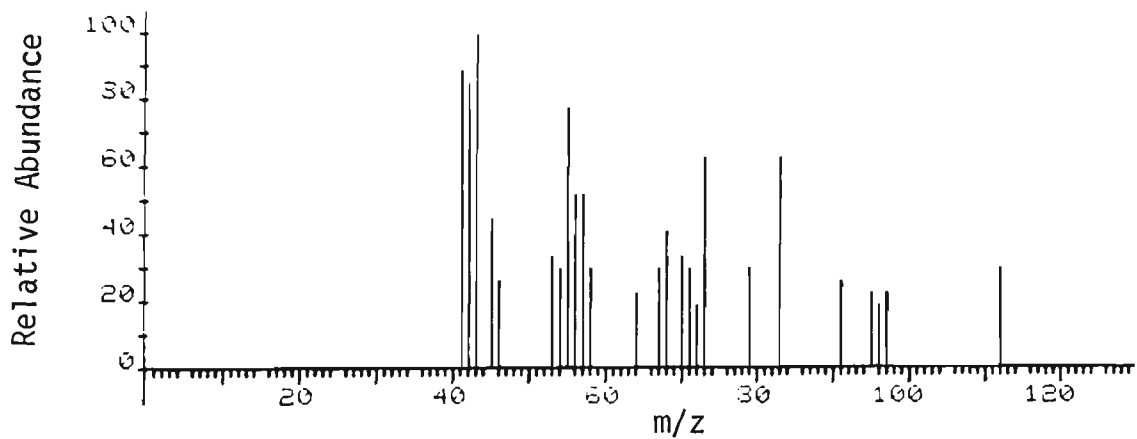
GC PLOT? N

APPENDIX C

Mass spectra of unknown peaks in Figure 8, page 56.

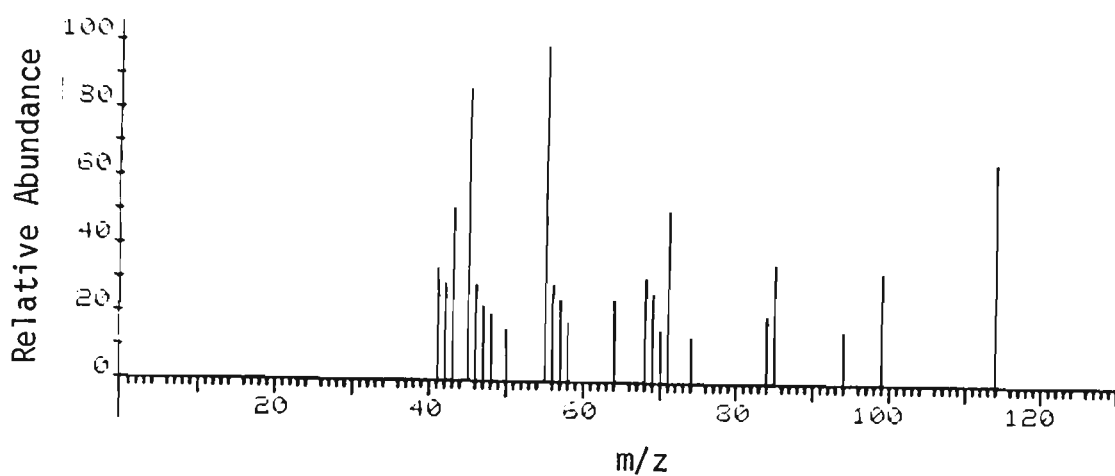


Unknown 1. Retention time 14.4 min (a furaldehyde).

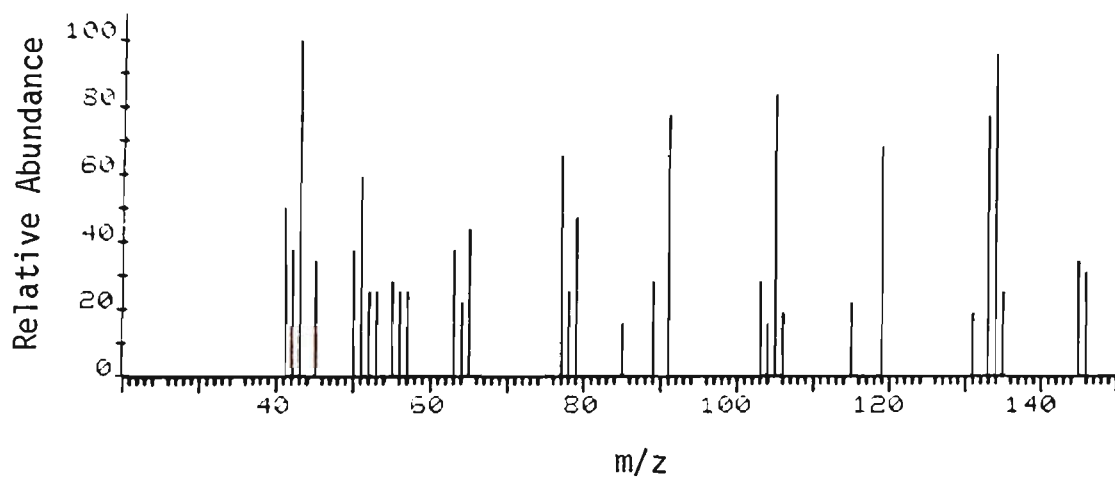


Unknown 2. Retention time 21.6 min.

Mass spectra of unknown peaks in Figure 8, page 56.

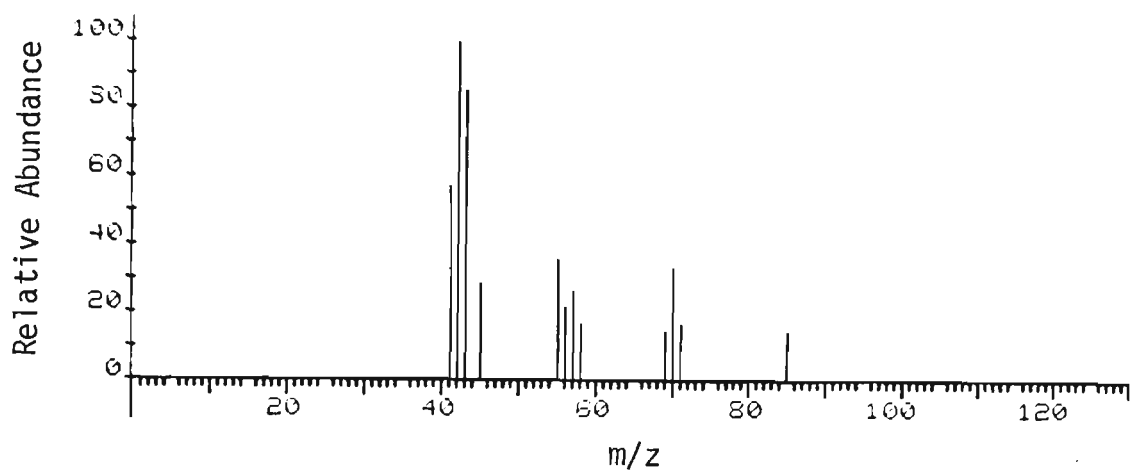


Unknown 3. Retention time 45.2 min.
(methyl-tetrahydrofurfural)

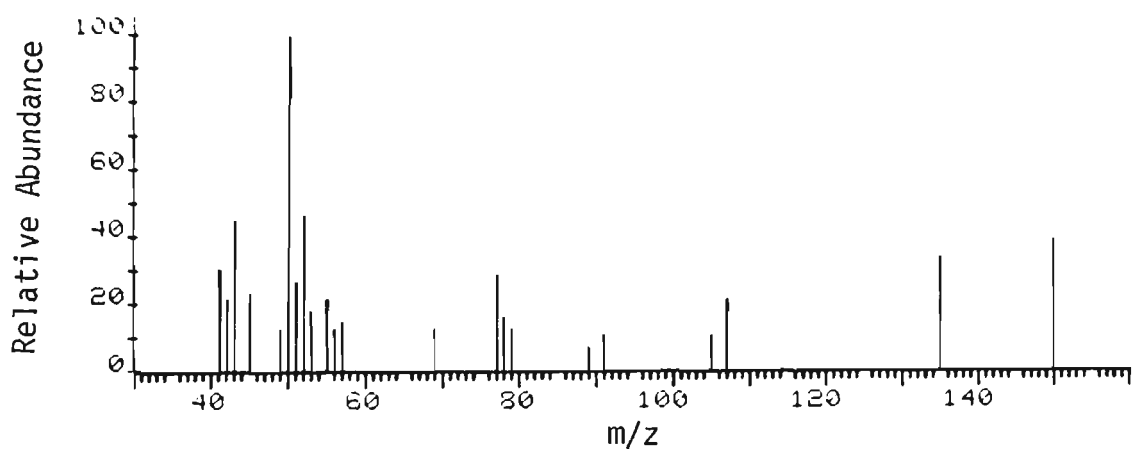


Unknown 4. Retention time 46.2 min.

