ABSTRACT

AUTOMATED METABOLIC PROFILING OF ORGANIC ACIDS IN HUMAN URINE BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

by

Stephen Carl Gates

The impetus for this research was the need to develop a method capable of both identifying and quantitating the large number of compounds typically present in biological fluids. The organic acid fraction of human urine was selected to test the resolution of the system, since at least 60 to 80 peaks, most of which are only partially resolved, are visible in this fraction when it is separated by gas chromatography.

The method chosen to measure the analyte pattern (''metabolic profile'') of urinary organic acids was separation on DEAE-Sephadex, followed by analysis of the derivatized acid fraction on a gas chromatograph-mass spectrometer-computer system. Data are collected with repetitive scanning of the magnetic field of the mass spectrometer accompanied by temperature-programmed gas

chromatography. The repetitive scanning data are then analyzed off-line by the mass spectral metabolite program (MSSMET). This program utilizes a reverse library search of the mass spectral data. Gas chromatographic retention indices are used both to limit the amount of data searched and to aid in identification of the substances. The intensities of a small set of pre-selected ions are used to judge the degree of match between each library spectrum and the experimental spectra. Compounds are quantitated by measuring the height and area of the peak of the "designate" ion, which is the ion which has been selected as being most likely to be differentiating for that compound. The relative concentration of each compound is calculated from the ratio of the area (or height) of the designate ion of the compound to the area (or height) of the designate ion of the internal standard. This relative concentration can be converted to absolute concentration by the application of an appropriate correction factor determined experimentally for each compound. For each substance positively identified as matching a library spectrum, MSSMET prints out information including name, concentration, degree of match to library spectrum, retention index, and retention time.

MSSMET was tested on spectra from a variety of pure compounds and urinary organic acid samples. All samples were analyzed as the trimethylsilyl derivatives on 10 ft 5% OV-17. These tests indicated that the retention indices were extremely precise (better than 0.2%) and that the compounds could be identified accurately almost to the limit of detection of the compound (usually to about 10 to 20 ng injected). Quantitation was linear over a 500 to 1000-fold range; this range was not extended by the use of isotope dilution techniques. Precision of the repetitive scanning technique was approximately 3% on isotope ratio determination, 5% on relative area determination with pure compounds, and 8% on relative area determination with urine samples.

Once MSSMET had been validated, the same techniques were applied to a variety of urine samples. These included urines from 9 "healthy" adults, 6 hospitalized children, and 5 children being treated for neuroblastoma. An average of 100 $\pm$ 30 compounds were reliably identified and quantitated in each urine sample, with up to 32 more compounds found less reliably. Statistical analysis of the reliably-found compounds indicated that the distribution of compound concentrations in adults was generally log-normal. Levels of twenty compounds were found to be significantly different

($p < .10$) between the adult and juvenile groups, while levels of 13 compounds were significantly different in the neuroblastoma urines compared to the other two groups. Differences at each level of significance tested (0.10, 0.05, 0.01, and 0.001) were found to considerably exceed those expected by chance. The concentration of one substance (caffeic acid) was also found to be related to the prognosis for survival of the neuroblastoma patients, although this finding is very tentative.

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry
1977

ACKNOWLEDGMENTS

In a project of this magnitude, a great many individuals necessarily make significant contributions to its success. Foremost among those whom I would like to acknowledge for their help in this project is Dr. Charles C. Sweeley, my thesis adviser. Dr. Sweeley not only suggested the original idea behind this project, but also has provided a constant flow of useful ideas and suggestions throughout my graduate career. He has provided an extremely well-equipped mass spectrometry laboratory for me to use in my research and has been especially helpful in teaching me to ask the proper analytical questions. I have also appreciated the opportunities he has given me to pursue ideas related to, but not directly contributing to, the development of MSSMET.

I also owe a special debt of gratitude to Nancy Dendramis for her help in the processing of urine samples and to Jack Harten for his help in the use of the LKB-9000. I have greatly benefitted from the example set by Bernd Soltmann, who has helped everyone in the laboratory to become more expert on the technical details of mass spectrometry. Bernd, Norm Young and Frank Martin have been extremely helpful in keeping all of the equipment in the mass

spectrometry facility repaired. Three programmers--Norm, Mike Smisko and Curt Ashendel--all made very significant contributions to the development of MSSMET, and I am appreciative of the strong foundations they built during the design and construction of early versions of this program.

A number of individuals also helped in planning and implementing the statistical analysis of the MSSMET data. Bob Wilson in the College of Education and Bill Brown in the Computer Center both provided valuable advice in planning statistical studies. Dr. B.E. Blaisdell in Dr. Sweeley's laboratory generously allowed me to use one of his statistical analysis programs and provided several useful criticisms of the MSSMET library.

Furthermore, I would like to thank Dr. John F. Holland for his general guidance, as well as his advice in technical matters. I sincerely appreciate the exposure to Dr. Arthur Kohrman's contagious enthusiasm for medical research, as well as his help in keeping a balanced perspective about the goals and needs of such research.

I genuinely appreciate the efforts of the personnel at the Bronson Clinical Investigation Unit in collecting the adult reference urine samples, and the generosity of the Upjohn Company in donating the urine samples to us.

Dr. William Krivit at the University of Minnesota has provided the urine samples from several groups of children; I sincerely appreciate his generosity in providing us with the samples and data on the subjects.

My father, Harry Gates, deserves special thanks for his superb job in typing the final draft of the dissertation. His typing this dissertation has made life considerably easier for me.

Finally, I would like to thank the one person most influential in my completion of this research--my wife, Jackie Cresswell. She not only provided encouragement throughout the five years, but also put up with a husband who frequently seemed to spend more time with Ms. Met than he did with her.

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INTRODUCTION

Man has long been interested in the possibility that his urine might provide clues to the state of his health or even of the world around him. From early recorded history, there are references to the uses of urine, and by Roman times a whole body of "knowledge" had developed concerning its medical importance. In medieval Europe the study of urine was viewed as a means of determining the reason for a person's ill health or forecasting his fortunes, and professional roving "uroscopists" were often paid for this service. In some modern-day primitive societies, urine remains a fluid believed to have magical or beneficial properties.

While most of these early uses of urine are rather non-scientific by modern standards, urine also has had its place in the development of science. For example, some of the earliest discoveries in analytical chemistry resulted from attempts to study this easily available fluid. Beginning with ammonium salts and elemental phosphorous, early chemists (and alchemists) isolated or derived a great number of chemicals from urine that were used extensively in early analytical and synthetic studies. A number of organic compounds, including creatinine, allantoin, hippuric acid,

leucine, cystine, xanthine, and uric acid were first discovered or isolated from urine.

Despite these early uses for and discoveries about urine, however, the detailed study of urine did not really begin until the invention of modern separation techniques. In particular, paper, thin-layer and column chromatographic methods made it quite clear that the compounds studied up to that time constituted only a small minority of the constituents to be found in human urine. With the application of gas chromatographic techniques to the separation of the low molecular weight components of urine, the number of known, albeit often unidentified, components grew even further.

Despite the plethora of components to study and the lack of techniques for studying more than a few closely-related compounds at one time, certain substances were detected and identified as being positively correlated with human disease states. This led early investigators to hope that compounds could be found that would be diagnostic of a wide variety of clinical abnormalities. Unfortunately, looking for a small number of compounds associated exclusively with each disease has proven to be an approach with utility only for a relatively few, generally rare diseases, notably the inherited metabolic disorders.

Beginning in the 1940's, a number of laboratories therefore

began to develop more general methods for measuring multi-component biological mixtures, especially urine, with the explicit goal of being able to apply these techniques to the diagnosis of human diseases. To date, these efforts have been only minimally successful, probably at least in part because only qualitative, rather than quantitative, extraction and analysis procedures have been utilized.

This problem of detecting a large number of components in urine is well illustrated by the problem of examining the low molecular weight organic acids by gas chromatography. Here, typical low resolution packed column gas chromatographic analysis of urine reveals approximately 30 to 50 clearly distinguishable peaks, but mass spectrometry of these peaks reveals that the actual number of components is over 125. Very few of these peaks are well resolved from neighboring compounds by the gas chromatograph, and it is almost impossible to quantitate more than a few of the most intense peaks with reasonable accuracy. Capillary (high resolution) gas chromatography, on the other hand, while adequately resolving components, presents considerable difficulties in providing enough sample over a long enough period to permit reliable identification by mass spectrometric or other techniques.

Hence, the current research was undertaken, using organic acids as the model substances, to determine whether a satisfactory technique could be found which would have the ability to do the

following:

Utilize gas chromatographic-mass spectrometric data.

Reliably identify components of complex mixtures.

Resolve all components adequately.

Quantitate all components.

Detect a wide range of concentrations of components.

Be amenable to almost complete automation.

Be adaptable to a number of different types of urinary constituents.

Provide results suitable for clinical studies.

The results of this research are described in the following chapters and in the papers listed in Appendix K.

CHAPTER ONE: LITERATURE REVIEW

Development of the concept of metabolic profiling

The concept that individuals might have a "metabolic pattern" that would be reflected in the constituents of their biological fluids was first developed and tested by Roger Williams and his associates during the late 1940's and early 1950's (51W1). Utilizing data from over 200,000 paper chromatograms, many run with techniques developed in his own laboratory for this purpose, Williams was able to show convincingly that the excretion patterns for a variety of urinary components varied greatly from individual to individual, but that these patterns were relatively constant for a given individual. He summarized his findings in 1951 as follows (51W1):

> "It appears that each individual we have studied has whenever tested exhibited a characteristic pattern of measurements which is distinctive for that individual alone. While there are in every case day-to-day variations in saliva and urine compositions and in taste thresholds, certain items, at least, stand out as grossly distinctive and the patterns as a whole remain nearly constant."

Williams went on to use his methods to examine samples from

a variety of subjects, including alcoholics, schizophrenics and residents of mental hospitals, and produced what he considered to be very suggestive evidence that there were characteristic metabolic patterns associated with each of these groups (51W1).

Williams' work, however, was apparently not duplicated by others, to whom his task must have seemed rather Herculean with few promises of tangible results. Hence, his ideas about the utility of metabolic pattern analysis remained essentially dormant until the late 1960's, when gas chromatography and liquid chromatography had advanced sufficiently to permit such studies with considerably less effort. Once these techniques became available, however, the rate of progress became extremely rapid. Thus, for example, in 1970 at least three different groups published papers describing multicomponent analyses of biological fluids and referred to the possibility of "considerable differences in excretion patterns of carbohydrates in disease" (70Y1), "personal blood 'profiles' " (70W1) and a "characteristic excretion profile" of organic acids in urine of patients with phenylketonuria (70B1).

However, the phrase most often used to describe the chromatographic patterns observed in biological fluids has been "metabolic profile." This concept was introduced by the Hornings in 1971 (71H2, 71H3). As originally defined, this term meant "multicomponent GC analyses that define or describe metabolic

patterns for a group of metabolically or analytically related metabolites'' (71H3). Commenting on the potential usefulness of this type of technique, the Hornings continue by suggesting,

> ''Profiles may prove to be useful for characterizing both normal and pathologic states, for studies of drug metabolism, and for human developmental studies.''

This definition of metabolic profile has been adopted by some workers essentially unchanged (73W3). Other workers have preferred just the term ''profile'' to mean the same thing (76M1). Johnson (72G3) has taken a more statistical approach by defining a profile as,

> ''... a vector of numerical values corresponding to measured characteristics or attributes of a given subject. In addition to clinical chemistry measurements, the profile may include measurements on demographic or physical variables such as age, weight, sex, exercise status, etc. Profile analysis is the study of several profiles for the purpose of characterizing the profiles of a given group of subjects or comparing the profiles of a different group.''

A number of hospital laboratories have experimented with a related technique, ''multiphasic screening,'' (reviewed in 71M1) designed to measure multiple components of a single serum or urine sample. The principal difference between multiphasic and

profile techniques has been one of technology: multiphasic testing has utilized single tests for each of the components, while profiling has used a single chromatographic run to analyze for multiple components. The underlying similarity of the two techniques is reflected in the recent literature; thus, for example, multiphasic testing was used to develop a "profile" that could differentiate drug-abuse and hospital-staff populations (74M4). Reece (74R1) has used an additional term, "uniphasic synthesis," for multiphasic screening when the test results are analyzed utilizing multivariate statistical techniques.

In general, then, interest in this type of approach is recent enough so that the terminology is in a rapid state of flux. However, in the material that follows, I will use a combination of the Horning and Johnson definitions: metabolic profiling is a means of obtaining, by chromatographic, physical examination, and demographic survey methods, a set of numerical values that can be used to estimate the chemical and health status of a given individual. In discussing metabolic profiling in the following sections, I will arbitrarily limit this definition to exclude studies of small numbers of components (e.g., less than 5 compounds), high molecular weight components (greater than 1000 amu) and all inorganic compounds.

Techniques for separating low-molecular-weight components of biological fluids

Although the terminology used to describe multicomponent analysis of biological mixtures is new, the techniques used are old. These have included paper chromatography, thin-layer chromatography, gas chromatography, liquid chromatography, mass spectrometry and a variety of more specialized techniques for unusual types of compounds; in short, almost all of the tools of the modern analytical biochemist have been used in this type of research.

Paper and thin-layer chromatography. Certainly the principal profiling effort utilizing paper chromatographic techniques was that of Williams, as described previously. While a great many other groups were successful in devising means of separating various fractions of urine and other biological fluids, these methods were not generally applied to producing human health profiles. However, paper and thin-layer chromatography have been, and continue to be, important tools in rapid screening procedures for gross excesses or deficiencies of individual components in biological samples (see, for example, the review by Scriver, Clow and Lamm (73S3) on screening procedures for aminoacidopathies).

Column chromatography. In contrast, column chromatography, particularly high-pressure liquid chromatography,

has proven to be a quite effective means for obtaining human health profiles. Thus, Young (70Y1) and Jolley and Freeman (68J1) reported one of the early attempts to use a high-resolution chromatographic apparatus to evaluate the health status of several types of individuals based on the profile of urinary carbohydrates. Young showed that considerable differences in the carbohydrate profile were apparent for several disease states, but that the pattern for a given individual was reasonably constant from day to day.

Similarly, Pitt et al. (70P1) and Scott et al. (70B1, 71M2), both groups from the same lab at Oak Ridge, described a system for analyzing as many as 150 ultraviolet-absorbing substances in body fluids. A minicomputer was used to resolve peaks and store data (70S1). Many of the compounds detected by this system were identified by gas chromatography-mass spectrometry (GC-MS) as aromatic organic acids and amino acids (71M2). Later work expanded this system to multiple column operation (72P2), use of a fluorometric detector for indolic compounds (72C5), sequential use of different column types to achieve more rapid separations (73S2), and more sensitive detection systems (73S1), including fluorescence monitoring of organic acids (73K1).

Other laboratories have also developed this type of system, but with computerized data processing (72C1) and other types of

detectors (74K2, 75K2). A highly sophisticated system of this type has also been developed for computerized analysis of clinical amino acid data (76S1, 76S2); this system will be discussed in more detail in the section on statistical analysis of data.

Gas chromatography. Although proposed as a possible technique in 1941 in a pioneering paper on liquid-liquid partition chromatography (41M1), gas chromatography (GC) was not successfully applied to complex biological mixtures on a practical basis until the introduction of lightly-loaded liquid phases by VandenHeuvel, Sweeley and Horning in 1960 (60V1). When coupled with the flame ionization or electron capture detectors developed at the same time, and suitable derivatization methods (see reference 73C3 for a history of GC), the number of uses of GC for the analysis of complex organic mixtures rapidly surpassed those of liquid chromatography, for which no comparable "universal" detector was then available and for which the speed of analysis was much slower than that of GC.

Early workers were quick to utilize the GC to aid in the diagnosis of disease. Thus, for example, by 1964 Williams and Sweeley (64W2) had published a general procedure for analyzing urinary aromatic acids, gross excesses of which could often be associated with specific diseases. Similar procedures were published in the same volume for other low molecular weight

substances (64S1).

Unfortunately, as numerous workers were quick to discover, the gas chromatograph by itself provided neither sufficient specificity of detector response nor adequate chromatographic resolution to permit unequivocal identification of most peaks in complex mixtures, and quantitative analysis of small to medium-sized peaks proved to be difficult at best. As a result, there were no reported Roger Williams-scale attempts to find the more subtle metabolic patterns present in biological fluids.

Gas chromatograph-mass spectrometer-computer systems. Since the mid-1960's, two fairly distinct approaches have been developed to overcome the shortcomings of using low-resolution GC for metabolic profiling: combined gas chromatograph-mass spectrometer-computer systems (GC-MS-COM) and capillary GC-computer systems. The GC-MS-COM approach will be discussed first.

Although individual GC eluates had been transferred manually to mass spectrometers for analysis for some time, and direct coupling of a GC and MS had been demonstrated in 1959 by Gohlke (59G1) and in 1961 by Henneberg (61H1), the introduction of the molecular separator by Ryhage (64R1) and Watson and Biemann (64W1), both in 1964, as a means of direct transfer of chromatographic material to the mass spectrometer allowed rapid

qualitative analysis of complex mixtures on a relatively routine basis. Thus, for example, Ryhage's first published use of the molecular separator was to obtain a profile of fatty acid methyl esters in butterfat (64R1). This was quickly followed by a study analyzing neutral fecal steroids in human subjects (64E1), and then a virtual flood of papers utilizing GC-MS systems for the analysis of a wide variety of biological, environmental and geophysical samples. Most of these early papers described uses of the GC-MS that were limited to qualitative analysis of a few peaks in a very small number of samples; researchers were limited principally by the lack of automated data processing equipment.

An alternative approach, this time emphasizing quantitative analysis of a very small number of peaks, was developed first by Henneberg in 1961 (61H1) and later, independently, in Ryhage's laboratory by Sweeley et al. (66S1) in 1966. "Selected ion monitoring," originally used for monitoring one and two ions, respectively, provides a means of obtaining highly precise measurements on a small number of GC peaks, including those unresolved from neighboring components in a mixture. However, even when expanded to include on-line computer control, more ions, computerized data reduction, and multiple-ion set-selected ion monitoring (see, for example, 73H2, 73H3, 73J1, 73W1, 75Y2) this general technique has been limited to measuring a very small

number of components of any one mixture, and hence, while extremely useful for other types of studies, has not seen particularly wide use for metabolic profiling. A notable exception to this has been Maume's group in France (73M1, 73M2) who have successfully examined mixtures of closely related steroids, catecholamines or amino acids by monitoring ions common to whole classes of these compounds.

Much more useful for profiling purposes is the computer-based technique called "mass chromatography," first described by Hites and Biemann in 1970 (70H1). In this technique, complete mass spectra are taken at frequent intervals and the entire data set stored in a computer. After the mass spectral data collection is finished for a given sample, the data are displayed by plotting the intensities of certain key ions for each of the scans during the run. These intensity versus time plots ("mass chromatograms") can be displayed for any ion of interest within the entire mass range of the mass spectrometer. Thus, the mass chromatograms are equivalent to the traces generated during selected ion monitoring, except that there is a much lower sampling frequency for any one ion in mass chromatography. This results in a much lower quantitative precision, but a much more generalized quantitative ability for mass chromatography compared to selected ion monitoring, and hence a higher degree of usefulness for profiling studies.

An approach that is intermediate between selected ion monitoring and mass chromatography was suggested by Axelson et al. (74A1) and Baczynskyj et al. (73B1). In this method, spectra are taken repetitively, but over a much shorter mass range. This technique, however, has been far surpassed in popularity by the selected ion monitoring type methods. In instruments with electrostatic mass filters (quadrupoles, dodecapoles), this technique, selected ion monitoring and repetitive scanning can often all be accomplished with essentially the same data algorithms and hardware, so for these types of instruments the distinction among techniques is more customary than meaningful. However, while any one of the three techniques can be, and has been, used for metabolic profiling in its broadest sense, the procedure of performing repetitive scanning of the full mass range, followed by analysis of selected mass chromatograms, has proven the most generally useful for analysis of mixtures containing a large number of unidentified components. For this reason, a large number of laboratories has developed GC-MS-COM systems utilizing repetitive scanning-mass chromatography techniques for performing metabolic profiling.

Principal advocates of this approach have been the Hornings at Baylor University and Eldjarn, Jellum and Stokke at the University of Oslo. The Hornings described their first studies on metabolic profiling in a pair of now-classic papers published in

1971 (71H2, 71H3). These papers not only defined metabolic profiling but also advocated use of methylene units (a measure of GC retention time that is virtually identical to retention indices, discussed below) for assisting peak identification and further proposed a series of techniques for sample isolation which are still followed in many laboratories.

In a similar fashion Jellum and his coworkers have utilized a GC-MS-COM system in the repetitive scanning mode for metabolic profiling studies. However, in contrast to the Hornings, Jellum's group has used this system as the final stage of a rather complete screening system for metabolic disorders (72J1, 72P1, 74E1). Hence, they have primarily relied on their system to identify major unknown peaks, rather than to spot abnormal profiles; only samples which have passed through the entire screening procedure and still found to be medically interesting are submitted for GC-MS analysis.

Common to both the Baylor and Oslo systems is an interest in identifying abnormal compounds or compounds present at abnormal levels. Little or no quantitative data has been published by either of these groups of workers. This same pattern of interest in qualitative, rather than quantitative, results has been followed by a number of other laboratories (70A1, 71H4, 71L1, 73W3, 74B1, 74D2, 74M2, 74M3).

Recently, more sophisticated systems have begun to appear. Sjovall, for example, has developed a method for location of steroid spectra and partial structure determination based on repetitive scanning data (73R1). This has been expanded to include some quantitation (74A1). McLafferty et al. (74M1) have described a microprocessor-based system for automated identification of compounds in mixtures, and Dromey et al. (76D3) have developed a method of resolving GC-MS data into contributions from separately identifiable peaks. These papers are all particularly germane to the present work and will be discussed in more detail in the section on computer techniques.

High resolution GC and GC-MS. Two other alternatives to the use of low resolution (packed column) GC-low resolution MS for metabolic profiling have been developed which still use GC or GC-MS, but with a greater resolution of either the mass spectrometer or the GC separation. Burlingame (74K1) has developed a high resolution MS-COM system which he has used to analyze major components of urine samples. He has also published a preliminary report on a system using high resolution GC-high resolution MS (HRGC-HRMS). However, most other researchers have concentrated on the less formidable task of coupling high resolution GC columns to either a computer or a low resolution MS-COM system. Pauling's group (71A1), for example has advocated use of capillary

columns with on-line data analysis using pattern recognition techniques and mass spectral identification of individual peaks when necessary.

Other groups, frequently using capillary GC-low resolution MS-COM systems, have been more interested in qualitative analysis. Zlatkis in particular has emphasized this approach in a series of papers describing the analysis of ether extracts of urine (71Z1), urine headspace samples (73Z1), organic volatiles in air (74B2) and serum and plasma headspace volatiles (74Z1). Politzer et al. (75P2) have even expanded this approach to examining volatile fractions of lung, brain and liver tissues; they have also published a review of GC-MS studies of underivatized volatile compounds in biological fluids (76P1). Maume and Luyten (73M2) have utilized a similar system in analyzing derivatized and underivatized steroids down to 10 ng injected, and down to picomole amounts when selected ion monitoring techniques were used. Horning et al. (74H2) have developed a method for the preparation of extremely high resolution (100,000 theoretical plates) thermostable capillary columns which they used for the analysis of a variety of biological fluid extracts. Luyten and Rutten (74L2) have used retention indices on the capillary columns to aid in compound identification. Novotny et al. have compared stationary phases of different polarity for their suitability in profiling studies (74N3), and have also

developed a method for concentrating samples prior to analysis (75N1). Recently, Knights et al. (75K4) have used a direct-coupled capillary GC-low resolution MS-COM system for the qualitative analysis of acidic urinary metabolites obtained by ether extraction. Hedfjall and Ryhage (75H2) have also published a method for obtaining much more rapid scans (1.4 second cycle time) from the mass spectrometer to accommodate the need for faster data collection rates when utilizing capillary columns.

Computer processing of GC-MS data for metabolic profiling

Forward library search methods. Despite the recent successes in utilizing capillary and high pressure liquid chromatography systems, most attempts at metabolic profiling to date have involved low resolution GC, repetitive scanning of the mass spectrometer and on-line data processing. This has typically been followed by either manual identification of spectra or computerized library search procedures, or a combination of both (see, for example, 71H4, 73W3, 75H3, 76L1). Whether manual or computerized, these spectrum identification procedures have typically been of the "forward" type: that is, comparing each sample spectrum of interest to a large library of reference spectra to find the best match. These forward searches have often utilized mammoth data bases and sophisticated pattern analysis algorithms (73H1, 73K2). While pre-ordering the library file can decrease the amount of time need for such library searches -- Dromey (76D2), for example, has proposed a method for identification of functional groups as an aid to such a search -- the forward library search method has proven to be very time-consuming and costly.

Reverse search methods. Beginning in 1974, several papers appeared which suggested an alternative approach for library search procedures. This approach, called the "reverse search"

by Abramson (75A1), has also been developed by McLafferty (74M1) and this author (74S2). The principal feature of this method is that spectra from the sample are searched for a match to a given library spectrum, rather than searching a library for a spectrum similar to the one of interest in the sample.

As noted by Abramson (74M1), in a forward library search,

> "... the presence of significant levels of interference may artificially suppress the relative intensity of relevant masses and produce a bad fit. Even more importantly, when data are compressed (e.g., saving only the two largest peaks in a 14 amu region), interferences of any nature may cause relevant masses to be excluded."

These disadvantages are then presumably avoided by a reverse search procedure.

There are several published variations of the reverse library search. Abramson's procedure (74M1) compares all spectra in a GC-MS run to each library spectrum, and then sums intensities for each positive match, so that an area is calculated. It makes decisions about a "match" based on a comparison of normalized intensities; for a match to be declared, the average match of normalized intensities between library and sample spectra must be within plus or minus 16%. His criterion for the selection of masses to be compared is generally peak intensity, so that he usually

selects the ten most intense masses of the library spectrum for comparison purposes.

McLafferty, however, has provided a more systematic method for the selection of comparison masses; as he points out, "the most abundant mass spectral peaks are not necessarily the most characteristic" (74M1). McLafferty has developed and commercially marketed a system employing "probability based matching of mass spectra," which examines the following factors: the uniqueness of a particular ion (m/e) relative to all of the m/e values in several thousand reference library spectra; the abundance of the ion in the reference spectrum; the degree of "dilution" of the spectrum by other spectra; and the "window tolerance," or degree of variability permitted compared to the reference spectrum. These criteria are used to compute a "confidence index," which must be above a certain value for a sample spectrum to be declared a match against a reference spectrum (74M1). McLafferty has also published a study of the uniqueness of various masses (75P1). Recently, deJong et al. (76D1) have applied information theory to the development of a somewhat different coding and library search algorithm, but their method has not been as thoroughly tested as that of McLafferty.

Retention indices. The reverse library search procedures described by Abramson and McLafferty necessitate searching an

entire GC-MS run for matches to each library spectrum. However, an alternative method to provide a more selective search was described by Nau and Biemann (73N1, 74N2). In their approach, GC retention indices (developed originally by Kovats, 58K1) are used to help further identify compounds located by a forward library search procedure. These retention indices, usually calculated by measuring GC retention times relative to a series of straight-chain hydrocarbons co-injected with the sample (see the methods section of this thesis for details), have also been used by Biemann's group to aid in identification of related compounds by correlating shifts in retention indices with addition of specific functional groups (74C4). Other workers have used retention indices in combination with mass spectral correlations to compute a combined match score; this approach has been used both for reverse library searches by this author (74S2, 76G1, 77G1) and for forward library searches by Blaisdell (77B1).

Other GC-MS-COM techniques for profiling. Three other approaches to the computerized analysis of GC-MS data are particularly pertinent to the work described in this thesis. The first of these is the system developed by Sjovall (73R1, 74A1), in which mass chromatograms are searched for locations where a number of ions are peaking. At each such location, a search is performed to identify potential molecular ions, and the general type of

compound (type of steroid skeleton in their usual case) identified where possible. A spectrum may then be compared to library spectra by a forward search procedure, or not, as desired. In addition, a compound amount is calculated by comparison of ion intensities to those of an internal standard. The printout from this system includes the retention time relative to cholestane as an aid in identification.

A related technique is that of Biller and Biemann (74B3). In an attempt to obtain spectra free from contributions of closely eluting compounds, their computer program examines all mass chromatograms for peaks. A new data file is then created which consists of the intensities of each peak found; these data are stored at the two scans corresponding to the apex and the immediately preceding scan of each mass chromatogram peak. They term this technique the production of "reconstructed mass spectra," and indicate that it improves the reliability of forward library search procedures.

Dromey et al. (76D3) have recently proposed a more sophisticated method for obtaining reconstructed mass spectra. This approach uses well-resolved peaks in mass chromatograms to resolve peaks of other masses occuring at approximately the same location in the GC-MS run, and hence it is able to obtain spectra free from background and neighboring peak contributions.

This approach, however, requires a much larger amount of time and computer memory to accomplish than does the Biller and Biemann technique.

Disease diagnosis by metabolic profiling

Regardless of the methodology used for metabolic profiling, the same problems must be confronted once the data are collected: how are the data to be analyzed and of what clinical significance are the data. In the case of some diseases, particularly metabolic disorders, where a few compounds are in gross excess, the data analysis need not involve statistics and the clinical significance is usually easy to discern. However, in the more common case, where the disturbance of metabolism is more subtle, complex statistical approaches may be necessary and the interpretation of the results may be correspondingly more difficult.

Non-statistical methods. A whole body of literature has developed concerning the detection of human disease based on the analysis of levels of one or a few compounds in urine or blood. Much of this literature has been listed in two recent bibliographies (68K1, 71O1). A more specialized sampling of such work is summarized in Table 1, which shows diseases detected by GC-MS methods because of excesses in one or more acidic metabolites in urine. While not truly "metabolic profiling," these early studies at least have given confidence to later workers that metabolic profiles will have some meaning in terms of specific disease states.

Figure 1. Diseases Where Organic Acids Have Been Detected at Abnormal Levels by GC-MS

Disease	Acids Most Affected	Reference
Burns and allergic diseases	pyroglutamic acid	68T1
Neural crest tumors	vanilmandelic acid	71V1
Tyrosemia	p-hydroxyphenyl acids	71C1
Maple syrup urine disease	branched chain α-keto acids	72G2
-Methylcrotonylglycinuria and -hydroxyisovalericaciduria	α-hydroxyisovaleric acid and others	72G1
Generalized ketosis	adipic and suberic acids	72P1
Isovaleric acidemia	isovaleric and α-methylbutyric acids	72T1
Pyroglutamic aciduria	pyroglutamic acid	73E1
Gastrointestinal disorders	acidic products of tyrosine and phenylalanine	73D1
Lactic acidosis	lactic, α-hydroxybutyric acids	73P2
Propionic and methylmalonic acidemia	propionic, methylmalonic and β-hydroxy-n-valeric acids	73S4
Phenylketonuria	aromatic acids	74C3

Figure 1 (cont'd.)

Disease	Acids Most Affected	Reference
Glycogen storage disease	C6 to C10 dicarboxylic acids	74D1
Periodic catatonia	malic and tartaric acids	74L1
3-Ketoacylthiolase abnormality	3-ethylhydracrylic acid	74M2
Parkinsonism	homovanillic, isohomovanillic acids	74N1
Uremia	lactic and several pentonic acids	75B1
Psychiatric disorders	5-hydroxyindoleacetic and indoleacetic acids	75H4
Hyperphenylalanemia	acidic phenylalanine metabolites	75K3
Ketoacidosis	branched short-chain hydroxy acids	75L2
α-Ketoadipic aciduria	α-ketoadipic and α-aminoadipic acids	75P3, 75W4
Mental retardation	phenylalanine metabolites; benzoic, α-ketoglutaric and citric acids	75W1
Neuroblastoma, pregnancy	catecholamine metabolites	75Z1
Unknown metabolic disorders	4-hydroxycyclohexane-1-carboxylic acid	76B1

Figure 1 (cont'd.)

Disease	Acids Most Affected	Reference
Dicarboxylic aciduria	dicarboxylic acids	76G2
Methylmalonic acidemia and isovaleric aciduria	short chain dicarboxylic acids	76N1
Glutaric aciduria	glutaric, lactic and other acids	76P2
Leucine metabolism defect	3-methyl substituted acids	76W1

Statistical treatment of data. Because only a few studies have been done with metabolic profiling, little has been published that deals specifically with statistical analysis of this type of data, and hence most of the literature in this area has developed from related studies in hospitals.

Most laboratories, especially hospital laboratories, have reported only mean values, or at best, means and standard deviations for individual compounds. Thus, for example, Young has reported "normal laboratory values" for over 200 blood, serum and urine constituents (75Y1). A growing body of literature suggests, however, that under some circumstances means and standard deviations may be misleading. Burnett (75B2) has suggested listing means and standard deviations with "outlier" values (those further than some predetermined number of standard deviations from the mean) removed, at least when reporting quality control data. He then recommends reporting an additional value, the "outlier frequency," to indicate the number of such outlying values removed. Reed et al. (72R1, 72R2) have suggested using estimates of normality which do not assume Gaussian distributions (i.e., using nonparametric estimates) and have provided tables for doing so. Gindler has similarly recommended several rapid nonparametric tests for method comparison and quality control studies (75G1). Westgard and Hunt (73W2) have evaluated several common least-squares methods for method-comparison studies.

A more general review of the statistical treatment of clinical laboratory data has recently been provided by Sunderman (75S1). This review carefully distinguishes between "normal values" and "reference values," favoring the latter term for most uses. He summarizes the types of information that should accompany reference values, and then provides a very useful review of the requirements for establishing a "discrimination value," or statistical cutoff point for distinguishing individuals in two different categories (e.g., healthy versus diabetic). Young has published a review of the computerized interpretation of clinical chemical data that discusses this and other problems of data treatment (76Y2). Werner and Marsh (75W2) have also provided a review of practical considerations when establishing normal values.

However, Harris (75H1) has recently suggested that the use of reference standards, even when stratified by age and sex, may frequently lead to an inability to detect other than extremely gross deviations from "normal." He has suggested criteria for deciding when use of such standards is inappropriate (74H1) and has recommended that, where possible, previous values from the same individual be used instead (75H1). This requires a different type of statistical approach (76H1).

A few workers have begun to apply more complex statistical methods to clinical labortory data; these methods have been fairly

widely applied to multiphasic screening data, but only in a very limited sense to metabolic profiling data. A pioneer in this area has been Winkel (72W1), who has suggested that multivariate statistical approaches could be used successfully to relate various test results. Winkel points out that it is possible to have values for a single variable that are univariate normal but abnormal in multivariate space, and conversely, that some univariate abnormal values, when taken in combination with other variables, can be shown to be normal. Johnson (72G3) has related the concept of the metabolic profile to multivariate statistics by suggesting that,

> "The profile could be thought of as a point in N space.
>
> Several profiles provide several points in the same N space.
>
> The statistical problem is to describe the cluster of points."

Mayron et al. have used multivariate statistics to develop a profile of drug-abuse populations based on routine hospital tests (74M3), and Reece (74R1) has predicted the use of this type of statistic as a principal feature distinguishing the hospital screening process of the 1980's.

Only a few, usually rather preliminary, studies have been completed with quantitative metabolic profiling. Young et al. (71Y1) reported a study of the effects of patients being given an artificial diet. This study, which measured levels of approximately 300 ninhydrin-positive, ultraviolet-absorbing or carbohydrate

components of urine and serum, led Young to conclude that many of the compounds in the volunteers' samples were of dietary, rather than endogenous, origin. He also noted that at least 4 days of the artificial diet were required to reach a stable set of values for many of the compounds. Interestingly, a few of the compounds, including creatinine, were excreted at the same rate regardless of the diet. Harris and DeMets (71H1) extensively studied a smaller number of compounds; they found serum ionized calcium to be constant, within their analytical precision, for single individuals over a period of 10 to 12 weeks even when there were considerable inter-individual variations. Witten et al. (73W3, 73W4) reported normal organic acid levels in young adults on a standard diet, and then determined the effect of ethanol ingestion on the profile. They found that levels of 2- and 3-hydroxybutyric, adipic, 3-methyladipic, p-hydroxyphenylacetic and 2,5-furandicarboxylic acids were affected by the intake of ethanol. However, it should be noted that these results were at best semi-quantitative, since an ethyl acetate-ether extraction procedure was used and quantitation was by peak area on low resolution GC. Bjorkman et al. (76B2) similarly determined levels of a number of major acidic metabolites in the urine from newborn humans, and Chalmers and Watts (74C3) have examined unconjugated aromatic acids in phenylketonuria, followed by a study of volatile fatty acids in several metabolic disorders (74C1). Liebich et al. (75L3) have

used a similar semi-quantitative method for measuring levels of low molecular weight aliphatic alcohols in normal and diabetic individuals. In a more quantitative study, Yamamoto et al. (76Y1) have found a seasonal variation in levels of urinary metanephrine, and a minor seasonal variation of vanilmandelic acid when these compounds were studied over a 5-year period.

Routh and Paul (76R1) found an effect of aspirin therapy on the levels of several serum constituents. Lawson et al. (76L1, 76C1, 76C2) reported qualitatively different excretion patterns of several organic acids in man, and qualitatively significant variations that depended upon the type of diet consumed by their subjects. Many of the compounds reported in this last study were quantitated in clusters because they were unresolved by the GC system used. Robinson et al. (73R2) have also reported very preliminary data suggesting general abnormalities among mentally retarded subjects, although this report was never amplified sufficiently to judge its importance. Blau et al. (73B2), in an interesting study of aromatic acid excretion in heterozygotes for phenylketonuria, found that heterozygotes could be distinguished

from normal subjects by the excretion of o-hydroxyphenylacetic acid.

Probably the most ambitious study so far has been that of Robinson and Pauling (74R2, 75D1). These workers have used an ion-exchange chromatography and capillary GC to profile several urinary fractions, principally head-space volatiles and free amines. A pattern recognition procedure is used to identify peaks and peak areas are normalized to a specially-selected subset of the peaks to reduce inter-sample variability. The data collected are compared to one another using the Wilcoxon test, a nonparametric statistical ranking procedure. This approach has been used to search for differences due to sex, ingestion of birth control pills, student grade point average, multiple sclerosis, Huntington's disease, fasting versus non-fasting, breast cancer and Duchenne dystrophy, with significant differences reported in each case except that of grade point average. However, they point out (75D1) that,

> "We have not proved that, for most of our sample groups, the only systematic property that contributes to the pattern for the group is that for which the group is labeled. We also have not shown how early the patterns for disease develop. We do not know whether or not the patterns are present before the disease is extensively developed, and therefore are useful for preventive medicine."

A less ambitious, but much better documented, system for

the statistical analysis of profiling data is that reported by Schoengold et al. (76S1, 76S2). This system was developed to allow processing of amino acid data, and it is noteworth in that it allows extensive inter-individual comparisons utilizing a variety of standard statistical methods on data routinely collected in the clinical laboratory. The authors, in describing this system, persuasively argue that a great deal of valuable data collected in the clinical laboratory is not utilized. They have therefore developed a relatively low-cost minicomputer-based system that makes information retrieval and comparison easy, and hence encourages such usage.

The most recent technique to be applied to profiling data is that of computerized pattern recognition. This approach, which requires a large data base and a correspondingly large amount of computer memory and processing time, has been proposed as a means of finding data patterns that are not apparent from traditional statistical analysis. Kowalski (75K4) has illustrated the use of such a procedure to distinguish patients suffering from two liver diseases on the basis of levels of 8 blood enzymes, but no one has yet published a similar study using pattern recognition on levels of low molecular weight substances.

CHAPTER TWO: MATERIALS

Reagents

General solvents	Hexane, acetone and methanol were redistilled by constant-flow rotary evaporation from reagent grade solvents.
Dry redistilled methanol	Reagent-grade methanol (Mallinckrodt, St. Louis, Mo.) was dried by distillation from magnesium turnings containing a catalytic amount of iodine and was stored over molecular sieves.
Dry pyridine	Pyridine (Mallinckrodt, St. Louis, Mo.) was dried by distillation from barium oxide after refluxing 1 hour and was maintained over potassium hydroxide pellets.
Acetic acid (glacial)	Allied Chemical Company, Morristown, N.J.
0.1M Barium hydroxide	Prepared on a weekly basis by dissolving 31.5 g of $Ba(OH)_2$ (Fisher Scientific Company, Fair Lawn, N.J.) in redistilled water to a final volume of 1.00 l.
Pyridinium acetate	Each of the following solutions was prepared by dilution to 1 l in a volumetric flask. Each was prepared fresh weekly.

	redistilled pyridine	glacial acetic
0.5M	40 ml	29 ml
1.0M	80 ml	58 ml
1.5M	119 ml	90 ml

Hydroxylamine hydrochloride — 75mg of $NH_2OH \cdot HCl$ (J.T. Baker, Philipsburg, N.J.) was dissolved in 1 ml redistilled H_2O.

Saturated picric acid — Picric acid (J.T. Baker, Philipsburg, N.J.) was added in excess to redistilled H_2O and stirred on a magnetic stirrer 1/2 hour. The solution was then allowed to saturate in the dark for 24 hours and filtered. The filtrate was stored in a dark brown bottle.

Creatinine standard — A 1.00 mg/ml solution was made by weighing 10.0 mg creatinine (Sigma Chemical Company, St. Louis, Mo.) on an analytical balance and then diluting to 10 ml in a volumetric flask.

Organic acids

Protium forms — Obtained variously from Dr. Clyde Williams, University of Florida; Sigma Chemical Company, St. Louis; and Aldrich Chemical Company, Milwaukee, Wisc.

Deuterium forms — Deuterated (α-d_2) forms of homovanillic, hippuric, 5-hydroxyindoleacetic and indoleacetic acids and 2,5,6-d_3-3, 4-dihydroxyphenylacetic (α-d_2) acid were obtained from Merck, Sharp and Dohme Canada Limited, Quebec, Canada.

Hydrocarbon mixture	64 µl decane, 70 µl undecane, 70 µl dodecane, 46 µl tetradecane, 51 µl hexadecane, 50 mg octadecane, 66 mg eicosane, 50 mg tetracosane, and 50 mg octacosane were dissolved in 10 ml hexane. Hydrocarbons were obtained from Applied Science, State College, Pa. and Aldrich Chemical Company, Milwaukee, Wisc.

Chromatography packings and supplies

Diethylaminoethyl (DEAE)-Sephadex A-25	Pharmacia Fine Chemicals, Piscataway, N.J.
Dimethyldichlorosilane	Pierce Chemical Company, Rockford, Il.
5% OV-17 gas chromatography liquid phase coated on Supelcoport 80/100 mesh	Anspec Company, Ann Arbor, Mi. (Distributor for Supelco, Bellefonte, Pa.)
Bis-trimethylsilyltrifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS)	Regis Chemical Company, Morton Grove, Il.; or Pierce Chemical Company, Rockford, Il.
Liquid chromatography columns with 200 ml reservoirs	Kontes Company, Vineland, N.J.
12 Foot x 2 mm id paperclip-shaped gas chromatography columns to fit Varian 2100 gas chromatograph	Glass shop, Department of Chemistry, Michigan State University, East Lansing, Mi.

10 Foot x 2 mm id coiled glass gas chromatography columns to fit LKB-9000	Glass shop, Department of Chemistry, Michigan State University, East Lansing, Mi.

Glassware

Disposable micropipettes, 100 ul	Dade, Miami, Florida
50 ml Glass centrifuge tubes	Pyrex, MSU Biochemistry Stores
Lyophilizer jars with ground glass joints	VirTis Company, Gardiner, N.Y.
Silanized glassware	Subjected to 5 min treatment with 1 to 5% solution of dimethyldichlorosilane in hexane, followed by washes with hexane, dry methanol and acetone.

Instruments

Gilford 300 spectrophotometer	Gilford Instrument Laboratories, Oberlin, Ohio.
Varian 2100 gas chromatograph with dual flame ionization detectors and Varian A-25 recorder	Varian Aerograph, Walnut Creek, CA.
Lyophilizer, Model 10-010	VirTis Company, Gardiner, N.Y. Operated at 0.1 to 0.5 Torr.
LKB-9000 gas chromatograph mass spectrometer	LKB Produktur, Stockholm, Sweden.

Data systems

PDP 8/e

Marketed by Systems Industries, Sunnyvale, CA., based on an original system by Sweeley et al. (70S3). The PDP 8/e has 16,000 12- bit words of core memory, a 1.2 million-word disk, DECtape magnetic tape storage, a Textronix 4010-1 cathode ray display device and a Tektronix hard copy unit number 4610.

PDP 11/40

The PDP 11/40 has 56,000 16-bit words of core memory, two 1.2 million-word removable disks and a 7-track magnetic tape drive. It also has a Tektronix 4010 display and shares the hard copy unit of the 8/e. The PDP 11/40 is capable of direct data data transfers to and from the PDP 8/e through an interface designed in this laboratory.

Computer programs

PDP 8/e

Programs were written by N.D. Young for the Systems Industries operating system. The programs are all based on those described by Sweeley et al. (70S3).