Spectra of peaks which could not be identified from normal urine.



Unknown 5. Retention time 50.0 min.
(a lactone or a tetrahydrofuran)

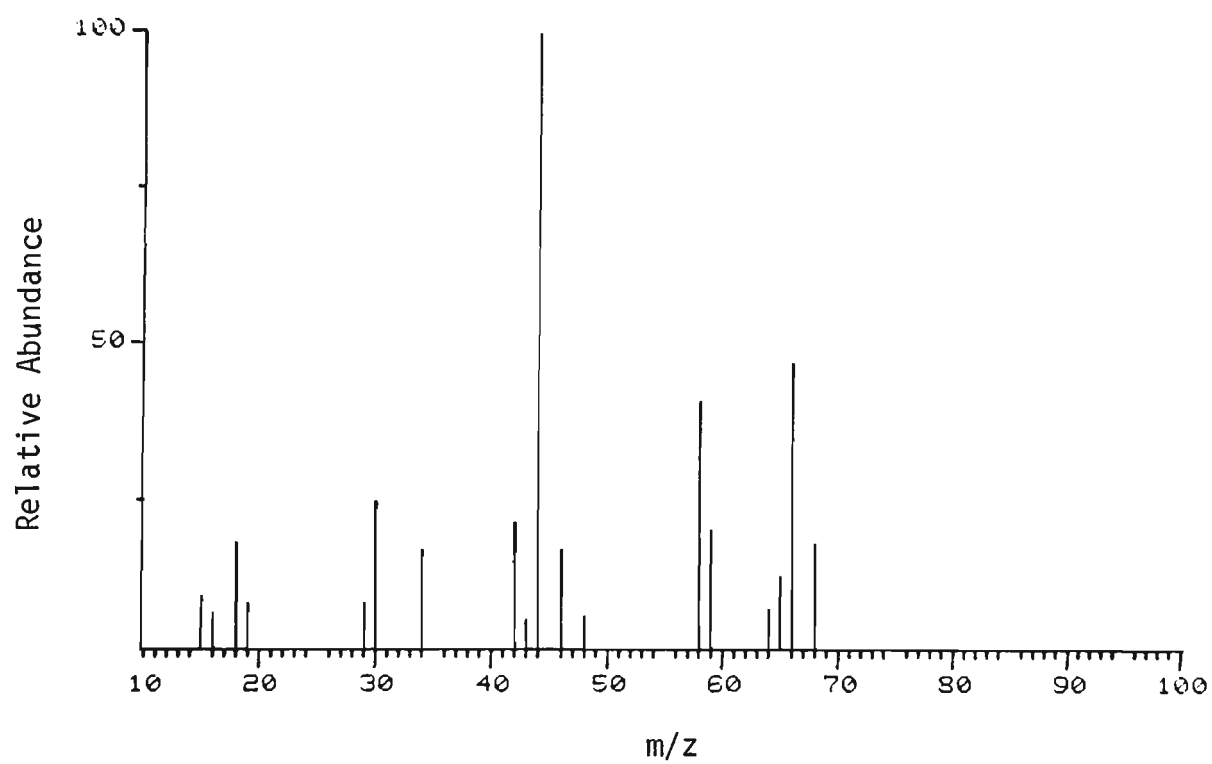


Unknown 6. Retention time 78.0 min.

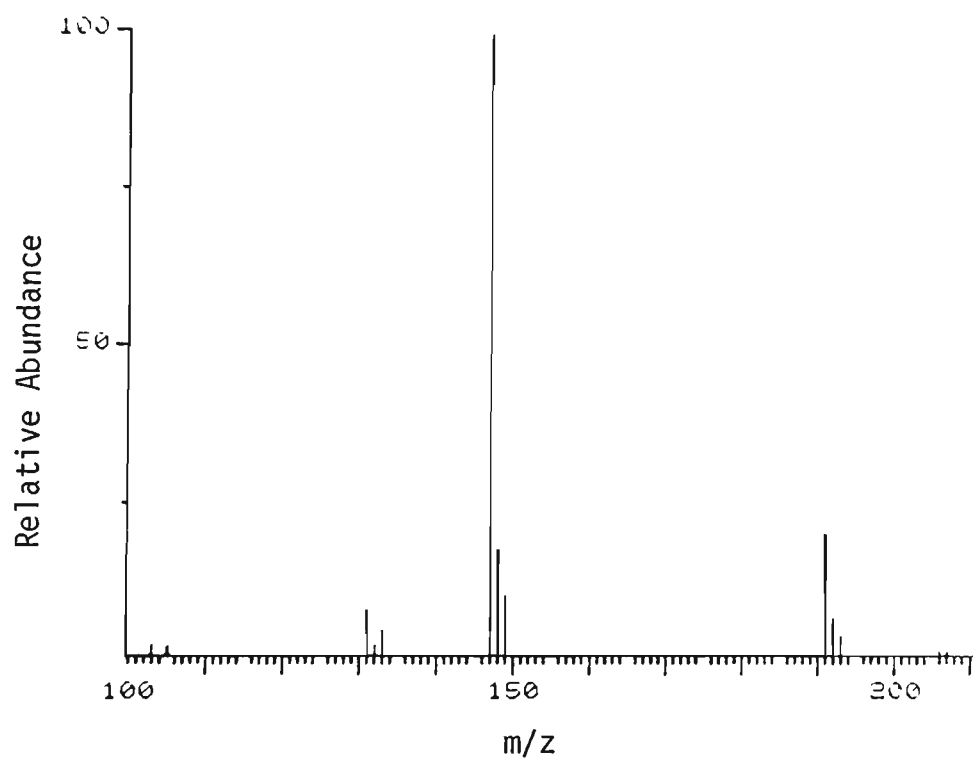
Spectra of peaks which could not be identified from normal urine.

APPENDIX D

Mass spectra of unknowns from Figure 14, page 71 and Figure 18, page 76.



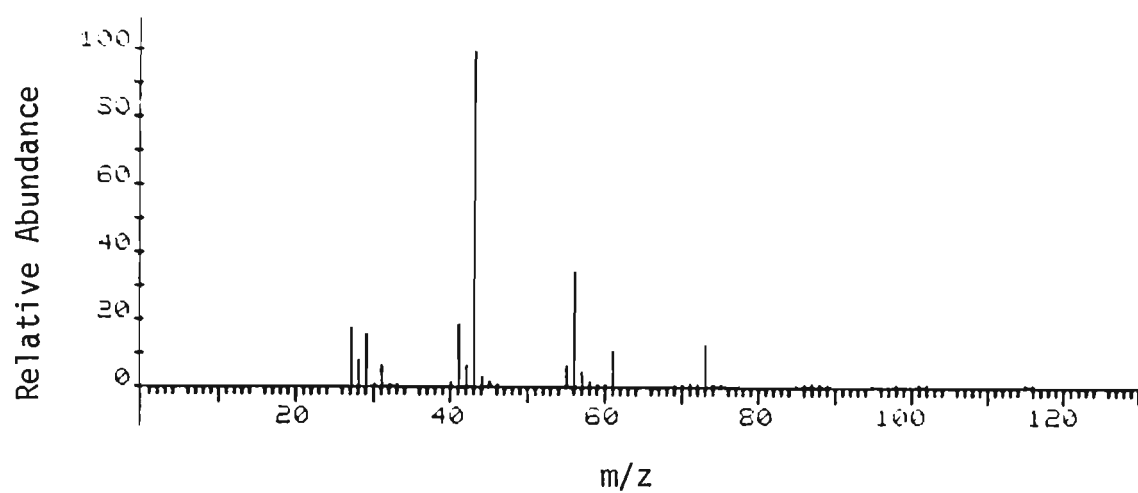
Mass spectrum of peak 3, Figure 14.