PDP 11/40

All programs were written to be used with the Digital Equipment Corporation timesharing system, RSX-11D, version 6B. General programs and assembly-language portions of MSSMET were written by C. Ashendel. Fortran portions of MSSMET were written by M. Smisko and S.C. Gates. All other programs were written in Fortran IV by S.C. Gates, except for FRGENL, which was written by Dr. B.E. Blaisdell. All of the programs on both systems

were written under the direction of Dr. C.C. Sweeley and Dr. J.F. Holland.

Miscellaneous supplies

Urine collection containers, plastic, No. 4013	Falcon, Oxnard, CA.
1 Dram vials	Kimax, MSU Stores, East Lansing, Mi.
Accutint pH 6.9-8.4 indicator paper	Anachemia Chemicals, Montreal, Canada.

CHAPTER THREE: METHODS

Collection of urine samples

Several collections were made of urine samples that were used in work discussed in this thesis.

Standard reference urine. A mixed urine was obtained by collections from a variety of individuals; no attempt was made to collect health or dietary information on these subjects, nor were they asked to fast. The sample consisted of approximately 3 liters of urine collected over a three-hour period from adult males visiting or working in the MSU Biochemistry Department. The sample was kept at 4°C during collection and then aliquoted into approximately 300 test tubes (13 x 100 mm) with Teflon-lined screw caps. The remaining urine was stored in a 1 liter plastic bottle. All of these fractions were stored at -80°C until used. The aliquoted samples served as reference and quality-control samples throughout the project.

BCIU collection. Approximately 200 morning fasting urines were obtained from adult "healthy" volunteer subjects by the Bronson Clinical Investigation Unit, a research unit of the Upjohn

Chemical Company and Bronson Memorial Hospital in Kalamazoo, Michigan. Each subject was asked to conform to a written protocol, a copy of which is included in Appendix A. These samples, referred to as the "BCIU urines," were refrigerated at -20°C as soon as they were brought by the volunteers to the Bronson Hospital and were subsequently transported in dry ice to MSU, where they were defrosted, aliquoted and stored at -80°C until used. Subjects were asked to complete a diet and health questionnaire illustrated in Appendix A. Subjects in this study may also have been subjects in other BCIU protocols, but not within a 72 hour period prior to the urine collection for this study. All subjects had had complete physical examinations within 6 months prior to the date they donated urine. The BCIU urines were used as a reference set of adult urines.

Juvenile reference and neuroblastoma urines. These urines were the generous gift of Dr. William Krivit, Department of Pediatrics, University of Minnesota. All were collected from children hospitalized at the University of Minnesota Hospitals. Diet and health questionnaires were not collected for this group. Urines were collected as early morning samples and subsequently shipped to MSU in dry ice. Information on each patient is summarized in Appendix D.

Foy urine. This urine was the generous gift of Dr. Robert Foy, Sparrow Hospital, Lansing, Michigan. It was collected from a newborn suffering uncontrolled seizures of unknown eitology.

Questionnaires

When appropriate, a diet, health and drug questionnaire was completed by subjects in this study. This questionnaire, shown in Appendix A, is based on the one used by the MSU Health Center in 1974, plus a simple dieter's checklist of foods. The questionnaire was pre-tested on a group of volunteers and a question about smoking habits added as a result of suggestions from these subjects. The questionnaires were encoded into computer files using a special program written for this purpose (MSSQST) to ensure consistent coding.

Isolation of organic acids from urine

Preparation of columns. DEAE Sephadex A-25 is swollen in an excess of freshly-prepared 1.0M pyridinium acetate for at least 48 hours. During this time, the supernatant is discarded and replaced with an equal volume of 1.0M pyridinium acetate, with stirring, at least twice. At the end of the 48-hour period, the supernatant is replaced with an equal volume of 0.5M pyridinium acetate. The DEAE-Sephadex is allowed to soak for an additional 24 hours, with at least two changes of solution.

Columns are prepared with a lightly-packed 1 cm plug of

glass wool at the bottom; too much or too tightly-packed glass wool results in extremely slow flow rates. Water is added and the glass wool poked with a stirring rod to dislodge air bubbles trapped in the glass wool. Most of the pyridinium acetate solution is then decanted from the DEAE-Sephadex, and the remaining material swirled to produce a thick slurry. This slurry is slowly poured into the column, allowing frequent periods of settling. This process is continued until a column packing with dimensions 8 cm by 1 cm is obtained. Once the column is completely poured, 21 ml of 0.5M pyridinium acetate is added and allowed to drain through the column until the fluid level is just above the level of the packing. Columns are left at this stage while the creatinine determinations are completed and the urine samples prepared; frequently the columns are prepared one day in advance and allowed to remain at this stage overnight.

Creatinine determination. Either at the time the urine is aliquoted or at the time the urine is defrosted for separation on the DEAE-Sephadex, the concentration of creatinine is determined(54H1). Fresh alkaline picrate is made by combining 7.5 ml 10% NaOH(aq) with 100 ml of saturated picric acid solution. A 25 ml volumetric flask is used for each of 5 standards and the samples, and 5.0 ml of the alkaline picric acid solution is pipetted into each flask. To each of the 5 standard flasks is added 0 µl, 62 µl, 125 µl, 188 µl and 250 µl, respectively, of the 1.00mg/ml creatinine standard solution, using a

100 ul syringe. The solutions are shaken and let stand for 10 min. Similarly, 50 µl of each urine sample is added to separate flasks. At the end of the 10 min period, all samples are diluted to 10.0 ml with water and thoroughly shaken. The absorbance of each sample is determined using the 0 µl standard as a blank. A graph of absorbance versus concentration of creatinine is constructed, with the 5 points on the abscissa from the standard solutions representing 0, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml, respectively. This graph should be roughly linear at low concentrations and match previous standard curves, or it is discarded. Concentrations of creatinine in the urine samples are read from the graph.

Preparation of urine sample. The procedure of Thompson and Markey (75T2) for the quantitative separation of organic acids from urine is used in a somewhat modified form.

First, the sample of urine, previously stored at -80°C, is brought to room temperature in a bath of warm water. Likewise, the tropic acid internal standard solution (1 mg/ml tropic acid in methanol, stored at -80°C) is brought to room temperature. For each urine sample to be prepared, 50 µl of the tropic acid solution is added to a silanized 13 x 100 mm test tube and evaporated to dryness under a stream of dry nitrogen.

Each urine sample is then shaken to achieve a homogeneous solution and an aliquot, calculated to contain 1.44 mg of creatinine

(approximately), is removed and added to the test tube containing the internal standard. Each tube is then sonicated to assure complete mixing of the tropic acid with the sample.

To each solution thus obtained is added 3.00 ml of 0.1M $Ba(OH)_2$ (aq). Each tube is mixed on a mechanical mixer to homogeneity and then centrifuged for no more than 30 seconds at maximum speed on a small table-top centrifuge. The supernatant solution is then removed by pipette and the precipitate washed and centrifuged twice, with an additional 1.0 ml 0.1M barium hydroxide solution added each time.

Oxime derivatives of oxo-acids in the mixture are prepared by the addition of 200 µl of a hydroxylamine hydrochloride solution to the combined supernatants, followed by heating at 80°C for 20 min. After cooling to room temperature in an ice bath, each sample is adjusted to pH 7 to 8 using 2N HCl (aq) or 2N acetic acid (aq). The pH is measured with pH 6.9-8.4 limited range pH paper; urine is pipetted in small amounts onto the pH paper rather than dipping it in the sample.

Once the sample has been prepared, it is slowly added to the top of a DEAE-Sephadex column with a Pasteur-type pipette. The stopcock of the column is opened to allow the sample to drain onto the column bed. Approximately 5 ml of redistilled water is pipetted onto the column to avoid disturbing the Sephadex, and then enough

water is added to bring the total amount of water to 50 ml. The eluate from the 50 ml water wash is discarded.

Acidic metabolites are eluted from the column into a clean, silanized 250 ml round-bottom flask with 40 ml of 1.5M pyridinium acetate. The sample is frozen in a dry-ice-acetone bath, using either hand or rotary-evaporator rotation of the flask to achieve a smooth, even coating of sample on the inside of the flask. If necessary, it is stored at -80°C until the lyophilizer is available. The sample is then dried on a lyophilizer at approximately 0.1 Torr (or lower) until about 5 ml of sample remain. At this point, it is melted and transferred to a silanized 50 ml conical centrifuge tube with a ground glass stopper. It is refrozen by submerging the stoppered tube in dry-ice-acetone (or liquid nitrogen). The tube mouths are covered with several layers of coarse-mesh cheesecloth attached with a rubber band to prevent losses of sample material and loaded into 1-liter lyophilizing flasks. They are lyophilized to complete dryness and removed immediately. The dried samples are silylated in the same tubes by the addition of 250 µl of the standard silylating mixture (BSTFA:TMCS:pyridine, 200:2:50, v/v). The stoppered tubes are heated at 80°C for 1 hour; an inverted test tube rack is placed over the ground-glass stoppers to hold them in place, reducing the likelihood of stoppers popping out during heating. In addition, the tubes are shaken at least once during the heating process

to ensure complete silylation.

Once the samples are silylated, they are immediately transferred to silanized glass capillaries. The capillaries are most conveniently prepared from commercially-available 100 µl disposable micropipettes broken in half and sealed with a flame at one end. Each capillary is filled about one-half full (approximately 20 µl) from a 500 µl syringe and sealed with a flame until a small bubble begins to form in the glass at the heated end. (Melting point capillaries are much harder to seal quickly and hence should not be used.) Sample capillaries are placed in 13 x 100 mm test tubes with screw-cap tops, labeled and stored at 4°C until used. The 500 µl syringe is cleaned with dry redistilled methanol, followed by redistilled hexanes between each sample. Residual methanol must be avoided since it will react with the trimethylsilyl derivatives.

Gas chromatographic analysis

All samples are analyzed by either GC or GC-MS. In either case, the chromatographic conditions are essentially the same: analysis on 5% OV-17 coated on 80/100 mesh Supelcoport, use of glass columns, and temperature programming from 60 to 260° at 4° /min.

Each time a GC or GC-MS column is packed, it undergoes the

same procedure. This begins with removal of all previous packing material, aspiration of concentrated H_2SO_4 through the column until it is filled, and removal of the sulfuric acid after 15 to 30 minutes. The column is then washed successively with redistilled water and acetone until free of sulfuric acid, and dried with a stream of nitrogen gas. It is then filled with a freshly-prepared 1% solution of dimethyldichlorosilane (DMDS) in hexane and allowed to sit 10 to 15 min. The solution is removed by aspiration and replaced by successive washes of hexane; dry, redistilled methanol; and hexane. The column is again filled with the DMDS solution and the same process repeated to ensure complete silanizing of the glass. The remaining hexane is then removed with redistilled acetone, and the column dried with a stream of nitrogen. If necessary, the column is heated at 100°C to ensure dryness.

The column is then packed in the following manner. A 3 cm glass wool plug is placed in the detector end of the column and the 5% OV-17 added via a small funnel while an aspirator is connected to the detector end. An electric vibrator is used to speed the process. The OV-17 is added slowly to avoid differential migration of fines, until the column is fully packed. The column is heated at 100°C for 10 min, and more packing added until it will not settle further upon vigorous vibration. The remaining 3 cm space is filled with silanized glass wool which has been stored in a desiccator. The

DMDS, OV-17 and glass wool are immediately returned to the desiccator for long-term storage.

Once the column is packed, it is conditioned with the detector end disconnected for at least 48 hours at 280°C. This is accomplished using normal carrier gas flow rates and programming the temperature from ambient to 280° at 2°/min, then holding at 280° or higher until the column is fully conditioned. Since Teflon ferules are used, the nuts holding the column must be tightened several times, especially before the column is cooled, to avoid having the column leak or drop out of position.

For GC analysis, the columns are 12-foot paperclip-shaped glass columns designed to fit the Varian Model 2100 gas chromatograph used in these studies. Since the columns frequently drop out of position, they are supported by specially-designed aluminum plates. Analysis on the GC is usually accomplished with the injector and detector heaters set at 300°C, the attenuation at 2, the gain at 10^{-10} amps/volt, the recorder at 1 mV full scale, and only a very small bucking voltage. Gases used are helium as carrier at 40 ml/min, hydrogen at 30 ml/min, and air at approximately 300 ml/min. Usually, 2 µl sample injections are used. A solution of fatty acid methyl esters is used to check the overall response of the GC prior to injection of urine samples. Septa are preconditioned at 300°C and changed frequently. The detector cylinder is cleaned after

each 2 to 3 injections by sonication in methanol and brisk brushing with a small test-tube brush.

Analysis of samples on the LKB-9000 GC-MS-COM system

A regular routine is adhered to in analyzing samples on the LKB-9000, as follows:

Column preparation. Glass columns 2.0 mm ID by 10 feet and containing 5% OV-17, are prepared exactly as for gas chromatography alone. Since the septa often do not stay in the septum holder, a special cage is fashioned from paperclips to keep the septa in place. Ferules are tightened regularly and replaced frequently. Septa are checked daily for leaks.

At the beginning of each day, electronic noise levels are determined with a special test routine on the PDP 8/e designed for this purpose (INTEST). Noise widths are checked, and the baseline is adjusted so that it is just above zero volts. A test is also made for high intensity random noise spikes. If the noise levels are above 300 mV (intensity of 15 out of a possible 500,000), or if spikes greater than 600 mV are detected, further analysis is halted until these problems are eliminated.

The ion source is then focused to a resolution of at least 500 (10% valley definition). A mass versus Hall effect calibration with

perfluorokerosene is then performed and the resulting calibration data are stored for use during the remainder of the day. Calibration is from m/e 51 to 700 at scan speed 8, with the mass marker reading 43 when scanning has ceased.

A standard reference capillary sample (described below) is then run to test all aspects of the system. In general, 6 to 8 μl of this sample are injected under the following conditions: the GC is temperature-programmed from 50-260°C at 10°/min; the sample is injected and the computer program started when the temperature reaches 60°C on the dial of the temperature controller. Other conditions are: ion source temperature, 290°; separator, 290°; GC injector, 150° (it reaches 270° by the end of the run); gain 8 on multiplier; scans at constant 4-second intervals at scan speed 8 over range m/e 49 to m/e 550 (the upper scan limit is set so that the magnet decays just to m/e 49 before beginning the next scan, lower limit remains the same as for calibration); accelerating voltage, 3.5 kV; trap current, 65 μA; box current, 30 μA; filament current, approximately 4A. The valve is opened and data collection begun at 5 min. At the end of the analysis, the data are examined for evidence of high column bleed, low response of hippuric or uric acids, low sensitivity, poor chromatographic resolution, abnormal GC retention behavior and other problems in comparison to previous runs with the same sample. All of these problems are eliminated

before proceeding with data collection.

Once the LKB-9000 is considered ready, the GC column is injected twice with 8 µl of the BSTFA-TMCS silylating mixture, and then cooled to 40°C. All data are removed from the data storage disk, and the computer is set up for data collection as for the standard sample, except that the run is set to end at 60 min (SR=60 command). A 0.5 µl aliquot of a mixture of straight-chain hydrocarbons in hexane is withdrawn with a 10 µl syringe, followed by a 0.5 µl air "spacer," and then 8 to 9 µl of the derivatized urine sample to be analyzed. The sample capillary that has just been opened is immediately discarded even if sample remains. The sample is injected under the same conditions as the reference capillary, except that the temperature programmer is set to 4°/min, and the separator valve is not opened until 6.5 to 8 min after injection, depending on the elution time of the last solvent peak.

At the end of each run, the GC column is injected with two 5-µl aliquots of the BSTFA-TMCS solution to prevent any carryover from sample to sample. The GC column is then cooled to room temperature to begin the next run. While the column is cooling, the data collected by the computer are examined for problems and transferred to the PDP 11/40 for storage and MSSMET analysis. During the transfer process, which takes approximately 10 min, the data are converted to the standard mass spectral data (MSD) format used on the

PDP 11/40 in this laboratory (76A1). Each run is stored with a six-digit identification code (month-day-run number), the sample number (month-day-year-initials of chemist-serial number), and other pertinent information or comments.

Preparation of samples for preliminary studies

Several studies were undertaken to test the analytical features of the DEAE-Sephadex procedure, the GC-MS-COM system, and the complete method, including statistical analysis of data. The samples prepared for these studies are described below: Approximately 10 ml of the standard urine sample was separated on a column 10 times larger than normal and 10 times more of each of the solvents was used. The 1.5M pyridinium acetate eluate was divided into 10 containers, lyophilized, and then the silylated urines combined in a single container. Approximately 100 capillaries, each containing 15 µl of sample, were prepared and sealed. These capillaries are referred to as the "reference capillary samples" in this thesis; they were used as reference standards to test the condition of the GC and GC-MS.

Capillary stability. A single urine sample was prepared by a scaled-up version of the standard procedure; all quantities were 5 times normal. A sample of the standard reference urine was used.

The silylated sample was carefully mixed in a single 50 ml centrifuge tube, and sets of capillaries were stored at -80°, -20°, -4°C and room temperature. Randomly selected samples from each temperature set were chromatographed at the following times: 0 days, 1 day, 2 days, 3 days, 1 week, 1 month, 2 months, 3 months and 6 months.

Urine stability. A freshly collected sample of urine was divided into 17 aliquots of approximately 5 ml each. All fractions were placed in standard 125 ml plastic urine collection containers. Four containers of each were stored at -80°C, -20°C, 4°C and room temperature. The remaining aliquot was analyzed immediately utilizing the DEAE-Sephadex procedure. One-ml aliquots of each of the samples were then analyzed at intervals of 1 day, 1 week, 1 month and 6 months from the starting date. Each sample was defrosted as rapidly as possible (if necessary), mixed thoroughly, an aliquot withdrawn, and any remaining urine discarded rather than being placed at the original temperature again. All samples were separated on the DEAE-Sephadex and analyzed on the Varian 2100 GC.

Silylating solvent. Four identical tubes were prepared containing 50 μg each of 5-hydroxyindoleacetic acid, indoleacetic acid and tropic acid. To each was added a silylating mixture consisting of the solvents shown in Table 1.

Table 1. Silylating mixtures

Tube no.	Silylating reagent	Additional solvent
1	75 µl BSTFA/TMCS	25 µl dimethylformamide
2	75 µl BSTFA/TMCS	25 µl acetonitrile
3	75 µl BSTFA/TMCS	25 µl pyridine
4	100 µl BSTFA/TMCS	none

All additional solvents were redistilled and stored with a drying agent. All four tubes were heated at 80°C for 1 hour and sealed in capillaries until analyzed on the gas chromatograph.

Recovery study. Solutions of several apparently pure compounds were prepared as shown in Table 2. A 400-µl aliquot of each solution (measured with a 500 µl syringe with no air bubble between solution and plunger) was added to a 5 ml volumetric flask, as was 200 µl of a 1.00 mg/ml solution of indoleacetic acid in n-butanol. All of the solutions of Table 2 were prepared using volumetric flasks. The mixture was diluted to 5.00 ml with redistilled dry methanol to form the "spike" solution. The spike solution was divided as shown in Table 3; the standard reference urine was used in tubes 1 through 11. All tubes containing urine were run through the complete DEAE-Sephadex procedure as usual. Tubes 12 through 14 were dried and silylated directly. All samples were run on the LKB-9000 and analyzed using MSSMET.

Table 2. Composition of recovery study solutions

Compound name	Dry weight (mg)	Final volume (ml)
Ascorbic	23.8	10.0
β-Hydroxy-β-methylglutaric	8.8	10.0
Succinic	11.6	10.0
Citric	12.6	10.0
Tropic	22.5	10.0
Vanilmandelic	10.1	10.0
α-Glycerophosphoric	13.0	10.0
Salicylic	6.4	50.0
Hippuric	24.2	10.0

Table 3. Recovery study samples

Tube no.	Amount of spike solution (µl)	Amount of urine (ml)
1 - 4	0	1.0
5 - 8	200	1.0
9 - 11	1000	1.0
12	200	0.0
13	100	0.0
14	500	0.0

Linearity and isotope dilution series. Stock solutions at 1.00 mg/ml in isopropanol were prepared from 5-hydroxyindoleacetic (5-HIAA), 3,4-dihydroxyphenylacetic (DHPA), indoleacetic (IAA), homovanillic (HVA) and hippuric (HIP) acids. A total of 10 stock solutions were prepared from these compounds: one each of the unlabeled compounds, and one each of the dideutero (α-d_2) forms of all except DHPA, which was available as the pentadeutero (α-d_2, 2,5,6-d_3) form. The DHPA required a small amount of added water to achieve complete dissolution. The unlabeled IAA and 5-HIAA required purification by recrystallization from hot chloroform to remove colored impurities. The unlabeled DHPA, while appearing impure, could not be purified further, and so was left as received. The labeled 5-HIAA, IAA and DHPA also appeared to require purification, as judged by color and crystal shape, but none was purified because of the cost of the substances.

In addition, 4 stock solutions (1.00 mg/ml in isopropanol) were made of compounds for which there were no deuterated standards: vanillic (VAN), p-hydroxycinnamic (PHC), ascorbic (ASC) and citric (CIT) acids. A 1.00 mg/ml solution of tropic acid in methanol was also used.

A stock "unlabeled mixture" was prepared by using a 500 µl syringe to remove 500 µl of each of the unlabeled compounds except tropic acid. These aliquots were combined into a single silanized

test tube and gently taken to dryness under a stream of dry nitrogen gas. The tube containing the unlabeled mixture was labeled tube number 1.

A separate "dilution mixture" was prepared from 500 µl of each of the labeled compounds and the tropic acid. This mixture was evaporated to dryness with nitrogen and then diluted to 20.0 ml with a 1:5 mixture of chloroform -methanol (both previously redistilled).

A series of 15 15-ml ground-glass-stoppered centrifuge tubes was then prepared as follows, using silanized tubes in each case. A 2-ml volumetric pipette was used to transfer 2.00 ml of the dilution mixture to the tube containing the dried unlabeled mixture (tube number 1). This tube was then sonicated and mixed mechanically until a homogeneous solution was obtained. A 1-ml volumetric pipette (the "transfer pipette") was used to transfer 1.00 ml of this solution to tube number 2. The contents of tube number 2 were diluted with 1.00 ml of the dilution mixture, using the "diluting" volumetric pipette. This tube was then sonicated and thoroughly mixed. The transfer pipette was cleaned by aspirating 20 ml of methanol and 10 ml chloroform (each interspersed with several sets of air bubble) through the pipette. It was then used to transfer 1.00 ml of the contents of tube number 2 to tube number 3, and the process repeated until tube number 14 was finished. Tube number 15 contained only 1.00 ml of the dilution mixture. The complete set

of tubes was then as shown in Table 4.

Table 4. Composition of linearity and isotope dilution series

Tube no.	Amount of each unlabeled compound (μg)	Amount of each unlabeled compound per 4 ul injection (ng)
1	250	10,000
2	125	5,000
3	62.5	2,500
4	31.2	1,250
5	15.6	625
6	7.81	312
7	3.90	156
8	1.95	78.1
9	0.976	39.0
10	0.488	19.5
11	0.244	9.76
12	0.122	4.88
13	0.061	2.44
14	0.030	1.22
15	0.000	0.00

The contents of all 15 tubes were dried under a nitrogen stream and 100 µl of a 9:1 solution of BSTFA-TMCS: pyridine added to each. The tubes were sealed with ground-glass stoppers and heated for 1 hour at 80°C. Each fraction was then thoroughly mixed mechanically and transferred to 4 to 5 silanized glass capillaries and sealed until analyzed. Four-µl injections from freshly-opened capillaries were used when the samples were analyzed on the GC-MS; approximately 0.5 µl of the hydrocarbon mixture was added to each aliquot at the time of injection.

Two separate sets of analyses were performed on these samples: repetitive scanning while the GC was programmed from 160 to 280°C at 4°/min, and selected ion monitoring analyses under the same conditions. Repetitive scanning was terminated 26 minutes after each injection. After the separator valve was closed, about 5 µl of BSTFA-TMCS were injected while the column temperature was kept at 280°. All injected substances were monitored by the repetitive scanning method; the substances and ion monitored by SIM are shown in Table 5.

Table 5. Compounds monitored by SIM during linearity study.

Group*	Compound	Ions (m/e)
I	tropic acid	280
	octadecane	254
	citric acid	273
II	3,4-dihydroxyphenylacetic acid	384,385,389,390
III	hippuric acid	206,207,208,209
IV	indoleacetic acid	319,320,321,322
V	5-hydroxyindoleacetic acid	407,408,409,410

*Note: each group corresponds to a different magnet field strength.

Urine studies. Once all of the preceding studies were complete, the entire procedure was tested on a series of urine samples. These included 9 BCIU urines, 5 neuroblastoma urines and 5 infant control urines. Each sample was prepared and analyzed by the procedures discussed in this thesis, with no difference in treatment among groups except that 1.0 ml of each of the neuroblastoma and infant control urines was used, whereas the BCIU urines were aliquoted so that 1.44 mg of creatinine equivalent was applied to the DEAE Sephadex column. In addition, one of the infant control samples was run on 3 separate days on the LKB-9000 to obtain a measure of the intrasample variability.

Analysis of data by MSSMET

At the end of real-time data collection, a urine sample is represented by approximately 700 to 800 mass spectral scans, each containing an average of 300 or more mass/intensity pairs. The goal of the mass spectral metabolite program (MSSMET) is to reduce these 1/2 million data to a set of 100 to 200 concentrations and compound names.

MSSMET exists in two versions. The older one was designed for use with a PDP 8/I computer with DECtape storage. Although successful in identifying compounds, this version, which was completed in late 1973, was limited by the small memory size and the slow tape access speeds of the PDP 8/I. It was abandoned when a more powerful computer, the PDP 11/40, became available. The second version of MSSMET was completed for the PDP 11/40 in late 1975 and was subsequently modified through a number of revisions until development was finished in the summer of 1976. Virtually all of the data reported in this thesis were provided by this last revision of MSSMET; hence, it will be the version discussed here. Documentation for the PDP 8/I version is available elsewhere (74S2).

The following section provides a description of the structure and operation of MSSMET in general. For a more specific description, the reader is referred to a separate document (76G3) which

also includes a copy of the program.

MSSMET has been designed as a general-purpose GC-MS analysis program. However, to date it has been utilized principally for the analysis of organic acids in human urine, and it is this use that will be described here.

In brief, MSSMET utilizes mass chromatography, GC retention indices and a reverse library search procedure to locate compounds of interest. Once located, the compounds are quantitated relative to an internal standard and the results provided to the user. This may be accomplished with almost no operator intervention. Flow charts of the normal operation of MSSMET are shown in Figures 2 and 3.

More specifically, MSSMET is a library-based search procedure that operates as follows:

Program initiation. This is the only portion of the program which absolutely requires operator interaction with the computer. The program begins by requesting a variety of information, such as the name of the library file, the name of the data file, the amount of urine extracted, the amount of creatinine per ml of urine and the amount of internal standard added. Once these data are read into the computer, the program can be set to run under manual control or, as is more often the case, under completely automatic control as directed by the library.

Reading the library file. The library contains operating

Figure 2. Generalized MSSMET flowchart.

The same type of analysis is performed by MSSMET for each biological sample. Repetitive scanning data are searched for spectra of retention standards, quantitative standards and the metabolites of interest. Compounds that are judged to match the library spectra are entered in a "found" file. Details of this process are given in Figure 3.

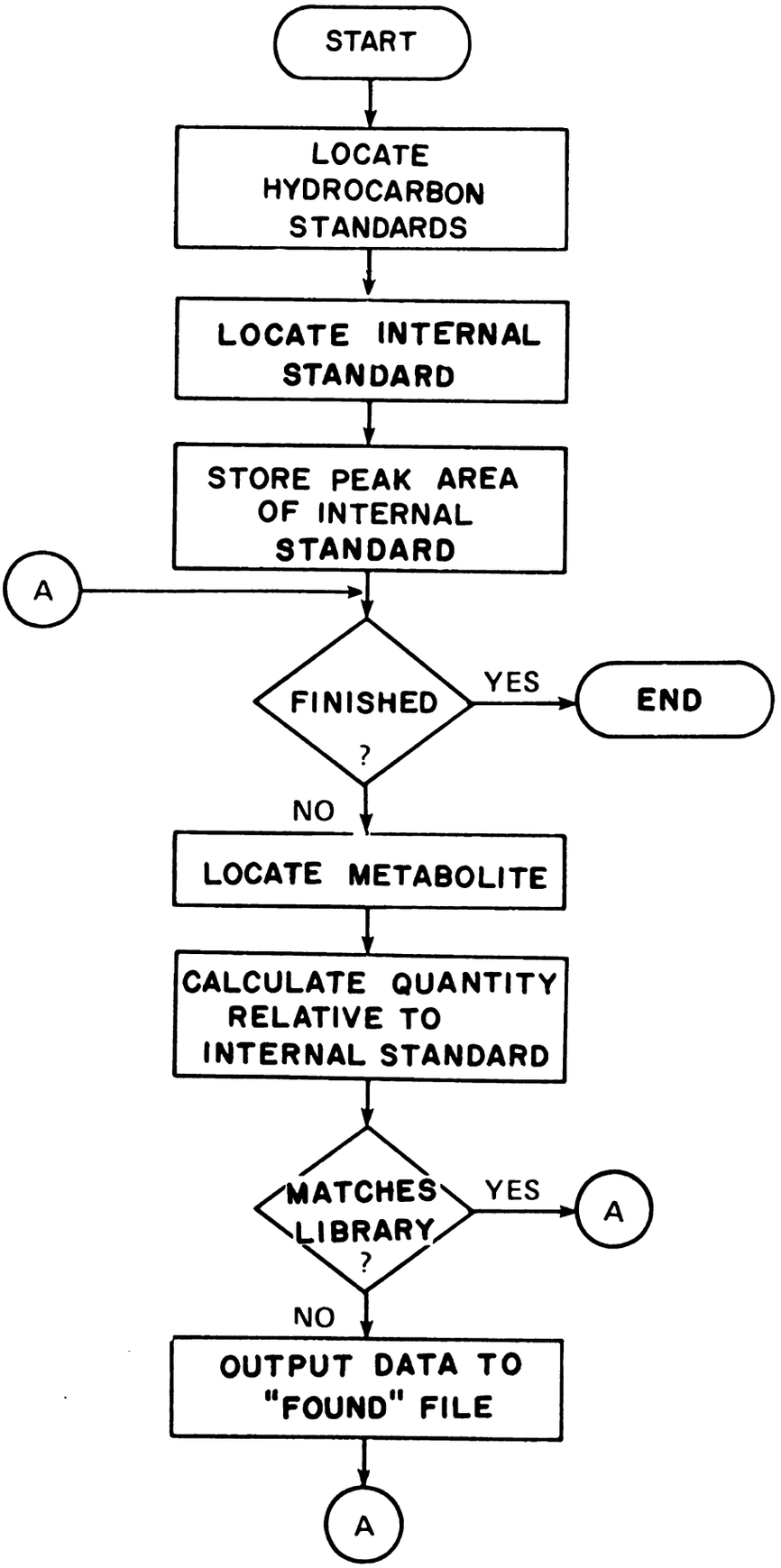
START
LOCATE HYDROCARBON STANDARDS
LOCATE INTERNAL STANDARD
STORE PEAK AREA OF INTERNAL STANDARD
A
FINISHED ?
YES
END
NO
LOCATE METABOLITE
CALCULATE QUANTITY RELATIVE TO INTERNAL STANDARD
MATCHES LIBRARY ?
YES
A
NO
OUTPUT DATA TO "FOUND" FILE
A

FIGURE 2

Figure 3. Detailed MSSMET flowchart.

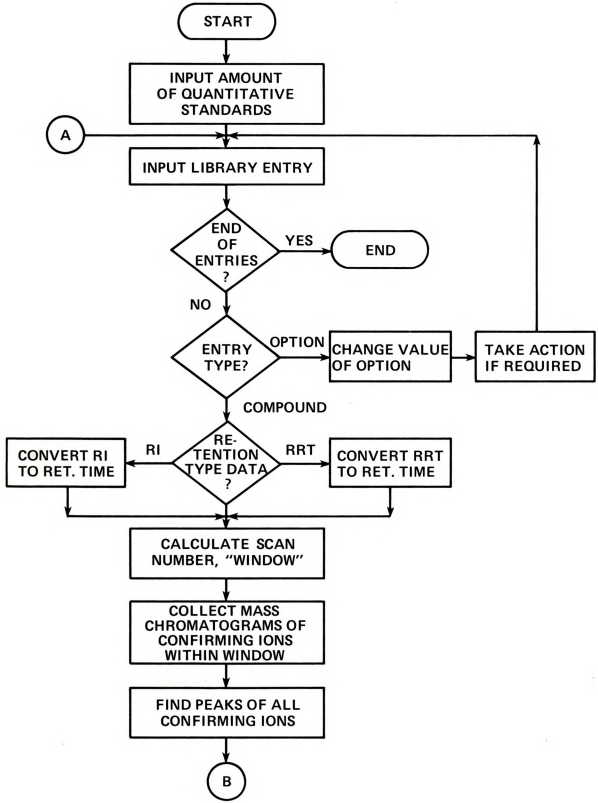
START
INPUT AMOUNT OF QUANTITATIVE STANDARDS
A
INPUT LIBRARY ENTRY
END OF ENTRIES ?
YES
END
NO
ENTRY TYPE?
OPTION
CHANGE VALUE OF OPTION
TAKE ACTION IF REQUIRED
COMPOUND
RE-TENTION TYPE DATA ?
RI
CONVERT RI TO RET. TIME
RRT
CONVERT RRT TO RET. TIME
CALCULATE SCAN NUMBER, "WINDOW"
COLLECT MASS CHROMATOGRAMS OF CONFIRMING IONS WITHIN WINDOW
FIND PEAKS OF ALL CONFIRMING IONS
B

FIGURE 3

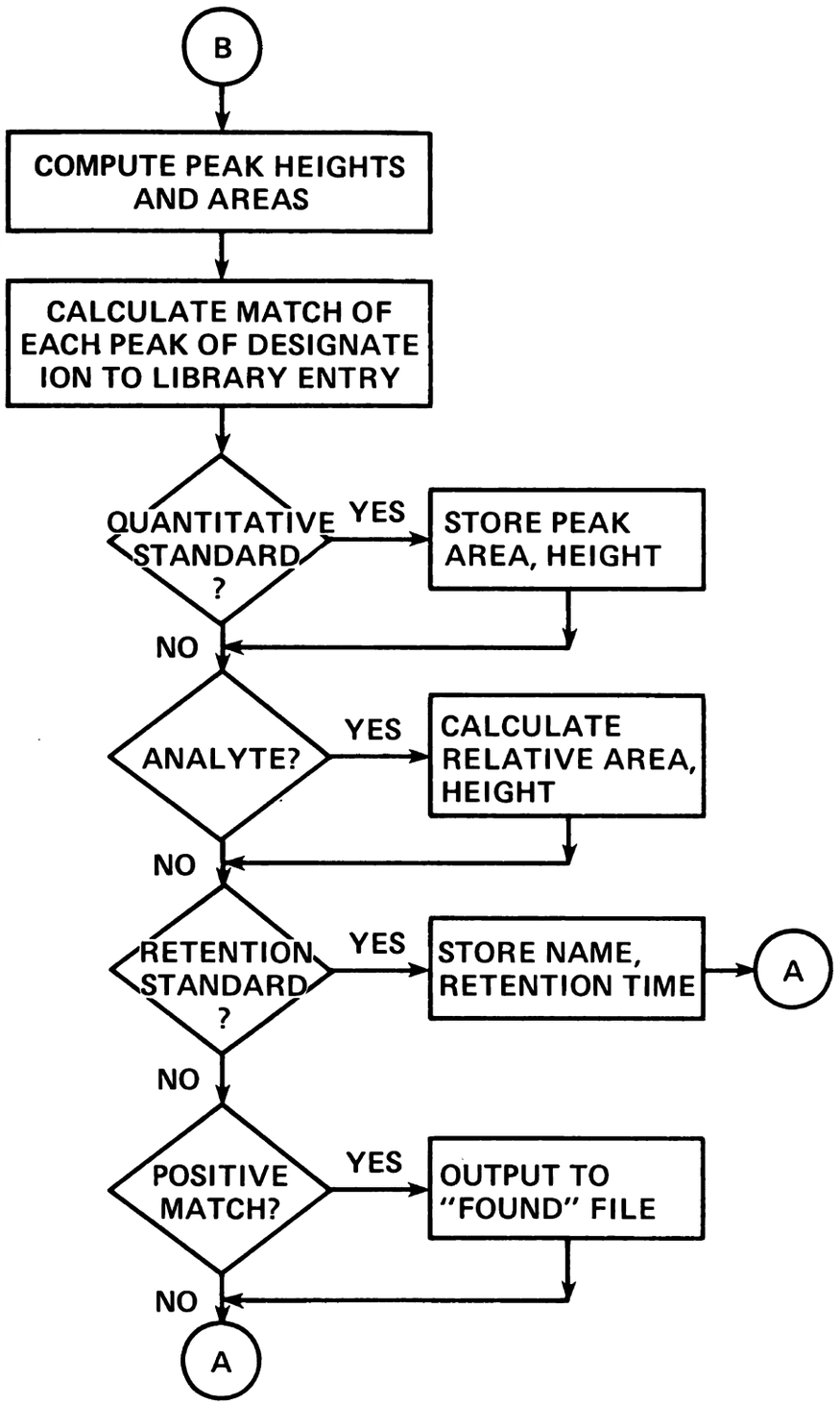

FIGURE 3 (Cont'd,)

parameters ("options") and sufficient information about the compounds of interest to allow them to be found in the GC-MS data of the urine sample. The options include such information as the criteria for peak detection, what kinds of outputs to generate and criteria for what constitutes an acceptable match between library and urine sample spectra. The compound information is of a fixed format and always includes the following:

Compound name,

Retention time,

Designate ion and k-factor, and

Confirming ions paired with intensities.

A sample library entry of this type is shown in Figure 4.

The retention time may be expressed in minutes and seconds (actual retention time), relative to a single standard (relative retention time), or relative to a set of hydrocarbons or other standards (retention index, originally defined in 58K1). The designate ion is that ion which is considered to be most likely to be differentiating of that compound, and the confirming ions are a set of up to 8 ions, always including the designate ion, that is used to judge whether a peak of the designate ion represents the compound of interest. The k-factor is used to convert the relative area of the designate ion to absolute concentration (mg/ml or mg/mg creatinine). A copy of the complete library used for the analysis of

Figure 4. Typical MSSMET library entry.

Two types of entries are found in the MSSMET library. The first is an "option," which changes the value of a particular program variable. Those illustrated change the peak detection threshold (TH), the window width (WM), and the minimum match coefficient required for a positive match (CF). The second type of entry gives information about a particular compound, including: its number in the library and IUPAC and common names, if known; GC retention index; m/e of the designate ion and the value of the k-factor; and the masses and relative intensities of the confirming ions.

```
      /TH 50,
      /WM 120,
      /CF 81,
* 129 M-HYDROXYPHENYLACETIC
 1728
 164,1.000,
 164,1000,252,  440,281,  990,296,  990
```

FIGURE 4

organic acids in urine samples is included as Appendix B.

Location of compounds. All types of compounds are located in the same manner. After reading the library options and the first compound entry, MSSMET calculates an "expected retention time" for the compound. This is converted to a scan number in the data file and a "window" is then determined by adding and subtracting a pre-specified number of scans from the expected retention time. This window is the region of the GC-MS run within which the compound is expected to elute and must be no narrower than the widest expected peak. Mass chromatograms--plots of ion intensity versus scan number (70H1)-- are collected within this window for all of the confirming ions, as shown in Figure 5. Peaks in each mass chromatogram are detected and measured to obtain peak areas and heights (see below).

For each peak of the designate ion found within the window, a "match coefficient" is calculated to judge the degree of match between the library and urine sample spectra. The formula for this match coefficient is given in Figure 6; it is a modification of a formula proposed by Grotch (73G1). Confirming ions must peak within a certain number of scans (specified by an option) of the designate ion in order to be included in the match coefficient.

In addition, the deviation of the actual retention time for each compound from the value expected is computed. Based on whether

Figure 5. Determination of retention index window.

Positions of the hydrocarbon standards injected with the sample are determined from the mass chromatogram of m/e 85. A "window" is centered at the retention index specified by the library entry for the trimethylsilyl derivative of m-hydroxyphenylacetic acid (Figure 4), and mass chromatograms of the confirming ions are collected within this window. The ratios of these ions are compared to the library entry to determine a match coefficient. Ions peaking at scans 382 and 407 are from the trimethylsilyl derivatives of tropic acid and p-hydroxyphenylacetic acid, respectively.

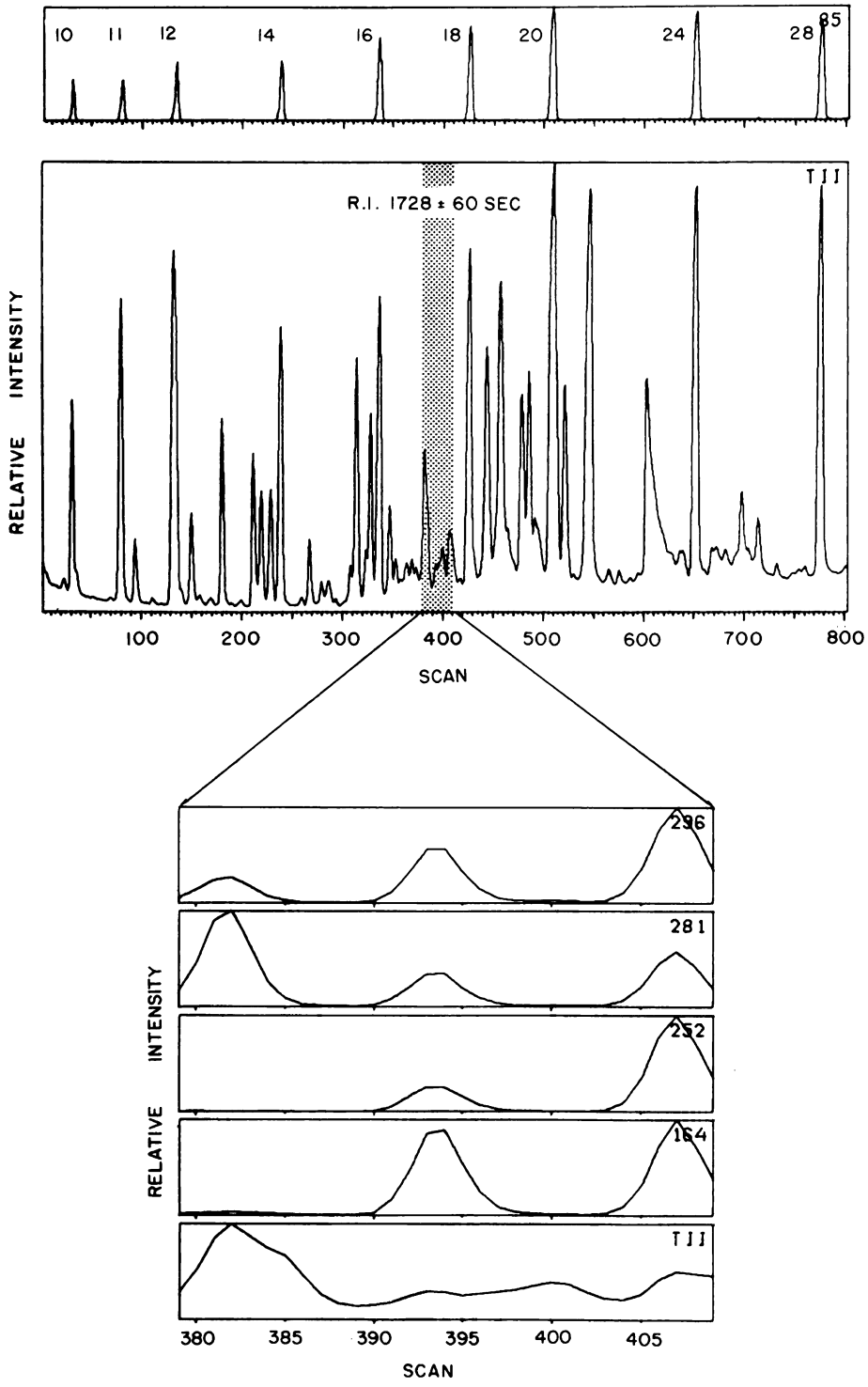
10
11
12
14
16
18
20
24
28
85
R.I. 1728 ± 60 SEC
TII
RELATIVE INTENSITY
100
200
300
400
500
600
700
800
SCAN
296
281
252
164
TII
RELATIVE INTENSITY
380
385
390
395
400
405
SCAN

FIGURE 5

Figure 6. Formula for calculation of match coefficient by MSSMET.

MSSMET calculates a match coefficient to measure the agreement between the library and experimental spectra. The formula is a modification of one proposed by Grotch (73G1). Separate match coefficients are calculated using peak area ratios and peak height ratios; these are referred to as the "peak area match coefficient" and the "peak height match coefficient," respectively.

$$MC = \left\{ 1 - \left[\frac{\sum_{j=1}^{N} | I_j^F - I_j^D |}{\sum_{j=1}^{N} (I_j^F + I_j^D)} \right] \right\} 100$$

MC=Match coefficient

N=Number of confirming ions

I_j^F = Intensity of j^{th} ion in library file

I_j^D = Intensity of j^{th} ion in data

FIGURE 6

the peak has a sufficiently high match coefficient and whether it elutes close enough to the expected retention time, the peak is designated as being in one of three categories: positively identified as matching the library entry ("+" category), identified as definitely not matching the library entry ("-" category), or of uncertain identity ("?" category). The range of values accepted for each of these categories is specified by the set of options. The use of these categories when retention indices are used to measure retention time (as is usually the case) is shown in Figure 7.