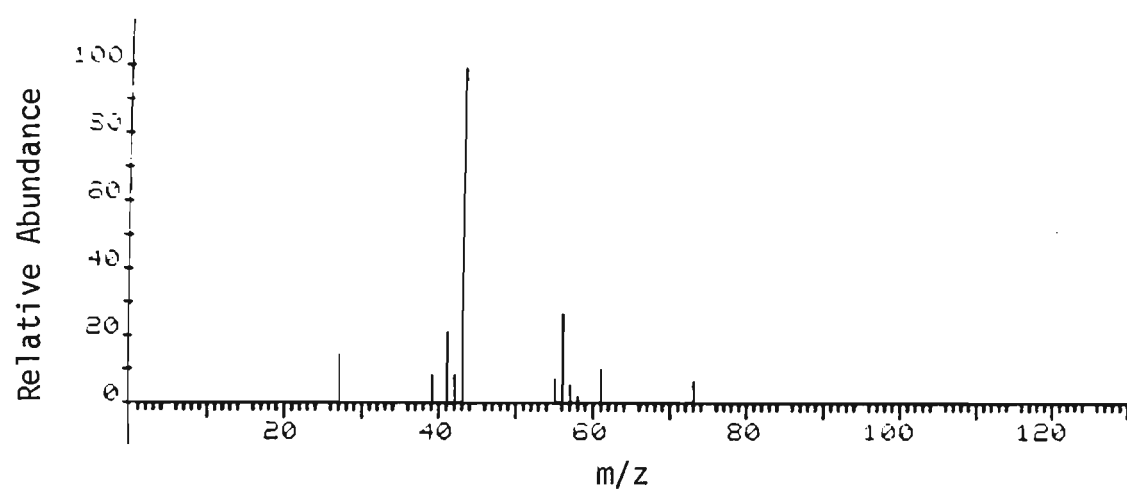
Mass spectrum at peak 2, Figure 18. The peak was collected as it eluted from a capillary column, derivatised with BSTFA, and the derivatised material analysed by GC-MS.

APPENDIX E

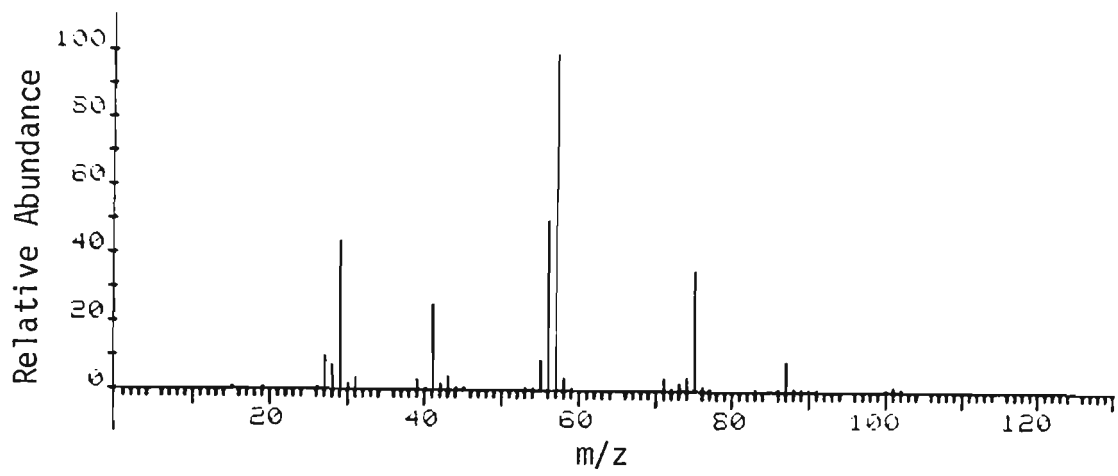
Mass spectra of the butyl esters of the seven short chain fatty acids
shown in Figure 23, page 89.



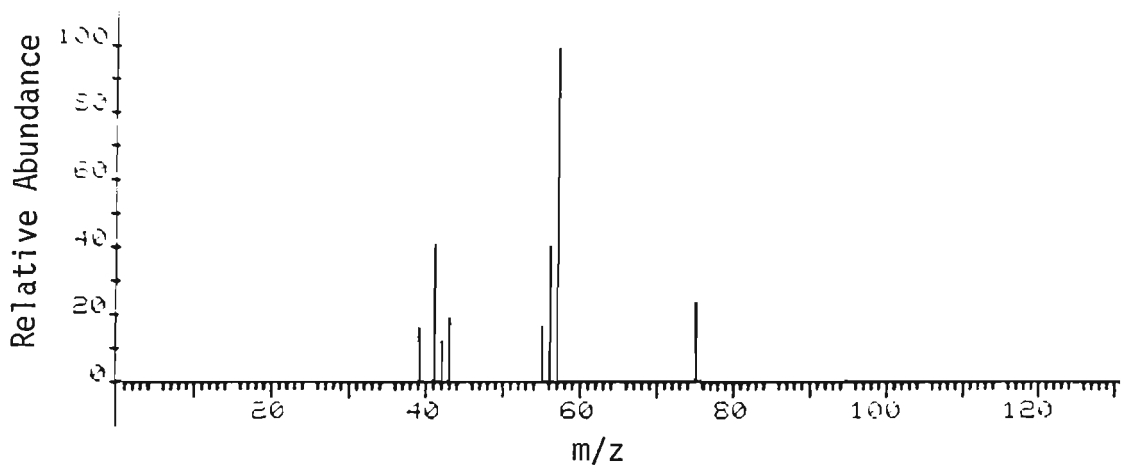
Library mass spectrum of butyl acetate.



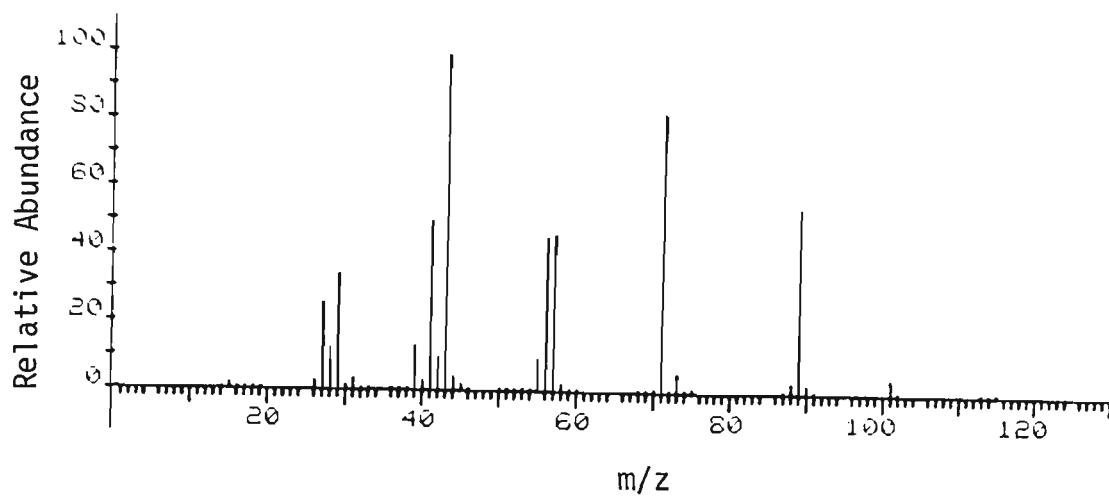
Mass spectrum of peak 1, Figure 23.



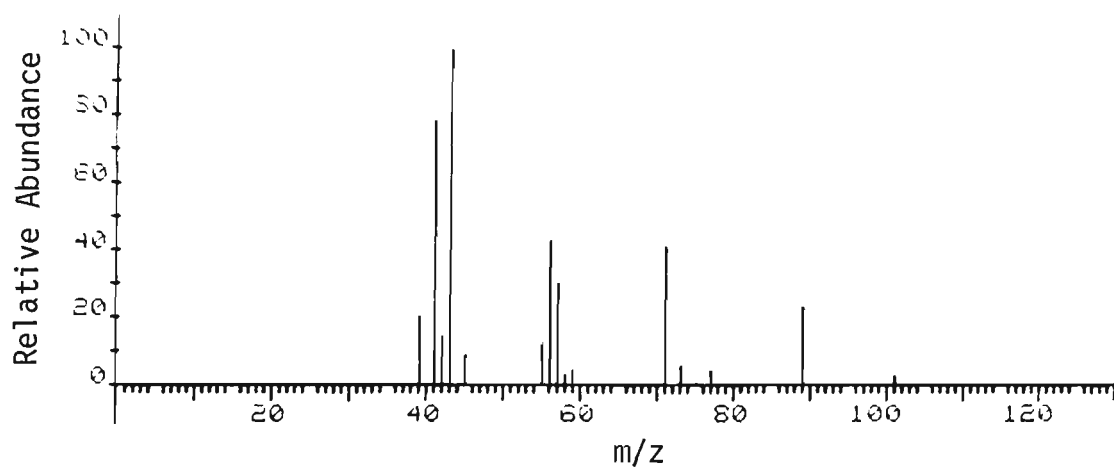
Library mass spectrum of butyl propionate.



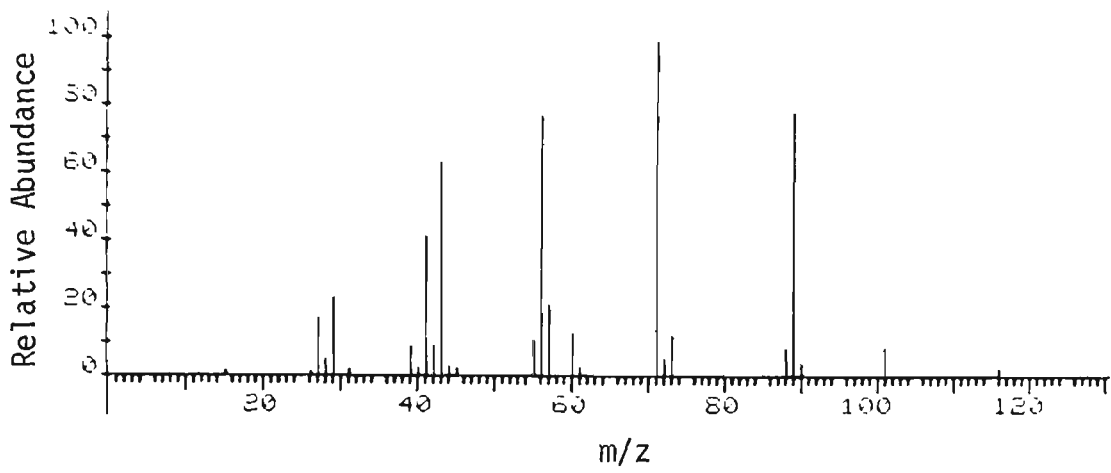
Mass spectrum of peak 2 Figure 23.



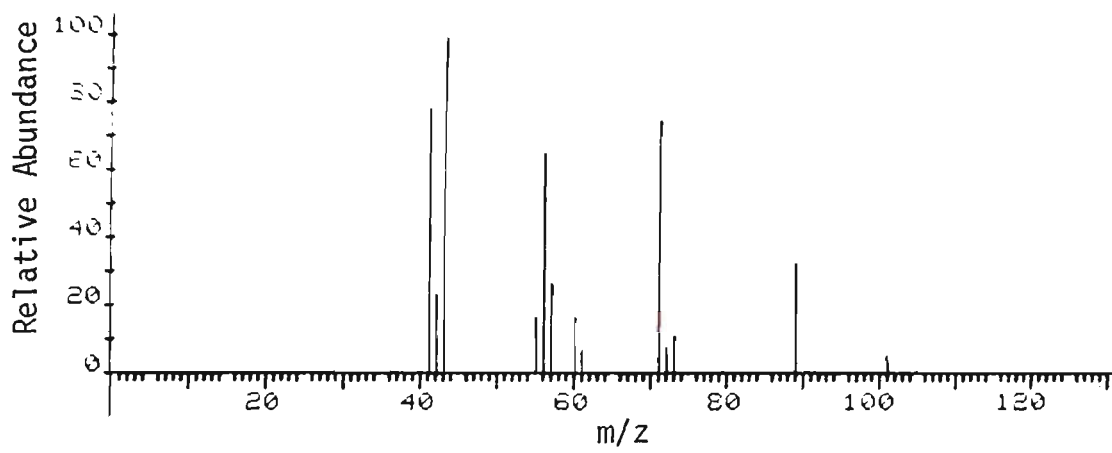
Library mass spectrum at butyl isobutyrate.



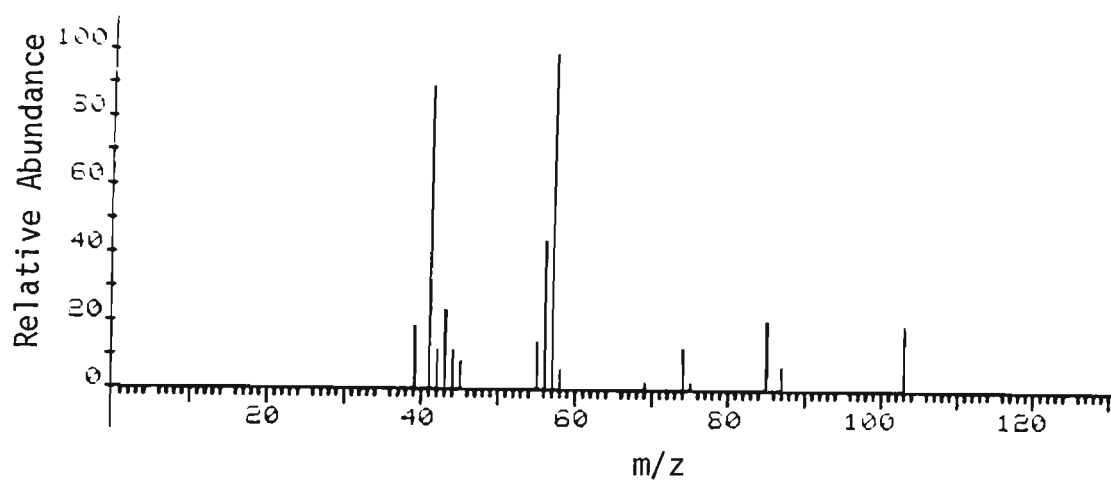
Mass spectrum at peak, Figure 23.



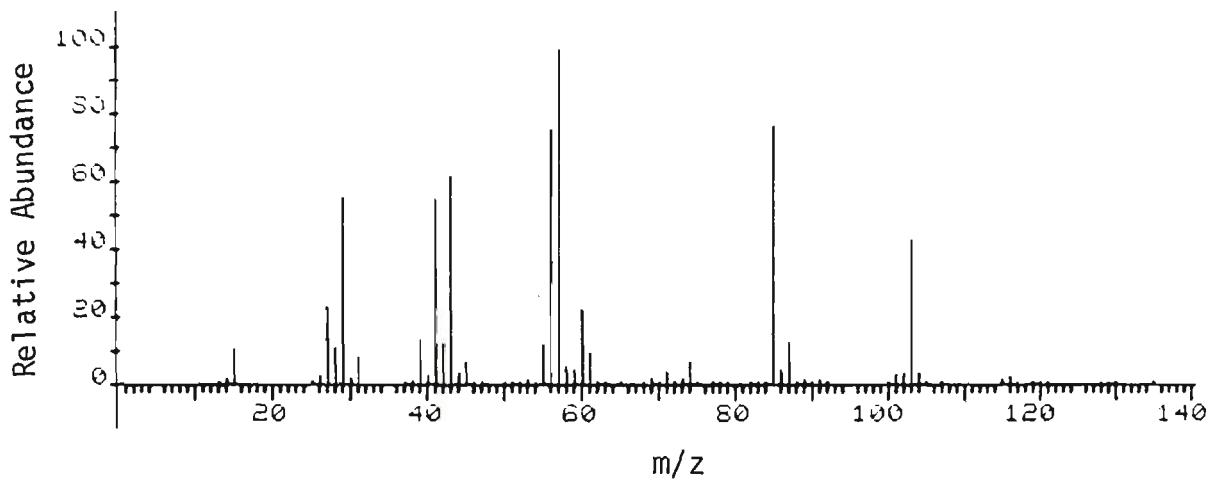
Library mass spectrum of butyl butyrate.