Location of ion peaks. Central to any automated chromatographic procedure is the ability to accurately locate and quantitate peaks. MSSMET algorithms for accomplishing this are necessarily complicated to allow for a variety of special situations.

As shown in Figure 8, the basic algorithm consists of identifying three regions in every peak detected in each mass chromatogram. In each region, a particular question is being asked:

Region I. Has a peak definitely started?

Region II. Has intensity reached a peak value and started to decrease?

Region III. Has the peak ended?

In order for a peak to be considered to have started, there must be either a certain number of points (specified by an option), each above the previous in intensity, or the slope of the curve must

Category	Match coefficient	|RI deviation|*
+	81 - 100	0 - 12
?	80	0 - 12
	81 - 100	13 - 16
-	0 - 79	16

* Absolute value of deviation of retention index from library value.

Figure 7. Criteria for positive match to the library entry.

The match coefficient (MC) and the measured deviation of the retention index from the library value ($|\Delta RI|$) are used together to determine whether a given peak of the designate ion represents the compound of interest. Both the MC and the $|\Delta RI|$ must fall within certain limits for the compound to be considered a positive match (+) to the library spectrum. Slightly wider limits determine the boundaries of the questionable match (?) region; all remaining possibilities are defined as negative matches (-). Only positive matches are placed in the "found" file.

Figure 8. Detection of mass chromatogram peaks.

The MSSMET peak detection algorithm distinguishes 3 regions of the peak; in these regions, data points are tested to detect whether the peak is starting, ending, or definitely ended. A peak is considered starting if either a minimum slope or a minimum number of consecutively increasing points is detected. The peak is considered ending if it has apexed and then dropped below a threshold value or a certain fraction of the peak height. The peak is considered ended if the ion intensity begins to increase again for a certain number of scans, or if the end of the window is encountered. If the end of the window is encountered before the "peak ended" criteria are met, the data points are not considered to represent a peak. All values of the peak detection parameters are set by the MSSMET library options.

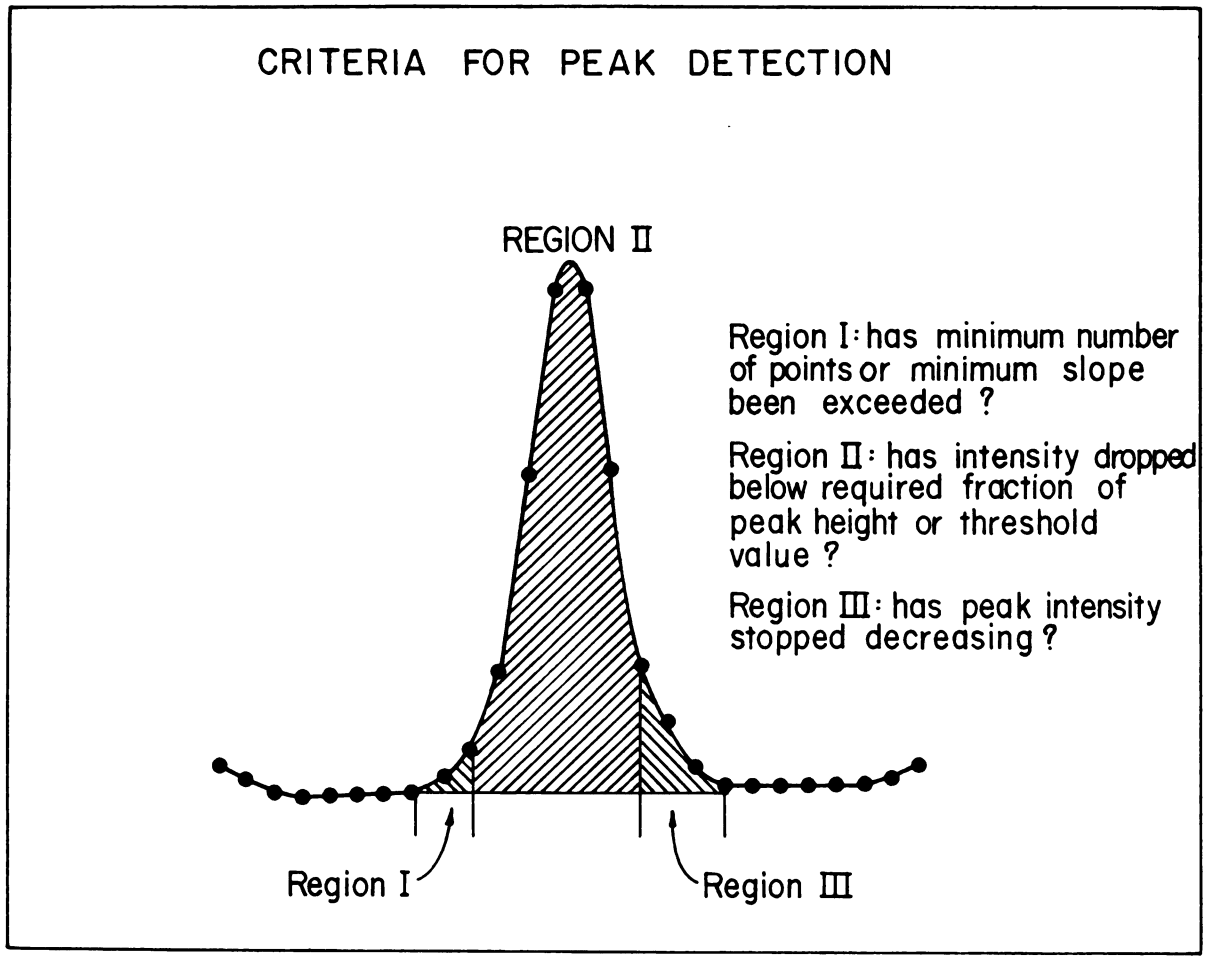
CRITERIA FOR PEAK DETECTION
REGION II
Region I: has minimum number of points or minimum slope been exceeded ?
Region II: has intensity dropped below required fraction of peak height or threshold value ?
Region III: has peak intensity stopped decreasing ?
Region I
Region III

FIGURE 8

be above a certain (option-specified) value. Typically, for organic acids, it is sufficient to simply look for 2 consecutive increasing points.

Once the peak has been categorized as definitely starting, each successive data point is examined until the peak intensity is less than one of two values: either a preset threshold, or a fraction of the peak height, whichever occurs first. Both the threshold and the peak height fraction are set by library options; generally, the latter criterion is the one met by most peaks.

After the peak intensity has dropped below one of these values, the peak is considered definitely ending; if the peak intensity increases at any succeeding scan and remains constant or increases for a certain number (typically two) of points in a row, the peak is considered ended. Succeeding points are then examined to see if another peak is starting. Alternatively, if the end of the window is reached once below the threshold or peak fraction values, the peak is also considered ended.

Baseline determination. Although the location of ion peaks is critical, baseline determination is equally important for accurate quantitation. In the current version of MSSMET, up to 20 baseline points are selected and used to determine a least-squares equation for the baseline. The order of the least-squares fit is determined by an option.

All points in the window are initially considered as candidates for baseline points. Points are discarded from consideration, however, if they meet any of the following criteria:

occurrence before the beginning of the first peak;

occurrence after the end of the last peak;

occurrence within the boundaries of a peak, except for the first and last points of the peak;

occurrence at a point common to two unresolved peaks, unless that point is lower than the last baseline point;

occurrence at the first point of the window, unless lower than the next baseline point; or

occurrence at the last point of the window, unless lower than the previous baseline point.

The use of these criteria is illustrated in Figures 9, 10 and 11.

Calculation of peak amount. The peak area is calculated for each peak of the designate ion of a given compound, regardless of the match category of the peak. This peak area of the designate ion is then divided by the peak area of the designate of the internal standard, so that a relative peak area is calculated, as shown in Figure 12. By a similar process, the relative peak height of each peak of the designate ion is also computed. From each of these is then calculated a "peak amount" utilizing the formula given in Figure 13. This formula uses a quantitation factor, k, to convert relative peak area

All points in the window are initially considered as candidates for baseline points. Points are discarded from consideration, however, if they meet any of the following criteria:

occurrence before the beginning of the first peak;

occurrence after the end of the last peak;

occurrence within the boundaries of a peak, except for the first and last points of the peak;

occurrence at a point common to two unresolved peaks, unless that point is lower than the last baseline point;

occurrence at the first point of the window, unless lower than the next baseline point; or

occurrence at the last point of the window, unless lower than the previous baseline point.

The use of these criteria is illustrated in Figures 9, 10 and 11.

Calculation of peak amount. The peak area is calculated for each peak of the designate ion of a given compound, regardless of the match category of the peak. This peak area of the designate ion is then divided by the peak area of the designate of the internal standard, so that a relative peak area is calculated, as shown in Figure 12. By a similar process, the relative peak height of each peak of the designate ion is also computed. From each of these is then calculated a "peak amount" utilizing the formula given in Figure 13. This formula uses a quantitation factor, k, to convert relative peak area

Figure 9. Baseline determination I.

Each black circle represents a baseline point which would be selected by MSSMET. The dotted line represents a second-order least squares fit of the baseline points. No baseline points are selected from the regions before the first peak and after the last peak. Note that small baseline fluctuations are not detected as peaks, and that peak 3 is not resolved into two components.

Figure 10. Baseline determination II.

Only five baseline points (circles) are selected by MSSMET in this 50 scan window. Since the starting point of peak 1 both occurs at the beginning of the window and is higher than the starting point of peak 2, the height of the second point is also used as the height of the first baseline point. The five baseline points are used to calculate a second-order least squares equation for the baseline; this calculated baseline is represented by the dotted line. The baseline is not allowed to go below zero.

Figure 11. Baseline determination III.

Points occurring between unresolved peaks are not included in the baseline. Hence, the baseline for the six peaks includes only three data points (dark circles). Depending upon the values of MSSMET library options, it is possible to include poorly resolved peaks (e.g., peak 4) as part of other peaks (peak 3 in this case). It is also possible to entirely exclude peaks whose heights are below a certain threshold value.

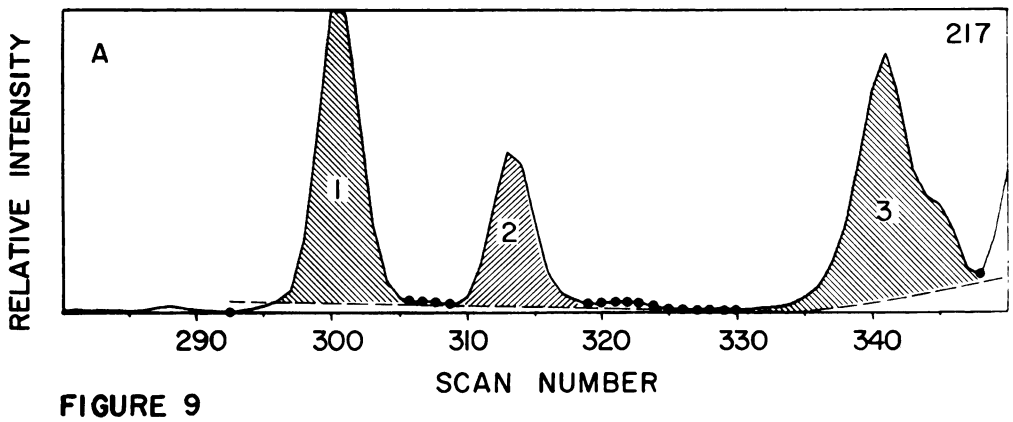

FIGURE 9

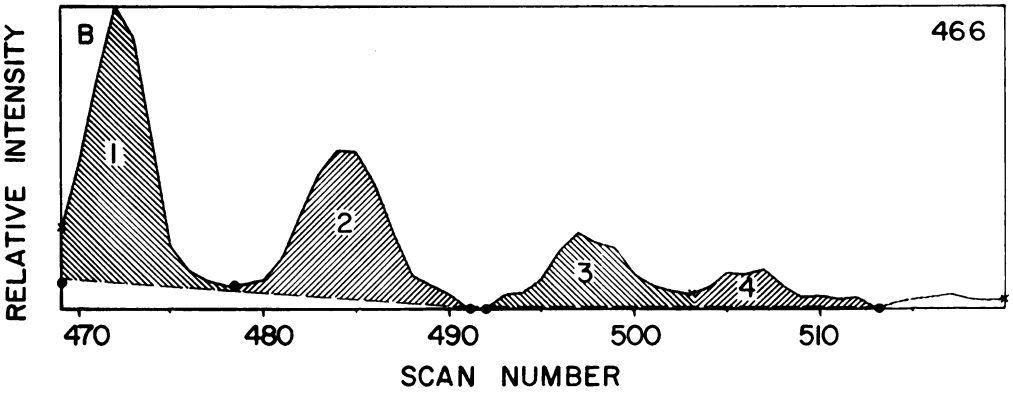

FIGURE 10

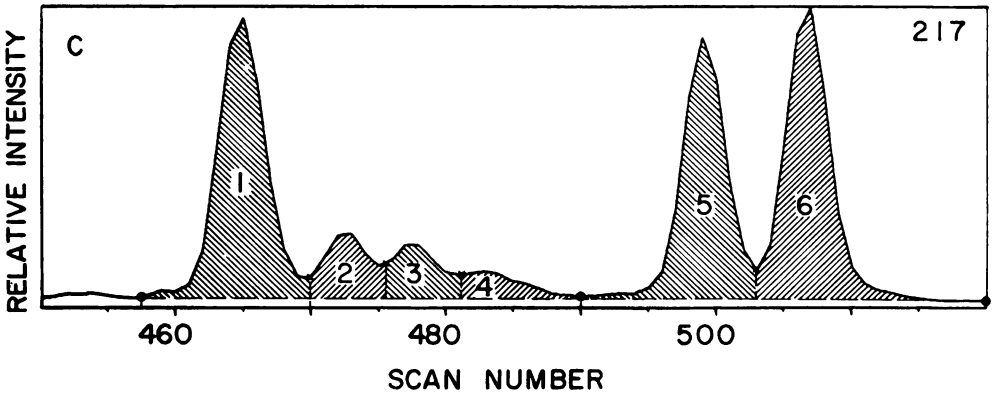

FIGURE 11

Figure 12. Determination of relative peak area.

The relative area of each substance detected by MSSMET is computed by measuring the ratio of the area of the designate ion of the compound (shaded) to the area of the designate ion of the tropic acid internal standard (shaded). The ratio of the peak areas is combined with a correction factor contained in the library entry to obtain the absolute concentration of the compound (Figure 13).

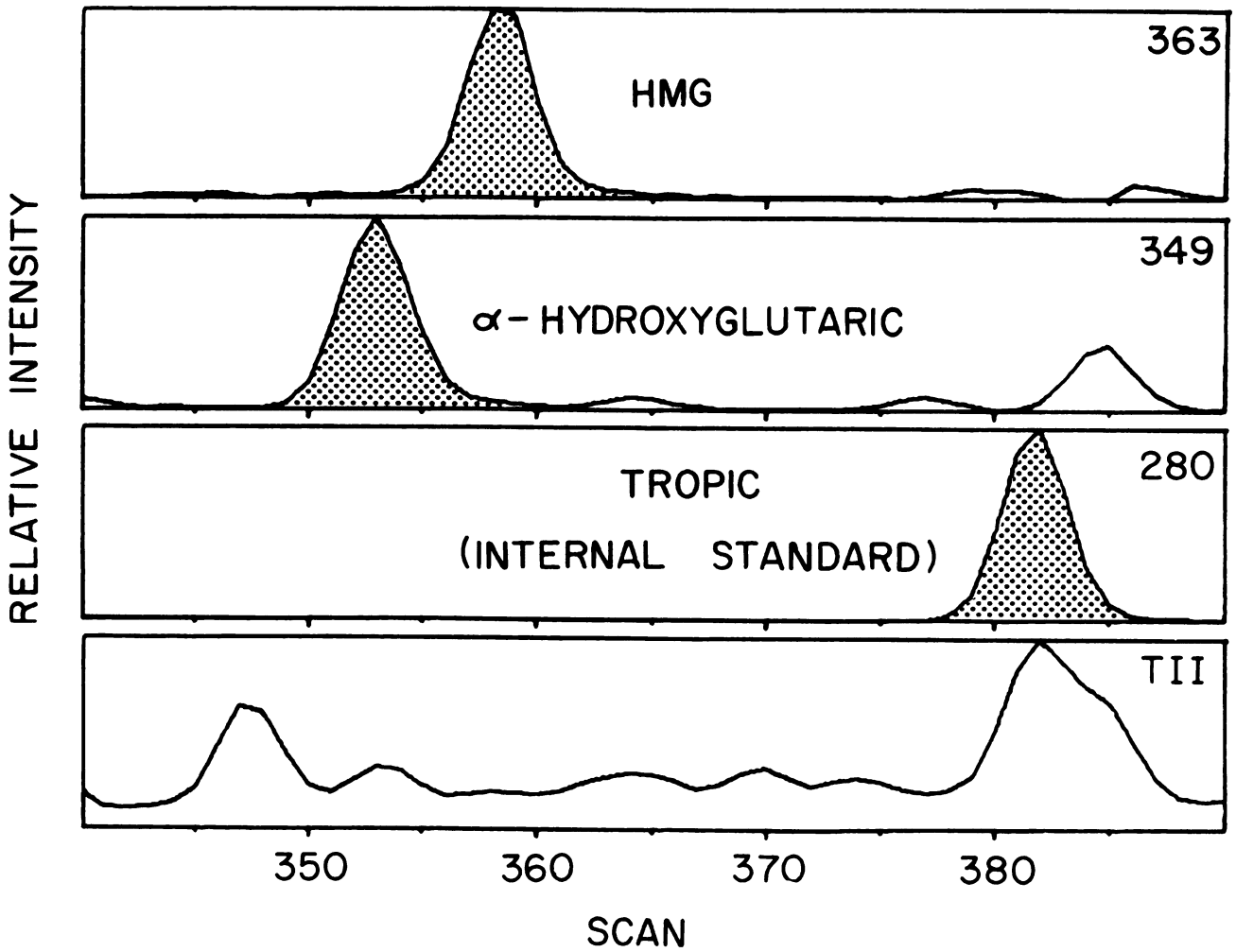
363
HMG
349
α - HYDROXYGLUTARIC
280
TROPIC
(INTERNAL STANDARD)
TII
RELATIVE INTENSITY
350
360
370
380
SCAN

FIGURE 12

$$C = \cdot \frac{A_x}{A_{is}} \cdot \frac{W}{V}$$

where C = concentration (mg/ml)

A = area of the designate ion

W = weight (mg) of internal standard added to the sample

V = volume (ml) of sample extracted

Figure 13. Formula for calculation of peak amount by MSSMET.

The ratio of the area (or height) of the designate ion of the compound of interest to the area (or height) of the designate ion of the internal standard is combined with a correction factor (k) to obtain absolute concentration. The value of k is obtained experimentally; it reflects recovery from the chemical separation process, losses during GC analysis, percent ionization in the ion source of the mass spectrometer, and the relative intensity of the designate ion in the spectrum of the compound. If k is not yet known, a value of 1.00 is used; the results are then termed "relative concentration" rather than absolute concentration. Such relative concentrations can be used for comparison of the levels of a substance present in different samples, but are not useful for comparisons of the amount of one compound to the amount of another.

or height into actual concentrations. The quantitation factor is determined from the area of the designate ions of the reference substance and internal standard of a known amount of pure compound taken through the entire DEAE-Sephadex procedure. If it is not yet known, the quantitation factor is assumed to be 1.0, and the peak amount is referred to as the relative peak amount. It is also possible to avoid the use of a specific value for the creatinine standard in this formula by setting M equal to 1.00, in which case results are reported as relative amount per ml urine. In any case, the peak height and peak area results are reported separately.

Printing of results. Two files of results are created and either printed immediately or stored on disk for later printing. The first of these is the "run" file, which contains information on all of the peaks of the designate ion of each of the compounds. The second is the "found" file, which contains only the best "+" match, if any, for each compound. It is this latter file which is used for later statistical analysis. The material printed in each file may include any or all of the complete dump of information shown in Figure 14.

Selection of designate and confirming ions.

The designate and confirming ions are those molecular or fragment ions which are expected to be the most differentiating for a

Figure 14. Typical MSSMET output.

Depending upon the value of a MSSMET option (the "print" option), any or all of the information shown can be displayed for each compound positively identified by MSSMET. The first line of the output includes an identifying number for the compound and both its IUPAC and common names, if known. The second line contains the following information: an off-scale indicator, if the compound area is above a certain value; a sequence number, which indicates which peak of the designate ion within the window is identified; the match category, which is based on the combination of the value of the match coefficient and the deviation of the retention index from the expected (library) value (Figure 7); designate ion peak area; substance concentration (or relative concentration if k is unknown), expressed in exponential notation; observed retention time in minutes and seconds; deviation of the retention time from the value expected; observed retention index; deviation of the retention index from the value expected; and the scan numbers corresponding to the beginning, apex and end of the peak of the designate ion. The next line of the output for each peak contains data corresponding to those printed directly above each one, except that all values are computed from peak heights instead of peak areas. In the example shown, the ions peaking at retention index 1759 are found to match the library ion ratios for 3 substances, but only the entry for p-hydroxyphenylacetic acid positively matches both the observed ion ratios and the observed retention index.

```
354 U62
  3 +   96    91668.  0.454E 01 30:21 + 0:05 1663     3   344   353   359
    +   95    19635.  0.348E 01
374 U82
  1 +   95    75408.  0.374E 01 30:25 - 0:05 1665    -3   345   354   364
    +   96    14220.  0.252E 01
111 M-HYDROXYBENZOIC
  2 +   97     9448.  0.468E 00 30:33 + 0:00 1669     0   351   356   361
    +   97     2344.  0.415E 00
112 2-PYRROLIDONE-5-CARBOXYLIC  (PYROGLUTAMIC)
* 1 +   99  2536068.  0.126E 03 30:41 + 0:01 1673     0   344   358   371
    +   99   484722.  0.858E 02
```

FIGURE 14

specific compound in a particular biological mixture. Hence, the best choices for these ions will vary with the particular choice of biological fluid, extraction method, and derivative. To facilitate the process of selecting these ions, a pair of methods has been tested. The first method was manual and involved intuitive selection of ions, which were typically intense and of high mass, with a bias toward high mass rather than intensity. The library of this set of ions was called MSSMETLIB; it evolved slowly as experience indicated which were poor choices.

A second, and more recent, approach is the computerized selection of ion sets, utilizing two programs called MSSDSG and MSSCHS, which were developed for this purpose. MSSDSG (acronym for mass spectral system-designate ion selector) is designed to select a designate ion and confirming ion set for each library spectrum. It does this by comparing the library spectrum to the average of a preset number of spectra (usually 16) taken from an actual urine sample. The key feature of this comparison, however, is that it compares the library spectrum to urine spectra centered at the retention index where the library compound would be expected to occur (i.e., its nominal retention index). A ratio is computed of the intensity of each mass of the library spectrum to the corresponding intensity at the same mass of the averaged spectra, using

the formula shown in Figure 15. The value of q in this formula is a small real number, typically slightly greater than 1.00, to allow weighting of more intense masses. A value of 1.05 is typically used for q. Library values below a certain threshold intensity (typically 50) are automatically assigned an R-value of zero, and a minimum intensity value in the urine sample of 1.0 is always assumed to avoid division by zero. The 20 highest R-values for each library spectrum are then selected. If any apparent isotope clusters exist (usually masses within 3 of one another are defined as isotope clusters), only the mass with the highest ratio is retained. The resulting list is stored in a permanent file for analysis by MSSCHS. A sample MSSDSG output is shown in Figure 16.

Construction of the MSSMET library from data supplied by MSSDSG is the task of MSSCHS. It may use up to 10 MSSDSG outputs, each representing a comparison of the library to a separate urine sample, to arrive at an optimum choice of designate and confirming ions. To provide a direct comparison, the list of the twenty (or fewer) ions from each library spectrum is ranked from low to high (1= lowest R value, 2= second lowest, etc.) All R-values below a user-defined minimum (typically 5.0) are discarded. Sets of ranked ions from each of the comparisons provided by MSSDSG are then summed to produce a tabulation of the ions that are ranked best overall. Masses below m/e 80 are automatically discarded from consideration.

$$R_m = \frac{L_m^q}{S_m}$$

where R_m = the ratio for the ion of mass m

L_m = the normalized intensity of the ion of mass m

S_m = the normalized intensity of the ion of mass m in the summed sample spectra

q = a factor used to weight intense ions more heavily

Figure 15. Formula for calculation of ratio by MSSDSG.

The ratio is calculated to measure the usefulness of a given ion in differentiating whether a particular compound is present in a sample of a biological fluid. The most discriminating ions will have the highest ratios. Typically, a value of q of 1.05 is used to weight the ratio to favor more intense ions.

Figure 16. Typical MSSDSG output.

MSSDSG selects a group of up to 20 ions that are found to be most differentiating in a given library spectrum when it is compared to spectra centered at the same retention index in a GC-MS analysis of a biological fluid sample. The masses of the ions with the highest ratios (Figure 15) are printed with their respective ratios. Several such outputs are used by MSSCHS to choose the best set of designate and confirming ions for a library entry.

TOP CHOICES FOR DESIGNATE AND CONFIRMING IONS FOR SCAN 280 ARE

73	607.	4.561	202	46.	5.598
218	18.	1.951	260	21.	2.406
274	15.	1.774	290	1000.	141.254
364	36.	4.281	392	70.	8.668
407	314.	41.910			

FIGURE 16

Finally, the 8 highest-ranking ions are selected and a library constructed in standard MSSMET library format. This library was identified as FINALLIB.

FINALLIB was further modified as experience indicated shortcomings. Ultimately, two libraries were established based on it: a library of compounds known to occur in urine (BESTLIB) and a more complete library including all compounds for which reliable spectra had been obtained (PUBLIB). Entries in both libraries are identified in one of several ways: by chemical name, if spectra were obtained from commercially-available standards; by the prefix UNK if the spectra were detected as arising from apparent impurities in the commercially-available standards; and by the prefix U if detected as spectra in urine samples. Tentative identification of some of the last class of "unknowns" has been made by reference to a variety of sources of published spectra; these compounds contain the suspected identity in parentheses after the identifying code name. PUBLIB has been published elsewhere (77G1); BESTLIB is included as Appendix B.

MSSMET analysis of urine samples

The final library for the MSSMET analysis of urine samples (BESTLIB) contains 155 compounds selected from a total library of

383 compounds upon the basis of a test run of the urine samples with MSSMET. Compounds have been eliminated from the library used for urine samples because they were not found in a sufficient number of urines or because they duplicated other entries. In addition, the library has been refined upon the basis of data from 4 of the urine samples to achieve an optimum set of ion ratios (except for compounds 342 to 383, which were added later). Library retention indices are based upon the average of values from several urine samples where possible.

All urines described in this thesis have been analyzed with BESTLIB. This library uses urinary metabolites as retention index standards, but also requires the co-injection of hydrocarbon standards with each urine sample. Nominal retention indices for the retention index standards are based upon their retention indices relative to hydrocarbon standards in a minimum of 12 urine samples.

Statistics

Statistical analysis of the data from the urine samples is performed on the PDP 11/40 utilizing two types of programs written specifically for this purpose.

MSSTAT. This is a series of computer routines, written in FORTRAN IV, designed to provide parametric statistics on the urine

profile data. Standard statistics provided by this program include mean, standard deviation, standard error, coefficient of variation, product-moment correlation, t-test of product moment correlations and t-test of samples means. Six categories of data are capable of being processed: peak area match coefficient, peak height match coefficient, retention index, difference of retention index from nominal (library) value, relative peak area and relative peak height. Data are prepared for this program by RAMAST, which creates a "master array" from MSSMET "found" files and by RASECD, which can perform simple data transformations (multiplication, division, logarithmic conversion) to create a "secondary array."

FRGENL. This program, also in FORTRAN IV, computes several parametric and non-parametric statistics on the urine data. It calculates means and standard deviations for the average of relative peak areas and relative peak heights; height match coefficients; and retention indices. It also uses peak amount data normalized to a subset of all of the compounds to compare different groups of urine samples using either the Wilcoxon statistic (non-parametric) or the "Student" t-test. Auxiliary routines include an outlier test and a logarithmic plot of peak amount data. Two preliminary programs, PURXAC and TRNSPO, are required to create a data matrix from MSSMET "found" files and to take its transpose, respectively. This package of programs was written by Dr. B.E.

Blaisdell specifically for use at the MSU Mass Spectrometry Facility.

Both MSSTAT and FRGENL use routine statistical techniques described in almost any standard text on statistics.

Clinical report form. Another type of statistical output that can be generated from a MSSMET output is the clinical report form. This report, generated by MSSRPT, is a comparison of an individual MSSMET output with a file of means and standard deviations for a group of reference subjects. The relative amount of each substance calculated by MSSMET is plotted in terms of the number of standard deviations it is from its respective mean value. A correction factor can be applied to the data to normalize the data to the sum of relative peak areas. If k-factors are known, these are multiplied times the appropriate data. Means, standard deviations and k-factors are kept in files which may be edited using the system text editor, and are dated so that the clinical report form includes the date each file was established.

CHAPTER FOUR: RESULTS

The results may be arbitrarily divided into three categories: evaluation of MSSMET and mass spectrometer-data system performance, tests of the urine extraction procedure, and clinical studies. Each of these topics is discussed separately below.

Evaluation of MSSMET and GC-MS-COM system

MSSMET and the GC-MS-COM system were tested to ensure that they were performing as expected.

Data collection reliability. Data collected by the LKB-PDP 8/e system were examined using MSSOUT, the general-purpose mass spectral output program, to detect anomalies which might contribute to an unreliable data analysis by MSSMET. Several such effects were noted and appropriate changes made in the data collection routines on the PDP 8/e. Most notable of these were problems associated with real-time ion peak detection. Extensive testing over a 10-month period finally revealed several problems. First, only one of the four intensity amplifiers was being tracked to determine the starting and ending points of the ion peak. For very intense peaks, especially if resolution was poor, this resulted in the peak at the

next higher mass being "missed" by the detection algorithm. Secondly, the peak-detection algorithm was too slow, so that relatively few points were collected during the sweep of the magnetic field across the ion. The third contributing factor was that the algorithm was too sensitive to small negative noise spikes in the beginning region of the peak; a peak was declared "ended" because of the noise spike, and the rest of the peak missed because of the delay involved in processing the spurious peak.

These problems were solved by tracking the most sensitive unsaturated intensity channel, instead of simply the most sensitive channel, streamlining the peak-detection algorithm and installing a criterion that a peak must have a certain minimum width before it is considered ended, respectively.

Data collection parameters. Also associated with the reliability of data collection were several operating parameters of the LKB-9000. Although the complexity of the interaction of these parameters with one another and with the data collection algorithm made a rigorous test of the influence of each factor impossible, several parameters were found to be especially important. The results suggested a set of minimum criteria without which reliable data collection should be considered impossible. This set of criteria, summarized in Figure 17, was implemented in all subsequent studies.

Figure 17. Minimum criteria for LKB-9000 operating parameters.

Whenever samples are to be analyzed on the LKB-9000, operating conditions are modified until all of the criteria listed have been met.

Figure 17. Minimum criteria for LKB-9000 operating parameters

Noise levels	
60 Hz and higher frequency noise on intensity from multiplier	Less than 0.4 mV (20 bits on data system) peak-to-peak
Random noise spikes	Less than 0.2 mV above or below normal noise level
Hall effect	Less than 40 μV (2 bits)
Mass resolution	500 (10% valley definition) throughout mass range
Ion source focusing	Should be optimized daily; no lens voltage should be at either extreme; peaks should be symmetrical
Hall effect vs. mass calibration	Should be optimized daily (more often if room temperature not constant); for masses 51-550 at scan speed 8 (4 seconds per scan cycle), calibration of each mass must be within 1/4 mass unit of value predicted by extrapolation from previous two calibration points. Day-to-day drift should be no more than 1 mass at highest mass, 1/4 mass at lowest mass.
Ion source leakage current	Should be stable.
Sensitivity	A 4 μl injection of test urine capillary should produce at least one peak at m/e 73 over 100,000 intensity.

Library spectra. Retention indices and complete spectra were needed for compounds expected to occur in the urine samples. Therefore, once the GC-MS system had been tested, reference spectra were made of almost 300 organic acids for which reasonably pure standards were then available. Many of these spectra are otherwise unavailable in the literature, but are too space-consuming to publish here; hence, the entire set is in the process of being added to the National Institutes of Health mass spectral library. These spectra form the basis of the more abbreviated MSSMET library spectra used in the following studies.

Calculation of retention indices and match coefficients by MSSMET. Over 100 retention indices were calculated both manually and by MSSMET; no cases of disagreement were found, except where an error had been made in the manual calculations. Likewise, both manual and computer calculations of match coefficients were found to agree completely, although only a few of these were calculated manually.

Mass chromatogram peak detection by MSSMET. One basic criterion was that MSSMET give peak heights and areas identical to those of MSSOUT, the general-purpose data display program. This was checked manually for a large number of peaks using identical integration parameters, and both programs were found to perform identically.

Sensitivity and linearity of system response. A serial dilution of several test compounds was performed, and the entire series analyzed by GC-MS and MSSMET. The results are displayed in Figure 18 for a typical compound. An expansion of the upper limit of this curve is shown in Figure 19. These serial dilution curves are usually linear from approximately 10 ng to 10 μg injected, but show marked non-linearity above 10 μg injected.

Precision of retention indices. Two separate tests were made of the precision of retention index determination with pure compounds, each with equivalent results. The combined results for 156 determinations of retention indices at a wide variety of concentrations are shown in Figure 20. The standard deviation of the retention index determinations on pure compounds was 2.20 retention index units, or approximately 1 scan.

Match coefficient reliability. Using the data for the linearity determination, plots were made of match coefficient versus concentration for pure compounds. One example is shown in Figure 21. These plots, which varied quantitatively from one compound to another, were qualitatively similar in every case. Specifically, the match coefficient appeared to be approximately constant above a certain minimum concentration (the limit of detection for that ion), but drop rapidly below that point.

Reproducibility. Two separate capillaries of the same urine

Figure 18 Quantitative working curves measured by repetitive scanning.

Samples of the trimethylsilyl derivative of p-hydroxybenzoic acid were tested at concentrations ranging from 1 ng injected to 64 ug injected. Intensities of three different ions were measured: m/e 267 (M-15), m/e 193 (M-89) and m/e 268 (isotope of m/e 267). The linear portions of the working curves obtained for each ion are shown. Relative areas are calculated by MSSMET from the areas of the designate ions of p-hydroxybenzoic acid relative to the designate ion (m/e 280) of the tropic acid internal standard.

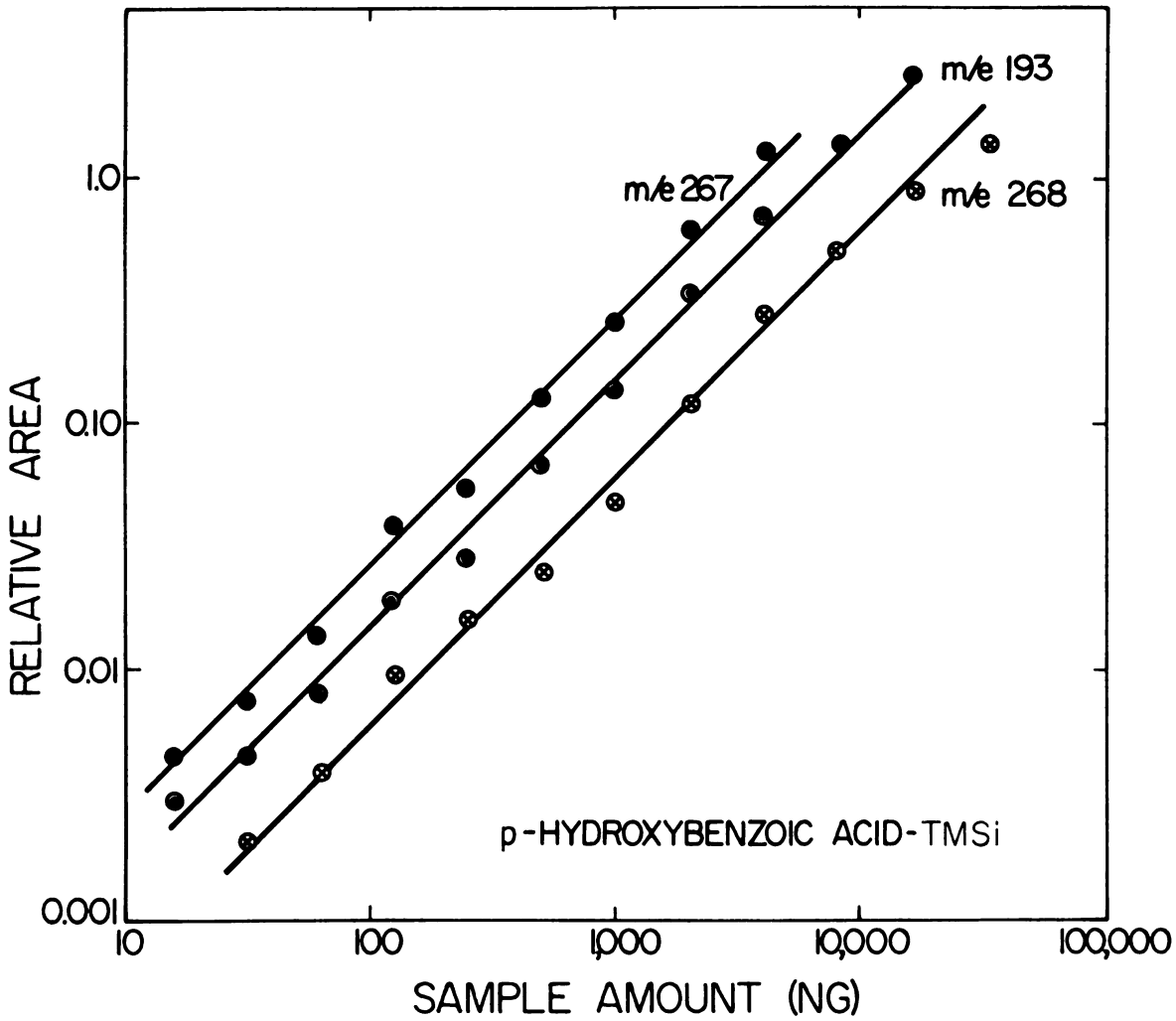
m/e 193
m/e 267
m/e 268
p-HYDROXYBENZOIC ACID-TMSi
RELATIVE AREA
1.0
0.10
0.01
0.001
10
100
1,000
10,000
100,000
SAMPLE AMOUNT (NG)

FIGURE 18

Figure 19. Quantitative working curve at high concentrations measured by repetitive scanning.

Most substances analyzed do not show a linear response on the GC-MS at high concentrations. A typical example is m/e 193 of the trimethylsilyl derivative of p-hydroxybenzoic acid. Response of this ion relative to the tropic acid internal standard (which is present at a fixed concentration) is shown for a portion of the serial dilution study pictured in Figure 18.

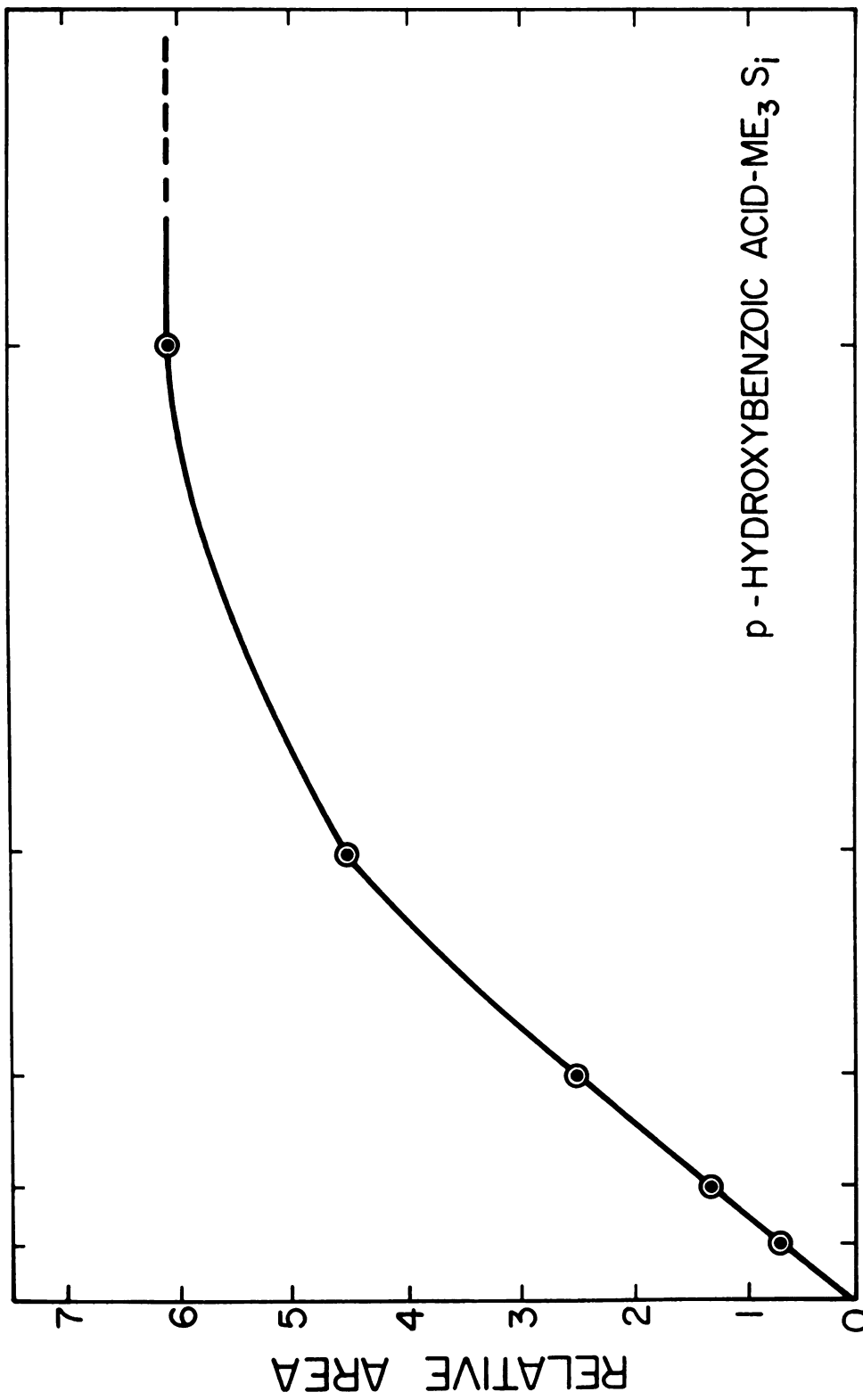
RELATIVE AREA
0
1
2
3
4
5
6
7
0
2000
8000
16000
32000
SAMPLE AMOUNT (NG)
p-HYDROXYBENZOIC ACID-ME3 Si

FIGURE 19

Figure 20. Precision of retention index determination on pure compounds.

Mixtures of 10 to 15 of the trimethylsilyl derivatives of reference organic acids were analyzed by repetitive scanning GC-MS and the retention index of each compound in the mixture determined by MSSMET. Retention indices are calculated using linear interpolation between flanking hydrocarbon standars coinjected with the sample. Each retention index is plotted as the deviation from the mean retention index for that compound, based on at least 10 determinations. Figures 27 and 28 picture comparable tests of retention index precision on compounds in urine extracts.