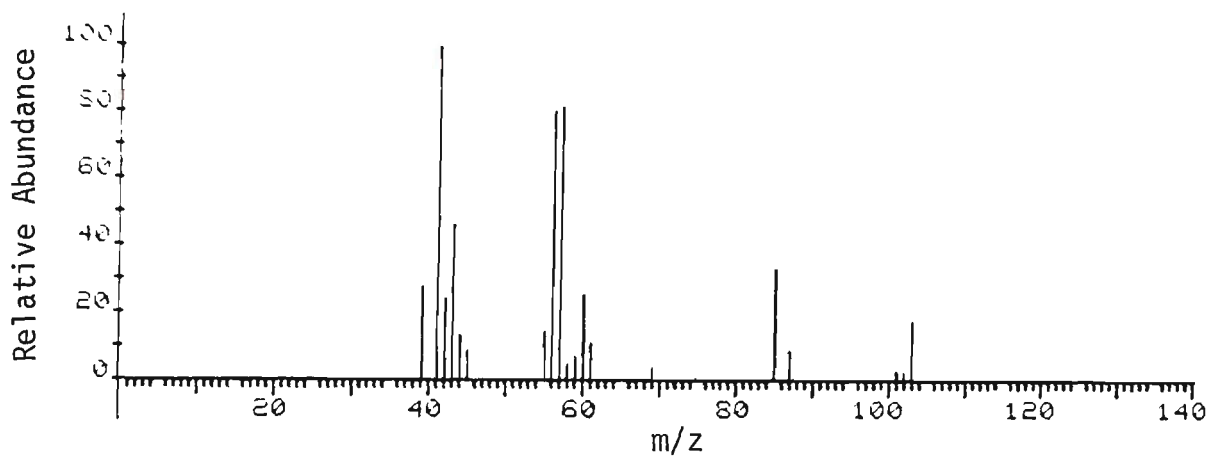
Mass spectrum of peak 4, Figure 23.



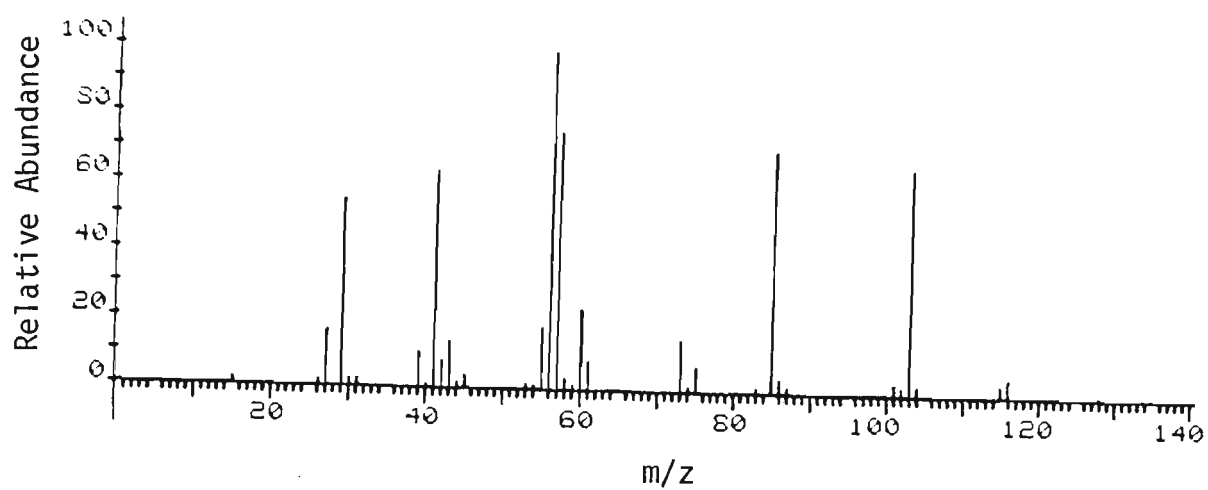
Mass spectrum of peak 5, Figure 23. A library mass spectrum of this compound (butyl 2-methylbutyrate) was not available.



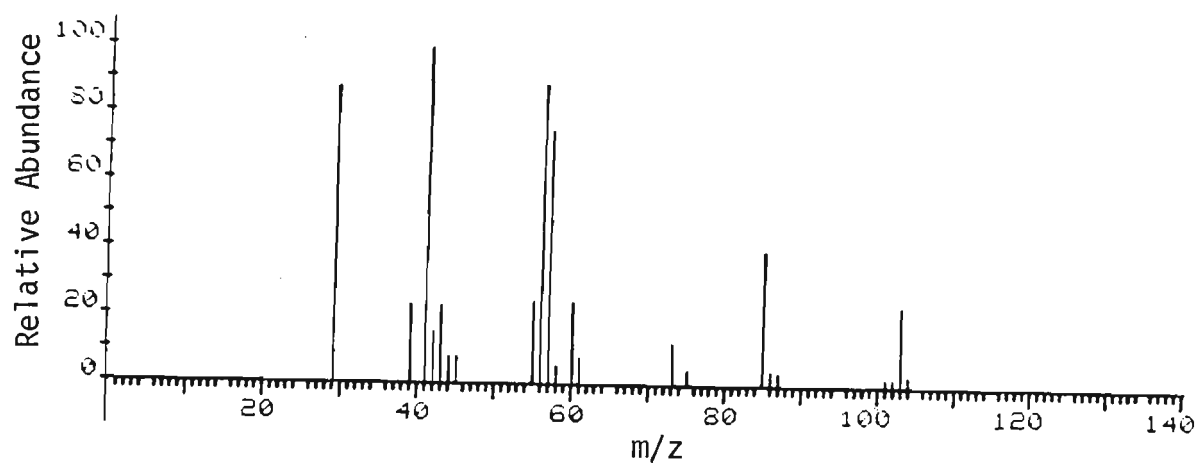
Library mass spectrum of butyl isovalerate.



Mass spectrum of peak 6, Figure 23.



Library mass spectrum of butyl n-valerate.



Mass spectrum of peak 7, Figure 23.

APPENDIX F

Tables from trimethylaminuria studies.

TABLE 5

Portion of the Computer print-out for the ratio of abundances of the ions
m/z 59 and m/z 60 for experiment HS840A.

NUMERATOR MASS = 59 DENOMINATOR MASS = 60

SPECT. NO.	NUM. INT.	DEN. INT.	RATIO
20	+0000002631	+0000001313	+.2003808E+01
21	+0000002564	+0000001285	+.1995331E+01
22	+0000002488	+0000001250	+.1990400E+01
23	+0000002422	+0000001215	+.1993415E+01
24	+0000002347	+0000001177	+.1994053E+01
25	+0000002275	+0000001141	+.1993865E+01
26	+0000002202	+0000001104	+.1994565E+01
27	+0000002132	+0000001071	+.1990663E+01
28	+0000002052	+0000001034	+.1984526E+01
29	+0000001990	+0000001000	+.1990000E+01
30	+0000001928	+0000000967	+.1993795E+01
31	+0000001862	+0000000937	+.1987193E+01
32	+0000001799	+0000000906	+.1985651E+01
33	+0000001751	+0000000879	+.1992036E+01
34	+0000001699	+0000000857	+.1982497E+01
35	+0000001644	+0000000834	+.1971223E+01
36	+0000001603	+0000000811	+.1976572E+01
37	+0000001560	+0000000789	+.1977186E+01
38	+0000001518	+0000000767	+.1979140E+01
39	+0000001475	+0000000749	+.1969292E+01
40	+0000001440	+0000000729	+.1975308E+01
41	+0000001408	+0000000716	+.1966480E+01
42	+0000001382	+0000000701	+.1971469E+01
43	+0000001351	+0000000684	+.1975146E+01
44	+0000001322	+0000000667	+.1982009E+01
45	+0000001287	+0000000653	+.1970903E+01
46	+0000001257	+0000000636	+.1976415E+01
47	+0000001230	+0000000621	+.1980676E+01
48	+0000001198	+0000000612	+.1957516E+01
49	+0000001167	+0000000596	+.1958053E+01
50	+0000001141	+0000000581	+.1963855E+01
51	+0000001114	+0000000566	+.1968198E+01
52	+0000001084	+0000000552	+.1963768E+01
53	+0000001059	+0000000539	+.1964749E+01
54	+0000001039	+0000000524	+.1982824E+01
55	+0000001012	+0000000514	+.1968871E+01

MEAN = +.1970487E+01

SD = +.1549162E-01

SD% = +.7861

HS 840A L20 DIS 5P EE19 5V

14/9/78

TABLE 6

Determination of Ratio Q, $\frac{[M + 1]^+}{[M]^+}$ of unlabelled TMA ($M^+ = m/z$ 59)

No. of scans	Ratio	Coefficient of variation (%)
80	0.063	1.7
76	0.062	2.1
64	0.061	2.8
71	0.052	2.3
50	0.061	3.1
\bar{x}	0.060	

TABLE 7

Determination of Ratio $S, \frac{[M-1]^+}{[M]^+}$ of labelled TMA ($M^+ = m/z 60$)

No. of scans	Ratio	Coefficient of variation (%)
50	1.24	0.3
50	1.29	0.4
50	1.28	0.5
50	1.26	0.3
50	1.33	0.4
50	1.34	0.5
50	1.30	0.2
50	1.26	0.2
50	1.33	0.3
50	1.39	0.3
50	1.42	0.3
50	1.26	0.2
53	1.29	0.4
51	1.32	0.7
49	1.29	0.2
49	1.29	0.2

$$\bar{x} = 1.31$$

$$\sigma = 0.05$$

Coefficient
of variation = 3.70%

TABLE 8

Determination of the Ratio S during the analysis of a batch of 4 duplicate samples. Each of the values reported below is the average of 50 scans

PRE*	MID†	POST‡	HS Nos.
1.32	1.29	1.29	818-821
1.30	1.27	1.28	822-825
1.29	1.31	1.33	826-829
1.30	1.37	1.36	830-833
1.31	1.29	1.31	834-837
1.34	1.31	1.30	906-909

*PRE = ratio S before any samples were analysed

†MID = ratio S after one of each of the 4 samples was analysed

‡POST = ratio S after the second of each of the 4 samples was analysed

Equations used in linear regression analysis

$$1. \text{ Slope } m = \frac{n \sum y_i x_i - \sum x_i \sum y_i}{n \sum x_i^2 - (\sum x_i)^2}$$

$$2. \text{ Intercept } b = \frac{\sum x_i^2 \sum y_i - \sum x_i \sum x_i y_i}{n \sum x_i^2 - (\sum x_i)^2}$$

$$3. \text{ Correlation Coefficient } r_{xy} = \frac{n \sum x_i y_i - \sum x_i \sum y_i}{\left[(n \sum x_i^2 - (\sum x_i)^2)(n \sum y_i^2 - (\sum y_i)^2) \right]^{1/2}}$$

$$4. \text{ Standard error of the estimate } \sigma_x^1$$

$$\sigma_x^1 = \sigma_x \sqrt{1 - r_{xy}^2}$$

where σ_x is the standard deviation of the x values predicted by the experimentally obtained y values.

Second Calibration Curve for Trimethylamine (TMA)

Amount of TMA (μ moles)	Peak Height Ratio (R_a)
0.09	0.29, 0.31
0.17	0.69, 0.73
0.26	0.98, 1.01
0.34	1.53, 1.58
0.43	1.94, 2.10
0.52	2.48, 2.58
0.60	3.06, 3.12

Y intercept = -0.269
slope = 5.430
correlation coefficient = 0.995

Third Calibration Curve for Trimethylamine (TMA)

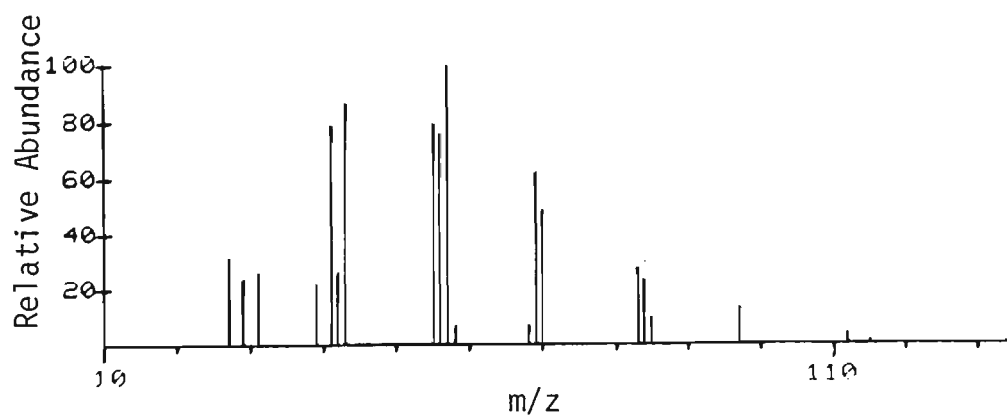
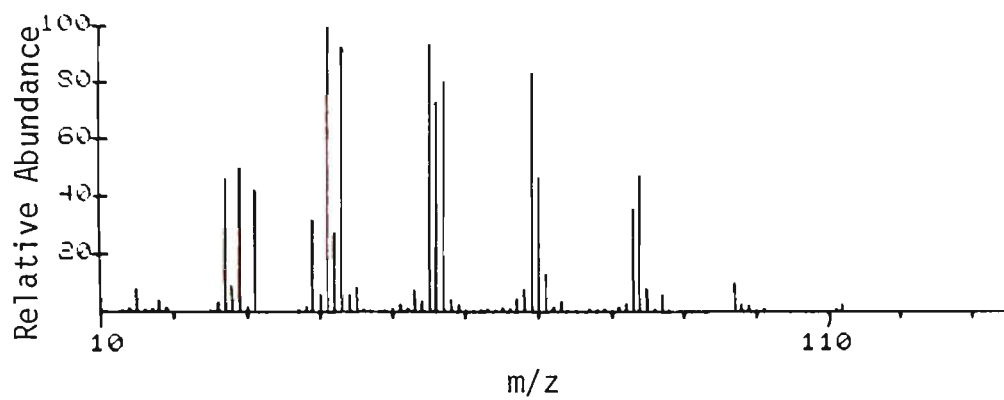
Amount of TMA (μ moles)	Peak Height Ratio (R_a)
0.30	1.29
0.50	2.37, 2.34
0.60	2.81, 2.79
0.70	3.25, 3.22

Y intercept = -0.098
slope = 4.810
correlation coefficient = 0.998

APPENDIX G

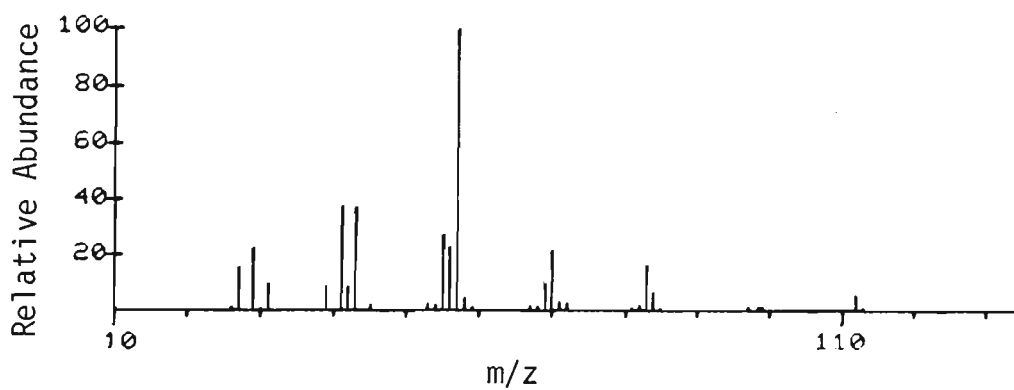
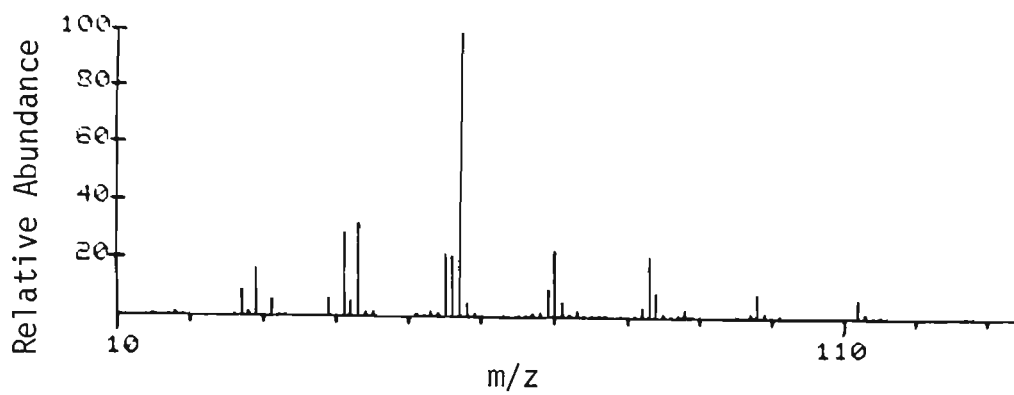
Mass spectra of peaks in Figure 39, page 144.