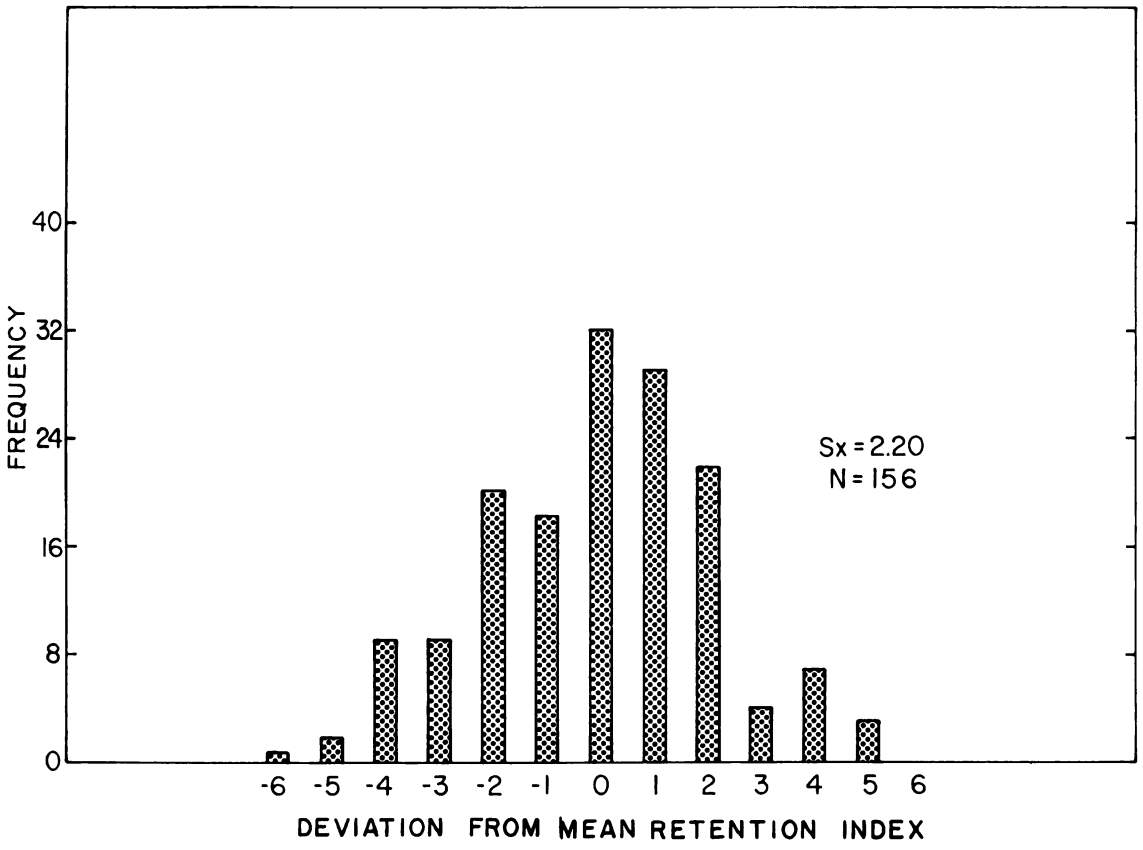
40
32
24
16
8
0
FREQUENCY
Sx = 2.20
N = 156
-6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6
DEVIATION FROM MEAN RETENTION INDEX

FIGURE 20

Figure 21. Dependence of match coefficient upon amount of sample injected.

Match coefficients were determined by MSSMET for varying amounts of the trimethylsilyl derivative of mandelic acid injected into the GC-MS. Analysis of other substances produces similarly-shaped curves, but the position of the inflection points depends upon the limits of detection for the particular compound being tested.

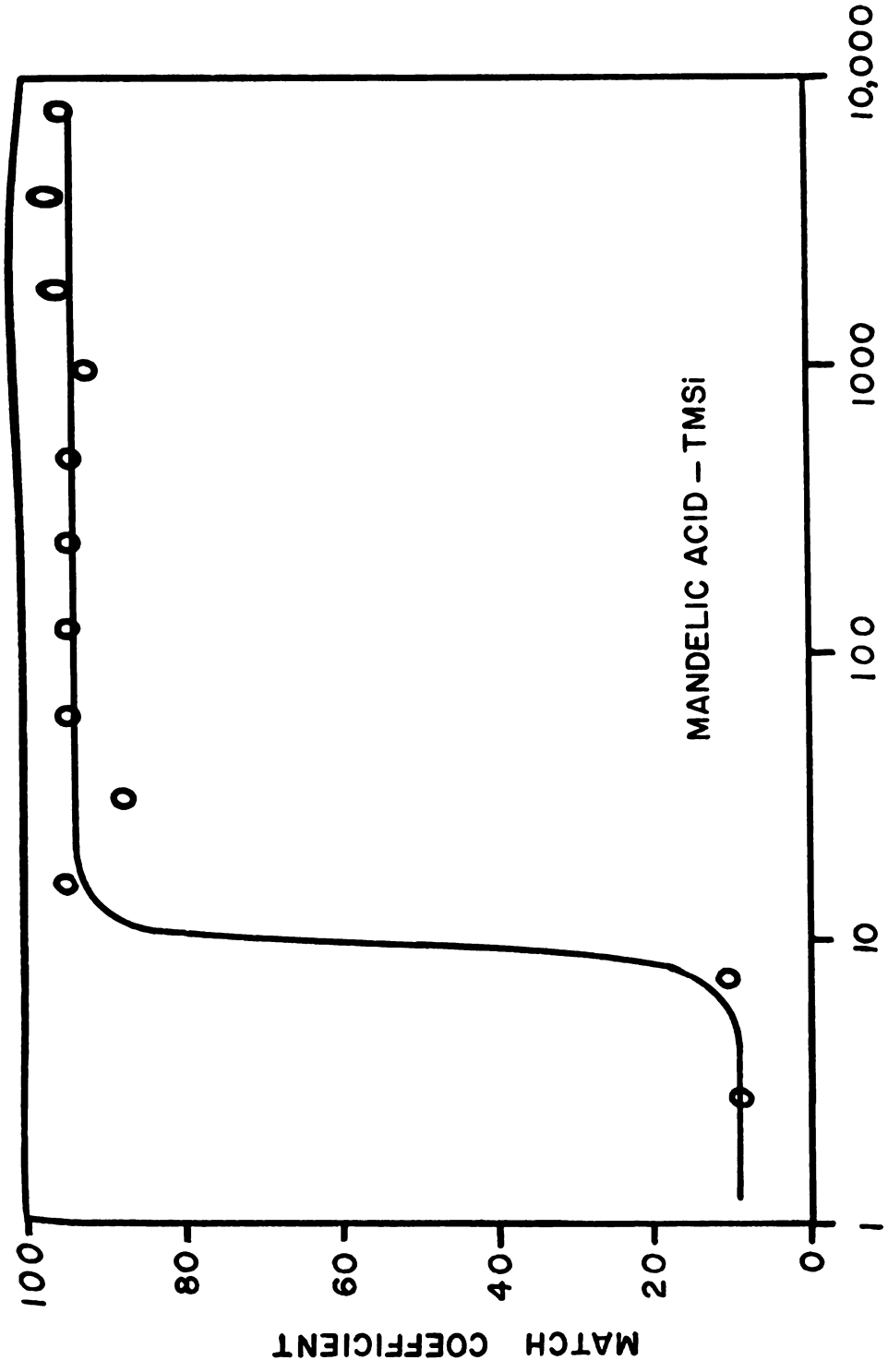
MATCH COEFFICIENT
100
80
60
40
20
0
1
10
100
1000
10,000
AMOUNT INJECTED (NG)
MANDELIC ACID – TMSi

FIGURE 21

sample were opened and injected one week apart. The MSSMET data were then compared to obtain a measure of GC-MS reproducibility. The results are shown in Figure 22. In addition, the sample was examined for the possibility that GC-MS conditions changed during the run, as shown in Figure 23. Data for both figures are peak areas relative to the quantitative internal standard; they have not been normalized to the sum of peak areas. In addition, the "unreliable" compounds listed in Appendix C, discovered by tests of 19 urines including one of the two tested here, have been removed from these plots. The median coefficient of variation between the two samples is 8%. Of the 14 compounds with a coefficient of variation greater than 35%, one is an artifact peak (probably from the pyridine solvent), 2 are substances just above their limits of detection, 3 have a retention index more than 6 retention index units from the library value (and 2 of these also have one of their match coefficients below 80), and 4 show evidence that the designate ion peak is poorly resolved. The problems with the remaining 4 substances are not explanable on the basis of the data contained in the MSSMET outputs.

Quantitative precision. Using the data from the linearity series, it was possible to determine the precision of quantitation by measuring the deviation of the data from a first-order least-squares fit of the data. For 20 samples, the mean coefficient of variation of the relative peak areas was 4.9%. Precision on the same samples

Figure 22. Reproducibility of repetitive scanning GC-MS on urine samples.

MSSMET was used to quantitate the relative peak areas of 106 urinary organic acids found in two injections of the same sample. The organic acids were separated from urine by the DEAE-Sephadex anion exchange procedure and analyzed as the trimethylsilyl derivatives. Data for the compounds with coefficients of variation greater than 35 were examined individually, as described in the text. The two samples were analyzed one week apart on the LKB-9000 using standard conditions.

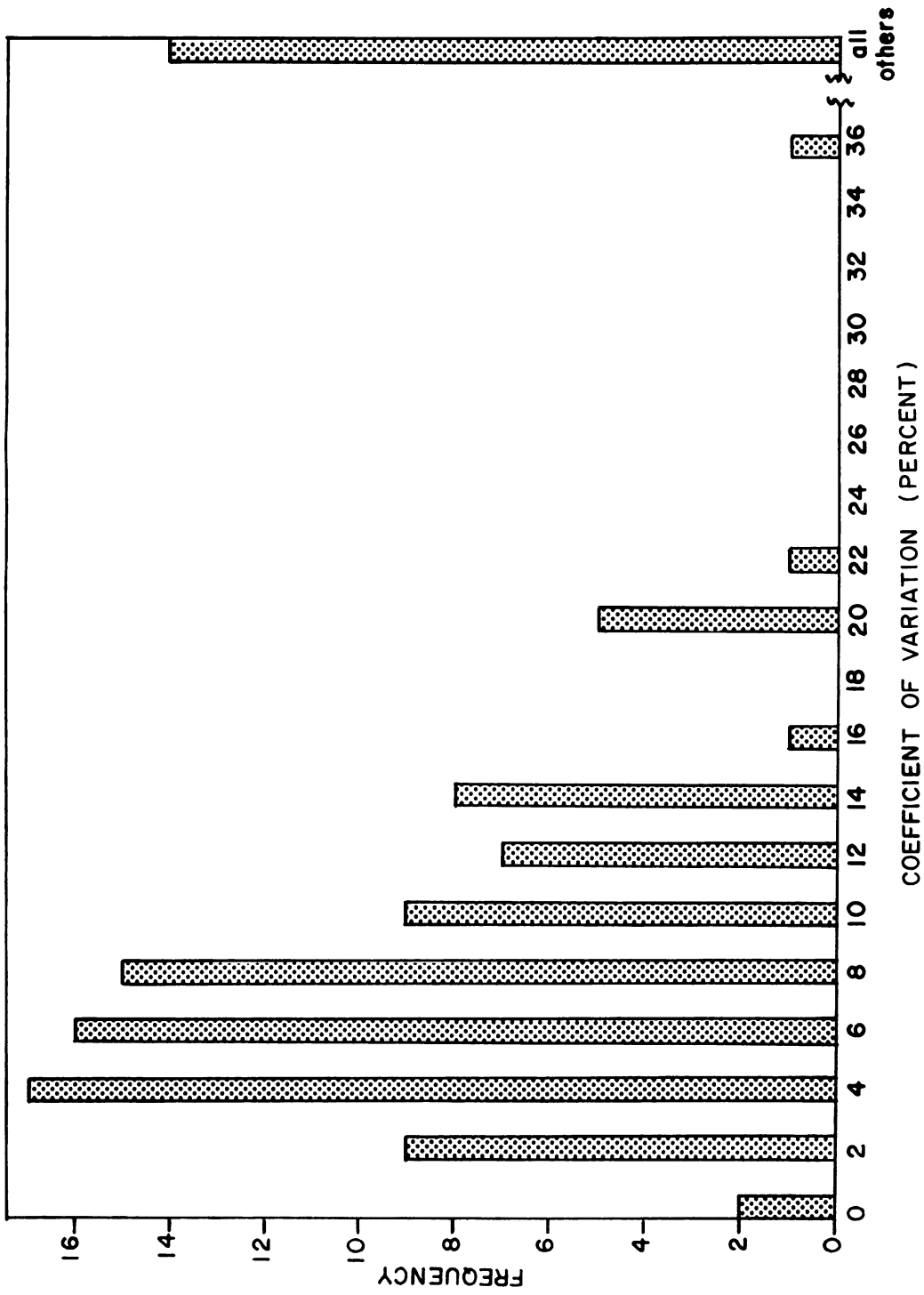
FREQUENCY
0
2
4
6
8
10
12
14
16
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
all others
COEFFICIENT OF VARIATION (PERCENT)

FIGURE 22

Figure 23. Dependence of GC-MS reproducibility upon retention index.

The individual data summarized in Figure 22 are plotted in order of increasing retention index. The sample marked with triangles was analyzed on the GC-MS one week after the sample marked with circles. Data from both samples are plotted as the percent each is of the mean value of the two samples. The apparent shift in the ratio of the relative peak areas between the two injections occurs at a retention index of approximately 1890.

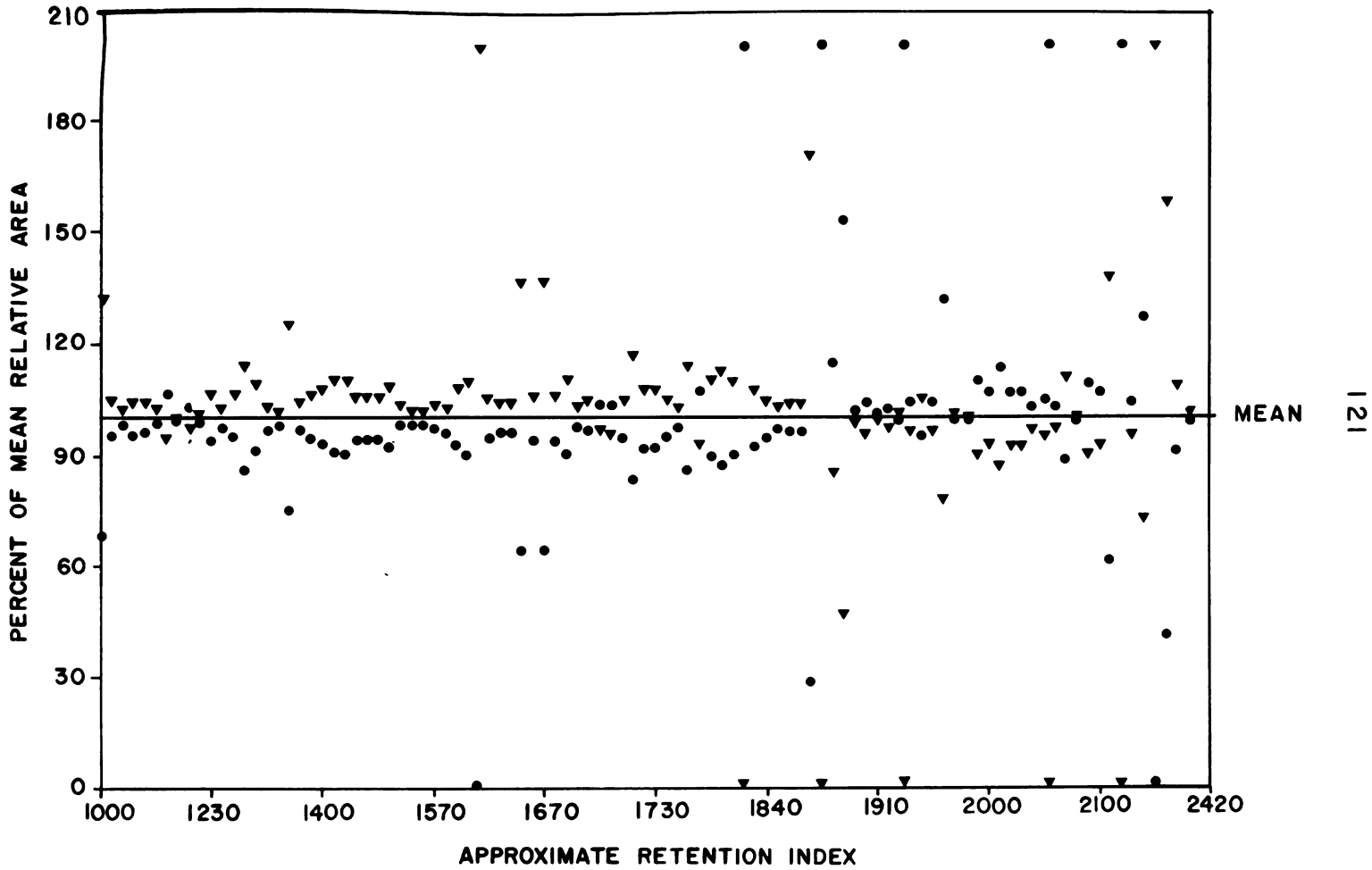
210
180
150
120
90
60
30
0
PERCENT OF MEAN RELATIVE AREA
MEAN
1000
1230
1400
1570
1670
1730
1840
1910
2000
2100
2420
APPROXIMATE RETENTION INDEX

FIGURE 23

Table 6. Precision of isotope ratio determination*

Compound	m/e	Coefficient of Variation	
		MSSMET	SIM
3,4-dihydroxyphenyl-acetic-d_5	389/390	± 2.2%	± 0.23%
indoleacetic-d_2	321/322	± 2.8%	± 0.36%
5-hydroxyindole-acetic-d_2	409/410	± 1.4%	± 0.33%

* Based on 10 injections of 1.0 µg of each compound.

Table 7. Accuracy of isotope ratio determination*

Compound#	m/e	Isotope ratio			Per cent error	
		Theory@	MSSMET	SIM	MSSMET	SIM
3,4-DHPA-d_5	389/390	2.92	3.25	2.81	11.3	-3.8
IAA-d_2	321/322	3.54	3.25	3.43	-8.2	-3.1
5-HIAA-d_2	409/410	2.72	2.56	2.56	-5.9	-5.9

* Based on 10 injections of 1.0 µg of each compound.

\# 3,4-DHPA-d_5 is labeled 3,4-dihydroxyphenylacetic acid, IAA-d_2 is labeled indoleacetic acid, and 5-HIAA-d_2 is labeled 5-hydroxyindoleacetic acid.

@ Calculated based on average isotope abundances.

using a selected ion monitoring (SIM) system ranged from 2 to 4%, depending upon the compound tested.

Quantitative accuracy. To obtain a measure of the accuracy of quantitation by MSSMET, as well as another comparison of quantitative precision, the same isotope ratios were measured by both MSSMET and SIM. These data, as summarized in Tables 6 and 7, indicate that the mean precision of isotope ratio determination is $\pm$ 2.1%, compared to the $\pm$ 0.3% precision of SIM. Mean percent error using MSSMET to determine isotope ratios was 8.5%, compared to a 4.3% mean error on the same samples using SIM.

Tests of the urine separation procedure

Although the general DEAE-Sephadex procedure had been studied extensively by several groups prior to our work (71H2, 71H3, 72C2, 72C3, 72C4, 75T2), it did not perform well in our hands, so considerable testing was undertaken to improve the procedure and to document it in such a fashion that other laboratories would be able to replicate our results. The revised procedure was then tested to gauge recovery, reproducibility and sample stability.

Recovery. Recovery data were computed by comparing the GC-MS results with a group of pure compounds to the results obtained with samples in which the same substances were added at

three levels of concentration to urine samples. The results from this study are summarized in Table 8. Recoveries for the derivatized hippuric acid are not given because of the difficulty in obtaining a reliable GC response with this substance in urine. Recoveries for the compounds ranged from 10% for ascorbic acid to 98% for vanilmandelic and ϱ-hydroxy-ϱ-methylglutaric acids. Only ascorbic and citric acids had recoveries below 50%.

Reproducibility. One of the principal measures of success of the separation procedure is the reproducibility of multiple separations of the same sample. Hence, conditions of the procedure were investigated and altered until a reproducible result could be obtained. Several tests of reproducibility were completed on the Varian 2100. GC profiles from a similar test on the LKB-9000 are illustrated in Figure 24. Chromatograms of m/e 73 have been plotted to show only the trimethylsilyl derivatives, so that hydrocarbons do not influence the plotted intensities.

Sample stability. Studies were undertaken to determine the stability of the unprocessed urines at various temperatures and to monitor the stability of silylated samples during storage in sealed glass capillaries. Examples of the former study are shown in Figure 25, and of the latter study in Figure 26. It should be noted that hippuric and uric acids (the two major peaks at the end of the run) varied with the GC column conditions, so that in some later

Table 8. Recoveries of organic acids using barium hydroxide-DEAE-Sephadex method.

Compound name	Mean recovery*
Succinic	92%
o-Hydroxybenzoic	67%
β-hydroxy- β-methylglutaric	98%
Tropic	91%
α-Glycerophosphoric	87%
Citric	16%
Homovanillic	94%
Vanilmandelic	98%
Ascorbic	10%
Indoleacetic	92%

* Based upon analysis of duplicate samples at each of two different concentrations in urine.

Figure 24. Reproducibility of analytical procedure on urine samples.

Two aliquots of the same urine sample were each separated on DEAE-Sephadex columns. The resulting organic acid fractions were lyophilized, derivatized and then analyzed on separate days with the LKB-9000. Hydrocarbon standards were coinjected with each sample; hence, m/e 73 is plotted to show only the trimethylsilyl derivatives present in each mixture.

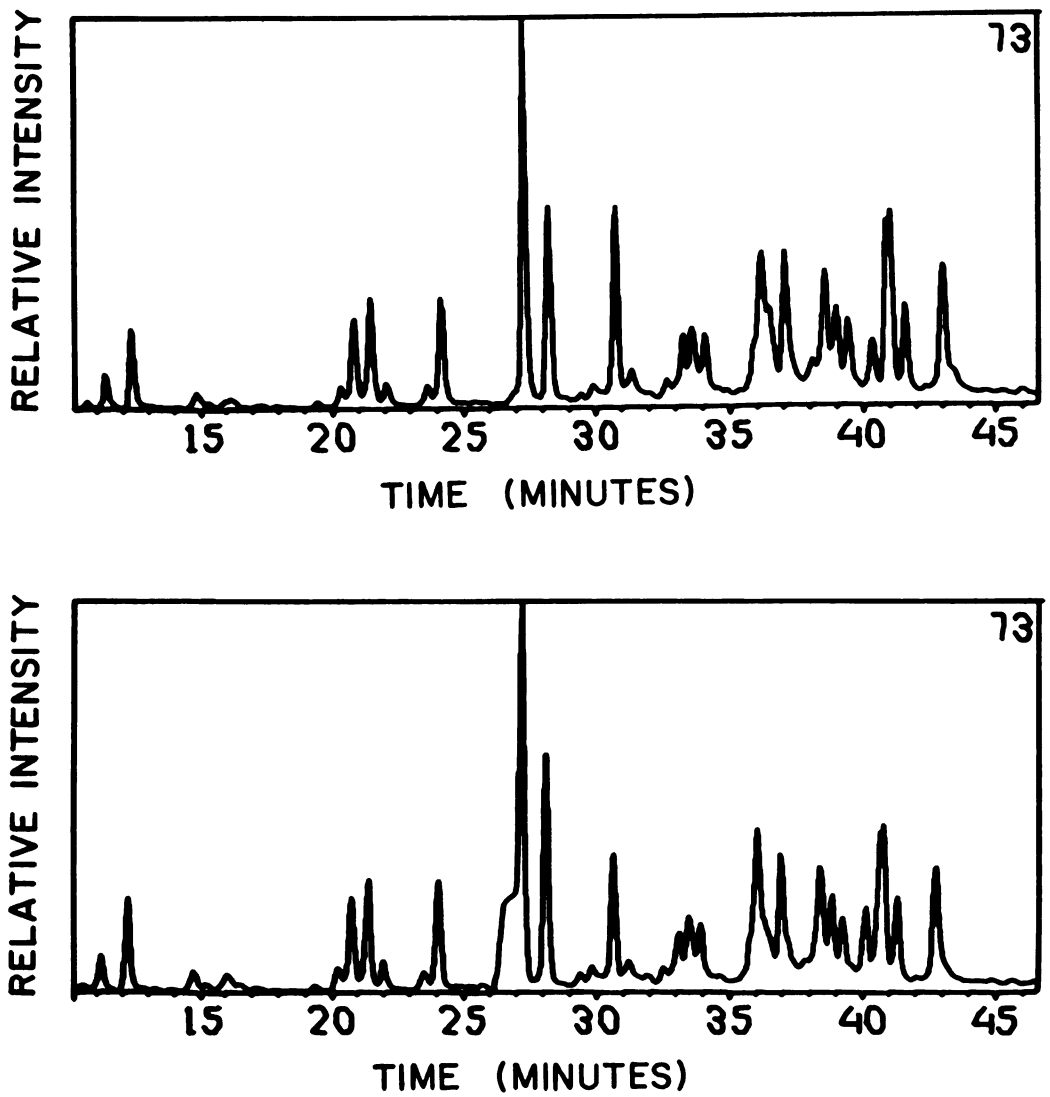
RELATIVE INTENSITY
73
15
20
25
30
35
40
45
TIME (MINUTES)
RELATIVE INTENSITY
73
15
20
25
30
35
40
45
TIME (MINUTES)

FIGURE 24

Figure 25. Stability of stored urine samples.

Four sets of aliquots of the same freshly-collected urine were stored at -80°, -20°, 4°, and room temperature. One aliquot from each set was analyzed at regular intervals, up to 6 months of storage. At the time of analysis, each sample was prepared by the complete DEAE-Sephadex procedure and chromatographed on a Varian 2100 GC. Chromatogram A is from a urine sample analyzed at the time the series was begun, while chromatogram B shows a urine sample after 6 months of storage at -80°C. Substances identified by MSSMET in similar samples are labeled. Conditions included temperature programming from 60 to 290° at 4°C/min on a 12 ft column containing 5% OV-17.

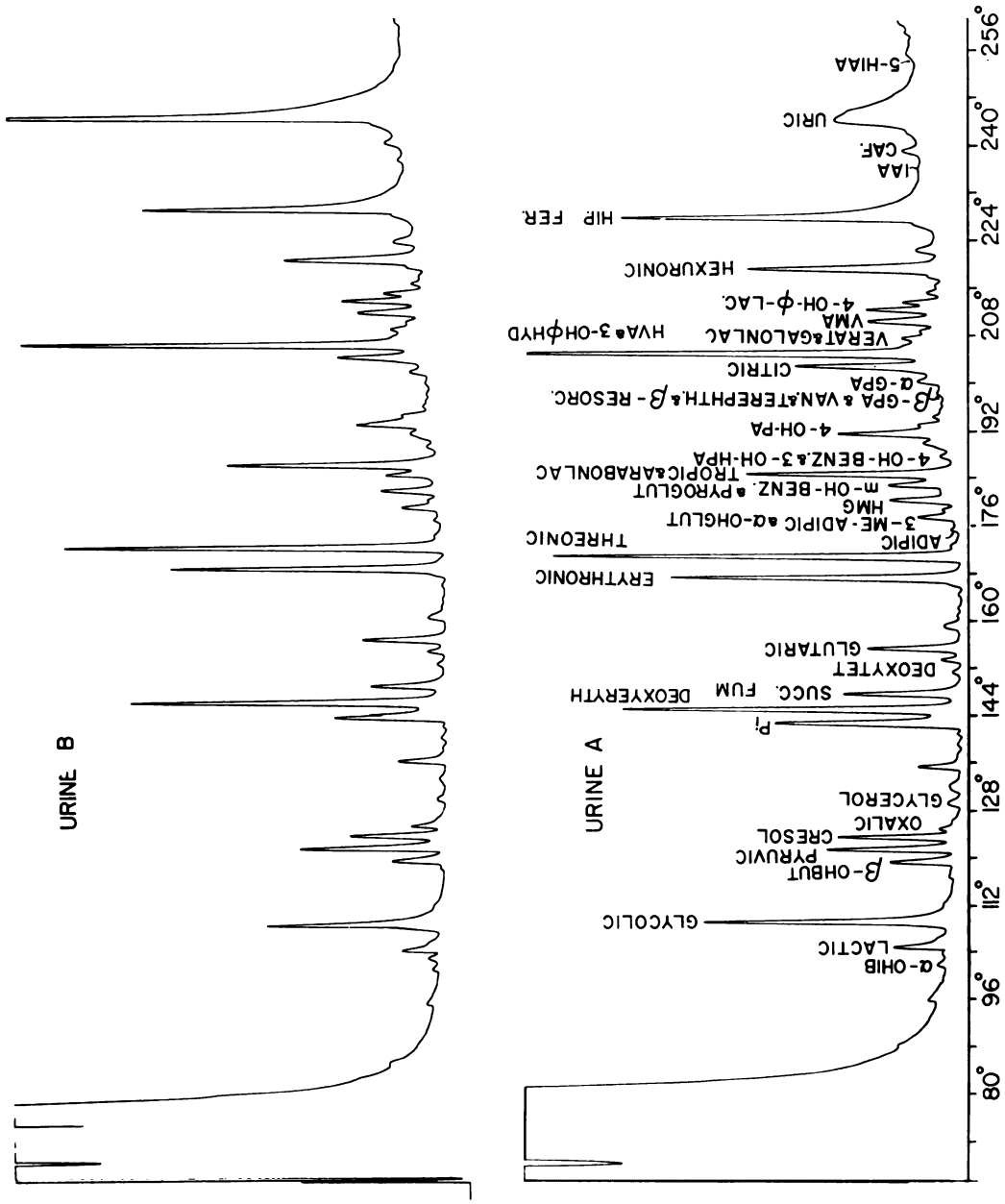
URINE B
URINE A
80°
96°
112°
128°
144°
160°
176°
192°
208°
224°
240°
256°
α-OHIB
LACTIC
GLYCOLIC
β-OHBUT
PYRUVIC
CRESOL
OXALIC
GLYCEROL
Pi
SUCC. FUM DEOXYERYTH
DEOXYTET
GLUTARIC
ERYTHRONIC
THREONIC
ADIPIC
3-ME-ADIPIC & α-OHGLUT
HMG
m-OH-BENZ. & PYROGLUT
TROPIC & ARABONLAC
4-OH-BENZ. & 3-OH-HPA
4-OH-PA
β-GPA & VAN. & TEREPHTH. & β-RESORC.
α-GPA
CITRIC
VERAT & GALONLAC. HVA & 3-OHφHYD
VMA
4-OH-φ-LAC.
HEXURONIC
HIP FER.
IAA
CAF.
URIC
5-HIAA

FIGURE 25

Figure 26. Stability of stored sample capillaries.

Samples of urinary organic acids are stored as the trimethylsilyl derivatives in silanized glass capillaries. Two capillaries of the same sample of urinary organic acids were analyzed as part of a series including storage of capillaries at -80°, -20°, 4° and room temperature. Capillary A was analyzed at the beginning of the series (0 hours of storage), while capillary B was analyzed after storage at room temperature for 6 weeks. Capillaries stored at the other three temperatures gave results indistinguishable from those stored at room temperature. The peak size of hippuric and uric acids is largely dependent upon the condition of the GC column; hence, variations of these peaks should not be considered to be due to the length or conditions of the sample storage. The samples were analyzed on a Varian 2100 GC, using a 12 ft 5% OV-17 column temperature programmed from 60° to 290°C at 4°/min.

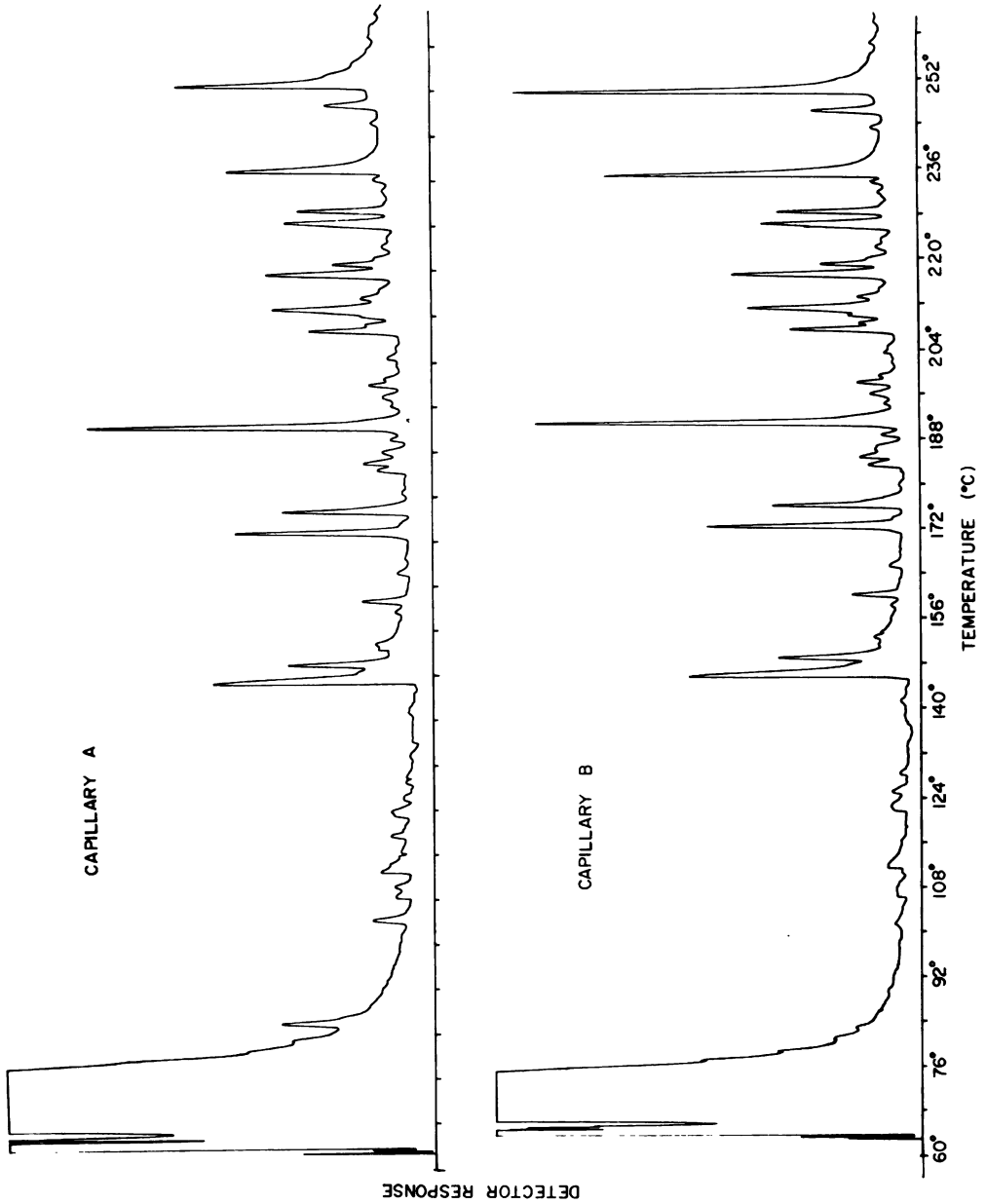
CAPILLARY A
CAPILLARY B
DETECTOR RESPONSE
60°
76°
92°
108°
124°
140°
156°
172°
188°
204°
220°
236°
252°
TEMPERATURE (°C)

FIGURE 26

samples better responses are obtained for these two compounds than found with the original sample. For the capillary stability studies, no apparent differences were observed between capillaries stored at -80°, -20°, 4° or room temperature for up to 6 months. Marked decomposition of unprocessed urine was observed except at -20° and -80°C. It should be noted, however, that none of these samples was analyzed by MSSMET (see Chapter 5).

Silylating mixtures. During early MSSMET analyses of urine samples, as it became apparent that silylation of indoleacetic and 5-hydroxyindoleacetic acids with BSTFA/TMCS alone (no solvent) frequently yields a pair of GC peaks from each compound. Mass spectral studies suggested that the first peak eluted was fully silylated compound and that the second peak was a compound with one less trimethylsilyl group. Addition of either dimethylformamide or acetonitrile to the silylating reagent usually reduced the amount of partially silylated indole observed. Only pyridine, of the three solvents tested, consistently produced a single peak and a solvent peak which eluted prior to the decane standard on 5% OV-17.

Clinical studies

Once the analytical method had been tested, a few urine samples were processed through the entire procedure to examine the

clinical significance, if any, of the data so obtained.

MSSMET analysis of urine samples. Twenty urines and one procedural blank were analyzed; the urines included 9 from the BCIU (adult control) group, 5 from infants undergoing therapy for neuroblastoma, 5 from the juvenile control group and the one from an infant with seizures of unknown etiology. A summary of the description of each subject is included in Appendix D. All samples were processed with the same conditions and the same MSSMET library. With 157 compounds in the library, 96 to 140 compounds were found in the samples. When 35 "questionable" compounds were removed (see below), 84 to 118 compounds were found. Some of these are duplicates, as discussed below. There is some evidence of a correlation between the sensitivity of the LKB-9000 (as measured by the absolute area of the internal standard) and the number of peaks found (correlation coefficient $\geq$ 0.50, significant at 3% level for the 157 compound group), although there also appear to be correlations between the number of peaks found and both the date of analysis and the type of sample run. Copies of the MSSMET library and the MSSMET results are included as Appendices B, E and F. The data in Appendix E are relative peak areas reported by MSSMET; Appendix F includes the same data, but normalized to the partial sum of peak areas, as described below.

Analysis of procedural blank. A procedural blank, consisting of a 1 ml aliquot of distilled water instead of urine, was extracted by DEAE-Sephadex. The resulting acid fraction was derivatized and analyzed on the GC-MS as usual. The results of the MSSMET analysis of this sample are presented in Table 9. Only 7 compounds were reported by MSSMET to be present in the blank.

Statistical analysis of urine samples. Results calculated by MSSMET from the mass spectral data underwent further analysis with a series of statistical programs.

Preliminary statistical analysis of the data indicated that 35 of the 157 compounds in the library were unsatisfactory for one or more of the following reasons:

1. The compound was found in less than 25% of the urine samples.

2. The match coefficient for the compound was too low in too many urines. Since the MSSMET library was set to automatically exclude a compound from consideration if its match coefficient was less than 80, any compound for which the mean match coefficient was significantly less than two standard deviations above 80 was excluded from further consideration.

3. The mean retention index was too far from the library value. Since deviations of the retention index from the library value, if more than 12 retention index units, were cause for considering a

Table 9. Analysis of procedural blank

Compound	Retention index	Relative area in blank	Mean relative area in BCIU samples ± SD
U1	985	7.76	1.18 ± 1.02
Lactic acid	1097	0.918	9.33 ± 5.49
U2	1107	1.13	1.75 ± 1.25
Glycerol	1269	0.722	2.26 ± 0.79
Palmitic acid	2097	0.318	1.16 ± 1.07
U37	2159	7.89	8.50 ± 5.50
3,4,5-Trihydroxy-cinnamic acid-peak 2	2400	0.860	0.27 ± 0.30

compound "not found," mean retention indices in urine samples were judged to be unreliable if more than 3% of the values might be expected to fall outside of the range, library value plus or minus 12 retention index units.

4. The mean relative area was too low. Compounds for which the mean relative area for all experimental groups was very close to the limits of detection of the system were excluded from consideration.

Compounds were eliminated by these four criteria in the order given. The number of compounds eliminated by each criterion was 11, 14, 4 and 6, respectively, excluding cases where a compound fell into more than one of these groups. Those 122 compounds remaining were used for all of the subsequent statistical analyses. The compounds excluded by these criteria are listed in Appendix C.

Retention indices. As shown in Figure 28, the distribution of retention indices about the mean retention index in urine samples had a standard deviation of 2.57 for the 861 values plotted. This value was obtained using urinary metabolites rather than hydrocarbon standards as retention index standards (except that tetracosane was used, since no one urinary metabolite was found to be consistently present in this region of the gas chromatogram). For these data, 77% of all retention indices fell within 2 retention index units of the

Figure 27. Precision of retention index determination on urine samples using hydrocarbons as standards.

Retention indices for the trimethylsilyl derivatives of organic acids were measured in 15 urine samples. Nine straight-chain hydrocarbons coinjected with the samples were used as retention index standards. Retention indices were measured by MSSMET and then compared to the mean retention index for all 15 urine samples. Each urine sample was analyzed on the LKB-9000 GC-MS, with repetitive scanning of the magnetic field at 4-sec intervals.

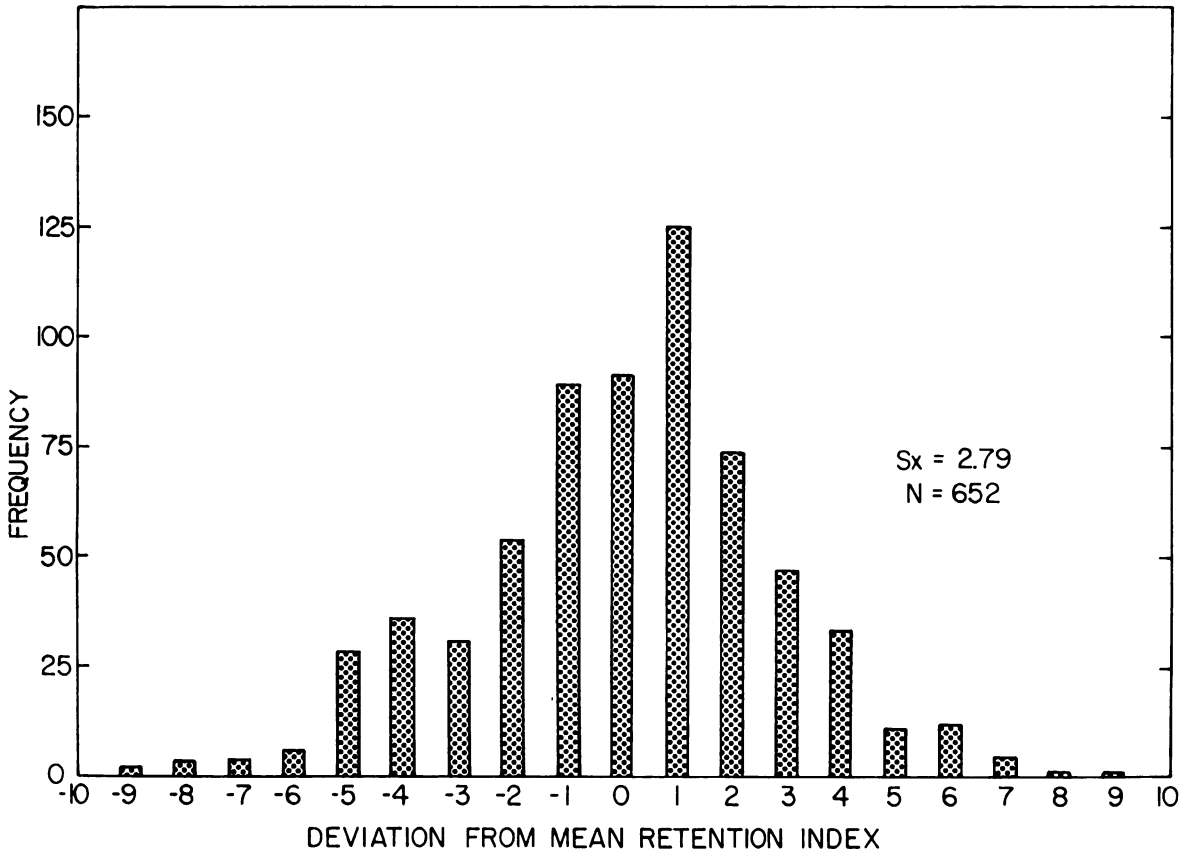
150
125
100
75
50
25
0
FREQUENCY
Sx = 2.79
N = 652
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
DEVIATION FROM MEAN RETENTION INDEX

FIGURE 27

Figure 28. Precision of retention index determinations on urine samples using metabolites as standards.

Retention indices were determined for organic acids in the same samples as those summarized in Figure 27, except that 12 naturally-occurring organic acids were used instead of hydrocarbons as retention index standards. A single hydrocarbon (tetracosane) was also used, since no routinely-occurring metabolite eluted in this region of the GC-MS run. Individual retention indices computed by MSSMET are compared to the mean retention index for the same compound in all 15 urine samples.

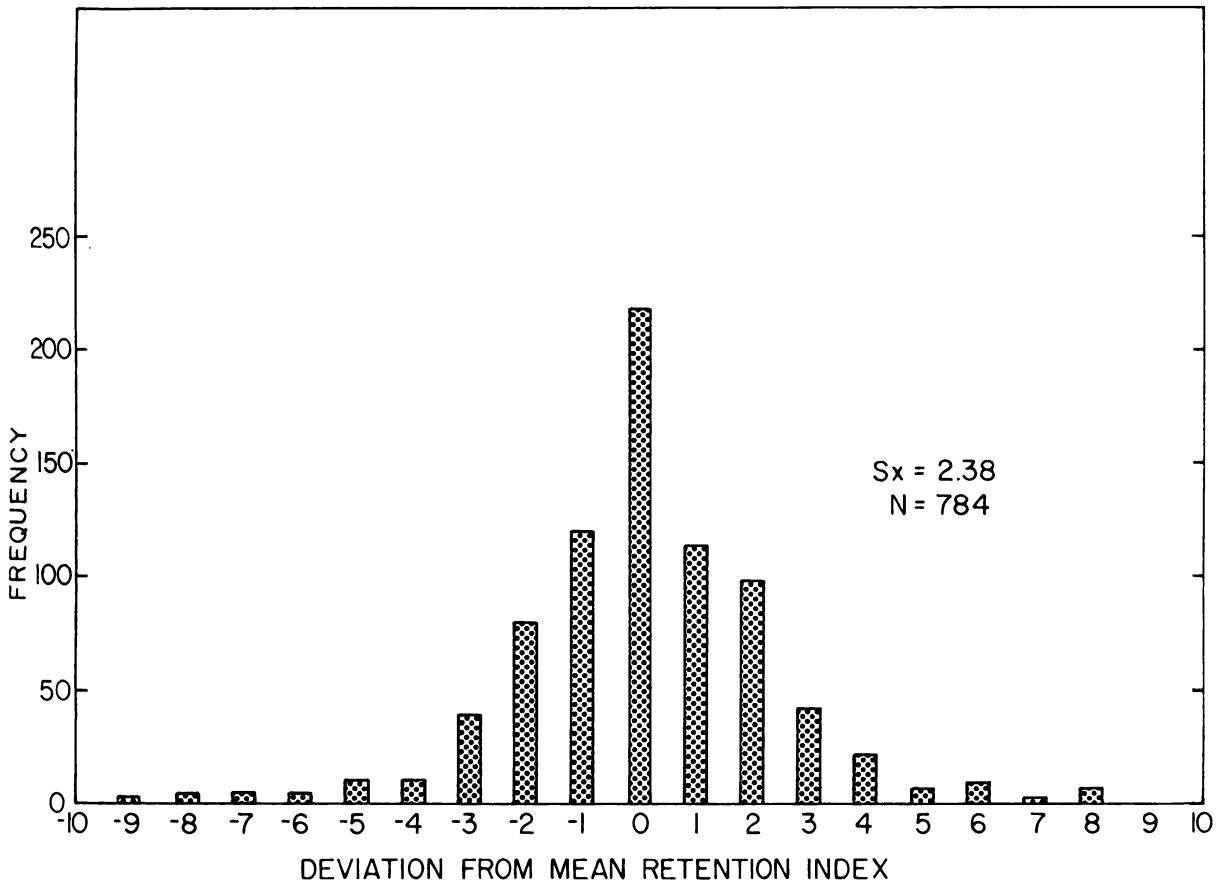
250
200
150
100
50
0
FREQUENCY
Sx = 2.38
N = 784
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
DEVIATION FROM MEAN RETENTION INDEX

FIGURE 28

mean and 95% fell within 5 retention index units. Matched data using hydrocarbons as retention index standards, instead of urinary metabolites, were less precise by approximately 0.59 retention index units, as shown in Figure 27. By either method, three of the most variable peaks were hippuric, uric and m-hydroxyhippuric acids, all of which occurred with varying degrees of GC peak tailing. Including these substances, the mean retention indices based on urinary metabolites averaged 0.28 retention index units higher than those based on hydrocarbon elution times; the retention indices never averaged further than 3.0 retention index units apart by the two methods.

Several tests were made of the correlation of retention index with other variables; the results of thse tests are shown in Table 10. Both measures of relative peak amount are highly correlated, as are both measures of the match coefficient. However, none of the correlations with retention index or retention index deviation from library value is above 0.25.

Match coefficients. Match coefficients were computed from both peak areas and peak heights. These two types of match coefficients were correlated with one another at the 0.78 level, as shown in Table 10, but did not correlate highly with any other measure of peak identity or amount. The correlations were all statistically significant, with $p < 0.001$ in each case. It was also

Table 10. Correlation of MSSMET output data.*

	MC (area)	RI	Del. RI	Rel. area
MC (height)	0.78			
RI	0.11			
Del RI	-0.01	0.15		
Rel. area	0.15	0.09	0.04	
Rel. height	0.19	NC	0.02	0.94

* Computed on raw data for 9 BCIU urines.

Key:

MC	match coefficient
RI	retention index in urine sample
Del. RI	difference between retention index in urine sample and library retention index
Rel. area	Relative area
Rel. height	Relative height
NC	Not calculated

Note: All correlation coefficients above 0.05 are significant ($p < 0.001$)

found that the match coefficient differed slightly depending upon whether the peak areas or peak heights were used. The peak height criterion gave significantly ($p = 0.025$) better match coefficients, but the actual difference in match coefficients was small (1.0 higher for the peak height criterion). The mean match coefficient computed from peak heights was 92 when the entire set of compounds was considered, with a range from 77 to 99, as shown in Figure 29. When only the 132 "reliable" compounds were considered, the range was 86 to 99, with a mean of 93. When both height and area match coefficients are plotted, the individual values range from 71 to 99, with a mean of 93, as shown in Figure 30.

Peak width in urine samples. As determined by MSSMET, the width of the designate ion peaks ranged from 4 scans (16 sec) to 28 scans (112 sec) when a window width of 120 sec was utilized; the distribution of peak widths is graphed in Figure 31. The mean peak width was 15 scans (1 min). Most of the very narrow (less than 8 scans wide) peaks were less than 200 area units (out of 500,000 possible on the PDP 8/e system), corresponding to very small peaks. The widest peaks were generally observed for very large asymmetric peaks (e.g., hippuric and pyroglutamic acids).

Relative peak areas and heights. These two measures of compound amount were found to be very highly correlated; the correlation coefficient, as shown in Table 10, is 0.94, with the

Figure 29. Distribution of mean match coefficients.

Peak height match coefficients were computed by MSSMET for 157 compounds found in 17 urinary organic acid samples. One of the criteria for a positive match to the library spectrum was that either the peak height match coefficient or peak area match coefficient must be greater than 80; hence, only a few cases were observed where the peak height match coefficient was below this value for compounds considered "found" by MSSMET. Data for compounds with mean match coefficients below 86 were later all judged to be unreliable and were therefore excluded from other statistical studies.

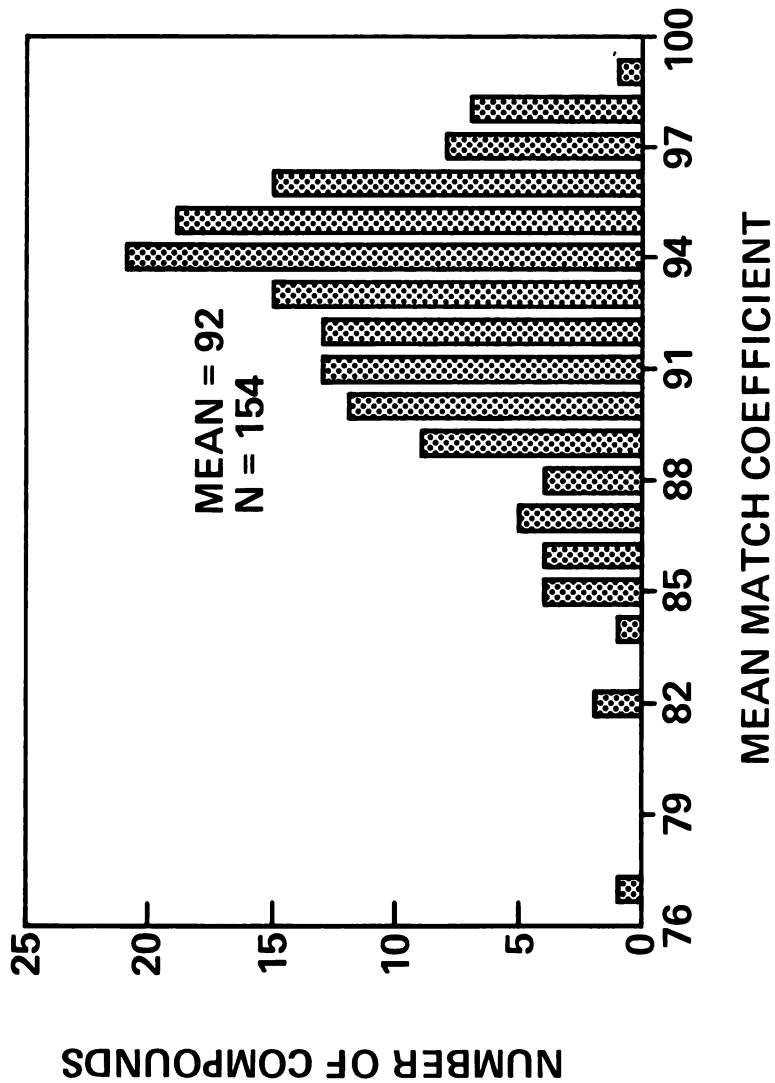
MEAN = 92
N = 154
NUMBER OF COMPOUNDS
MEAN MATCH COEFFICIENT
0
5
10
15
20
25
76
79
82
85
88
91
94
97
100

FIGURE 29

Figure 30. Distribution of individual match coefficients.

Peak height and peak area match coefficients were calculated for 157 compounds found in 4 urinary organic acid samples by MSSMET. Both types of match coefficients are plotted. The mean match coefficients for each of the same compounds are plotted in Figure 29.

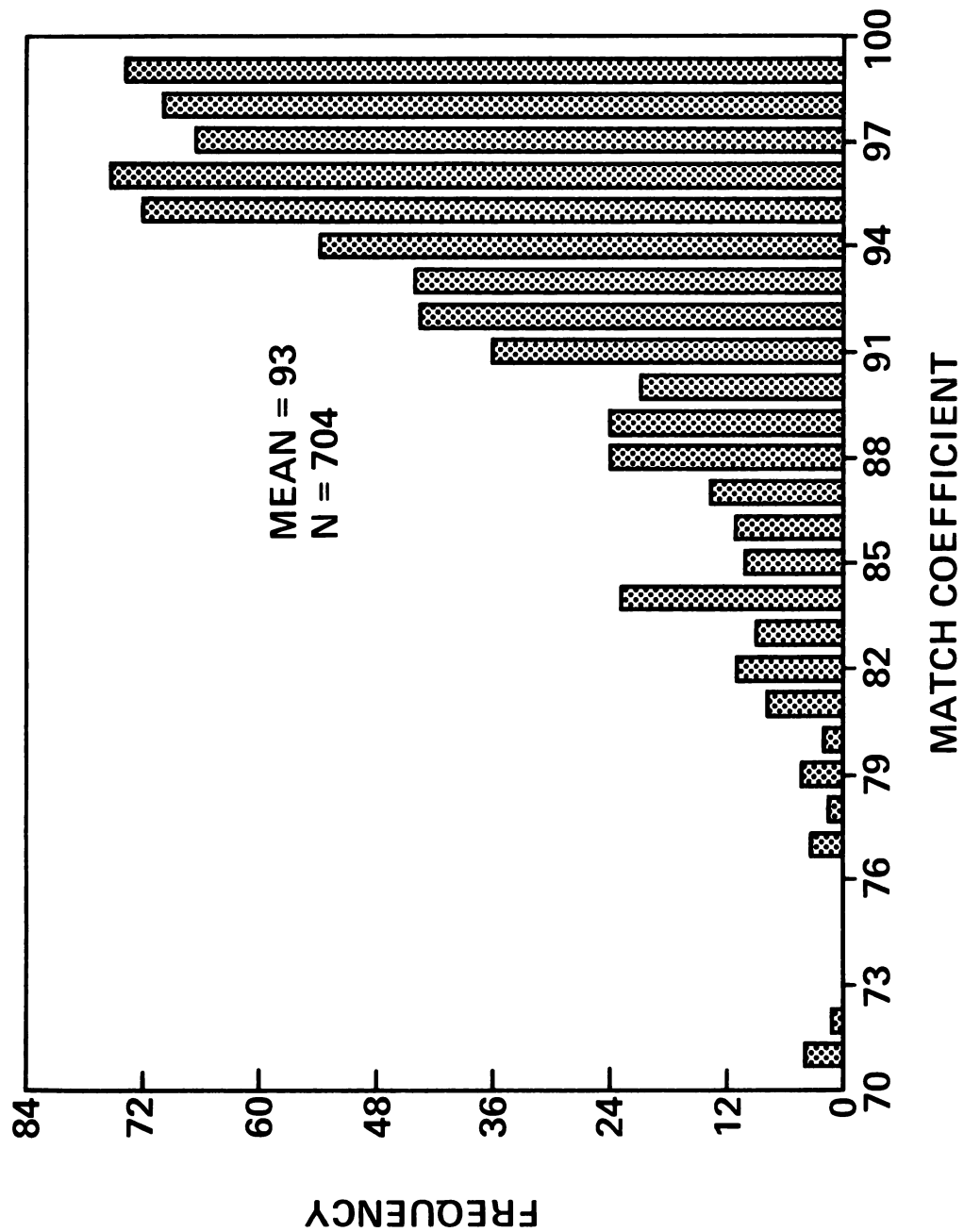
MEAN = 93
N = 704
FREQUENCY
MATCH COEFFICIENT
0
12
24
36
48
60
72
84
70
73
76
79
82
85
88
91
94
97
100

FIGURE 30

Figure 31. Distribution of designate ion peak widths.

Part of the data reported by MSSMET in both the "run" and "found" files is the starting and ending scan numbers for the designate ion peak of each compound. The "found" file limits reported for 157 compounds in four urine samples were used to calculate the width of the designate ion peak in each case. The peak width is a function of both the amount of substance present and the extent to which the designate ion peak is resolved from other peaks of the same ion.

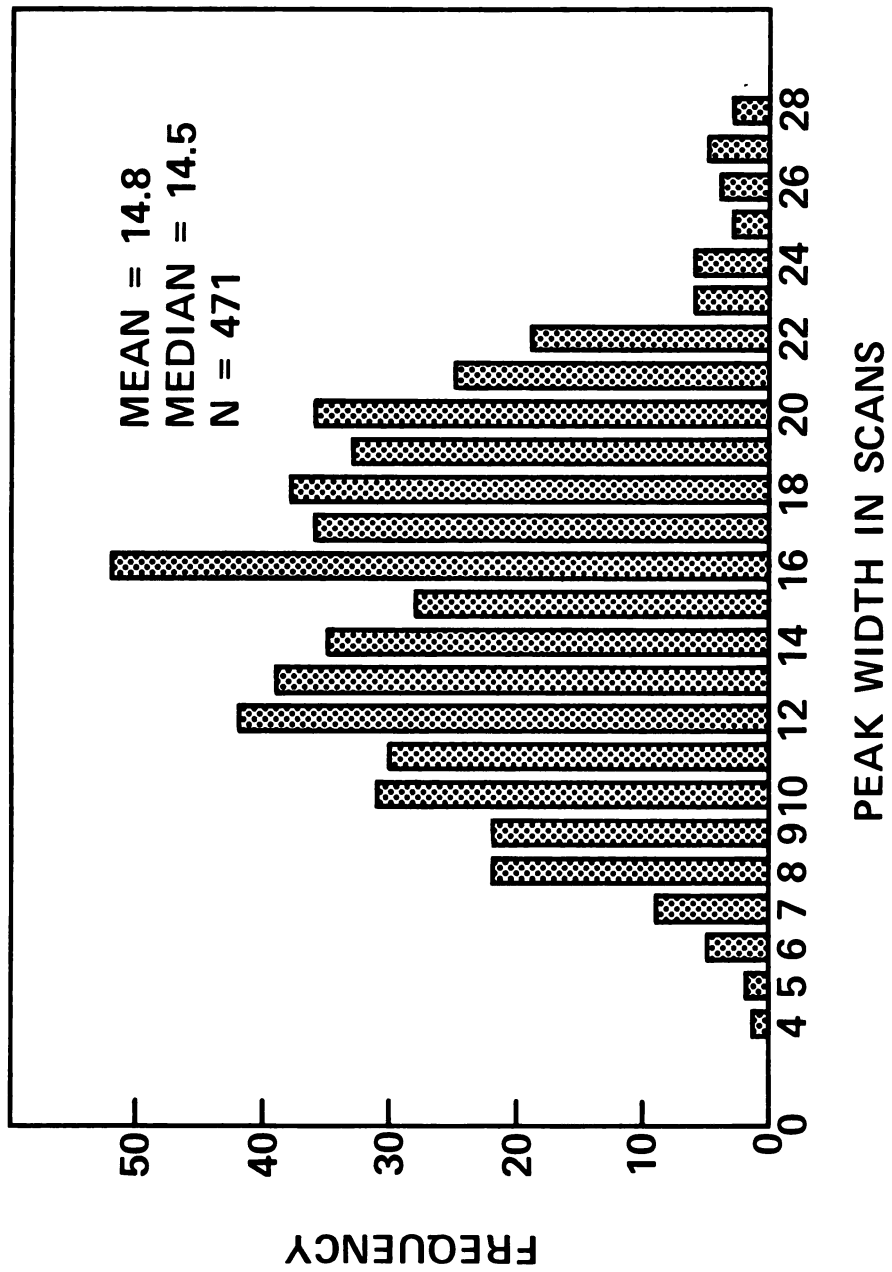
MEAN = 14.8
MEDIAN = 14.5
N = 471
FREQUENCY
0
10
20
30
40
50
4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28
PEAK WIDTH IN SCANS

FIGURE 31

values ranging in individual urines from 0.935 to 0.999.

Distribution of peak amounts. To check whether the peak amounts for a given compound were normally distributed within members of a group, the relative peak areas were plotted for several substances in the BCIU urines that had no values of zero (to avoid the problem of taking the logarithm of zero for the second plot, described below). The data are plotted in Figure 32, as the normalized relative peak areas, each expressed as a fraction of the mean value for that compound. Since the distribution of these values appears to be log-normal rather than normal a second plot was constructed, using the same data converted to their logarithmic forms and then expressed as a fraction of the mean of the logarithmic values. This plot is included as Figure 33.

Outlier test. A test for outliers (abnormally high or low values) was performed on the normalized relative peak amounts (listed in Appendix F) of both the neuroblastoma and BCIU groups. For a group of n subjects, the test compares the difference between values for subjects (n-1) and (n) to the difference between the values for subjects (1) and (n). A similar test is made for outliers at the lowest values. A table of outlier values significant at least at the 5% level is included as Table 11. One of the 5 neuroblastoma patients and three of the 9 BCIU subjects had a large number of substances identified by the program as outliers.

Figure 32. Distribution of concentrations in a group of reference urine samples.

Relative peak areas reported by MSSMET for organic acids in 9 BCIU urines were normalized to the sum of all but the 32 largest relative peak areas. The normalized relative peak areas for each compound were expressed as a ratio to the mean normalized relative peak area of that compound. Only substances which MSSMET identified as being present in all 9 BCIU urines were included in the data. The same data are displayed in Figure 33.

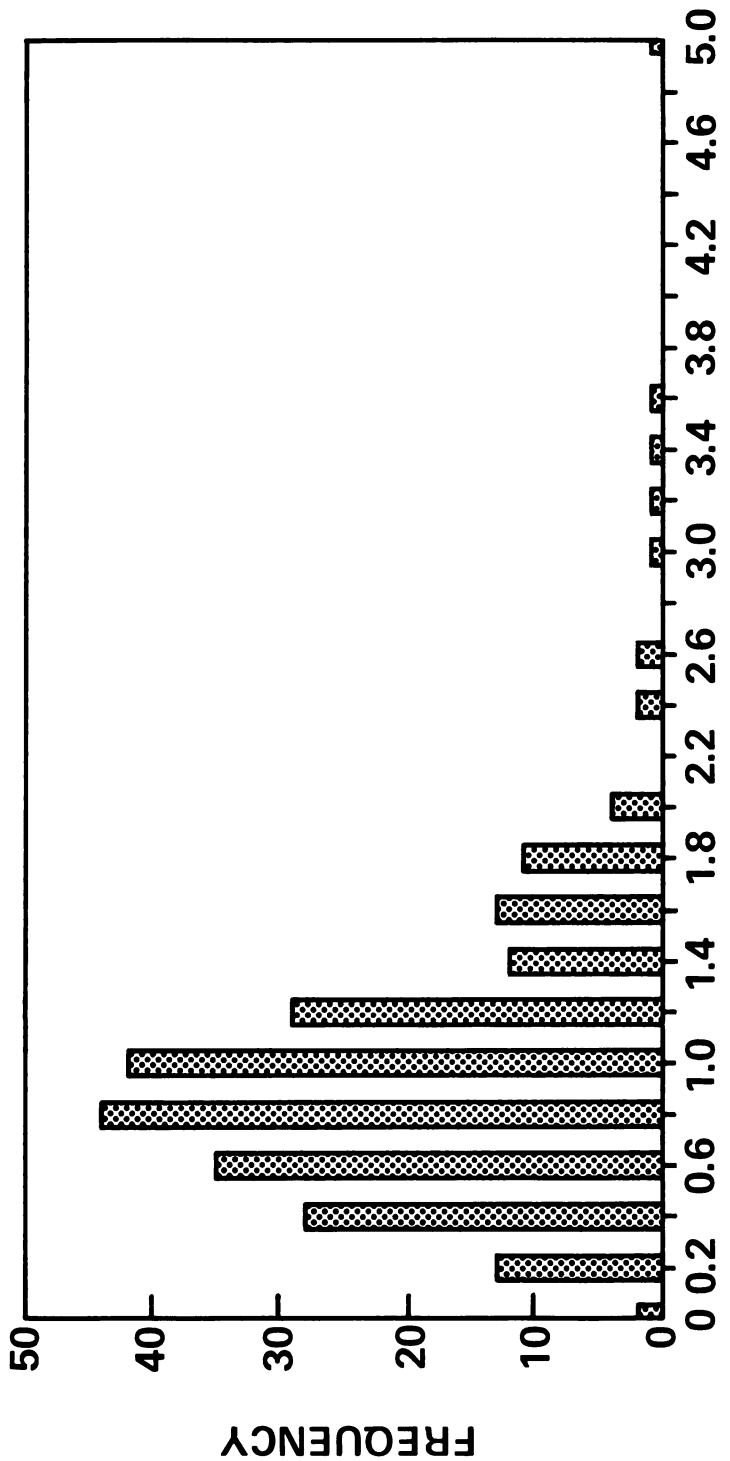
FREQUENCY
50
40
30
20
10
0
0 0.2 0.6 1.0 1.4 1.8 2.2 2.6 3.0 3.4 3.8 4.2 4.6 5.0
FRACTION OF MEAN NORMALIZED RELATIVE AREA

FIGURE 32

Figure 33. Distribution of logarithms of concentrations in a group of reference urine samples.

Because the data displayed in Figure 32 did not appear to follow a "normal" distribution, each normalized relative peak area was converted to its logarithm (base 10) and then expressed as a ratio to the mean of the logarithms of the normalized relative peak areas for the same compound. No substance with a concentration of zero in any of the 9 BCIU urines was used, in order to avoid the problem of trying to take the logarithm of zero.

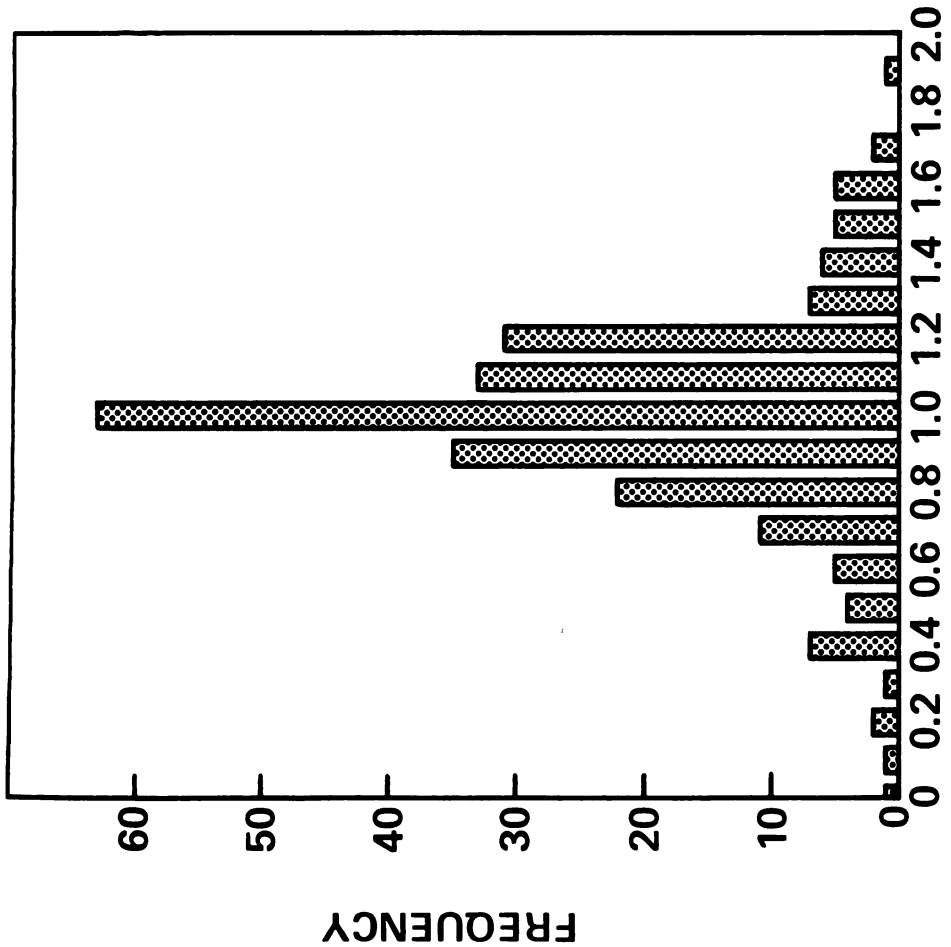
FREQUENCY
60
50
40
30
20
10
0
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
FRACTION OF MEAN LOG10
NORMALIZED RELATIVE AREA

FIGURE 33

Table 11. Test for outlying substances

Patient identification		Numbers of outlying compounds
Neuroblastoma urines		
MG	09076N3	18*, 77, 98, 105, 207*, 246*, 266*, 298*, 349, 353, 359*, 364, 367, 369, 371*, 374, 375*, 376, 383*
MM	09076N5	104, 135, 301, 176*[@]
MM	09076N1	none
JJ	09076N8	23, 114*, 136*, 293
RG	09076N7	none
BCIU urines		
056	06306N7	8, 215[@]
074	07086N1	152[#], 306, 77[@]
052	06306N8	215, 325, 218[@]
060	06306N4	328, 381[#]
120	07076N6	23[#], 42[#], 104[#], 112[#], 124, 310[#], 342[#], 349[#], 352[#]
115	07076N4	17[#], 294
087	07086N2	172, 321, 359, 369[#]

Table 11. (Cont'd.)

Patient identification	Numbers of outlying compounds
113 07076N2	98, 223, 235, 257#, 258, 266
080 07086N7	125#, 176, 291, 357

Note: Substances listed are those which have less than a 5% likelihood of belonging to the same population of values as the values of the same compound for the other urines in the same group (neuroblastoma or BCIU). Values tested are the average of the relative peak area and relative peak height for each substance, normalized to the sum of all such values except for the 12 substances listed in Appendix H. The names for each of the substances are listed in Appendix E.

Key: * Values are abnormal for the group of neuroblastoma urines but are very close to the mean value of the BCIU group of urines.

@ Unusually low value; all other values are unusually high.

Values are abnormal for the group of BCIU urines but are very close to the mean value of the neuroblastoma urines.

Comparison of subject groups. Samples from three groups of subjects (BCIU, neuroblastoma, juvenile controls) were analyzed in sufficient numbers to permit a comparison of group means utilizing the "Student" t-test. Three types of comparison were made: raw data; data normalized to the sum of intensities of a subset of the peaks; and the logarithm of data normalized to the summed intensities. In each case, relative area was used as the raw datum. This was relative area per mg creatinine for the neuroblastoma and BCIU groups, but for the infant controls it was relative area per ml urine. The list of compounds omitted from the sums is given in Appendix G. Tables 12 and 13 summarize the results of these studies by showing the compounds found to be statistically significant in differentiating adults from children and neuroblastoma patients from both control groups. Using the t-test on the logarithms of normalized data, concentrations of 20 compounds were found to be significantly different at the 10% level or better between the juvenile and adult groups; similarly, 13 compound concentrations were significantly higher or lower in the neuroblastoma group than in the two types of control subjects. A complete table of the t-test results on the logarithms of the normalized data plus the means, standard deviations, standard errors, and coefficients of variation, is included in Appendix J. For this t-test, a value of 0.005 has been arbitrarily assigned in place of all data that are zero; this

value represents an estimate of the minimum relative concentration detectable in our system. This value is converted to -2.30 during the logarithmic transformation.

In addition, the non-parametric Wilcoxon test was applied to some of the same data. Table 14 illustrates the comparison between the Wilcoxon test and two parametric tests on essentially the same data. Table 15 compares the Wilcoxon on normalized data to the t-test on data which has been normalized and then undergone logarithmic (base 10) transformation.

Clinical report form. A physician-oriented summary sheet was also developed that displays data for a single sample in comparison to a set of reference values. This form allows data to be corrected for k-factors where known, and it indicates the minimum value detectable (approximately) with the system. A copy of such an output is contained in Appendix H.

Table 12. Substances differentiating urines of adult subjects from urines of juvenile subjects*

Compound name	Level of significance**	
	BCIU vs. neuroblastoma	BCIU vs. juvenile control
Glyoxylic	4 -	3 -
Oxalic	4 -	3 -
Unknown OXB-1	3 -	3 -
Fumaric	2 -	2 -
Malic	2 -	2 -
α-Hydroxyglutaric	1 -	1 -
β-Hydroxy-β-methyl-glutaric	3 -	3 -
Pyroglutamic	3 -	2 -
Tartaric	4 -	1 -
Homovanillic	4 -	1 -
Veratric	1 +	4 +
Hydrocaffeic	2 +	4 +
Caffeic-peak 2	3 -	2 +
Ferulic-peak 2	1 +	2 +
m-Hydroxyphenyl-hydracrylic	2 +	4 +
Unknown U6	3 +	1 +

Table 12 (Cont'd.)

Compound name	Level of significance	
	BCIU vs. neuroblastoma	BCIU vs. juvenile control
Unknown U26	4 +	4 +
Unknown U30	4 +	4 +
Unknown U36	2 +	2 +
Unknown U51	3 -	3 -

* Based on t-test on $\log_{10}$ of data normalized to sum of relative peak areas, excluding 35 large peaks. See Appendix G for list of compounds omitted from sum. Groups tested include 9 BCIU urines, 5 neuroblastoma urines and 6 urines from juvenile controls.

** Levels of significance:

1	$0.05 < p < 0.10$	(10%)
2	$0.01 < p < 0.05$	(5%)
3	$0.001 < p < 0.01$	(1%)
4	$p < 0.001$	(0.1%)

\- Mean adult concentration lower than mean juvenile concentration.

\+ Mean adult concentration higher than mean juvenile concentration.

Table 13. Substances differentiating urines of subjects with neuroblastoma from urines of all control subjects*

Compound name	Level of significance** Neuroblastoma vs. BCIU	Neuroblastoma vs. juvenile control
α-Hydroxyisobutyric	4 -	2 -
Lactic	2 -	2 -
Glycolic	3 -	2 -
Phosphoric	1 +	2 +
Tartaric	4 +	1 +
Citric	1 +	1 +
Homovanillic	4 +	3 +
Vanilmandelic	4 +	1 +
Caffeic-peak 2	3 +	4 +
m-Hydroxyphenyl-hydracrylic	2 -	4 +
Unknown U6	3 -	3 -
Unknown U65	4 +	1 +
Unknown U79	4 -	4 -

* Based on t-test on $\log_{10}$ of data normalized to sum of relative peak areas, excluding 35 large peaks. See Appendix G for list of compounds omitted from sum. Groups tested include 9 BCIU urines, 5 neuroblastoma urines and 6 urines from juvenile

Table 13 (Cont'd.)

controls.

** Levels of significance:

1	$0.05 < p < 0.10$	(10%)
2	$0.01 < p < 0.05$	(5%)
3	$0.001 < p < 0.01$	(1%)
4	$p < 0.001$	(0.1%)

\- Mean neuroblastoma concentration lower than mean control concentration.

\+ Mean neuroblastoma concentration higher than mean control concentration.

Table 14. Comparison of three statistical tests*

Compound# number	Level of significance**		
	t-test	t-test normalized	Wilcoxon normalized
6	1	2	2
8	-	-	1
10	-	1	2
17	2	2	3
18	1	1	-
23	1	2	3
28	-	2	1
34	-	1	-
61	1	2	1
65	-	1	-
77	1	1	1
107	1	1	-
110	1	2	2
112	2	1	2
123	1	2	2
125	2	2	2
127	2	2	1
135	-	1	1

Table 14. (Cont'd.)

Compound# number	Level of significance** t-test	t-test normalized	Wilcoxon normalized
136	-	1	1
152	1	1	1
168	-	1	1
184	-	2	1
200	1	1	-
216	-	2	3
218	-	2	1
223	-	-	1
236	-	1	-
237	-	1	-
251	1	2	2
290	-	2	1
298	3	2	3
303	-	1	1
310	-	1	-
314	-	1	1
316	-	1	-
318	2	2	3

Table 14. (Cont'd.)

Compound# number	Level of significance**		
	t-test	t-test normalized	Wilcoxon normalized
322	2	2	3
324	-	1	3
328	-	-	1
343	1	2	1
344	-	1	-
345	-	2	-
346	-	1	-
349	1	-	1
357	2	2	1
361	-	1	-
369	1	-	-
371	2	1	1
374	1	1	-
381	-	1	1
383	-	1	1

* Test of 9 BCIU samples against 5 neuroblastoma samples.

T-tests and Wilcoxon use two-tailed test of significance.

Table 14. (Cont'd.)

Normalization is to sum of all but largest 12 peaks. (See Appendix G for list of compounds omitted from sum.)

\# Refer to Appendix B for names of each compound.

** 1 refers to compounds whose concentrations are significantly different at the 5% level; 2, at the 1% level; and 3, at the 0.1% level for the t-test and the 0.2% level for the Wilcoxon.

Table 15. Comparison of Student t-test on log-transformed data with Wilcoxon test*

Compound number#	Level of significance**	
	Wilcoxon@	T-test&
6	2	3
8	1	1
10	2	2
17	3	3
23	3	3
28	1	2
61	1	1
65	-	3
77	1	-
79	-	1
86	-	1
110	2	2
112	2	2
114	-	2
123	2	3
125	2	2
127	1	1

Table 15. (Cont'd.)

Compound number[#]	Level of significance**	
	Wilcoxon[@]	T-test[&]
135	1	2
136	1	1
152	1	1
168	1	-
184	1	3
187	-	2
216	3	3
218	1	-
223	1	1
229	-	1
236	-	1
251	2	2
290	1	1
298	3	2
303	1	1
310	-	1
318	3	3
322	3	3

Table 15. (Cont'd.)

Compound number#	Level of significance**	
	Wilcoxon@	T-test&
324	3	-
328	1	1
343	1	2
344	-	3
345	-	2
349	-	1
357	1	3
371	1	3
381	1	-
383	1	1

* Test of 9 BCIU urines against 5 neuroblastoma urines.

\# Refer to Appendix B for names of each compound.

** 1 refers to compounds whose concentrations are significantly different at the 5% level; 2, at the 1% level; and 3, at the 0.1% level for the t-test and the 0.2% level for the Wilcoxon.

@ Wilcoxon test on data normalized to sum of relative peak amounts, excluding 12 large peaks. See Appendix G for list of

Table 15. (Cont'd.)

compounds omitted from sum.

& T-test on $\log_{10}$ of data normalized to sum of relative peak areas, excluding 35 large peaks. See Appendix G for list of compounds omitted from sum.

CHAPTER FIVE: CONCLUSIONS AND DISCUSSION

The metabolic profiling of urine samples can logically be divided into a series of steps: chemical separation, GC-MS-COM analysis, MSSMET analysis, and statistical analysis. Each of these steps will be discussed separately.

Organic acid separation procedure

The first step in the metabolic profiling process is the separation of the fraction of interest from the remainder of the constituents of the biological fluid. In the work described here, this consisted of separating the organic acids from human urine.

Reproducibility. The most important criterion for the separation procedure is that it be reproducible; otherwise, any variability in recovery will be superimposed on the inter-subject variability. Hence, a considerable amount of effort was devoted to perfecting a separation method that would result in reproducible GC traces of the same urine. Originally, ether-ethyl acetate extraction, charcoal adsorption, lyophilization of unextracted samples, and several other techniques were tried, but none of these

was sufficiently reproducible, usually because recoveries were quite low for some types of compounds. The DEAE-Sephadex procedure of Horning et al. (71H2) was also tried, but was discarded because the column eluate often contained so much of some unidentified residue (perhaps a mixture of inorganic salts and some protein) that it could not be lyophilized to dryness, thus preventing complete silylation.

However, the barium hydroxide precipitation suggested by Thompson and Markey (74T2) significantly altered the contents of the column eluate so that it could be more readily lyophilized. Hence, the Thompson and Markey procedure was tested extensively. Unfortunately, it also did not give reproducible results, although it was better than other procedures tested. This was particularly puzzling because Thompson and Markey claimed that the recoveries were high by their procedure, and Chalmers and Watts, in a previous study, had shown that recoveries were near 100% for most of the substances they examined (72C3, 72C4). Chalmers and Watts had not used a barium hydroxide precipitation, however, and many of the conditions of the separation differed between the two groups.

By careful examination of the procedure, we discovered that reproducibility improved significantly with an increase in the quantities of elutants (water and 1.5M pyridinium acetate). The

ratios of these elutants to column size that we determined to be adequate are very similar to the ratios suggested by both the Horning and Chalmers and Watts papers.

In addition, Domino and Mathews (77D1), while using our exact procedure, discovered that the barium hydroxide precipitation reduced the amount of residue left after lyophilization and that less residue was produced when acetic acid was used instead of hydrochloric acid to adjust the pH of the urine.

We also discovered from studies of pure compounds, particularly indolic and amino acids, that addition of pyridine to the silylating reagent avoided multiple GC peaks of these types of compounds. The use of sealed glass capillaries for storage of silylated samples significantly improved the procedure by increasing sample stability. Another useful, but less critical, change has been that creatinine concentrations are used to judge how much urine to apply to the DEAE-Sephadex column. Since 1 ml of the standard reference urine contained 1.44 mg creatinine, and this seemed to give acceptably concentrated samples on the GC-MS, and attempt was made to place urine containing approximately 1.5 mg of creatinine on the DEAE-Sephadex column for all other urine samples. This seemed to produce an acceptable level of concentration for all but urine from children; for children under age 5, at least, a smaller amount of urine might be more appropriately applied to the column.

Other studies of extraction procedure. Once the above changes (except the addition suggested by Domino) were made, the method proved to be acceptably reproducible, as shown in Figure 24. Part of the variability observed is from the GCMS, as shown by Figure 22, rather than the chemical separation. After making certain that the method was reproducible, a series of studies was begun to test the various analytical features of the extraction procedure. It was concluded that urine samples are stable at -80° and even at -20°C for as long as 6 months (the maximum tested), as illustrated in Figure 25. Not unsurprisingly, the samples are not stable at 4°C or room temperature, where bacterial and enzymatic degradation can be expected to occur. The entire procedure appears to yield acceptable, although less than complete, recoveries (Table 8). More recovery studies, particularly of the various sugar acids, are needed; tracer methods would probably be preferable to mass spectrometry for studying losses at each step of the procedure, however.

The barium hydroxide precipitation step was originally proposed by Thompson and Markey (75T2) as a means of reducing the amount of phosphoric and sulfuric acids in the final sample eluted from the DEAE-Sephadex column. In their opinion these peaks obscured the adjoining peaks on the GC because they were large and tailed markedly. However, in a recent exchange of published letters Chalmers et al. (77C1) have argued with

Thompson's claim (77T1) that there is any reason to justify the use of the barium hydroxide precipitation. They suggest that Thompson may have been using injector temperatures which caused the phosphoric acid to partially decompose on the column. Since both groups use GC peak area as their measure of compound amount, the amount of GC peak overlap is indeed an important problem, and hence, the amount and shape of the phosphoric acid peak may be critical. However, even in Chalmer's letter, samples from patients with glycolic aciduria, lactic acidosis and severe acidosis were used as examples of urines containing aliphatic acids well-resolved from the phosphoric acid despite the lack of a phosphate precipitation step; these are all cases in which the acids in question are excreted in abnormally high amounts. In urines from other types of subjects, both groups are forced to measure several of these types of substances together, because they are unresolved on the GC (76L1, 75T2). Thus, arguments about accurate quantitation and percent recoveries are largely moot for these two groups, since the GC-based method is, by its very nature, inaccurate to the point where results are largely qualitative rather than quantitative.

By comparison, the method developed here, using MSSMET, does provide accurate quantitation, and therefore questions of resolution and recovery become important. There is no apparent interference between the ions produced from phosphoric acid and

those of any other nearby organic acids on OV-17 (at least not of any ions used in confirming ion sets). Hence, for any method such as MSSMET, which uses a small set of differentiating ions rather than peak area on the GC as a measure of compound identity and amount, it is of little importance whether a given compound is resolved from another compound on the GC as long as the ions chosen are sufficiently differentiating of the compounds of interest. When using MSSMET-type approaches, therefore, there is no reason to precipitate out a majority of the phosphoric and sulfuric acids. In addition, the poor recovery of some organic acids (citric especially) produces variable results for these compounds when the barium hydroxide precipitation is used. Therefore, it seems logical to omit the barium hydroxide precipitation, unless it is found to significantly reduce the amount of material lyophilized to dryness. (As was mentioned earlier, the Horning method, which uses the DEAE-Sephadex, but without oxime formation or barium hydroxide precipitation, seems to produce a large amount of very-difficult-to-lyophilize material, while the Thompson method does not.) This question requires further study.

Procedural blank. The procedural blank was surprisingly free from peaks, as shown in Table 9. While there were other peaks present on the GC trace, particularly from the new septum used, these did not correspond to any substances in the MSSMET library,

and hence are not reported. From the data listed, it would seem likely that unknowns U1 and U2 are impurities in one or more of the solvents used rather than urinary metabolites. The lactic acid level is probably increased slightly by some procedural contribution (from the acetic acid?), but is probably not seriously affected since endogenous levels are usually much higher than the level of contamination. The peaks identified as glycerol and palmitic acid are very small, and the procedure may be a major contributing source of these compounds. The compounds U37 and 3,4,5-trihydroxycinnamic acid-peak 2 are probably from septum bleed or packing bleed peaks, although it is possible they are also solvent impurities; the latter compound is one of those deleted from consideration because of a low match coefficient, implying that the substance is probably misidentified by MSSMET. There is no evidence of any "carry over" from previous samples, since all three of the late-eluting peaks are present at approximately the same levels in both blank and urine samples.

Hence, it would seem reasonable to designate U1, U2, glycerol, palmitic, U37, and 3,4,5-trihydroxycinnamic-peak 2 acids as "procedural contaminants" and exclude them from statistical comparisons. However, lactic acid should probably be retained as a urinary metabolite in the MSSMET library.

Choice of GC column type and derivatizing agent

Once the organic acids have been separated from the urine and derivatized with a silylating reagent, they are then subjected to GC-MS analysis. Early experience showed that interesting metabolites could be found over an extremely wide temperature range on any of the silicone-based GC liquid phases. In my early work, 1% SE-30 was used as the standard liquid phase, but later 3% and then 5% OV-17 were adopted to achieve a better separation of components. The column length was originally 12 feet, to achieve maximum resolution, but was reduced to 10 feet when the 12-foot columns were temporarily unavailable. Once having begun collecting data with the 10-ft 5% OV-17 columns, no further attempt was made to experiment with other column types or conditions since each type of liquid phase produces its own set of retention indices and, hence, all standards must be rerun on each new column type to obtain new values for the MSSMET library.

Similarly, early experiments indicated that methyl ester formation using diazomethane produced non-quantitative derivatization in some cases, as did the formation of methyl esters-trimethylsilyl ethers. Hence, the formation of per-trimethylsilyl derivatives was selected as the method of choice, preceeded by oxime formation of the few oxo-acids present in the urine samples.

In retrospect, both of these decisions are at least open to question. It has been claimed by some investigators that bleed from the OV-17 liquid phase rapidly degrades ion source optics, particularly at high temperatures. In addition, GC resolution does not appear to be an important factor when using MSSMET, so considerations such as stability, capacity, and range of metabolites separated in a reasonable length of time may be more important. Similarly, per-trimethylsilyl derivatives, while usually satisfactory, may be less desirable than methyl derivatives for some compounds which tend to produce only a few, low-intensity fragment ions other than m/e 73 and 147. Since these latter two ions are present in virtually all trimethylsilyl derivatives, they are useless for either qualitative or quantitative analysis. This does not appear to be as serious a problem in permethylated or methyl ester-trimethylsilyl ether derivatives, and therefore higher sensitivity might be obtained for some compounds using these derivatives.

In addition, compounds with only one or two distinctive ions are more difficult for MSSMET to identify properly. Unfortunately, however, there is no uniformly better choice, to my knowledge, for either the GC column type or the derivatization method.

Use of repetitive scanning data

Once the column packing and derivative types were chosen, attention was focused on the actual collection of data. An early decision had already been made to use repetitive scanning methods rather than selected ion monitoring techniques, since we were interested in obtaining data on a large number of components.

The one major drawback to the use of repetitive scanning GC-MS techniques, however, is the difficulty in obtaining reliable data. In order to achieve maximum precision and sensitivity, I used the LKB-9000 set at scan speed 8 (4 seconds per scan cycle from m/e 50 to 550 and back) and intensity gain 8, since tests showed that faster scan speeds resulted in significantly reduced peak intensities and that higher multiplier gains resulted in no increase in the signal-to-noise ratio. The LKB itself showed an amazing ability to withstand these conditions. Unfortunately, the data system frequently was unable to collect data reliably at these rates, particularly when system noise levels were high or mass resolution was less than optimal.