6-METHYL-1-HEPTANOL

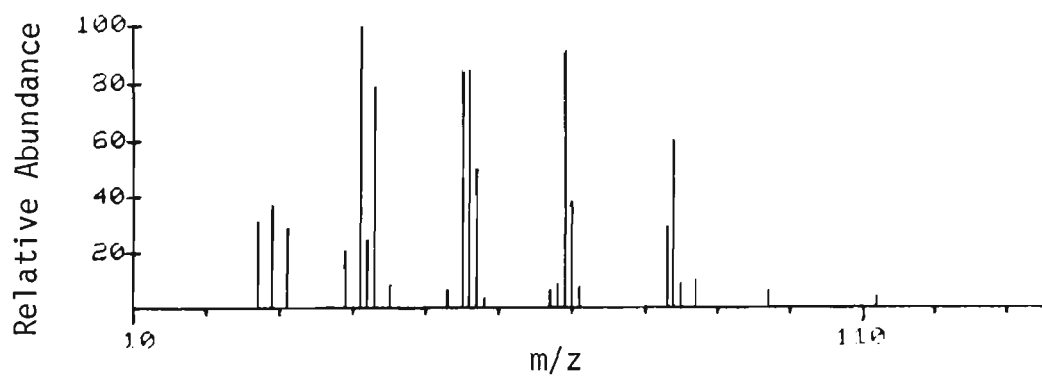
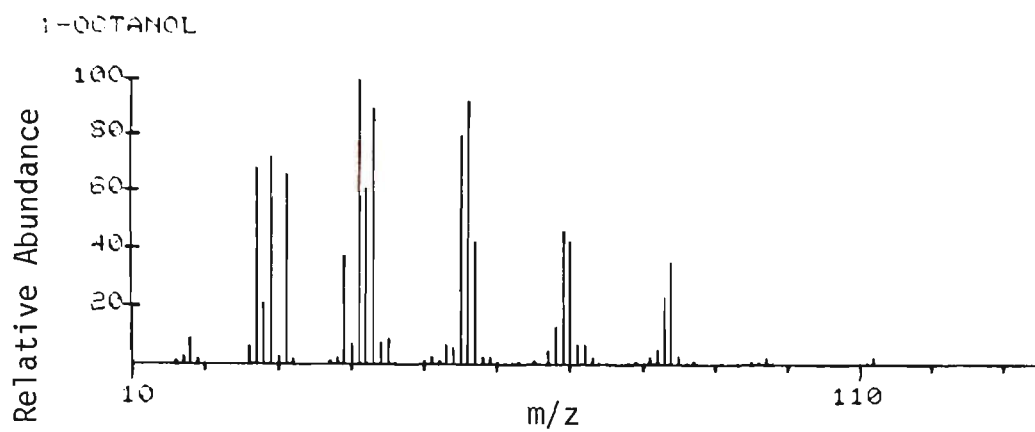


Mass spectrum of peak 1, Figure 39 (bottom) and library mass spectrum of 6-methyl-1-heptanol (top)

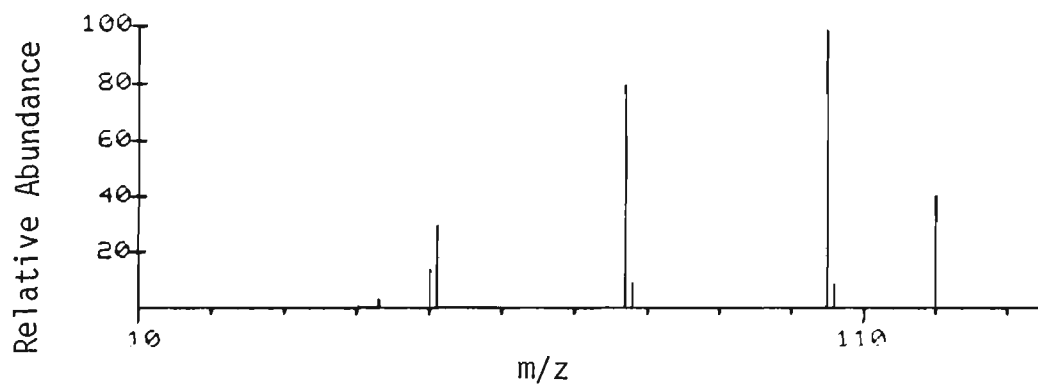
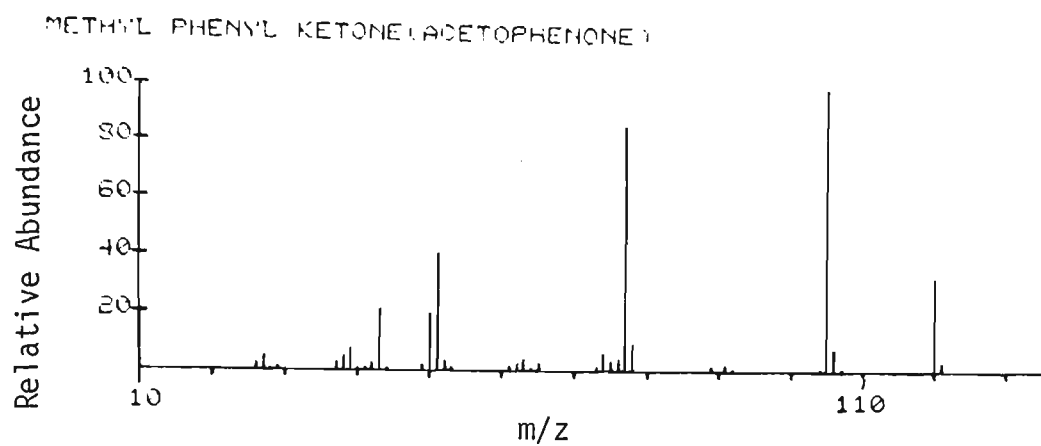
2-ETHYL-1-HEXANOL