Regardless of the instrument used, there are going to be similar limiting factors in the operation of any mass spectrometer. Therefore, I recommend use of an objective test procedure on a routine basis to check the overall performance of the mass

spectrometer system. The test that I have settled upon, although not fully satisfactory, does give a measure of overall performance as well as a measure of a few of the individual variables. Noise levels are examined both with and without the multiplier turned on at the normal operating gain. If the noise levels are not markedly different, then about 75 scans are taken (under usual operating conditions) of the calibration compound (PFK). The data are examined for several ions at low, medium and high mass ranges, and then a group of ions selected (e.g., masses 350 to 360) with a variety of intensities. Mass chromatograms of all of these ions should be linear, with no more than about ± 10% variability. Extremely low intensity ions, of course, will vary considerably more than this; the reliability of data on these ions should be used to set the "minimum height" (MH) criterion of MSSMET to ignore peaks at these low intensities as being too unreliable. For our present system, this level is usually approximately 50 (where 500,000 is the maximum value detectable in the system), but it may be as high as 150 under some circumstances.

It should be noted in this context that achieving increased sensitivity is a task of considerable importance. Daily focusing of the ion source and frequent cleaning of the separator and ion source parts are the most obvious ways to achieve optimum sensitivity. Three other methods of improving sensitivity or detection limits

should not be overlooked, however. The first of these is improved amplification systems. Thus, for example, the replacement of a single intensity amplifier with a quadruple amplifier system resulted in a much wider linear range and increased the amount of sample which could be introduced into the ion source without saturating the amplification system.

A second method is to eliminate sources of electronic noise from the system. "Ground loops" between the LKB and the CH-5 or the PDP 11/40 were the cause of a great deal of 60 Hz noise for a long time, although this problem now seems to be solved. The GC oven heater frequently gives rather large negative spikes on the intensity channel of the data system; this problem is only now being solved. Loose connections to the multiplier preamplifier have been, and continue to be, a source of noise ready to destroy the data of the unwary. Probably a number of other sources of noise have yet to be identified (for example, there occasionally seem to be positive noise spikes from some source). A third way to improve the effective signal-to-noise ratio is to design more noise-resistant peak detection algorithms for the data collection routines. The current algorithm is the result of a great deal of effort by several people, but could stand even more work to improve its reliability.

The quality of repetitive scanning data might also be improved

by increasing the scan rate. With magnetic sector instruments, such as the LKB-9000, there is often a considerable time period required for allowing the magnet to decay to the starting mass; of the four-second scan period on theLKB-9000, almost 2 seconds are spent in magnet decay. During this time, no data are collected, although processing and storage of data just collected does continue. One way of increasing the rate might be driving the magnet rapidly back to the starting mass (rather than simply turning off the magnet current). Under these conditions data could be collected during both the scanning up and scanning down processes. On our system, a change to more rapid scanning could also be accompanied by addition of a higher-speed clock and perhaps some decreases in the post-scan processing time, or transferal of some of the data processing functions to another computer. These changes would probably make possible at least a twofold improvement in data collection rates without changing the basic frequency of analog-to-digital conversion in the system. This, in turn, would mean that twice as many points could be collected across a GC peak, thus improving overall data reliability. Of course, even more sophisticated means can (and should) be devised (using faster A/D's with microcomputer controlled peak detection, for example), and these would all greatly improve the precision of the final data. While data collection rates (and hence precision) can never equal those of SIM of a small number

of ions, nonetheless, current data rates are certainly nowhere near the theoretical maximum rate possible or desirable.

GC conditions

Another area that can cause considerable difficulty to the novice is the use of GC columns. The production of reproducible results is dependent upon obtaining stable GC columns which are fully silylated and properly conditioned. For example, until I began using multiple silylations of the glass columns, I found that column coatings were often incomplete (producing tailing peaks) and prone to rapid degradation. Hence, it is important that the column packing procedure be followed exactly.

The best method for achieving reproducibility is to test each GC column (including the one in the GC-MS) at least once at the beginning of each day of use. For this purpose, I prepared a series of 100 identical capillaries of silylated urinary acids and carried out GC separations at 10°/min. The operator should check sensitivity and peak symmetry, in particular. For the samples used here, uric and hippuric acids often proved to be the best indicators of column condition; column deterioration was often first apparent from a decrease in peak height and a marked peak asymmetry of these two compounds. Regardless of the test sample, a plot of test results for

each day is an extremely useful guide to column conditions.

Some of the most frequent causes of problems on the GC are incomplete conditioning (up to 48 hours may be needed), leaking septa (avoid by changing after a fixed number of injections), and failure to adequately clean the detector parts (or separator, in the case of the GC-MS) of silicate deposits (sonication in methanol on a regular basis, followed by mechanical brushing or polishing, is an excellent cure). Other potential problem sources are improper cleaning of syringes (clean with methanol followed by hexanes after each injection, then air dry), insufficient support of the GC column, (design additional supports for long columns) and improper gas flow rates (monitor frequently and record carefully).

Data storage and transfer

Early attempts to store and transfer the huge quantities of data generated by the repetitive scanning of urine samples were very disappointing. Data were frequently lost permanently because of faulty storage or transfers that were unsuccessful. This problem, first noticed on the PDP 8/I, was subsequently encountered with the PDP 8/e and the PDP 11/40, and is still occasionally a problem despite considerable attempts to eliminate it. It seems to be an almost inevitable problem that is associated with storage of data on

magnetic tape, at least with present technology.

There are at least two possible solutions to this kind of problem. One is to make provisions for verification of all data transfers (by comparing transferred data to the original, for example). This approach, while the most certain, is also very time-consuming. A second possibility is to reduce the amount of data transferred and hence presumably reduce the chance of an error occuring in a given data set. For example, I have developed a method for producing "area reduced mass spectra" that reduces the size of the data file to 15 to 20% of the original size while retaining all of the information needed by MSSMET. However, I am generally opposed to throwing away some information from urine data files, since subsequent programs developed for metabolic profiling may require some of this information. Hence, at the present time, at least, the first choice seems preferable.

Time required for GC-MS analysis

A major disadvantage of metabolic profiling by repetitive-scanning GC-MS is that it is very time-consuming. Typically, it takes approximately 1 hour of preparation time before samples can be run. This includes column installation, ion source focusing, mass calibration, and noise level and resolution checks. Any one of these

steps may, and frequently does, present problems which take large amounts of time to solve. Running and analyzing a test sample takes one hour. Once finished with preparative steps, the operator can expect to average 1.5 hours of data collection and data transfer time per sample. This means that an experienced operator can expect to average only 4 samples per 8-hour day, not including time spent on MSSMET or other data reduction techniques, and then only if he encounters no major problems. This, almost by definition, severely limits the uses to which metabolic profiling by GC-MS can be put under the conditions of our present methodology.

Performance of MSSMET

A series of tests was designed to check the performance of MSSMET. One of the principal advantages of the design of MSSMET became apparent during these tests. Using the "debug" feature, it is possible to find the source of problems very rapidly. This feature allows inspection of a variety of information such as the raw data collected, the baseline points and least-squares equation, and the areas and heights of all ions.

More useful than the debug option, however, even in routine use is the ability to change peak detection parameters at any point in the program, either manually or automatically via special commands

inserted in the library. Other useful features allow the operator to view as little or as much of the data processing procedure as he desires, and to make modifications of operating parameters whenever a special problem is encountered. For example, the window can be widened to increase the allowable deviation of the retention index for compounds such as hippuric and pyroglutamic acids, which frequently are either very broad or whose retention indices tend to be more variable.

Although MSSMET could be somewhat streamlined by deletion of these two special features, they are well worth retaining, in my opinion.

MSSMET baseline determination and peak detection. Central to the functioning of MSSMET are its baseline and peak detection algorithms. The two problems of baseline determination and peak detection can really be viewed as a single problem in pattern recognition. Can the computer recognize which data points are parts of peaks and which are not---the latter, by definition, being the baseline points? Conventional wisdom holds that the human eye/mind is still better at pattern recognition than any sophisticated computer algorithm. Unfortunately, the human eye is somewhat slower and more subject to fatigue. Hence, the justification for computerized pattern recognition is that it is faster and more reliable than the human eye, although not necessarily as accurate.

Thus, in evaluating the peak detection algorithm of MSSMET, the principal features of interest are its speed and accuracy in comparison to human spectral interpretation methods, as well as to other mass spectral interpretation techniques.

Speed. MSSMET currently requires about 15 min to process the 157-compound library, or an average of about 6 sec for the computer to identify and quantitate each compound. Excluding the time required to set up files and locate the retention index and quantitative standards, the time required is approximately 4 sec per compound. MSSMET was not designed for maximum speed, so that, with relatively minor changes, this rate could easily be doubled or tripled, with the limiting factor being the amount of core memory available. Clearly, this is far faster than the manual interpretation of the data. In any case, the overall rate-limiting factor in the present system is certainly not the speed of MSSMET, but the speed of data collection on the mass spectrometer and the wet processing steps. A further consideration is the level of other demands upon the data system by programs running concurrently; this would seem to be a more important consideration than the absolute speed of MSSMET. Clearly, increasing need for the system by other users (or the cost of renting computer time) would dictate that MSSMET be redesigned to run more rapidly in the future.

Accuracy. Extensive manual examination of data has

confirmed that MSSMET is currently able to accurately measure peaks, given the set of data within the search window. However, during development a few exceptional cases were noticed for which the algorithms were inadequate. Where possible, appropriate changes have been incorporated into the operating parameters or the algorithms themselves. Thus, special techniques had to be evolved for treating peaks at the extreme edges of the window, poorly resolved peaks, and situations where small, widely-separated peaks were observed.

The only known remaining inaccuracy that exists in MSSMET peak area determination is a consequence of using small window sizes. Currently, a window width of 120 seconds is used for almost all compounds. This window width was tested against a procedure where the baseline was chosen based on the entire run (55 min), and where the same integration and baseline algorithms were used. The difference was small but noticeable for most of the 41 peaks integrated by both methods: on the average, MSSMET areas were 4% smaller than those obtained by the complete-run baseline determination method. The largest differences between the two methods were observed in the region between retention indices 1750 and 2050, which is clearly the region containing the highest number of compounds. Since increasing the size of the window increases the length of time needed to process each compound, an appropriate

solution to this problem might be to use a smaller window in situations where few peaks are expected and a larger window elsewhere. An alternative solution might be to test for unresolved peaks and widen the window until a stable baseline is achieved; depending on the number of such cases encountered, however, this approach might prove to be very time-consuming.

Retention indices

Precision of retention indices. Clearly, utilization of retention indices is the principal advantage of MSSMET over conventional library search procedures. Most of this advantage lies in the extremely high precision of retention index determination. As shown in Figure 20, precision is such that, for pure substances, compounds elute within two to three scans of the expected (mean) value in every case tested. This is of course far more precise identification than the match coefficients (or any other measure of special match to the library) can provide. It is the best single criterion for determining peak identity, but does not, by itself, provide unequivocal identification.

Accuracy of retention indices. No other published data is available which can be used to compare retention indices from MSSMET to those of other laboratories on an exact basis. Butts

(72B1) has published an extensive set of data for compounds under similar, although not identical, conditions; these represent the single best source for comparison data. Table 16 lists a comparison of compounds that have been measured in both laboratories. It should be noted that there are several marked anomalies in the comparisons, especially for indoleacetic, p-hydroxyphenylpyruvic, α-ketoglutaric, glycolic, urocanic and nicotinic acids. These differences appear to be the result of typographical errors (indoleacetic, α-ketoglutaric) or differences in chromatographic conditions. Exclusive of these apparent errors, the mean difference in retention indices is 12 retention index units.

It should be mentioned that retention indices, while quite consistent, may vary somewhat depending upon GC column conditions. Thus, for example, we noticed a shift in almost all retention indices of -8 that occurred sometime during the summer of 1976. Although we cannot be certain, it appears that this change was associated with a change in the lot of OV-17 being used. Although both lots of OV-17 were from the same supplier, there may have been a minor shift in the chemical composition of the OV-17 or support material which resulted in a corresponding shift in retention indices.

Two changes were made in MSSMET to correct for small shifts in retention indices of the type observed. One was to include

Table 16. Comparison of literature and experimental retention indices.

Compound name	RI* lit	RI** exp	Difference
Benzoic	1363	1362	1
2-Methoxybenzoic	1654	1642	12
4-Methoxybenzoic	1702	1688	14
2-Hydroxybenzoic	1627	1621	6
3-Hydroxybenzoic	1690	1669	21
4-Hydroxybenzoic	1736	1724	12
3,4-Dimethoxybenzoic	1947	1934	13
3-Hydroxy-4-methoxybenzoic	1929	1921	8
3-Methoxy-4-hydroxybenzoic	1907	1900	7
3,4-Dihydroxybenzoic	1918	1904	14
2-Aminobenzoic	1747	1736	11
4-Aminobenzoic	1996	1975	21
2,5-Dihydroxybenzoic	1878	1862	16
2,4-Dihydroxybenzoic	1918	1905	13
2-Amino-3-hydroxybenzoic	1979	1967	12
Phenylacetic	1439	1428	11
3-Methoxyphenylacetic	1706	1699	7
4-Methoxyphenylacetic	1735	1718	17
2-Hydroxyphenylacetic	1695	1684	11
4-Hydroxyphenylacetic	1768	1756	12
3,4-Dimethoxyphenylacetic	1959	1945	14
3-Methoxy-4-hydroxyphenylacetic	1936	1923	13
3,4-Dihydroxyphenylacetic	1938	1929	9
2,5-Dihydroxyphenylacetic	1940	1929	11
Phenylpropionic	1561	1543	18
4-Methoxyphenylpropionic	1854	1846	8
Mandelic	1595	1583	13
2-Methoxymandelic	1800	1790	10
3-Methoxymandelic	1837	1816	21
4-Methoxymandelic	1873	1860	13
3-Methoxy-4-hydroxymandelic	2024	2004	20
3,4-Dihydroxymandelic	2015	2004	11
Phenyllactic	1693	1683	10
4-Hydroxyphenyllactic	2000	1983	17
Cinnamic	1713	1704	9
2-Methoxycinnamic	2014	2008	6

Table 16. (Cont'd.)

Compound name	RI^*_{lit}	RI^{**}_{exp}	Difference
3-Methoxycinnamic	1997	1980	17
4-Methoxycinnamic	2064	2045	19
2-Hydroxycinnamic	1963	1955	8
4-Hydroxycinnamic	2086	2072	14
3-Methoxy-4-hydroxycinnamic	2274	2268	6
3,4-Dihydroxycinnamic	2245	2238	7
Phenylpyruvic	1834	1818	16
4-Hydroxyphenylpyruvic	2158	2110	48
Lactic	1115	1101	14
Glycolic	1113	1128	-15
Malic	1554	1537	17
Tartaric	1719	1693	26
Citric	1902	1874	28
Oxalic	1254	1231	23
Malonic	1296	1281	15
Methylmalonic	1311	1285	26
Succinic	1417	1397	20
Fumaric	1409	1401	8
Glutaric	1496	1489	7
Adipic	1600	1594	6
Glyoxylic	1293	1287	6
α-Ketobutyric	1222	1216	6
α-Ketovaleric	1291	1276	15
α-Ketoglutaric	1942	1682	260
Uric	2259	2260	-1
Picolinic	1526	1526	0
Nicotinic	1432	1436	-4
Kynurenic	2318	2300	18
Urocanic	2235	2240	-5
Indoleacetic	2093	2189	104
Indole-3-propionic	2306	2279	27
Indole-3-lactic	2356	2344	12
Indole-3-pyruvic	2635	2624	11
5-Hydroxyindoleacetic	2440	2418	22
Indole-3-acrylic	2648	2634	14
Uracil	1450	1445	5
Hippuric	2108	2102	6

Table 16. (Cont'd.)

Compound name	RI^*_{lit}	RI^{**}_{exp}	Difference
m-Hydroxyhippuric	2378	2371	7
Glutamic	1689	1678	11
Orotic	1865	1852	13
Pipecolic	1407	1400	7
∝-Aminobutyric	1200	1192	8
∝-Aminoisobutyric	1160	1157	3

* Retention index according to Butts (W.C. Butts, Anal. Biochem., 46:187, 1972). Conditions: 6 ft by 0.25 in. o.d. column containing 3% OV-17 on 80/100 mesh Chromosorb W-HP, temperature programming 100 to 325° at 10°/min.

** Retention index as determined for MSSMET. Conditions: 10 ft 5% OV-17 on 80/100 mesh Supelcoport, programmed 60-290° at 4°/min.

an option in MSSMET to allow library retention indices to be shifted by a fixed amount as they are read into core memory. This approach requires operator intervention whenever retention indices shift, but successfully solved the observed problem. A second change was to build a new library which based retention indices upon the elution times of acidic compounds known to be present in every urine sample, rather than upon hydrocarbons. This latter approach proved to have additional benefits, as discussed below, and was used for all of the data reported here. A third approach, which was not tried here, but which might prove very useful, is that of Dromey et al. (76D3), in which a running average is kept of the most recent occurrences of important values (retention indices, in this case, but the concept could be expanded to include match coefficients, ion intensity ratios, and any other important information). This updated average could then be used for subsequent analyses, but would probably require occasional manual inspections to be certain that data from some new compound had not been substituted for data from a similar compound. Probably the best long-term solution to the problem of retention index variability, however, is to purchase a very large quantity of a GC packing material which has been tested and found to give satisfactory retention indices; in this manner, lot-to-lot variability, at least, can be avoided.

One other slight improvement might also be made in the

calculation of retention indices. Since most compounds elute within one scan of the nominal retention index (Figure 20), the scan duration (4 sec, or approximately 2 RI units in our system) tends to limit the resolution of retention indices that can be achieved. Dromey et al. (76D3) have used corrections for scan duration to estimate retention time to within 1/3 scan instead of the integer scan value used here. Blaisdell (77B2) has advocated using curve-fitting algorithms to obtain a better estimate of the position of peak maxima. However, the Dromey and Blaisdell approaches may be more useful with quadrupole systems than they are with magnetic sector instruments, where most of the ions of interest occur within a one-second portion of the 4-second scan. In any case, the rather wide limits within which retention indices vary in urine (Figure 28) would suggest that other factors than the scan duration may also be important when analyzing urine samples.

Match coefficient

Although retention indices are clearly the best indicators of compound identity, ion intensity ratios provide an excellent and largely independent test for compound identification. Thus, as shown in Figure 21, the match coefficient is relatively constant at any level of compound and, as shown in Figure 30, averages well over the

cut-off value for a positive match, even in urine samples.

One way to judge the efficiency of the match coefficient is to realize that the retention index can be used to eliminate all but about 1% of the scans in a typical urine sample analysis, whereas the match coefficient must be used to decide whether the compound of interest exists within that remaining region. Although a retention index and the presence of a single ion may be all that will be needed if this method is applied to chemical ionization (or other "soft" ionization methods) spectra, the electron impact spectra of the type used here must be matched to library spectra on the basis on more than one ion plus the retention index.

It should be pointed out, however, that the type of match coefficient used here suffers from a few shortcomings. The most serious of these is an over-sensitivity to intense ions. Thus, for example, a compound whose confirming ion set is (267,1000)(282,50) will have a match coefficient of 95 or higher whenever m/e 267 is present, even if m/e 282 is completely missing. Hence, as is often the case, small "noise" peaks at the mass whose intensity is highest in the library (267 in this case) will have a match coefficient near 100 (perfect score), even though no ion peak is found for the other mass at all.

This problem has been avoided in large part by making the weaker ion the designate ion, so that a peak of that ion must be

present within the search window before the match coefficient is even computed. Unfortunately, this decreases MSSMET's sensitivity for that compound by a considerable factor (20 in the above case). Attempts to find other library matching algorithms which are less sensitive to this kind of problem, but still adequate over the whole range of possible ion intensities, have proven unsuccessful thus far. Fortunately, this problem does not occur very often, and usually, when it does occur, the retention index does not match the library value anyway. Furthermore, only a few compounds occur for which it is necessary to have a library entry of this type; most compounds have several reasonably intense ions which can be used in the library, and hence the problem is not frequently encountered. Further research is needed, however.

K-factors

As noted earlier, the relative peak areas and heights calculated by MSSMET can be converted to actual concentrations of compounds if the appropriate conversion factors are known, since the dose-response curves are linear and go through the origin. Hence, it should be possible to obtain a series of conversion (k) factors, one for each compound of interest. The 200 or so individual purification and recovery studies necessary to obtain these k-factors,

however, are clearly outside the scope of this thesis project. Only a few such k-factors were obtained for compounds that were pure enough as obtained commercially to justify such a study, and none of these included corrections for recovery data.

However, the fact that k-factors have not yet been determined is by no means a serious disadvantage to the MSSMET approach. Statistical studies of the data will provide exactly the same results whether data are given in mg/ml or relative area/ml, as long as the same k-factor is used for all occurrences of the same compound. Only if the data are to be used in other laboratories, or in method-comparison studies, are k-factors important. Indeed, given the time and effort needed to determine k's, an argument can be made that k should only be determined for those few compounds determined to be of clinical significance; for any other compound, an approximate value of k can be determined from the proportion the designate ion is of the total ion intensity in a spectrum of the compound. This latter approach makes two assumptions: 100% recovery relative to the internal standard and a constant total ionization for a given amount of any compound. While both of these assumptions are clearly not completely accurate, they are adequately followed in many cases, as shown in part by Figure 34.

It seems likely that all k-factors, including those for compounds whose chemical identity is uncertain, will not be known

Figure 34. Determination of the quantitative correction factor (k) by two methods.

The quantitative correction factor (k) can be used to convert relative peak areas to absolute concentrations. Normally, k-factors are computed by measuring the response of the designate ion from a known amount of compound to the response of the designate ion from a known amount of internal standard. When the amount of substance present is not known, however, an estimate of the k-factor can be obtained by measuring the total ion intensity (TII), or sum of all of the ion intensities of a compound and using this value in place of the compound amount. The correction factor calculated based on the weights of pure compounds is shown in the left-hand column; that calculated from the TII is shown in the right-hand column.

Compound	Value of k	
	By weight	By TII
β-Hydroxy- β-methylglutaric	1.9	2.6
Vanilmandelic	0.41	0.20
Succinic	1.8	1.3
o-Hydroxybenzoic	0.31	0.34
∝-Glycerophosphoric	0.86	0.85
Citric	1.5	2.5
Hippuric	2.2	0.58
Indoleacetic	1.7	1.0
Ascorbic	9.0	10.0

Figure 34. Determination of the quantitative correction factor (k) by two methods.

to a high degree of accuracy for a period of several years, at least. Hence, the practice I have followed in developing statistical studies (q.v.) is to use a k-factor of 1.00 for all compounds throughout the analysis, and to apply the k-factor, where known, only when data are being displayed for publication or use by others. This allows me to keep a separate computer file of k-factors which can easily be updated without recalculating all previous results. In addition, every time this file is used, the computer printout contains the date it was last modified, so that there is never any question about which k-factor was used for a given calculation.

Ion intensity variability

One of the more serious problems of any library search procedure is the variability of ion ratios in spectra. This poses two difficulties: accuracy of quantitative analysis may vary, and the spectra matching algorithm must be relatively insensitive to such changes if the compound is to be found.

The fluctuations giving rise to these problems generally fall into two groups, random short term noise and long term drift. The ion current for most ions varies by about 10% from scan to scan as a result of the random noise and intensity fluctuations which are the inevitable concomitant of a sampling technique (repetitive scanning)

which takes a single value each four seconds (of the ion peak apex) rather than averaging values over an extended period of time (SIM, for example). Thus, short-term noise is important, particularly for low-intensity peaks, but does not seriously hamper collection of reasonably accurate data. Of considerably greater concern, however, are the long-term fluctuations in ion intensity ratios. The ratio of two ions has been observed to vary as much as 50% over a long period of time; usually this is associated with changes in ion source temperature or other variables under operator control. Unless compensated or corrected, these long-term variations can result in considerable inaccuracy of quantitation and great difficulty in obtaining high match coefficients.

There are several ways that long-term variability can be compensated. One is to keep a record of ion intensities for each compound found and constantly update the library values based on recent experience. A second is to run a control sample each day, and use values from it to correct for systematic fluctuations from previous values. A third is to assume that the deviations within a given run will be systematic, rather than random, and devise a "self-correction" procedure for these deviations. A fourth method is to use multiple designate ions, rather than one for each compound, to obtain better estimates of true peak areas. Each method, of course, has its disadvantages. The first requires

extensive bookkeeping by the computer and is of relatively little value for sudden, marked shifts in intensity ratios (I have observed at least one such sudden shift). The second method will only work for those compounds contained in the control sample. The third alternative, while attractive, is based on an as-yet-unproven assumption of systematic errors, and the fourth did not seem particularly promising when briefly tested on an early version of MSSMET.

None of these alternatives has been formally implemented in the current version of MSSMET, although the library has been manually updated by inspecting several urine runs to achieve optimum intensity ratios. With this new set of averaged ratios, very few situations were encountered for which the ion intensity ratios varied by more than 10 to 15% from the mean. Hence, the entire set of ratios from each run was accepted as containing only a small number of significant errors.

However, in the long run it will be necessary to implement a vigorous correction procedure, since data will be collected over a much longer time, by several individuals, and perhaps even on several instruments. The procedure I would recommend is a combination of several of the above methods. Specifically, I would suggest running a daily control sample, which should be evaluated by MSSMET against previous control runs before proceeding. If a certain number of compounds fall outside of acceptable limits

(e.g., more than 20% of mean), then the operator should be warned to inspect the instrument and correct the source of variation if possible. However, if correction of the deviant behavior is not possible, then the control sample could serve to provide corrective data for the MSSMET library.

Comparison of MSSMET to SIM

The major technique used by mass spectrometrists for quantitative analysis is selected ion monitoring (also referred to as mass fragmentography by some practitioners). Hence, if MSSMET is to be considered a viable technique, it must yield comparable results to those of SIM on the same samples. As shown in Tables 6 and 7, and by comparison of Figures 18 and 35, MSSMET does indeed yield quantitatively comparable results with known standards. Unquestionably, MSSMET does have some disadvantages: it is not as precise and sensitivity is at least one order of magnitude (probably two orders of magnitude) better using SIM with isotope dilution techniques than with MSSMET. The real advantage of MSSMET, however, lies in its use for the analysis of a wide variety of sample components; whereas SIM is typically limited to one or at best 6 or 8 compounds per sample, MSSMET routinely quantitates over a hundred separate components in a single sample. MSSMET can be

Figure 35. Quantitative working curve measured by selected ion monitoring.

Isotope dilution and selected ion monitoring techniques are often used for quantitative analysis by GC-MS. An example of this type of analysis is the measurement of various amounts of 3,4-dihydroxyphenylacetic acid present in mixtures that contain a fixed amount (1 μg injected) of the pentadeutero-3,4-dihydroxyphenylacetic acid. The data are displayed for the entire range of sample concentrations tested. Both compounds were analyzed as the trimethylsilyl derivatives on a 10 ft column containing 5% OV-17. Four ions were measured simultaneously for the compounds: m/e 384, 385, 389 and 390.

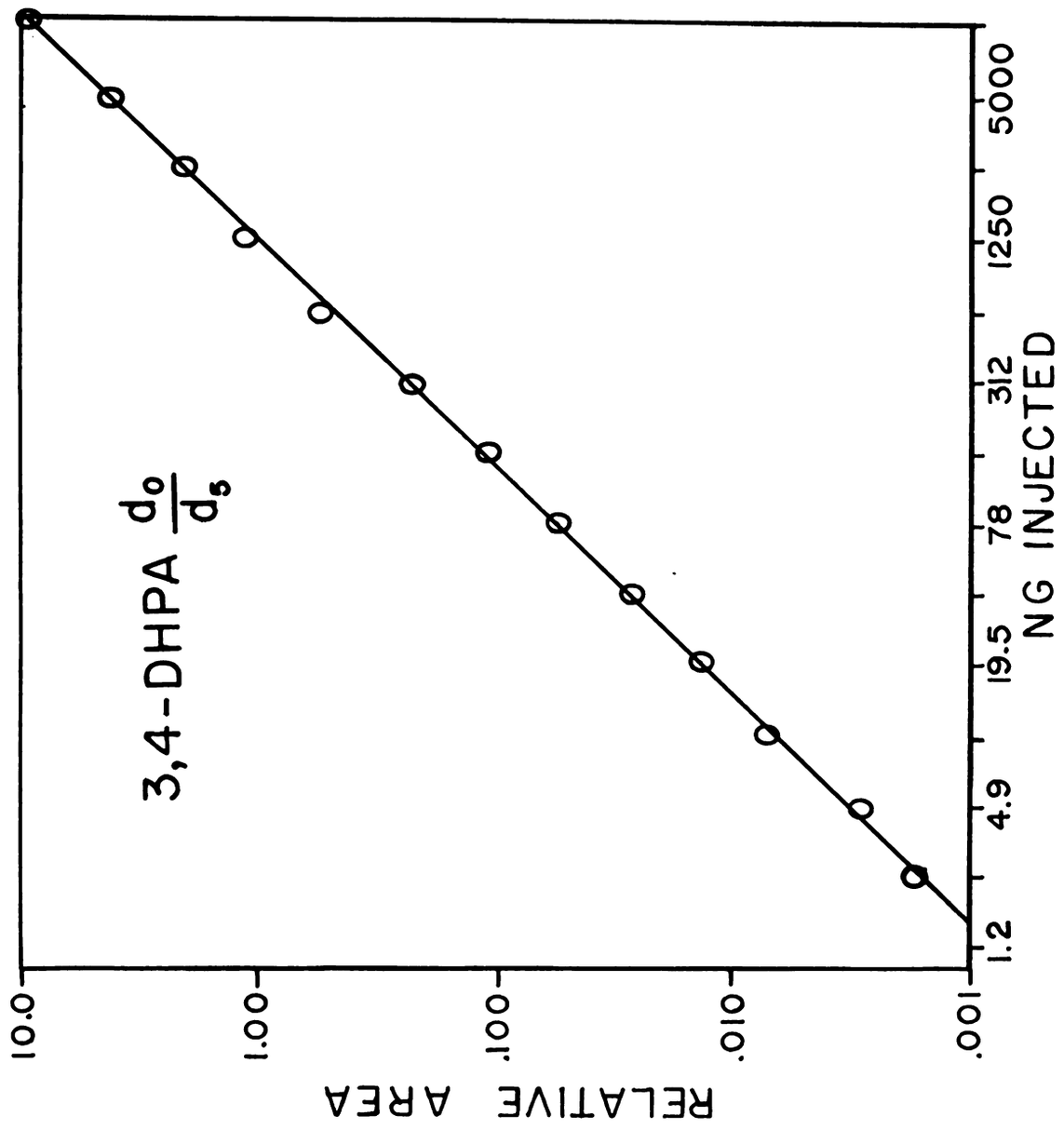
3,4-DHPA $\frac{d_0}{d_5}$
RELATIVE AREA
10.0
1.00
.100
.010
.001
1.2
4.9
19.5
78
312
1250
5000
NG INJECTED

FIGURE 35

used to quantitate compounds whose identity (chemical name) is unknown, and, because it is not a real-time analysis system, can even be used to quantitate compounds discovered months after the actual GC-MS data collection is complete. By comparison, SIM can only be used to quantitate compounds for which differentiating ions are known in advance, and for full sensitivity to be achieved, labeled isotopes of the compound must be available.

Thus, SIM and MSSMET can be seen as complementary techniques. SIM is appropriate for rapid, high-precision, high-sensitivity studies of a very small number of well-characterized compounds. MSSMET and similar techniques are most suitable for the analysis of complex mixtures when the investigator is less certain of what he is looking for, or when he is interested in a large number of components.

Ease of operation of MSSMET

One of the proposed features of MSSMET was that it should be highly automated, and hence easy to operate; therefore, it is important to ask how well this goal has been achieved. Based on my own experience, as well as that of 3 other individuals in the same laboratory who have used the program, MSSMET is as easy to collect data for and use as SIM, but, like SIM, requires some care

in the planning stages. The primary difficulty in using MSSMET is the establishment of a working library containing all of the compounds of interest plus retention time standards and internal (quantitative) standards. I approached this by running pure standards, 6 to 12 at a time, over a period of close to two years. In retrospect, I would have been much better advised to spend a smaller amount of time looking at urine samples to obtain spectra of compounds actually present in the samples, rather than finding spectra of a much larger number of compounds, relatively few of which occur routinely in urine.

The other major impediment to the routine use of MSSMET is the method used to locate retention index standards. This portion of the program is fully automatic only if retention times of the standards are relatively close to those expected. Major deviations--caused by changes in carrier gas flow rate or variations in the actual starting temperature--may necessitate some operator intervention to achieve proper location of the retention time standards. There are two ways to make this process more automatic. One is to standardize the operating conditions of the GC-MS so that carrier gas flow rates can be measured directly and by using a digital temperature gauge in the GC oven. The other is to use a different algorithm for the location of the standards. In any case, this difficulty can be easily overcome by an operator who has a minimum of training with MSSMET.

Studies on urine samples

Once MSSMET had been successfully tested on pure compounds and standard urine samples, a series of urine samples was collected and prepared to fully test the ability of MSSMET and to check for preliminary indications, at least, that MSSMET would produce clinically useful results.

Selection of subjects. The first step was to collect the urine samples. The major effort in this regard was the collection of some 200 "BCIU" urines from a group of reasonably healthy adult subjects of both sexes. In no way was the group of urines collected from the BCIU group intended to be "normal." As shown in Appendix D, the data obtained from the questionnaires of several subjects in this group indicate that this group is not a random sample of the U.S. population by age or sex or health status (or probably any other variable). Such a sample is not even available. Clearly, one does not go from house to house and collect samples of urine from randomly selected individuals across the country. In fact, it is simply impossible from a practical standpoint to obtain a completely random sample of urine. However, what was chosen as a viable alternative was to select a group of individuals who had been carefully examined by physicians within the last 6 months, who were willing to fill out a rather lengthy questionnaire, and who were distributed at least

somewhat by age and sex (an earlier collection of urine from personnel within the Department of Biochemistry met almost none of these criteria).

In addition, as a test of the ability of MSSMET to detect patients with a known abnormality, urines were collected from a group of patients undergoing therapy for neuroblastoma, a disease in which those affected, typically children under the age of three years, form a large tumor which is frequently observed to secrete large quantities of one or more metabolites of tyrosine. High levels of vanilmandelic and homovanillic acids, in particular, as well as several non-acidic metabolites, have been detected in conjunction with this disease (64W2). In addition, several urines were collected from an age-matched group of hospitalized patients to serve as control samples for the neuroblastoma patients.

Selection of MSSMET library for urine samples. One of the most time-consuming aspects of this project has been the compilation of a suitable library of mass spectra of organic acids. As noted previously, this library was originally based solely on individual spectra of pure compounds, but was later expanded to include spectra from the urine samples themselves. This expansion has proven to be of critical importance for two reasons: first, because almost half of the compounds routinely found in urine samples have been ones for which we currently have no commercial source of reference

compounds; and second, because it provided a valuable clue that led to a considerable improvement in the library. This clue was that match coefficients for library entries of spectra taken from urine samples were much higher than match coefficients for library entries taken from pure compounds. This suggested that the ion ratios taken from spectra of pure compounds were inappropriate for urine samples, a suggestion that was confirmed by inspection of ion ratios for all compounds in the urine sample using a MSSMET "debug" option. Hence, all ion ratios in the library were updated based on the urine spectra, and a new library created. Success with this new library (BESTLIB) was far superior to that with the earlier libraries, and it has therefore been used in all urine analyses reported here.

It should not be inferred, however, that spectra differ depending on the chemical environment in which they are taken; while this may be true to a very limited extent, it is much more likely that a shift in ion source conditions caused the shift in ion ratios, and that this shift occurred coincidentally at approximately the time the current set of urine samples was run on the GC-MS. Regardless of the source of the shift in ratios, it is obviously important to continually check library entries against spectra of the urinary metabolites to maintain a set of valid ion ratios, as has already been noted.

Selection of designate and confirming ions from urine samples.

The problem of selection of designate and confirming ions for the library to be used with the urine samples deserves further comment. McLafferty (74M1) has properly pointed out that some ions are more useful than others in differentiating a particular compound from others in the same spectra library. He has developed criteria for the selection of differentiating ions based on comparison of the thousands of spectra in a general-purpose spectral library (75P1). However, little attention has been given to the fact that ions differentiating of a given compound in one chemical environment are not necessarily differentiating in another. This is most clearly illustrated by Figures 36 and 37, where ion intensities at various regions in a GC-MS analysis of the organic acids in human urine are plotted. Thus, for example, in Figure 36, where the sum of all scans is plotted, any of the ions at m/e 205, 217, 220, 292, 333, 441 or 456 would appear to be poor choices for designate or confirming ions. Yet, as illustrated in Figure 37, any of these ions would be excellent choices in the first 100 scans, where they appear at low levels, if at all. In general, there is an increase in the number and intensity of occurrences of higher mass ions at high scan numbers, but this effect is by no means uniform enough to be relied upon. The distribution of given ions in fact varies markedly over much shorter regions than those plotted; ion composition may change over even

Figure 36. Distribution of ions in a typical urine sample GC-MS analysis.

In order to measure the likelihood that a given fragment ion would occur during the GC-MS analysis of the organic acid fraction of a human urine sample, the intensity of each ion was summed for all scans in a GC-MS run. These summed intensities can be used to aid in selecting appropriate designate and confirming ions for MSSMET.

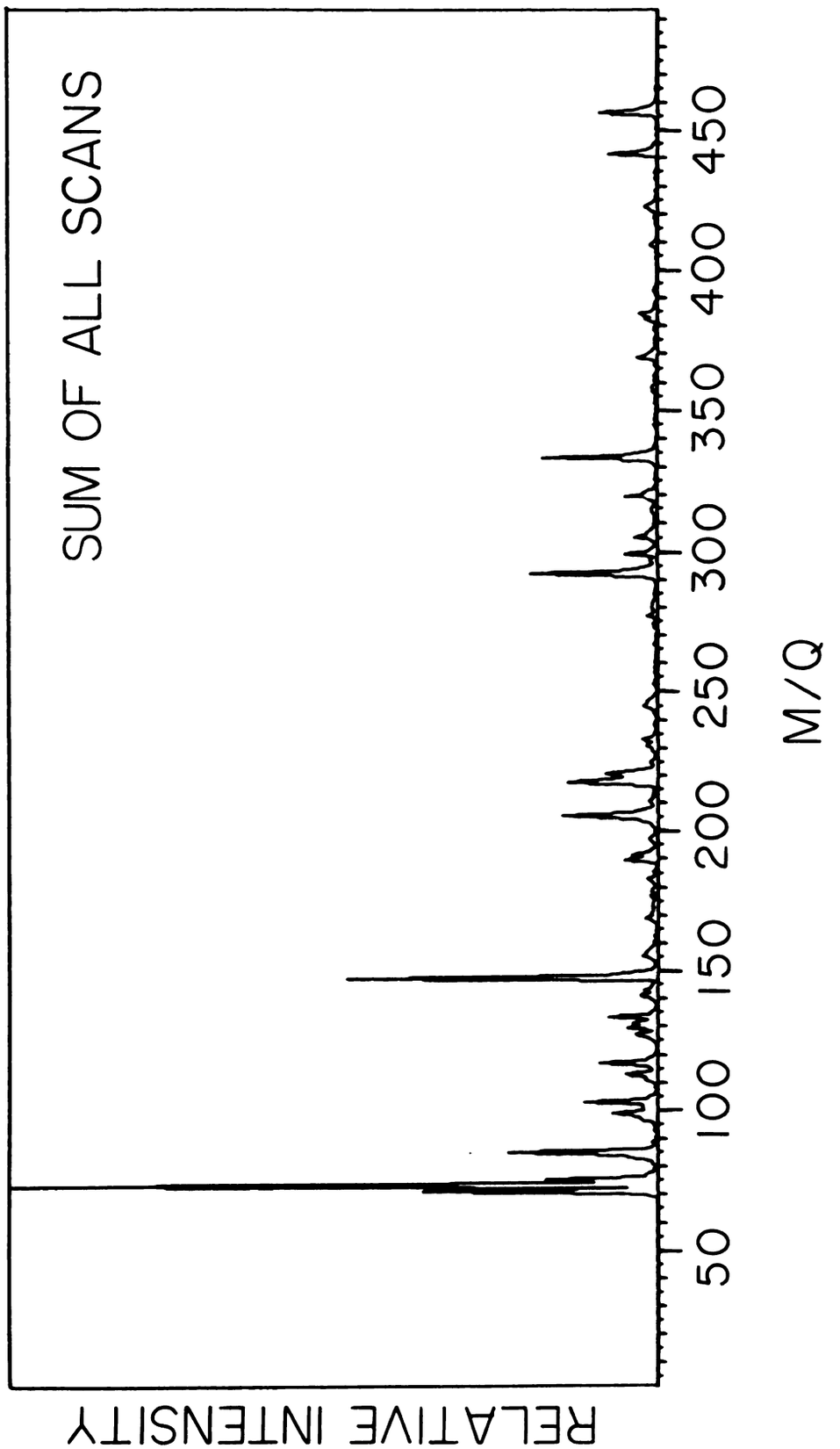
SUM OF ALL SCANS
RELATIVE INTENSITY
50
100
150
200
250
300
350
400
450
M/Q

FIGURE 36

Figure 37. Dependence of ion distribution upon the region of the GC-MS analysis.

Plots similar to that shown in Figure 36 are illustrated for limited regions of the GC-MS analysis of the trimethylsilyl derivatives of the organic acids in a human urine sample. Ion intensities have been summed for selected scans within the scan range marked on each plot; scans including spectra of hydrocarbons have been specifically excluded. Each plot therefore represents the relative proportion of each mass ion found within the scan range indicated on the plot, and the likelihood that a given ion from a reference spectrum will be differentiating for that compound in the region shown can be judged by the ratio of ion intensity in the reference spectrum to that in the plots shown.

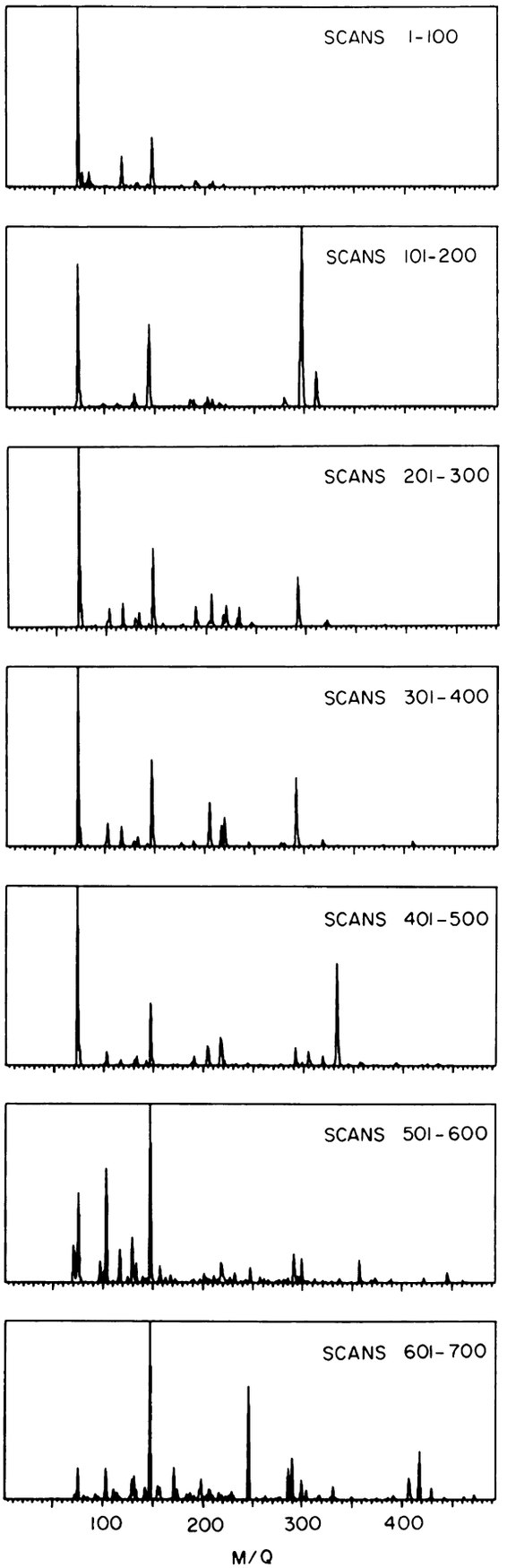
SCANS 1-100
SCANS 101-200
SCANS 201-300
SCANS 301-400
SCANS 401-500
SCANS 501-600
SCANS 601-700
100
200
300
400
M/Q

FIGURE 37

two or three scans by as much as 80 to 90%, with the exception of the ubiquitous m/e 73 and 147 ion clusters characteristic of virtually all trimethylsilyl derivatives. Hence, the computer programs (MSSDSG and MSSCHS) designed to distinguish differentiating ions had to compare each library spectrum to precisely that region of the GC-MS run where the compound was expected to elute; retention indices were used to select the appropriate region.

Although computerized selection of designate and confirming ions is a definite improvement over manual selection, the library constructed by MSSCHS (the library was called FINALLIB, somewhat prematurely) was mildly disappointing, in that many of the choices had to be modified when they subsequently proved to be less than completely differentiating. There may be several contributing factors to this relatively poor performance. Probably the most important factor is the choice of spectra of library compounds. As noted earlier, these spectra were all recorded on pure samples prior to a significant change in ion ratios; hence, the choice of differentiating ions was based on a less than an ideal set of starting spectra. A second factor is the choice of retention indices. At the time of construction of FINALLIB, many library retention indices were based on single determinations from pure samples; subsequently, much more reliable values for most of them have been obtained. Thirdly, the program was not designed to deal with

compounds which were major urinary components, since the ratio of library spectrum to averaged urinary spectra had to be greater than 5.0 for the selection process to succeed. A final factor, recently suggested by Blaisdell (77B2), may be that differentiating ions should be selected on the basis of comparison to a "worst case," rather than an average set of urinary spectra.

Fortunately, most of the shortcomings of FINALLIB have been detected and eliminated in BESTLIB, which was used for studies here, but it would be very interesting to build a new file of complete spectra, add the new set of retention indices, and then test them against urine samples with MSSDSG and MSSCHS once more.

GC-MS analysis of urine samples. It should be noted at the outset that actually running urine samples on the mass spectrometer has proven to be a difficult task, at best. I would conservatively estimate that about 80% of the data I collected on urine samples in the past two years has been discarded or lost for one or more of the following reasons: misassigned m/e values; data lost during transfer to magnetic tape; mass spectrometer malfunction; inadequate GC-MS sensitivity; poor quality GC analysis; insufficiently concentrated sample; or operator error during chemical preparation of the sample or during the GC-MS analysis itself (these are

listed in approximate order, from most important to least important). Hence, only a small number of samples are reported here, although the number actually run was much larger. The frequency of such problems decreased markedly as I gained more experience and learned how to prevent them from occurring.

Good quality runs on the GC-MS, however, gave data that proved to be extremely interesting. As shown in Figures 38, 39 and 40, the organic acids fractions from the three groups of subjects are highly complex, but seem to show many of the same peaks. Further analysis by MSSMET, however, reveals a much larger number of compounds to be present than the 60-80 visible on a typical GC trace.

MSSMET analysis of urine samples. MSSMET was used to check the first 15 urine samples, using FINALLIB, the complete library containing over 300 entries. However, as noted earlier, many of the entries in this library were found to contain inaccurate ion ratios or retention indices, since they were based on spectra taken before a change in the ion source conditions. In addition, many of the compounds were not found in any of the urines examined; many of these were compounds that would never be expected to occur in the acid fraction of urine (e.g., aminobutyric, methyl esters of acids, phytol, etc.). Hence, the entire library was corrected to reflect current conditions (forming PUBLIB), and then all compounds that did not occur in the urine samples were removed leaving BESTLIB.

Figure 38. GC analysis of typical BCIU urine sample.

Analysis on the LKB-9000 of organic acids from a typical BCIU urine (subject 120). The sample was isolated by DEAE-Sephadex anion exchange procedure, and then analyzed as the trimethylsilyl derivatives on a 10 ft 5% OV-17 column programmed from 60° to 260° at 4°C/min. Portions of the sample are not plotted so that Figures 38, 39 and 40 can be shown to the same scale.

Figure 39. GC analysis of typical juvenile control urine sample.

Organic acids from the urine of subject SW (who has retinoblastoma). Conditions of analysis are the same as those described for Figure 38.

Figure 40. GC analysis of typical neuroblastoma urine sample.

Organic acids from the urine of subject JJ were analyzed on the LKB-9000. Conditions are the same as those described for Figure 38.

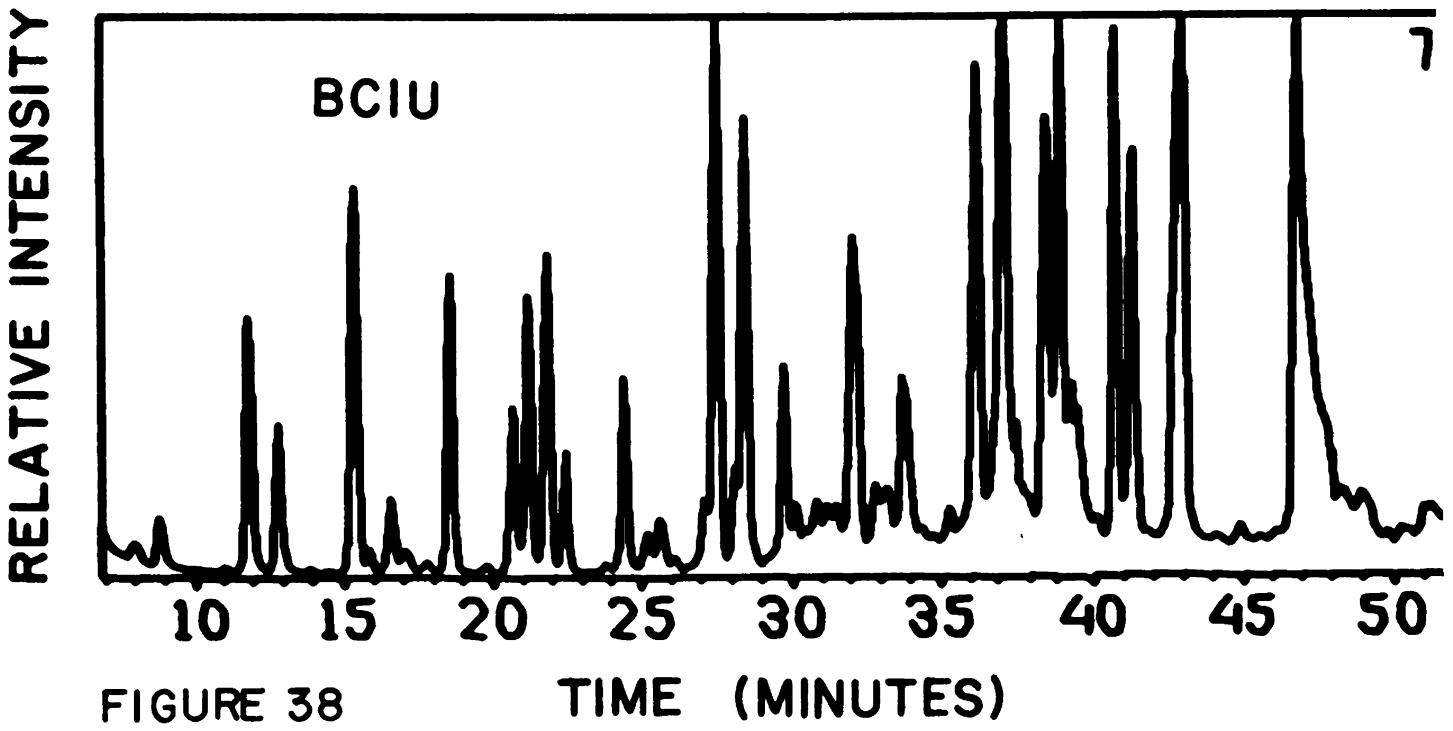

FIGURE 38

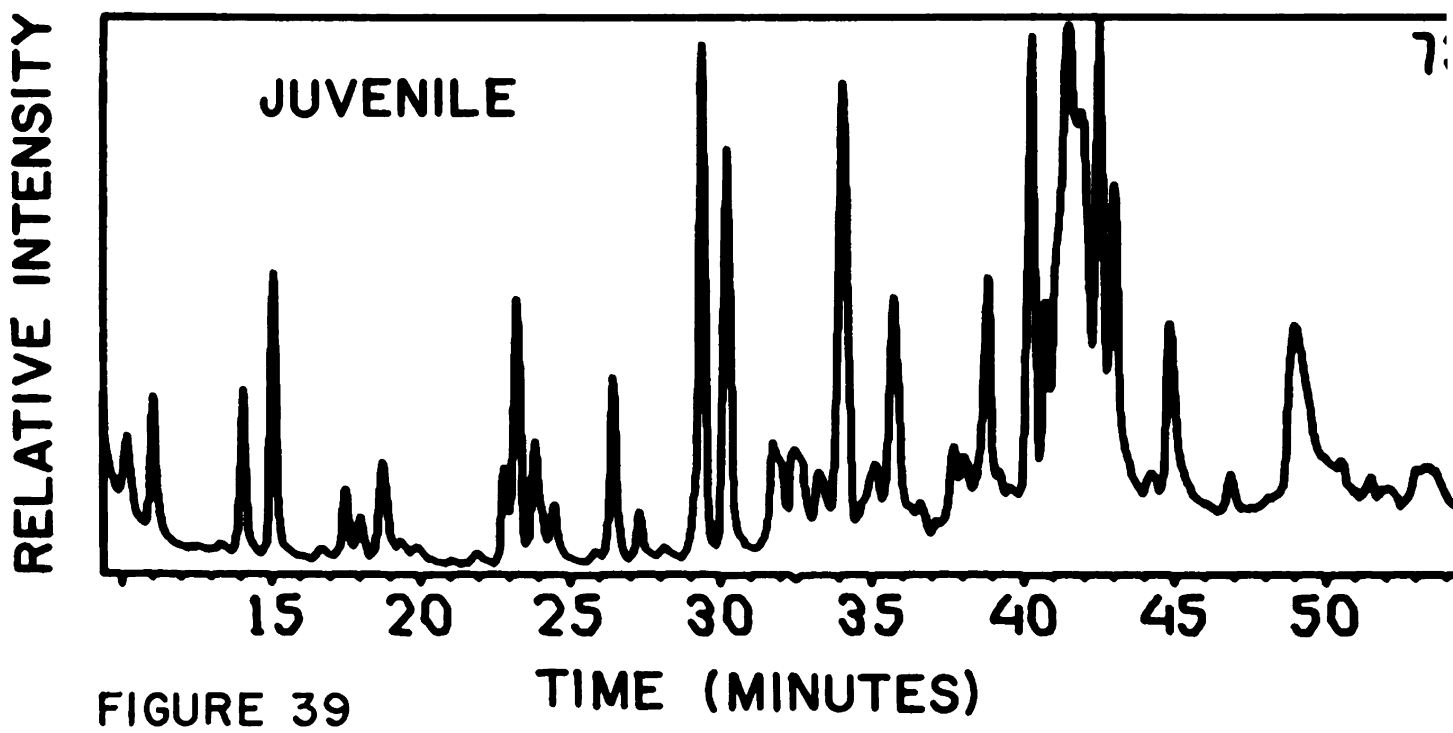

FIGURE 39

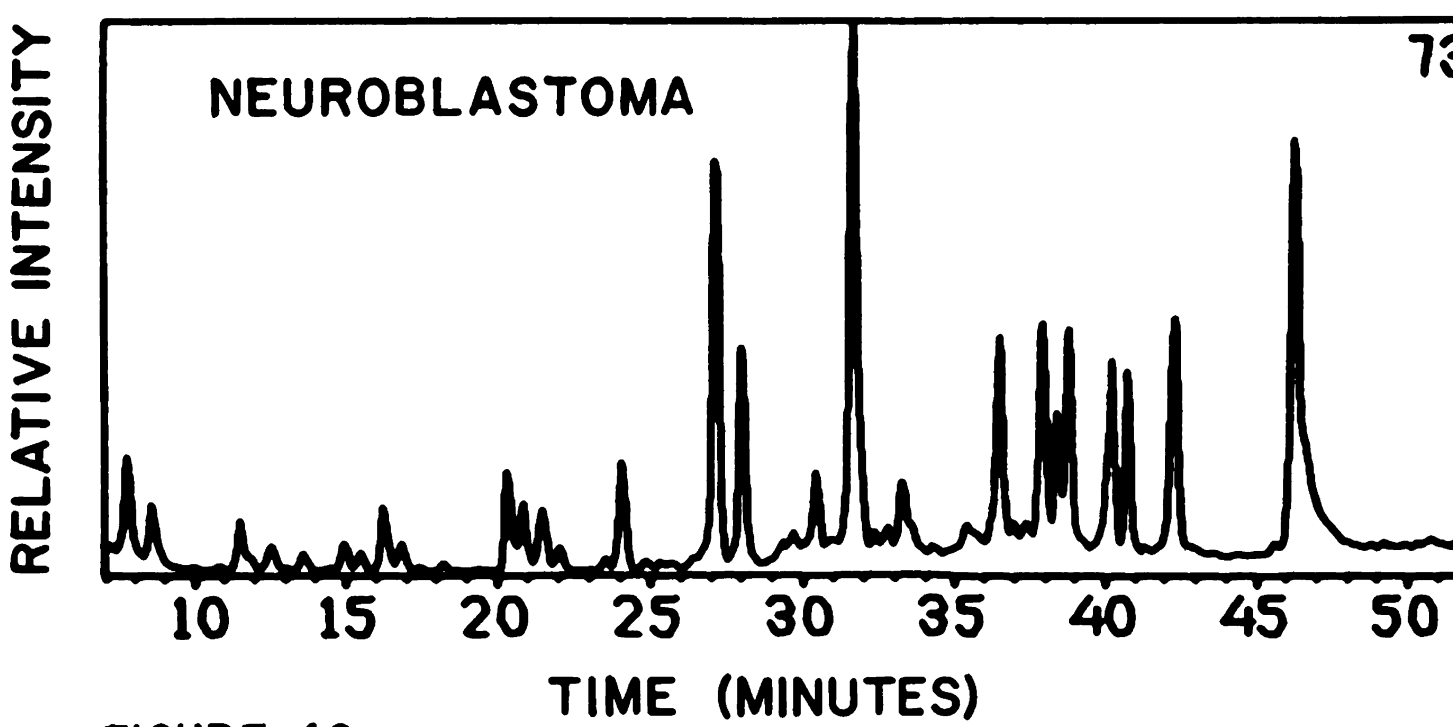

FIGURE 40

All samples reported here were analyzed with the last library (BESTLIB); however, as more types of urine samples are analyzed and more of the pure compounds are reanalyzed under present ion source conditions, it may be advisable to add some substances in PUBLIB back to BESTLIB. For the urines analyzed here, however, there is no evidence that any compounds present in the urines have been ignored because a library entry present in PUBLIB was not transferred to BESTLIB.

A number of compounds are missed by MSSMET using BESTLIB, but these are compounds which are not present, as yet, in any of our libraries. As will be discussed later, these "missing" substances will eventually have to be added to the library using programs or procedures specifically designed for this purpose. It should be recognized by anyone using these libraries that BESTLIB is exactly what its name implies: it is currently our best library for metabolic profiling of urinary organic acids. Because each of the compounds has been found in a large number of urine samples, the retention indices and ion ratios are average values that have been found to be reliable. Other compounds, which do not occur in urine samples measured so far, have most often been measured only once, and thus cannot be considered as precise or reliable. Hence, the user of PUBLIB or the other libraries would be well advised to utilize wider retention index and match coefficient

acceptance limits than normally used with BESTLIB.

This question of reliability is well illustrated by the problems encountered using PUBLIB. Of the 157 compounds in BESTLIB, 35 were discarded from futher statistical consideration because their retention indices or ion ratios were not considered to be sufficiently accurate or because they appeared in only a couple of urines. Many of the 35 compounds were newly added to the library and had never been tested; these could easily be corrected with careful examination of values from three or four more urines run with the debug option set to display the full set of ion ratios. Others of the 35 compounds had been left in the library only because they were known to be of importance in special diseases (e.g., α-ketoadipic and α-aminoadipic acids), and hence it was not surprising that they were not found in any urines.

Of the remaining 122 compounds, an average of approximately 100 were found in each urine sample. As work on the expansion of the library with newly-detected compounds continues, this number will probably rise so that at least 145 to 150 compounds are found in each sample at sufficiently high levels to quantitate reliably. An additional 30 or 40 compounds will probably be identifiable, but will usually be present at such low levels that accurate quantitation by repetitive scanning methods will be impossible. There is no way of knowing how many more compounds

are present at even lower levels.

In fact, although MSSMET analyses could in theory be performed intermixed with the data collection process on the GC-MS (i.e., during real time), one of the advantages of MSSMET has turned out to be that the data can be reanalyzed as often as needed, rather than being limited to a single real-time analysis. Thus, as the library is improved or new compounds added, old samples can be rerun with the new library or the same library can be used with a new set of values for the operating parameters of MSSMET. Indeed, it is important to recognize that MSSMET is still evolving, and that the GC-MS data that has been obtained with such difficulty should not be discarded until fully exploited (which will probably not be for some years).

Number of substances found. As shown in Table 17, the number of substances found in each urine varied markedly. While there is a 0.50 correlation coefficient between the number of peaks found and the intensity of the internal standard (a measure of overall GC-MS sensitivity, since approximately the same amount of internal standard was injected in each case), other factors, such as urine concentration before extraction of acidic components, may also be important. Recent evidence suggest that a partially blocked molecular separator in the GC-MS may account for some of the decreased sensitivity, but changes in the ion source itself must also

Table 17. Number of compounds found in urines by MSSMET.

Run number*	Area of internal standard (in thousands)	Number of peaks found out of 157**	Number of peaks found out of 122***
072502	290	99	90
072506	263	93	83
072601	202	114	100
072802	194	119	105
072902	96	115	103
091802	473	143	111
091901	274	131	112
091902	528	139	117
102903	276	130	112
103101	103	127	109
103102	154	124	107
103103	119	118	108
111001	142	102	91
031603	111	123	105
031604	110	112	98
032302	91	115	101
033004	29	101	89

* Run number consists of month (first 2 digits), day (third and fourth digits) and sequence number. All were run during 1976, except the last 4, which were run in 1977.

** Using BESTLIB.

*** Using BESTLIB after 35 least reliable compounds were removed.

be of some importance. Despite the decreased sensitivity in some cases, however, most substances were still found at concentrations high enough for reliable quantitation.

Statistical analysis of urine data

Originally, three types of output from the MSSMET analysis of urine samples were saved: the "found" file, the "run" file and the "not found" file. The run file contains the complete set of information on all peaks detected by MSSMET; the found file is a subset of the run file consisting of complete information on all peaks positively identified as matching a particular library entry; and the not-found file is the subset of all compounds not located by MSSMET, but it contains only substance names. The not-found file was eliminated from MSSMET when it proved to be of limited utility, in order to decrease the size of the program. The run file has generally only been used when testing the library and must be deleted after each sample is run.

The found file is therefore the only MSSMET output that has been consistently useful. An example of a typical MSSMET found file is illustrated in Appendix I. Visual inspection of a single such output does not provide much new information beyond that already discovered in studies of pure compounds and reference

urine samples; hence, when a number of such outputs were available, the entire set was carefully examined using univariate and to a lesser extent, bivariate statistical methods.

Distribution of concentrations. One of the basic questions to be answered before beginning advanced statistical studies is whether the data are distributed "normally" or whether the distribution is skewed or otherwise non-normal. In the latter case, non-parametric statistical methods must be used; in the former, parametric statistical approaches are sufficient. Since the group of reference (BCIU) adult urines analyzed numbered only 9, it is difficult to be certain whether any one compound is distributed normally within this group. However, when normalized concentrations of compounds which are present in all of the BCIU urines are plotted (Figure 32), it is clear that, as a group, they are not normally distributed (i.e., the distribution is non-Gaussian). When the logarithm of the normalized concentrations are plotted (Figure 33), the distribution is much more Gaussian in appearance, suggesting that most of these compounds follow a log-normal, rather than a normal, distribution. This is not at all unexpected; the same distribution has been observed for many other substances in biological samples (e.g., trace metals in human hair (76M2)). It should be noted, however that individual compounds may follow other distributions (for example, be bimodal), but without more data, this is

impossible to judge with any certainty. Therefore, as a first approximation, at least, the concentration data reported here may be assumed to be distributed log normally. Hence, the data are tested using an assumed log normal distribution and, where possible, by non-parametric methods as well.

Distribution of retention times. As shown in Figure 41, the retention times of a given compound vary widely from urine sample to urine sample, but the general shape of the retention index versus retention time curve seems to remain approximately constant. Since the data illustrated are from samples run over a one-year period under a variety of conditions, some of this variability is expected. However, the two major sources of variability in the retention time are probably GC carrier gas flow rates and temperature programming conditions. There is currently no convenient way to measure gas flow rates on the LKB-9000; I have attempted measuring the effluent from the forevaccuum pump with a flow meter but found this method to be very imprecise. The flow rate is a complex function of gas pressure, the tightness of column packing, any leaks in the gas connections or septum, and the pressure at the detector end of the column. The flow gauge on the LKB is uncalibrated and unreliable. I have typically used the method of gauging the flow rate by the vacuum pressure at the first stage of the separator, but this method is clearly not very satisfactory.

Figure 41. Variability of retention times of retention index standards.

Retention times were found by MSSMET for each of the retention standards in 5 samples of urinary organic acids analyzed on the LKB-9000 over a one-year period. Other urine samples analyzed during the same period were similar to those shown. The retention indices of the standards were defined to be the mean retention indices previously calculated from 17 urine samples using hydrocarbons as retention index standards.

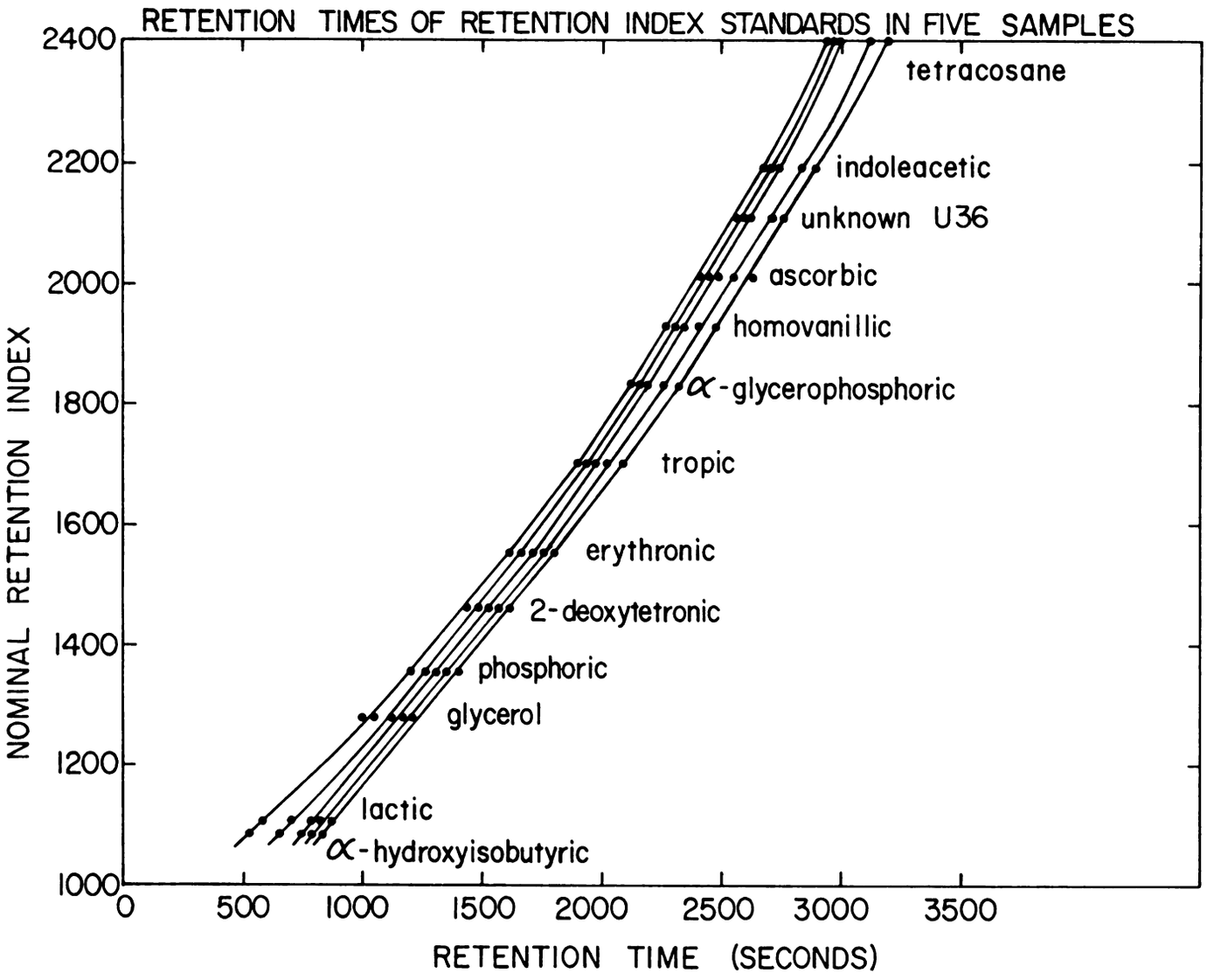
RETENTION TIMES OF RETENTION INDEX STANDARDS IN FIVE SAMPLES
NOMINAL RETENTION INDEX
2400
2200
2000
1800
1600
1400
1200
1000
0
500
1000
1500
2000
2500
3000
3500
RETENTION TIME (SECONDS)
tetracosane
indoleacetic
unknown U36
ascorbic
homovanillic
α-glycerophosphoric
tropic
erythronic
2-deoxytetronic
phosphoric
glycerol
lactic
α-hydroxyisobutyric

FIGURE 41

Likewise, the temperature programmer on the GC of the LKB-9000 is rather imprecise, and neither the precision nor accuracy of the programmer have, to my knowledge, ever been measured. A computer interface to the existing thermocouple in the GC oven was designed and built, so that the computer would be able to record actual temperatures, but the noise levels and short-term drift of the thermocouple were too high to achieve useful results. As a consequence of this problem, I have ignored the temperature gauge completely and started the programmer 10° below the temperature at which the sample is injected, so that at least the temperature programming has been done consistently.

A great deal of time could be spent improving the reliability of these measurements. Such efforts would undoubtedly result in much more reproducible retention times, especially if both parameters are computer controlled. However, I am not at all certain that the time and money spent would be reflected in a significant improvement in the precision of the retention indices, which are much more precise than retention times, as discussed below. The only justification for these improvements would be that the location of retention index standards would be much easier; this is very important because they represent the least automated aspect of the MSSMET program. However, it may be possible to devise an algorithm to predict the retention times of the standards

based on the curves of Figure 41; success of such an algorithm would obviate the necessity for improving the precision of the temperature and flow rate controllers.

Distribution of retention indices. A comparison of Figures 20, 27, and 28 leads to several conclusions. The first is that even for the urine samples, approximately three-fourths of all retention indices fall within 2 retention index units (approximately 1 scan) of the mean value, and that over 90% fall within 4 retention index units (2 scans) of the mean, suggesting that the precision of retention index determination is excellent. Secondly, about 3% of the retention index values are more than 6 retention index units from the mean in the urine samples, while none is that far from the mean in the samples of pure compounds. This is probably the result of several factors: greater retention index variability of some compounds (notably hippuric and uric acids) at the high concentrations encountered in urine samples; collection of data over a considerably longer period of time (1 week for the pure compounds, 1 year for the urine samples); and errors by MSSMET in identifying compounds in the urine samples, where identification is often less certain. Careful inspection of the original GC-MS data reveals that there are some instances of each of these problems.

A comparison between using urinary metabolites versus

hydrocarbons as retention index standards (Figures 27 and 28) suggests that the former method is more precise by about 0.4 retention index units. However, what is not apparent from the data is that the metabolites are more difficult to use than hydrocarbons, since isomeric compounds are often present which may be misidentified as the standard. It should be noted, however, that even the location of hydrocarbon standards occasionally presents some difficulties, particularly to the novice MSSMET user. Hence, I would suggest that the use of hydrocarbons as standards be abandoned with implementation of a more automated search procedure that uses endogenous metabolites as standards.

Furthermore, the current library options are set so that a compound is considered to be a positive match to the library entry if it falls within 12 retention index units of the library value. I would recommend that, when sufficient values have been recorded, the allowable limits be reduced to 8 or 9 retention index units. However, I would recommend that the limits for hippuric, uric and similar acids be widened to plus or minus 20 retention index units from the mean and that new additions to the library be allowed to vary plus or minus 15 retention index units until a reliable mean has been established.

This does not mean, however, that the "window" width should be decreased. (Recall that the window is not the same as the

allowable limits of retention index variation). Currently the window typically used is 120 seconds (30 scans). Peak widths average about 15 scans, but range from 4 to 28 scans within the window (Figure 31). Allowing a small margin for error in the library retention index, it is apparent that the window width is adequate for most peaks, but might even need to be increased for a few very large peaks. In addition, as noted earlier, wide windows, while more time-consuming for MSSMET to use, generally lead to more accurate baseline determination.

Interitem correlations. Correlations between several different combinations of variables were examined, as shown in Table 10. As expected, a very high correlation of 0.94 exists between relative peak area and relative peak height, confirming that relative peak area and relative peak height are almost completely equivalent measures of peak amount. It is interesting to note that within each urine, the correlation coefficient ranged from 0.935 to 0.998, averaging about 0.98; this suggests that within-urine correlation of peak height and area may be better than the correlation of peak heights and areas among various urine samples.

Similarly, a fairly high (0.78) correlation between the peak area match coefficient and the peak height match coefficient suggests a reasonably good agreement between these two measures. However, this value is probably somewhat lower than what might be expected because of the elimination of all compounds with match

coefficients below 80. Thus virtually all values of the match coefficient included in the calculation of the correlation coefficient are between 80 and 100. Inclusion of match coefficients of compounds considered "not found" by MSSMET would probably significantly improve this correlation.

All of the remaining correlation coefficients in Table 10 are quite low. It is interesting to note the slight trend toward decreasing match coefficients with increasing retention index, as well as the tendency toward higher match coefficients with increasing peak area. These correlation coefficients, although fairly small, are probably most influenced by extreme cases: peaks measured near the limit of detection for the system do appear to have smaller match coefficients, while moderately small to very large peaks seem to have approximately the same match coefficients. Likewise, match coefficients are relatively high for most peaks with the exception of a few peaks at retention indices above 2100.

Correlations with the difference between library and observed retention indices (the value reported by MSSMET) are difficult to interpret because the difference is a signed value. Hence, two sets of correlations are reported: correlation to the retention index difference for all occurrences in the found file and correlation to the retention index difference only in those cases where the retention index difference is non-negative. In either case, the correlation

with other variables is extremely small, suggesting that retention index and match coefficient are independent measures of peak identity.

The absolute difference between the two types of match coefficients is small. As shown in Table 18, the mean difference between the two is about 1 (out of 100), with the height match coefficient averaging slightly higher. However, the standard deviation of the difference between the two is somewhat larger (3 to 4), suggesting that there is a considerable variation in the match coefficients. Inter-compound match coefficient variability is also evident, as illustrated in Figure 30; although the median match coefficient in the urine samples is 94, only 91% of the values are above 85. A few values even occur below 81, the cutoff point for a positive match, because one of the two types of match coefficients is above this value and the other below (only one need be above the cutoff to be a positive match). In general, this suggests the need for further refinement of library ion ratios. Such refinement will probably also be accompanied by an increase in the correlation between the two types of match coefficients as their overall reliability improves.

Outlier test. The outlier test provides a means of checking for unusually low or unusually high values of the relative peak amount. It identifies values that have only a small chance (less

Table 18. Agreement between peak area match coefficient and peak height match coefficient.

Urine run number	072505	102902
Mean difference between peak area match coefficient and peak height match coefficient	-1.08	-0.89
Standard deviation of mean difference	3.99	3.05
Value for t-test	-2.32	-3.03
Number of values tested	74	109
Probability that the mean difference is not different from zero	0.02	0.01

than 5%) of belonging with the remaining values. Assuming an average of 110 substances found in each urine sample, we could expect to find, by random chance, 5 to 6 substances that would be identified as outliers by this test even if they were not in fact outliers. Table 11, viewed in this context, suggests several interesting conclusions. Among the BCIU urines, each has at least one outlying value. However, the number of outlying values is generally what would be expected by chance, except for urines 120, 113, and 080. BCIU urine 120 is especially interesting because 7 of the 9 outlying values would not be considered outliers if the subject were part of the neuroblastoma group. Similarly, 8 of the 19 abnormal values for neuroblastoma patient MG would not be considered abnormal if the patient were part of the BCIU group. I can discern no pattern to the abnormalities in BCIU subjects 113 and 080. The number of outliers in the other neuroblastoma patients does not exceed those expected by chance.

It is tempting to speculate that BCIU subject 120 has some kind of disease, other than neuroblastoma, which produces abnormally high values of these "outlier" compounds. Inspection of the health questionnaire for this subject reveals no drug ingestion or disease state to account for the observed abnormality. However, it is extremely difficult to draw any firm conclusiions from such limited data. Similarly, neuroblastoma patient MG is still alive,

although he has suffered a relapse; patient MM (two samples were run from this patient) is the only one that has been judged cured, but nothing unusual was detected about this patient by the outlier test.

What is apparent from these data is that the outlier test is of little value in the current study. It does not identify abnormally low values very well: only 4 abnormally low values were discovered by this approach. Furthermore, it does not identify whether a particular outlier value would also be an outlier for other subject groups; this must be done manually. It may also be misleading for other reasons. For example, the urine from neuroblastoma subject MG appears more normal than it probably is, because 11 of the 19 abnormally high compounds are numbered above 341; this means that they were taken as library compounds from this one urine sample. It is not surprising, therefore, to find the same urine considered to be abnormally high in these substances. Presumably, as these entries are improved they will be found in more of the urine samples; likewise, as more urines are examined for new spectra, more unusually high or low values will be discovered. It should be remembered also that many compounds were removed from the library because they occurred in only a few urines and that these would be detected as abnormal values by the outlier test in many cases.

Blaisdell, after examining the outlier data, is of the opinion

that the outlier test results suggest that it may be possible to find compounds unique to each individual subject (77B2). I do not believe this conclusion to be warranted based on the urines examined so far. However, Blaisdell has not removed data that I consider unreliable, so he is examining outliers for all 157 compounds. He also considers even one outlier per urine sample to be significant, which I do not believe can be supported on statistical grounds.

Comparison of subject groups. Once sufficient data were available, it became possible to compare various subject groups using the "Student" t-test and the Wilcoxon test. Reference to Tables 12 and 13 suggests several interesting conclusions. Approximately 29 compounds are significantly different between adults and the two groups of juveniles (juvenile controls and neuroblastoma patients). Most significant of these appear to be unknowns U26 and U30, and pyroglutamic, β-hydroxy-β-methyl-glutaric, fumaric, oxalic and glyoxylic acids. Several patterns also emerge. First, short-chain aliphatic dicarboxylic acids seem to be lower in adults than juveniles (e.g., oxalic, fumaric, glutaric, malic, α-hydroxyglutaric and tartaric acids). Secondly, several aromatic dihydroxy or dimethoxy acids seem to differ; levels of veratric (dimethoxybenzoic), hydrocaffeic (dihydroxyphenyl-propionic), and caffeic (dihydroxycinnamic) acids are all significantly different between the two groups. Also of interest is

the difference in pyroglutamic acid levels, since this acid has been implicated in several disease states (68T1). Unfortunately, many of the compounds which differentiate the two groups are as yet unidentified; U26 and U30 seem especially interesting to identify.

Of more interest from a medical viewpoint is the difference between neuroblastoma patients, all of whom are under 5 years of age, and the two control groups (adult and juvenile). Only the compounds which distinguish them from both control groups are listed here. The four most consistently differentiating substances listed in Table 13 appear to be homovanillic and caffeic acids, which are high in neuroblastoma patients, and unknowns U6 (probably an isomer of α-hydroxyisovaleric) and U79, both of which are lower in the neuroblastoma patients. It is interesting to note that homovanillic and vanilmandelic acids are both significantly different between the two groups of subjects, since these acids have typically been associated with the disease. However, neuroblastoma patient MM is the only one with extremely elevated levels of these two compounds, yet she is the one who is currently judged to be cured (Table D1) by Dr. Krivit, the physician who is treating the patients. Patient MM seems to have essentially normal levels of almost all metabolites. For example, that patient is the only one to exhibit normal levels of unknown U79, which is missing entirely in all of the other neuroblastoma patients but present in all other subjects

(except an infant suffering uncontrollable seizures). Interestingly, caffeic acid is elevated in all of the neuroblastoma patients, but the two who have died since the collection of the urine sample had much higher levels than the two who are still living, who had levels very close to those of the control subjects. It is difficult to suggest a biological reason for this difference, since caffeic acid is a tyrosine metabolite found primarily in plants. Furthermore, this may simply be a chance association, rather than one directly related to the disease, so considerably more work needs to be done before this result can be understood properly.

The above generalizations about differences among or between groups must be followed by a series of caveats. First of all, the differences between subject groups vary depending upon which normalization or transformation is used. This makes interpretation of the data more difficult, since no one method of data manipulation can be defended absolutely. Normalization to urine volume is clearly unsatisfactory since urinary volume may fluctuate markedly without reflecting any change in metabolism. Creatinine levels have frequently been used by others to normalize excretion data, but recent literature (75A2) suggests that this procedure is particularly unsuitable for infant urines, because the ratio of excretion of many metabolites relative to creatinine changes markedly with age. In any case, creatinine levels have not been

considered anything other than a rough estimate of overall metabolic rate (or body mass), since it reflects primarily the rate of energy utilization in large muscle masses. Robinson and Pauling have suggested normalization to the sum of the compounds present in the biological fluid, excluding those compounds excreted at the highest concentrations (74R2); a variation of this procedure has been used to normalize our data. This type of normalization makes some biochemical sense, since the largest peaks seem more likely to represent compounds of dietary origin, which may fluctuate markedly. However, this type of normalization has not been well-studied, nor has it been validated by studies of known differences among subjects. Robinson and Pauling have not even justified this procedure on statistical grounds, other than that it reduces the variances of sample concentrations. While I agree that some type of normalization is required, I believe that it must be justified on a somewhat different basis. Does it decrease inter-group variance while increasing between-group variance? Simply decreasing overall variance, as proposed by Robinson and Pauling, may lead to a corresponding decrease in diagnostic information. (The extreme example of this would be to multipy all values by zero. Overall variance would be minimized, but the ability to differentiate profiles of various groups of subjects would be reduced to zero.)

We have used a normalization procedure similar, although not

identical, to that of Robinson and Pauling. This procedure can be justified on the basis that it does increase the number of detectable differences between the neuroblastoma and BCIU groups (Table 14). Similarly, the choice of appropriate statistical tests must be defended on the same basis. Thus, for example, when the t-test is compared to the Wilcoxon test, a test that uses ranks rather than actual concentrations, the results, shown in Table 15, suggest that neither method is clearly superior. Since the "true" differences between subject groups are not known in advance, there is no way to judge whether a compound judged to be significant by one method but not by the other is indeed significant in distinguishing one group of subjects from another. It is interesting to note, however, that even when slightly different normalizations are used, the Wilcoxon test on normalized data gives results comparable to the t-test using log-transformed, normalized data. This would suggest, since the data appear to be log-normally distributed, that either of these two methods may be used almost interchangeably; a better procedure perhaps, might be to use differences detected by both tests.

In attempting to make a choice between the t-test and the Wilcoxon (or other distribution-free) test, the comments of Armitage (71A1) are relevant:

> "Distribution free tests are supported by remarkably strong theoretical arguments.....If the distributions are normal with the same variance, the

t-test is the most efficient test, but the rank test (Wilcoxon, Mann-Whitney or Kendall) has a relative efficiency of 0.96. If the distributions are not normal, the relative efficiency of the rank test is never less than 0.86 and may be infinitely high. For detecting a shift in location, therefore, the rank test is never much worse than the t-test, and can be very much better.

"Furthermore, the distribution-free test based on normal scores has a relative efficiency against the t-test which is never less than unity and may be infinite. Why, then, should one not always use either the rank test or the normal score test in preference to the t-test? The first point to make is that significance tests form only a part of the apparatus of statistical analysis. The main purpose of an analysis is usually to provide as much information as possible about the nature of the random variation affecting a set of observations. This can usually be done only by specifying a model for that variation, estimating the parameters of the model in a reasonably efficient way and informing oneself about the precision of these estimates.

"Distribution-free methods are basically tests and are not easily adapted for purposes of estimation. It is true that the statistics can often be said to estimate something, but the parameter estimated may be of limited interest.....

"A second point about the theoretical results on power is that they refer to one particular form of difference between two distributions, namely a displacement or difference in location. In other situations the position is less clear.

"In general, then, distribution-free methods are best regarded as a set of techniques to fall back on when standard assumptions have particularly doubtful validity; it is often useful to be able to confirm the results of a normal-theory significance test by performing an appropriate distribution-free test."

It is also possible that differences between subject groups are due to some other difference than the one being tested. For example, all of the neuroblastoma patients have been undergoing intensive chemotherapy, although urines were collected at least 3 weeks after

the most recent medication, thus eliminating short-term drug effects at least. The difference between neuroblastoma patients and the two control groups (adult and juvenile) may be due entirely or partly to the long-term effects of the medication, or perhaps some other less obvious factor differentiating the two groups (e.g., differences in diet). Similarly, the adult versus juvenile differences may in fact be differences between the well and the sick, since no healthy juvenile controls are included in the urines tested here. The fact that the juvenile controls are hospitalized for various diseases (see Appendix D) is itself both an advantage and a disadvantage. It is an advantage because the differences between neuroblastoma patients and juvenile controls are likely to be due to the neuroblastoma, not hospital diet or general non-health. For example, there is both internal and published (68T1) evidence suggesting that elevated pyroglutamic acid levels are associated with at least some types of infection. Sweeley and Dendramis (77S1) have indicated that some pyroglutamic acid may arise from spontaneous degradation of glutamine in the urine sample. Regardless of the source of the pyroglutamate, however, it may prove a useful means of measuring infection levels. Before the juvenile control urines became available, one of the most interesting contrasts between the neuroblastoma patients and the BCIU groups was that the pyroglutamic acid levels were markedly elevated in the

neuroblastoma patients--so elevated, in fact, that we prematurely speculated that it was a newly-discovered concommittant of neuroblastoma. Subsequently, it has become clear that in fact the BCIU subjects are the only ones studied so far with low levels of this metabolite, perhaps since they are the only "healthy" group studied. Hence, it has been an advantage to have the "unhealthy" juvenile controls to remind us that there may be other sources of observed chemical differences than the disease in question.

However, having a mixed group of "unhealthy" controls is also a disadvantage, because there is considerable danger that one or more of these samples may reflect a markedly different metabolic profile characteristic of that disease, and thus obscure the similarities of the remaining members of the control groups. Furthermore, it would be extremely interesting to be able to distinguish compounds that differentiate the well from the sick, if such compounds existed. Obviously, the solution is therefore to have samples from both types of control groups--the healthy, unhospitalized subjects, and the unhealthy, hospitalized subjects with a variety of other diseases.

It is appropriate at this point to add another cautionary statement about using metabolic profiling for detecting diseases. It is entirely likely that "healthy" control groups will contain some individuals who exhibit the biochemical symptoms of one or

more as yet undetected (for them) diseases. (In fact, we probably all are suffering from one or more mild viral or bacterial infections, hardening of the arteries, aging and other mild diseases, even when we are "healthy".) For example, as noted earlier, subject 120 of the BCIU group appears to have some of the characteristics of an unhealthy person. Statistical comparisons should also be examined carefully to identify such individuals, although they should not be discarded from statistical consideration on the basis of an abnormal profile; to do so without confirmation of disease by other means may lead to an artificially narrow "normal" range.

Clinical report form. In anticipation of the need to provide metabolic profiling data to clinicians in an easily understandable format, a "clinical report form" has been developed to compare urine from a given individual to an appropriate group of reference urines. This form, shown in full in Appendix H, is based on the report forms used with many of the multi-channel analyzers in hospital laboratories. It is designed with the idea that data from a single sample are graphed to show how many standard deviations from the mean each value is. A useful modification to this plot might be to allow all data to be normalized and converted to their logarithm, so that advantage is taken of the log-normal distribution of the data. This would require maintenance of a separate file of log-normal mean values, however.

The clinical report form must be interpreted with some caution. While easy for a physician to understand, the report oversimplifies in several ways. One is that data for a given compound may not be distributed normally (or log-normally). A second problem is that only a very small number of reference samples are available so far; hence, the mean and standard deviation may contain a considerable degree of uncertainty. Third, the report completely ignores the possibility of inter-relationships among variables. Fourth, because the standard deviation of the data for a given compound frequently approaches the size of the mean (i.e., since the coefficients of variation are often 100%), values of zero will not usually be considered abnormally low, so the form may be insensitive to unusually low values.

Thus, in general, the clinical report form should not be viewed as an alternative to more complete statistical analysis, but rather as a useful report when speed of analysis or reporting simplicity are primary objectives (MSSRPT, the program which creates the report form, takes only 1 minute to run, plus a few minutes additional printing time.)

Other statistical considerations. It may seem that prospects for learning about disease processes by metabolic profiling are poor, given the data presented so far. However, there are several hopeful aspects to make metabolic profiling worthwhile pursuing. One is

that, even with limited data sets, marked differences are detectable. This would suggest that, utilizing larger data sets, more subtle differences will become apparent. Even more encouraging, however, is the prospect of utilizing higher-order statistics. Thus far the limited number of samples has prevented use of multivariate statistics, but even bivariate statistical methods would appear to be an improvement over univariate statistics. Two illustrations of preliminary tests with this type of statistic are shown in the 2-dimensional plots of Figures 42 and 43. Thus, for example, while homovanillic acid (HVA) by itself is useful in distinguishing control subjects from neuroblastoma patients, the combination of HVA with vanilmandelic acid (VMA) is even more informative. Similarly, m-hydroxyphenylhydracrylic acid adds information to a plot of caffeic acid levels.

In addition, some limited experience has been gained with inter-compound correlations. These correlations appear very exciting, but the problem of proper normalization of the data is again apparent. Further research with this type of statistic must be gained before it can be used with confidence; however, it is my suspicion that this type of statistical approach will in the end be more fruitful than simple univariate statistical analysis.