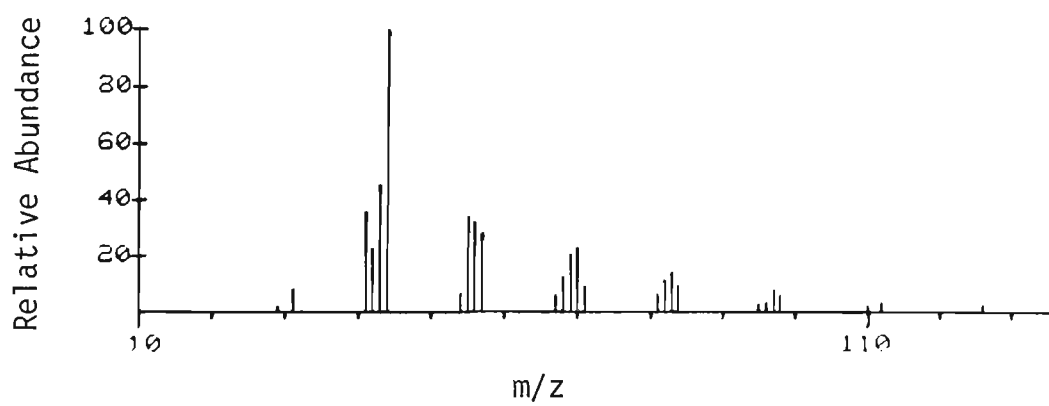
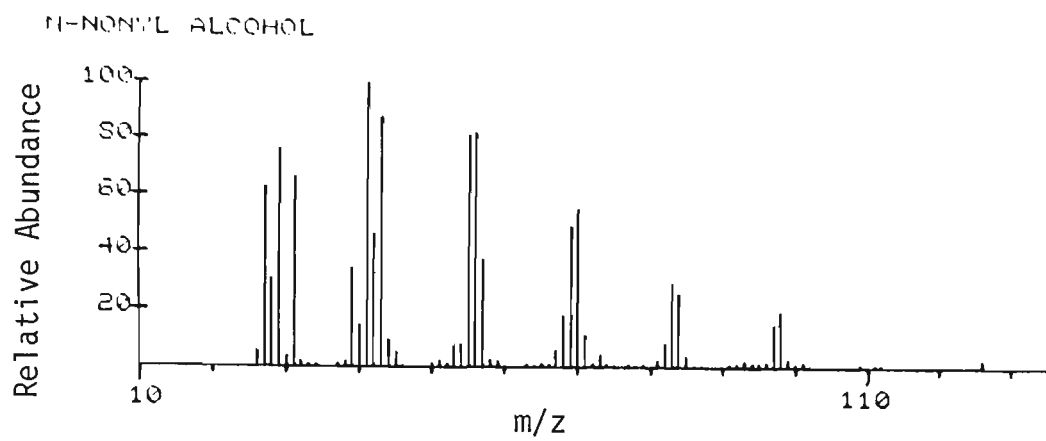
Mass spectrum of peak 2, Figure 39 (bottom) and library mass spectrum of 2-ethyl-1-hexanol (top)



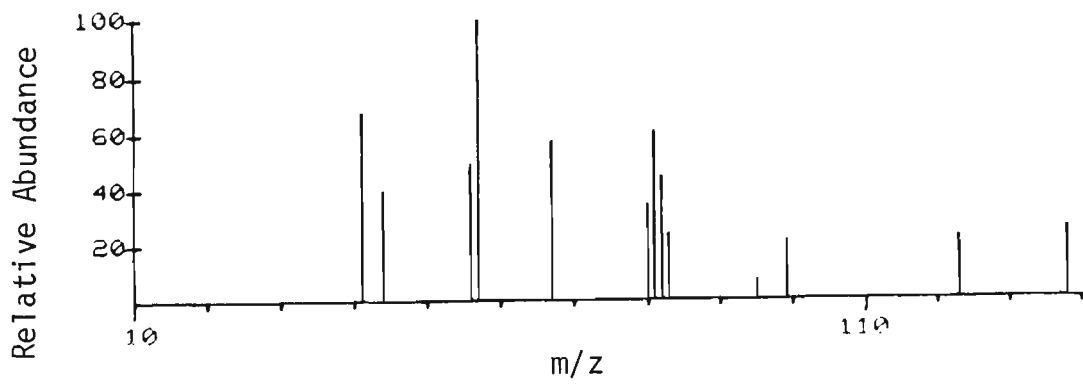
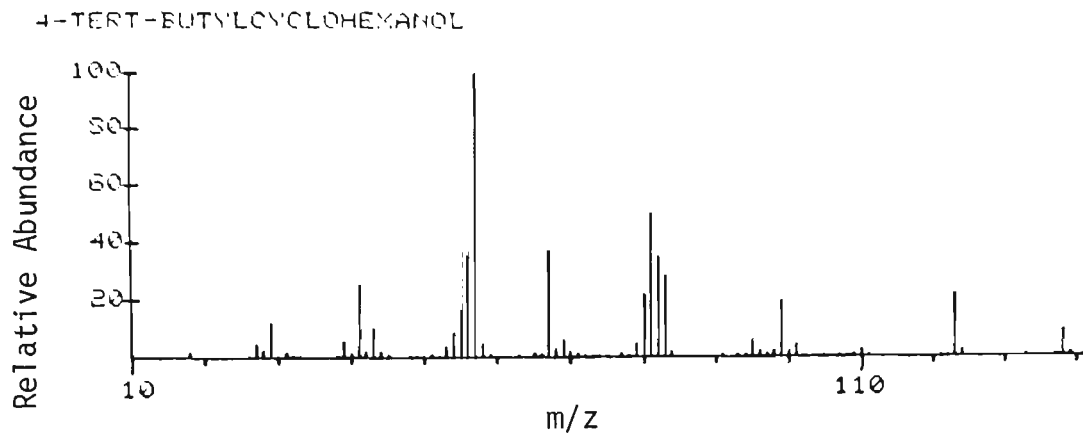
Mass spectrum of peak 3, Figure 39 (bottom) and library mass spectrum of 1-octanol (top)



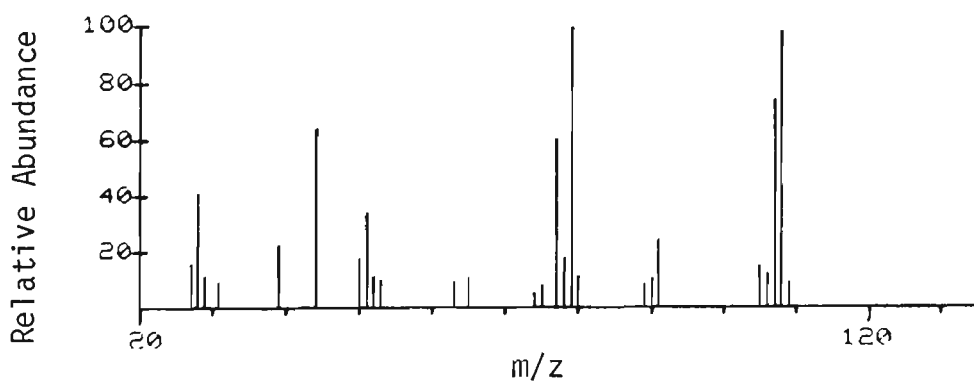
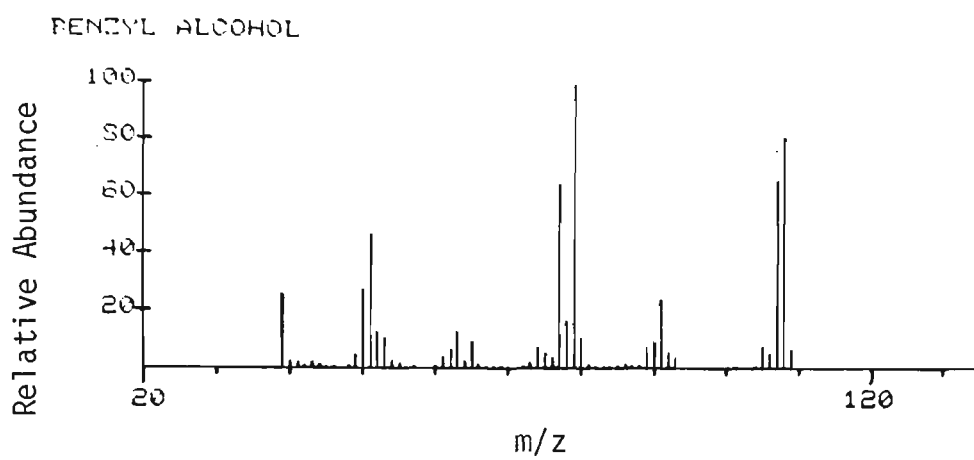
Mass spectrum of peak 4, Figure 39 (bottom) and library mass spectrum of acetophenone (top)



Mass spectrum of peak 5, Figure 39 (bottom) and library mass spectrum of n-nonyl alcohol (top)



Mass spectrum of peak 6, Figure 39 (bottom) and library mass spectrum of 4-tert-butyleyclohexanol (top)



Mass spectrum of peak 7, Figure 39 (bottom) and library mass spectrum of benzyl alcohol (top)

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