In general, my evaluation is that the normalization process is indeed central to the correct interpretation of the clinical data.

Figure 42. Concentration of VMA and HVA in four types of urine samples.

Normalized relative concentrations of homovanillic acid (HVA) and vanilmandelic acid (VMA) were determined by MSSMET for 20 urine samples. The acids were measured as the trimethylsilyl derivatives after separation on DEAE-Sephadex. The levels of each were normalized to the sum of relative peak areas of all of the other compounds found in the urine samples, except that the 32 largest relative areas were excluded from the sum. Both metabolites were found to be statistically significant in differentiating subjects with neuroblastoma from the two groups of control subjects.

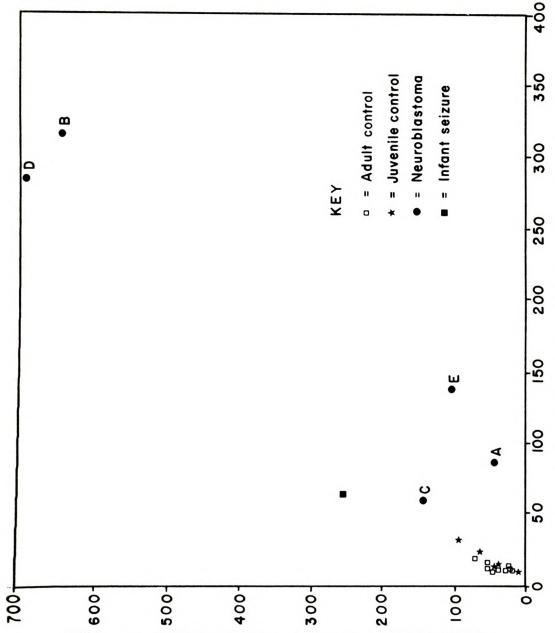
KEY
□ = Adult control
★ = Juvenile control
● = Neuroblastoma
■ = Infant seizure
A
B
C
D
E
NORMALIZED RELATIVE CONCENTRATION OF VMA
NORMALIZED RELATIVE CONCENTRATION OF HVA
0
50
100
150
200
250
300
350
400
100
200
300
400
500
600
700

FIGURE 42

Figure 43. Concentrations of caffeic and m-hydroxyphenyl-hydracrylic acids in 4 types of urine samples.

Normalized relative concentrations of caffeic and m-hydroxyphenylhydracrylic acids were determined by MSSMET on the same samples as those described for Figure 42. Both metabolites were found to be statistically significant in differentiating subjects with neuroblastoma from the subjects in the two control groups. Of the four patients with neuroblastoma only those marked A and B and D (the latter two being the same patient at different times) are still alive. Subject A has suffered a relapse, however.

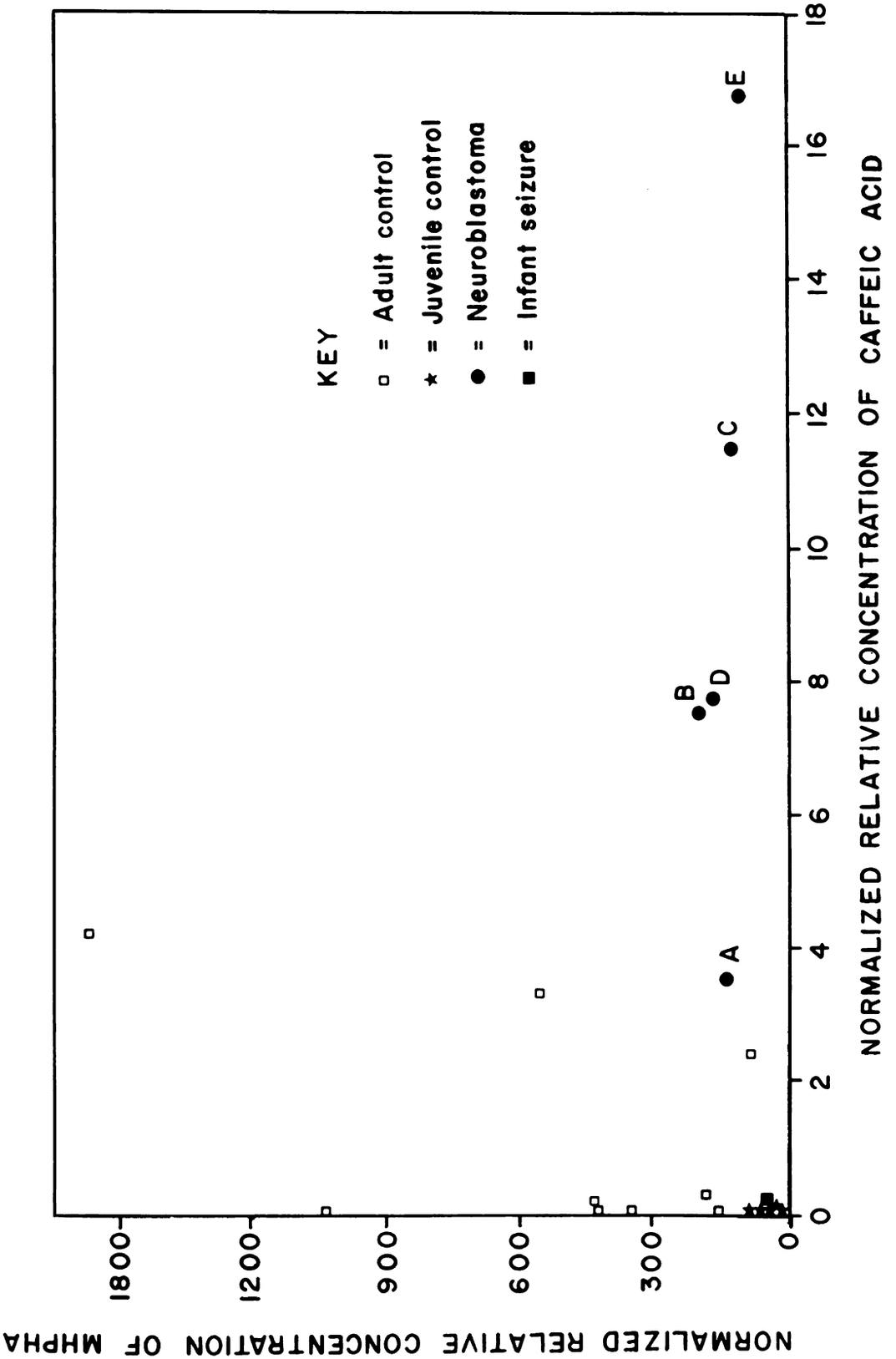
KEY
□ = Adult control
★ = Juvenile control
● = Neuroblastoma
■ = Infant seizure
A
B
C
D
E
0
300
600
900
1200
1800
0
2
4
6
8
10
12
14
16
18
NORMALIZED RELATIVE CONCENTRATION OF MHPHA
NORMALIZED RELATIVE CONCENTRATION OF CAFFEIC ACID

FIGURE 43

The overall objective of any of these data transformations is to increase inter-group variance while decreasing intra-group variance for each of the subject groups. Hence, it should be possible, given a sufficiently large group of urine samples, to test each type of transformation for its success in achieving this objective. Unfortunately, it may happen that transformations of univariate data will initially appear to meet this objective, but fail miserably when tested by bivariate or multivariate methods.

Ultimately, it may prove more reasonable to separately normalize different classes of compounds (e.g., citric acid cycle intermediates, sugars, amino acid metabolites, bacterial by-products, etc.). This normalization must be done carefully, however, so that it can be defended from a biochemical and physiological, as well as a statistical, point of view. It must also be done in such a way that important information is not lost; for example, if one class of compounds is abnormally elevated relative to others, this must not be obscured by the transformation process.

CHAPTER SIX: EVALUATIONS AND RECOMMENDATIONS

Overall, I believe that MSSMET and its associated extraction techniques and statistical programs accomplish the original goal of this project: to prove that a highly automated system could be designed for the quantitative and qualitative profiling of low molecular weight organic acid metabolites from urine. While by no means completely finished, MSSMET, even at its current state of development, has proven to be easy to use, precise, sensitive, and capable of processing an extremely large number of compounds. It has been successfully tested on a variety of human urine samples with results that suggest a great amount of medically-significant information can be gained from this type of approach. MSSMET has not yet been tested on the more subtle diseases, principally because of time and equipment limitations, but there is currently no reason to think that MSSMET will not be equally useful in examining these types of diseases.

While current technology does not encourage the hope of using metabolic profiling by GC-MS in a routine clinical setting, it does suggest that MSSMET and similar programs may find considerable

use in detecting new biochemical relationships which can then be used as the basis for new, hospital-oriented test procedures utilizing other kinds of instrumentation. MSSMET would also be very useful in such specialized situations as drug overdose screening and some forensic applications.

For the future, I can suggest several general improvements over current methodology which I believe would make significant improvements to MSSMET or the general metabolic profiling process.

Chemical separation procedure

While satisfactory as a research method, the DEAE-Sephadex procedure unfortunately has few of the characteristics of an ideal clinical separations method. It is neither rapid, nor easily automated, nor tolerant of operator errors, nor pleasant-smelling, nor 100% effective. It takes approximately 48 hours to run and requires a great deal of manual manipulations, so that operator time per analysis is high. An upper limit of 10 to 15 samples per day per technician seems to be the maximum achievable. Samples require constant attention from the technician, so that the opportunities for forgetting a crucial step are high. The use of pyridine solutions requires that the entire procedure be performed in a hood. Recoveries of some substances (e.g., citric acid) are low. It is, however, reproducible,

which none of the liquid extraction procedures tested are, and this is its principal redeeming virtue.

Despite its good overall reproducibility, the method has several disadvantages even in a research lab, where time requirements may be less critical. One is the need for lyophilization, which not only is the most time-consuming step, but also one of the least well-controlled. Chalmers and Watts have shown (72C2) that the vacuum pressure and external temperature are critical variables when lyophilizing pure compounds. Although they may be less critical for urine samples than for pure compounds, a much more careful control of lyophilizing conditions than is possible with common laboratory equipment would appear to be desirable.

An even more serious problem is the use of a barium hydroxide precipitation step prior to separating the urine sample on a DEAE-Sephadex column. While this precipitation eliminates a large fraction of the phosphoric and sulfuric acids present in typical urine samples, it also eliminates at least a portion of citric and other acids. In addition, it may affect recovery of other compounds as well. As mentioned earlier, (Chapter 5), this step in the procedure should be omitted if possible in future studies.

In general, the DEAE-Sephadex procedure, while adequate, is considerably less than ideal. The best solution is probably development of some new batch separation method quite different from the

laborious column chromatography used here. Even if the DEAE-Sephadex procedure is retained, however, it can be improved by careful studies of all of the parameters involved. These studies should be completed using the entire MSSMET analysis system, since many of the preliminary tests on stability and reproducibility were performed with GC assays (before MSSMET was perfected) and hence may not be valid for small peaks not resolved on the GC traces.

Quantitative precision.

One of the most difficult, yet important, problems to be faced when evaluating a new procedure is to sort out which facets of the procedure are most in need of improvement, and which can be considered acceptable for the time being, at least. The former must be confronted immediately, while the latter may wait until some later date to be remedied. Influencing this decision is a variety of factors: ease of improvement, comparison to currently available methods, resources, external pressures (competition, funding sources), dependence of other portions of research upon progress in this area, and so on.

An example of this kind of dilemma is the need for quantitative precision. MSSMET, which uses a repetitive-scanning

based approach, has a quantitative precision of 5 to 10%. This is relatively poor compared to that of selected ion monitoring (0.5%-3%), but comparable to that of many current clinical laboratory techniques. Does it therefore require improvement?

For this particular case, a series of guidelines for answering the question may be established, based on statistical considerations. Assuming that the precision of the method is plus or minus 10% at all concentrations (this is probably not a valid assumption at low concentrations), and that typical biological inter-variation is approximately 100% (coefficient of variation), then how much do each of these terms contribute to the overall variability of the data?

Assuming that the absolute mean of the group of individuals is 100, then (75W2, p88), the standard deviation, σ, is

$$\sigma_{observed} = \sqrt{(\sigma_{method})^2 + (\sigma_{group})^2}$$

$$\sigma_{obs} = \sqrt{(100)^2 + (10)^2}$$

$$\sigma_{obs} = 100.5$$

Hence, the method variability, which is 10% of the inter-individual variability, contributes only 0.5% more variability to the "true" variability under these circumstances. (It should be noted that this calculation assumes that the two sources of variance are independent, which is probably true). However, similar analysis for

an intra-individual comparison study where the variability of the individual is only plus or minus 10% (coefficient of variation), would suggest that $\sigma_{obs} = 14.14$ that is, the overall results are 41% more variable because of the addition of method variability to the "true" variability of the individual being tested.

Usually, then, the methods developed for MSSMET are adequate and will give normal ranges insignificantly different from those using much more precise techniques when measuring inter-individual variations. However, results from MSSMET for intra-individual studies will be significantly less precise than selected ion monitoring-based methods, and will require repetitive analyses of each sample on the GC-MS to achieve reasonably precise data. Whether a given degree of precision is unacceptable of course will depend upon the use to which the data is to be put.

Other recommendations. Another major improvement to the current procedure, I believe, would be the development of a capillary GC-computer system for screening purposes. The cost of such a system should be much lower than a GC-MS-computer system, and the reliability of the instruments much higher. Such a system could be used to inspect large numbers of samples for the presence of interesting patterns or differences, which then could be examined in more detail utilizing a GC-MS-computer system and MSSMET.

MSSMET itself should also be redesigned. Two of MSSMET's

disadvantages are that it cannot find compounds which are not in its library, and it may mistakenly identify a single compound by two different library entries, since there is no provision in MSSMET which prevents the same data from being identified twice. A prototype program which would solve both of these problems has been designed by the author and preliminary tests completed; this program subtracts any positively identified spectrum from the mass spectral data file so that spectra left over at the end of the analysis are those NOT identified by the program; these can be added to the library for use in subsequent analyses. For example, this program was the means by which unknowns U50 to U91 were selected. This approach also prevents the same data from being identified twice, since it is removed when first identified. A third advantage is that it improves baseline detection accuracy by examining the whole run to choose baseline points, rather than using a "window." The program needs further testing before routine use is possible, however.

Another improvement needed is continued work on the MSSMET library, perhaps with an automated history-taking of data. A quantum jump in the quality of the library resulted from detailed inspection of MSSMET outputs utilizing the "debug" feature to examine ion ratios for all peaks found. Unknowns U50 to U91 would be considerably improved by further inspection of this type, since they are based on data from a single urine sample. Other compounds

(for example, those listed in Appendix G, plus other compounds in PUBLIB) may also be re-added to the library as this process continues.

Yet another improvement might well be achieved by designing a program to inspect raw GC-MS data for errors. The most common error on our system is a sudden drop in ion intensity for a single scan, sometimes associated with an abnormally high intensity in the next higher mass at the same scan. It should be easy to design a program to inspect raw data for such errors and correct them by interpolation. A better solution, of course, would be to correct the data collection algorithm so that such errors do not occur, but this particular type of error has resisted such corrective efforts in the past.

Another recommendation related to overall quality of data collection is the design and implementation of a quality control program. While the human eye is reasonably good at recognizing abnormal results on large peaks, it tends to ignore fluctuations in small peaks. I recommend that a computer program be written to compare runs of the quality-control sample and to make a "go-no go" decision on purely objective grounds. I would similarly recommend that any gas chromatographs used in this program be interfaced to a computer and a computerized quality control program be designed for them as well. A history of past runs of the control sample should

be available to examine for trends in sensitivity, column degradation, and so on, similar to quality control procedures in clinical laboratories.

Another project which should be undertaken by those interested in metabolic profiling is the purification of reference compounds. Either preparative GC or HPLC could be used advantageously to purify large numbers of compounds, since individual purification by more classical procedures would be very time-consuming. It might even be possible to purify compounds from urine samples in the same fashion. Pure compounds could then be used in recovery studies, to establish k-factors, and to obtain high-quality mass spectra. The establishment of k-factors, especially, would be useful for comparison to other quantitative methods, and would make clinical reports more meaningful to others outside this laboratory. While some k-factors have already been established, they are not yet sufficiently reliable to be published in this thesis, so k-factors are needed for all compounds in the library.

Once many of the technical difficulties listed above have been solved, however, it should not be assumed that the task of the metabolic profilist will be an easy one. A great many philosophical and practical problems remain. For example, the problem of handling new urine samples needs to be confronted. Hilman (77H1) has suggested that a referral system be established, so that all

samples are screened by routine hospital screening methods and then sent to a clinician who will decide on the proper disposition of each. It is important, according to Hilman, to establish, in advance, the time period within which results are needed, and the likelihood of therapy being undertaken based on positive or negative results being presented to the referring physician. In essence, these suggestions are simply that we follow the guidelines of any well-run genetic screening center. In my opinion, it is well to establish a formal procedure of this type as soon as possible. Lack of such a system can easily result in chaotic sample processing, over-burdened staff, invasion of patient privacy, and unmet expectations on the part of both the referring physician and the group attempting to interpret the profile. The system must necessarily provide a series of procedures, including an exact protocol for the referring physician to follow in collecting the urine, selection of appropriate control samples, sample storage, transmission and secure storage of patient health and dietary data, provision of estimated processing times, computerized report forms and staff consultation on the specific meaning of results. It is especially important that a reliable means of communication with referring physicians be established, both to insure that urine collection protocols are followed and to insure that results are properly interpreted and followed up with appropriate treatment or further testing of the patient.

Another problem area which will have to be considered is a means of examining the effect that intake of specific dietary items by the patient will have on the metabolic profile of the subject. Hilman (77H1) has already noted several specific foods and food additives which can affect urinary organic acid content, but a detailed study of such effects needs to be made. It is especially important, therefore, that referring physicians provide a completed diet and health questionnaire for each patient, even those with supposedly well-diagnosed diseases. Failure to keep a permanent, computerized record of such data can result in loss of a great deal of valuable information.

One other suggestion about statistical studies is warranted. Some workers have cautioned against using metabolic profiling in a "shotgun" approach; this is, examining a wide variety of diseases until one is found which can be diagnosed by this means. I do not believe such caution is necessary. In fact, I believe it is in just such "shotgunning" where metabolic profiling is most promising, with a very high chance of positive results. However, I think that it is important to be sure that the person aiming the shotgun knows where he (or she) is aiming, and in the case of metabolic profiling, I think it is very important that the question be clearly defined in terms of what is possible. Thus, for example, it is not enough to ask, "can I detect disease X by metabolic profiling." It is much better to ask

such questions as:

"Can I find more than n compounds that differ significantly between the group with the disease and the appropriate control groups?"

"Can I detect changes in the levels of more than n compounds when a single subject (or group of subjects) is stressed in some fashion (onset of disease, exercise, unusual diet, drug ingestion, etc.)?"

"Can I detect changes in the relationships of more than n pairs of compounds between subject or treatment groups?"

In each case, it should be possible to predict in advance the likelihood of a given number of significant differences occurring by chance; this number must be considerably exceeded to have confidence that real differences do indeed exist. Some differences or relationships may prove to be more interesting than others, because of known or suspected biochemical relationships; hence, the problem is not entirely one of statistics but requires significant interaction between statisticians and biochemists if progress toward understanding test results is to be made.

Speculation on long-term prospects

At the outset, the prospects for relating urinary metabolite concentrations to the diagnosis of specific disease states may appear to be minimal. After all, urine is the "dumping ground" of the body metabolism, so that what is observed in urine is a mixture of metabolic wastes from all of the body. In addition, there is the further complication that the process of urine formation in the kidney is itself quite complex, with wide differences in rates of diffusion, secretion and readsorption among various types of compounds. The major complicating factor, however, is the variation in excretion levels among individuals due to differences in both diet and enzyme activities. This inter-individual variability is the apparent bane of the metabolic profilist, making anything but longitudinal studies of single individuals seem almost pointless.

However, there is hope. Looked at from a biochemical point of view, man is little more than an extremely complex set of chemical reactions, each related to one or more others, which in turn are related to yet others. In this context, most disease is the imbalance of one or more pathways, whether produced by dietary deficiency, inherited enzyme dysfunction, bacterial toxin production, viral infection, or almost any other causative factor or combination of factors. When these imbalances become sufficiently large, they

appear as gross physical symptoms that can be detected by the physician or patient. Very few diseases have the visible symptoms localized only at the site of the imbalance; effects are frequently observed at numerous locations in the body. From this it is possible to infer that diseases must be affecting a variety of biochemical pathways in a number of tissues or organs. Presumably, at least some of this imbalance will be reflected in the urine of the individual involved. While direct sampling of the affected tissues would be preferable, and sampling of serum constituents a good second choice, nonetheless, urine sampling may in the end be preferred because of the ease in collection of urine and the higher concentrations of many metabolites in urine compared to other body tissues.

An extremely important point in this vein is that regardless of the tissue or fluid sampled, it will probably be relationships, not absolute levels, that are important. Undoubtedly, some diseases will be indistinguishable from one another because they have the same generalized biochemical effects; others will be undetectable because the changes are too localized or at such low concentrations that more sensitive methods must be used. Yet others will be indistinguishable from dietary effects. However, for many diseases, it may be that the real value of metabolic profiling lies not in the "shotgun" effect, but rather in the potential for sampling some of the complex

inter-compound relationships that exist. Since all humans have essentially the same metabolic pathways, it may be expected that certain relationships will occur repeatedly in urine samples from "healthy" individuals, but be deranged in certain types of illnesses.

Pauling (68P1) has argued for a concept he calls "orthomolecular psychiatry," of which the most famous (or infamous) example is his ascorbic acid therapy. He suggests that some mental diseases may be cured by obtaining the "optimum molecular concentrations" of substances normally present in the body. While I do not share his optimism about being able to use "orthomolecular therapy," I nonetheless believe his approach of examining health from a biochemical viewpoint to be very useful, and in the long run, very productive. I would further suggest, however, that there is no such thing as the optimum concentration, but rather, an optimum level consistent with the current pattern of concentrations of biochemically related substances in the body. It becomes, then, the task of the metabolic profilist to discover those optimum relationships, and to measure how they have been altered in each specific disease state.

APPENDICES

APPENDIX A

Diet, health and drug questionnaire

The following set of materials was provided to each subject where possible. The materials include a set of instructions, a medical history questionnaire, a diet questionnaire for the day preceeding the urine collection, a 72-hour drug ingestion history, a survey of whether the protocol was followed by the subject, and a consent form. Subjects were each assigned a unique number prior to the collection time. Completed questionnaires were coded into computer-readable format by a program specifically designed for this purpose.

INSTRUCTIONS TO VOLUNTEERS

We appreciate your interest in this project. As we explained on the first set of materials you received, we are concerned with the effects of diet, sex, and medical and drug history on the urine of normal and diseased individuals. Hence, IT IS MOST IMPORTANT THAT YOU COMPLETE ALL QUESTIONNAIRES AND FOLLOW THE INSTRUCTIONS BELOW EXACTLY.

1. If possible, do not take any non-prescription drugs or alcohol for 72 hours (3 days) prior to the time you will collect urine. However, it is important that you do not discontinue any drugs important to your health. Since even simple drugs like aspirin are known to affect the contents of urine, it is actually much better for us to know that you did take drugs or alcohol than to have you take drugs that may affect your urine without our knowing about it. If at all possible, please give us a complete drug history (we remind you that it will be kept totally confidential).

2. The day before you donate your sample, eat breakfast, lunch and dinner as normal. However, we ask that you refrain from eating or drinking (except water) after 7 p.m. If for any reason something is consumed after this time, please make a note of the item(s) on the questionnaire.

3. Complete the food, drug and health questionnaire before going to bed.

4. If you need to urinate in the middle of the night, you may do so, but note this on the questionnaire.

5. In the morning before eating breakfast, collect the urine sample as follows: Start to urinate, allow first portion of urine to go directly into the toilet. Then use the container provided to collect the remainder of the urine. Allow any excess urine to go directly into the toilet. Do not worry about having too little urine for us to use as a very small amount is needed for the analysis. Various people urinate various amounts, so do not worry if the container seems too large or too small.

6. Put the plastic cap on the container of urine; make certain it is snapped completely on. You may rinse the outside of the container with water if you wish. Place urine sample into the bag provided.

7. If you will not arrive at work for an hour or more, store the sample in a cold place (outside in your car, for example). Otherwise, place it where you will be sure to remember to bring it to work.

8. Eat breakfast as usual.

9. Bring the sample to work with you. You will be told what to do with the sample there.

MEDICAL HISTORY QUESTIONNAIRE

NUMBER_____

Please complete the following as fully as possible. It is vital that you answer every question; omitted information makes computer analysis of the data more difficult. Ignore small numbers; these are for coding information in the computer.

1) Age ^{1}( ) 0-10 ^{2}( ) 11-20 ^{3}( ) 21-30 ^{4}( ) 31-40 ^{5}( ) 41-50 ^{6}( ) 51-60
^{7}( ) 61-70 ^{8}( ) 71-80

2) Sex ^{1}( ) M ^{2}( ) F

3) In general, would you consider the foods you eat (your diet)
^{1}( ) better, ^{2}( ) about the same, or ^{3}( ) poorer in quality than an "average" American's meal?

4) Considering your general body build, are you ^{1}( ) underweight, ^{2}( ) within 15 pounds of your "ideal" weight ^{3}( ) overweight?

5) Have you recently lost more than 5 pounds in your weight?
^{1}( ) yes ^{2}( ) No. If yes, how much did you lose?_____lbs.
Over what time period did you lose this weight?_____weeks. Were you on a diet during this time period?_____

6) List any medicines you have taken regularly or occasionally within the last year. Include non-prescription medicines (such as aspirin, vitamins, blood builders and cough syrup), as well as prescription drugs (birth control pills, hormones, tranquilizers, etc.)._____

7) Are you on a special diet? ^{1}( ) Yes ^{2}( ) No. If yes, briefly describe (Vegetarian, low-calorie, low fats, etc.)_____

8) Have you had any major operations? ^{1}( ) Yes ^{2}( ) No. If yes, please describe briefly._____

9) Do you currently have any disease(s)? ^{1}( ) Yes ^{2}( ) No. If yes, please describe briefly (diabetes, heart disease, etc.)._____

10) Are you currently being treated by a doctor for any disease ^{1}( ) Yes ^{2}( ) No. If yes, please specify what disease_____

11) Do you smoke cigarettes or use other tobacco products? ^{1}( ) Yes^{2}( ) No. If so, specify product and how often used_____

FIGURE A1. Diet, health and drug history questionnaire

MEDICAL HISTORY QUESTIONNAIRE

Number_____

Please complete the following checklist of diseases or problems which you have now or have had at any time in the past. Be certain to answer each question either "yes" or "no"; leaving a blank makes analysis of the data more difficult.

Yes	No	Check at left	Yes	No	Check at left
		1. Measles			42. Pain in chest
		2. German Measles (3 day)			43. Shortness of breath
		3. Mumps			44. High blood pressure
		4. Chickenpox			45. Low blood pressure
		5. Smallpox			46. Pounding of heart
		6. St. Vitus Dance (Chorea)			47. Heart trouble
		7. Diphtheria			48. Heart murmur
		8. Scarlet Fever			49. Swelling of feet
		9. Rheumatic Fever			50. Asthma
		10. Infectious Mononucleosis (Glandular Fever)			51. Food allergy
					52. Backache
		11. Brucellosis (Undulant Fever)			53. Dizziness
					54. Overweight
		12. Typhoid Fever			55. Underweight
		13. Influenza			56. Recent loss of weight
		14. Kidney Disease			57. Food allergy
		15. Sugar or albumin in urine			58. Skin disease
		16. Sugar Diabetes			59. Athletes foot
		17. Venereal Disease (syphilis, gonorrhea)			60. Encephalitis
					61. Meningitis
		18. Poor appetite			62. Paralysis
		19. Stomach, liver, or intestinal trouble			63. Poliomyelitis
					64. Epilepsy or convulsions
		20. Gall bladder trouble			65. Fainting or loss of consciousness
		21. Jaundice			
		22. Appendicitis			66. Ever been knocked out?
		23. Amebic Dysentery			67. Nervousness
		24. Chronic Diarrhea			68. Emotional trouble
		25. Constipation (requiring laxatives)			69. Poor concentration
					70. Loss of interest
		26. Frequent, prolonged sore throats			71. Tendency to worry
					72. Feeling of inferiority
		27. Running ear			73. Moodiness
		28. Deafness			74. Depression
		29. Poor vision			75. Sleepwalking
		30. Hoarseness			76. Frequent nightmares
		31. Thyroid trouble			77. Loss of memory (amnesia)
		32. Rectal Trouble			78. Are you very shy or sensitive?
		33. Easy fatigue			
		34. Sense of exhaustion			79. Are you clumsy?
		35. Cold hands or feet			80. Speech defect
		36. Decreased tolerance to cold weather			81. Lameness
					82. Arthritis or "rheumatism"
		37. Tendency to bleed easily			83. Swelling or painful joints
		38. Dry skin or hair			84. Painful feet
		39. Tuberculosis			85. "Growing pains"
		40. Pleurisy			86. Varicose veins
		41. Pneumonia			

FIGURE A1. (Cont'd.)

DIETARY SURVEY

Why the Need for a "Fasting Morning Urine"?

Please note that the survey contains no space for foods eaten after 7 p.m. This is to remind you that we have asked that you not eat or drink (except water) after 7 p.m. the night before the urine collection. This is because the content of urine is obviously largely influenced by what you eat and drink. By not eating after your normal dinner, you are giving your body time to digest dinner and eliminate products of that meal. Much of what appears in your urine in the morning, then, will be a reflection of your body's natural night-time activities rather than the contents of any snacks or other food or drink consumed the night before. However, should you forget and eat anyway, note that information in the space provided on the last page of the survey.

Instructions:

Please fill out the survey as completely as possible for the food you have eaten the day before you collect your urine. Simply check each food that you eat in the column that gives the approximate time of day when it was consumed (ignore the numbers--they are for the computer coding of information). If a food you have eaten contains two or more types of food, check each one as appropriate (for example, for a hotdog in a bun, check both "sausage, frankfurter" and "rolls, frankfurter or hamburger.").
If an item is not listed, please fill in the blank with that item.

4-10 a.m. | 10 a.m.-3 p.m. | 3 - 7 p.m.

DIETARY SURVEY

Number_____

Milk, Cheese, Cream, Eggs

1. Milk, whole
2. Buttermilk
3. Cheese
4. Cream, and cream products
5. Milk, chocolate flavored drink
6. Baked custard
7. Ice cream or ice milk
8. Eggs and egg products
9. Yoghurt
10. Other milk products _____

Meat, Poultry, Fish

11. Bacon, Pork, Ham, Pork sausage
12. Beef(braised, simmered, pot-roasted; hamburger, steak)
13. Chicken
14. Lamb
15. Liver
16. Veal
17. Fish or fish products
18. Shrimp, tuna, sardines, other sea foods
19. Other meat or poultry or fish_____

_____

Fruits & Fruit Products

20. Apples
21. Apple juice
22. Applesauce
23. Apricots
24. Avocados
25. Bananas
26. Blueberries
27. Cantaloup
28. Cherries
29. Cranberry sauce
30. Cranberry juice
31. Dates
32. Fruit cocktail
33. Grapefruit
34. Grapefruit juice
35. Grapes
36. Grape juice
37. Lemonade
38. Orange
39. Orange juice
40. Peaches
41. Pears
42 Pineapple
43. Pineapple juice
44. Plums
45. Prunes
46. Prune juice
47. Raisins
48. Raspberries
49. Rhubarb
50. Strawberries
51. Watermelon
52. Other fruit or fruit products

_____

_____

Grain Products

53. Bagel
54. Biscuits
55. Breads
56. Brownies
57. Cakes
58. Apple pie
59. Cherry pie
60. Custard pie
61. Lemon meringue pie
62. Mince pie
63. Pumpkin
64. Other pies_____

FIGURE A1. (Cont'd.)

4-10 a.m. | 10 a.m. - 3 p.m. | 3 - 7 p.m.

Grain Products (cont.)

65. Pizza
66. Popcorn
67. Pretzels
68. Rice
69. Rolls
70. Rolls, frankfurter or hamburger
71. Rye wafers
72. Spaghetti
73. Spaghetti & meatballs
74. Waffles
75. Beans, navy
76. Beans, canned with pork & tomato sauce
77. Peanuts
78. Peanut butter
79. Other grain products, beans or nuts

_____

Vegetables & Vegetable Products

80. Asparagus
81. Beans, lima
82. Beans, snap or wax
83. Beets
84. Broccoli
85. Brussels sprouts
86. Cabbage
87. Carrots
88. Cauliflower
89. Celery
90. Corn, sweet
91. Cucumber
92. Lettuce
93. Mushrooms
94. Okra
95. Onions
96. Peas, green
97. Peas, canned
98. Potatoes or potato chips
99. Radishes

FIGURE A1. (Cont'd.)

4-10 a.m. | 10 a.m.-3 p.m. | 3-7 p.m.

Number_____

Vegetables & Vegetable Products (cont

100. Sauerkraut
101. Spinach
102. Squash
103. Sweet potatoes
104. Tomatoes
105. Tomato catsup
106. Tomato juice
107. Turnips
108. Other vegetables_____

_____

Fats, Oils & Salad Dressings

109. Butter
110. Fats, cooking
111. Margarine
112. Oils, salad or cooking
113. Salad dressings, Blue cheese
114. Salad dressings, French
115. Salad dressings, Mayonnaise
116. Other fats_____

_____

Sweets and Drinks

117. Candy
118. Soft drinks containg cola
119. Soft drinks (other)
120. Coffee
121. Tea
122. Alcoholic beverage (beer, wine; mixed)
123. Other sweets or drinks_____

_____

124. Diet drinks (low calorie)_____

_____

Number_____

DRUG AND MEDICATION SURVEY

In the 72 hours preceding the time of urine collection, did you consume any of the following?

Yes	No	
		1. Vitamins
		2. Diet pills
		3. Birth control pills
		4. Sedatives
		5. Aspirin or Anacin or Bufferin
		6. Marijuana
		7. LSD
		8. Heroin
		9. Alcohol or alcoholic beverages
		10. Other non-prescription drugs (specify which)
		_____
		11. Prescription medication (specify if known)_____
		_____
		12. I have consumed one or more items from #6 to 10 above. However, I would prefer not to specify which drugs.
		13. At what time(s) did you urinate during the evening or night before you collected your urine_____p.m._____p.m. _____a.m._____a.m.

CHECKLIST FOR URINE COLLECTION

Yes	No	
		1. Did you fast after 7 p.m. the night before the urine collection? If not, please write what you ate on the reverse side of this page.
		2. Did you complete the health and food questionnaires?
		3. Did you follow the instructions on collecting urine in the morning?
		4. Did you collect the urine before eating breakfast?
		5. Did you include all of the information that might be useful in interpreting the results of the study of your urine?

If any of the above questions were answered "no", please give details on the reverse side of this page.

Please write any suggestions or comments on anything relating to this project on the back of this form. Your comments will be appreciated.

Yes( ) No( ) Would you like a copy of any publications that may result from this study.

CONSENT FORM

I give my consent for the use of urine samples from_____(donor)
In giving my consent, I agree that all of the following statements are true:

1) I understand that this urine is to be used for purposes of scientific research. This research has been explained to me, and I am fully aware of any risks inherent in the process of donating this sample.

2) I have not been coerced in any way to participate in this experiment, and freely consent to participate. I understand that I am free to discontinue my participation in this experiment at any time, without recrimination.

3) I understand that this research will not produce any results which will be to my direct benefit.

4) I understand that all results will be treated with strict confidence and that the donor will remain completely anonymous.

5) I understand that, if I so desire, I will be given a summary of the results obtained during this experiment upon conclusion of the experiment.

_____
Signature of donor (or parent or guardian, if donor is a minor)

Date_____

_____

For Office Use:

Number Assigned:_____

FIGURE A1. (Cont'd.)

APPENDIX B

BESTLIB (MSSMET library)

This is the complete MSSMET library used for most of the studies in this dissertation. A sample explaining the format of each entry has been shown previously (in Figure 4). BESTLIB contains entries for 15 retention index standards, but the octacosane standard (2800) was not used to compute any retention indices, and the standard at retention index 989 was frequently not found in urine samples. Notice that the expected retention indices of the standards (second line of the entry) differ from the nominal retention indices (first line), to accomodate the location of standards by linear extrapolation. BESTLIB also contains entries for one relative retention time standard (eicosane), the internal standard (tropic acid) and 157 metabolites. All entries are based on the analysis of compounds as their trimethylsilyl derivatives on 10 ft columns of 5% OV-17 temperature programmed from 60° to 260° at 4°/min.

```
      /NG 2,
      /LU 0,
      /OF 400000,
      /IS 1,
      /FH 0.75,
      /RQ 95,
      /RX 94,
      /CQ 80,
      /CF 90,
      /CC 65,
      /PR -1,
      /TY 1,
      /TM 2,
      /WM 192,
      /NP 2,
      /SL 50,
      /TH 50,
      /TS 1,
      /NU 2,
      /MA 100,
      /MH 2000,
      /WS -1,
      /DF 2,
      /CR 0,
      /ST 2,
*2000
40,30
71,1
71,1000,85,625
      /ST 3,
      /TM 1,
      /TY 2,
      /RQ 20,
      /RX 19,
      /MH 200,
*1923
0,95
326,1
326,282,311,153,295,53
      /ST 4,
*2009
1,01
332,1
332,296,374,34,261,30
      /ST 5,
      /TM 3,
*1834
1837
299,1
445,546,357,2183,387,245,299,1851
*1700
1686
280,1
280,339,295,45
*1552
1548
292,1
292,117,205,57,220,47
*1457
1451
231,1
233,155,189,113,231,60
*1149
1343
299,1
299,1000,314,280,283,52,211,48
*1268
1262
205,1
205,170,218,50,284,20
*1101
1097
190,1
190,1287,219,740,88,621,133,672
*1078
1082
131,1
131,1000,233,66,205,120,95,57
*989
978
207,1
207,400,295,130
*2110
2100
297,1
297,280,428,28
*2189
2188
202,1
202,1000,319,172,384,51
*2400
2395
71,1
71,1000,85,650
*2800
2750
71,1
71,1000,85,650
      /ST 7,
      /TM 3,
      /TY 3,
      /LU 1,
```

FIGURE B1. BESTLIB (MSSMET library)

```
     /CF 85,
     /RX 12,
     /DQ 16,
     /MH 1000,
     /CR 0.
*TROPIC
1700
280.1
280.1000,295,133,310,65
     /ST 8,
     /TV 4,
     /MH 50,
     /TH 50,
     /WM 120,
     /CF 81,
* 293 U1
 990
 207,1.000,
 207,1000,295, 225
* 342 U50
 1004
 208,1.000,
 122, 300,142, 787,155, 125,193, 498,208,1000
*   6 2-HYDROXY-2-METHYLPROPANOIC  (A-HYDROXYISOBUTYRIC)
 1078
 131,1.000,
  95,  57,131,1000,205, 200,233,  66
*   8 2-HYDROXYPROPANOIC  (LACTIC)
 1101
 190,1.000,
  88, 320,133, 300,190,1000,219, 640
* 294 U2
 1110
 281,1.000,
 281,1000,269, 545
*  10 HYDROXYACETIC  (GLYCOLIC)
 1128
 205,1.000,
 161, 300,177, 570,205,1000
*  17 OXOACETIC OXIME  (GLYOXYLIC OXIME)
 1195
 190,1.000,
 190, 130,218,1000
*  18 3-HYDROXYBUTANOIC  (B-HYDROXYBUTRYIC)
 1196
 191,1.000,
  88, 256,117,1000,130, 170,191, 551
* 296 U4 (PYRUVIC OXIME)
 1211
 204,1.000,
 130, 812,204,1000,247, 968
```

FIGURE B1. (Cont'd.)

```
* 297 U5 (CRESOL)
 1223
 165,1.000,
 165,1000,166, 142,180, 425
* 371 U79
 1227
 117,1.000,
 117,1000,191, 132,218, 224
*  23 OXALIC
 1231
 190,1.000,
 175, 160,190,1000,219, 770
* 298 U6
 1234
 247,1.000,
 204, 299,205, 368,247,1000
* 343 U51
 1246
 208,1.000,
 193, 274,208,1000
* 344 U52
 1246
 216,1.000,
 188, 974,216,1000,231, 408
* 299 U7
 1264
 152,1.000,
 152,1000,177, 333
*  28 1,2,3-PROPANETRIOL  (GLYCEROL)
 1268
 205,1.000,
 177,  86,205,1000,218, 238
*  29 4-OXOPENTANOIC  (LEVULINIC)
 1279
 173,1.000,
 145, 900,155, 113,173,1000
*  36 MALONIC
 1281
 248,1.000,
 233,1000,248, 280
*  33 4-HYDROXYBUTANOIC  (G-HYDROXYBUTRYIC)
 1282
 233,1.000,
 143, 333,218, 500,233,1000
*  35 METHYLMALONIC
 1285
 247,1.000,
 218, 700,247,1000
*  34 UNK RA183 (GLYOXYLIC??)
 1287
 191,1.000,
 191,1000,221, 400,265,  86,293,  76
```

```
*  42 UNK-OXB1
1321
174,1 000,
174,1000,248,  174,279,  70
* 301 U9   (2-METHYLGLYCERIC)
1326
219,1 000,
129,  312,219,1000,233,  341,306,  125
*  50 PHOSPHORIC
1349
299,1 000,
211,   48,283,   52,299,1000,314,  200
* 302 U10   (4-DEOXYERYTHRONIC)
1362
292,1 000,
220,  199,292,  828
*  51 BENZOIC
1362
179,1 000,
105,1000,179,  600
* 303 U11   (4-DEOXYTHREONIC)
1380
292,1 000,
220,  357,292,  757
*  60 SUCCINIC
1397
247,1 000,
172,  200,247,1000,262,   92
*  59 UNK-NF2
1397
261,1 000,
186,  209,217,  695,232,  270,261,1000
*  61 FUMARIC
1401
245,1 000,
115,  252,143,  390,245,1000
*  65 CIS-BUTENEDIOIC   (MALEIC)
1410
170,1 000,
170,   96,245,1000
* 345 U53
1422
201,1 000,
186,  503,201,1000
*  66 PHENYLACETIC
1428
164,1 000,
164,1000,193,  875
*  67 3-PYRIDINECARBOXYLIC   (NICOTINIC)
1436
180,1 000,
106,  364,136,  408,180,1000,195,   39
```

FIGURE B1. (Cont'd.)

```
* 346 U54
1440
219,1 000,
129,  221,219,1000,321,  183
* 305 U13 (3-DEOXYTETRONIC)
1444
219,1 000,
147,  630,219,  1000,321,  200
* 306 U14   (2-DEOXYTETRONIC)
1457
231,1 000,
189,  729,231,  387,233,1000,246,  258,321,  354
* 347 U55
1457
321,1 000,
189,  795,233,1000,246,  172,321,  258
* 348 U56 (DEOXYTHREONIC)
1469
292,1 000,
221,  372,292,1000,321,  362
* 349 U57   (THREONOLACTONE ISOMER?)
1481
247,1 000,
217,  286,233,  177,247,1000,262,  477
*  77 PENTANEDIOIC   (GLUTARIC)
1489
261,1 000,
158,  330,186,  145,261,1000
*  79 3,3-DIMETHYLPENTANEDIOIC   (3,3-DIMETHYLGLUTARIC)
1500
289,1 000,
199,  347,214,  121,289,1000
*  82 2-HYDROXY-2-METHYLBUTANEDIOIC   (CITRAMALIC)
1510
321,1 000,
247,1000,321,   94,349,  160
*  86 HYDROXYBUTANEDIOIC   (MALIC)
1540
233,1 000,
190,  350,233,1000,335,  505
* 350 U58 (3-METHYLGLUTACONIC-PEAK 1)
1528
143,1 000,
143,1000,198,  319,273,  383
* 308 U16   (ERYTHRONIC)
1552
292,1 000,
205,  586,217,  190,220,  484,292,1000,489,  184
* 372 U60 (3-METHYLGLUTACONIC-PEAK 2)
1560
129,1 000,
129,1000,157,  236,170,   73,273,  122
```

```
* 351 U59  (THREONO-1,4-LACTONE)
 1573
 247,1.000,
 189, 732,219, 435,247,1000,262, 382
* 389 U17 (THREONIC)
 1579
 292,1.000,
 205, 590,220, 478,292,1000,489, 110
*  96 PHENYL-2-HYDROXYACETIC  (MANDELIC)
 1582
 253,1.000,
 105,  32,179,1000,253,  54
*  98 HEXANEDIOIC  (ADIPIC)
 1594
 275,1.000,
 159, 286,172, 425,185, 147,275,1000
* 373 U81
 1602
 117,1.000,
 117, 531,129, 392,156, 344,183,1000,218, 282,219, 292,333,  76,456,  63
* 105 3-METHYLHEXANEDIOIC  (3-METHYLADIPIC)
 1618
 269,1.000,
 155, 440,289,1000
* 104 O-HYDROXYBENZOIC
 1621
 267,1.000,
 193,  90,209,  80,267,1000
* 352 U60
 1622
 189,1.000,
 171, 422,189,1000,261, 625
* 210 U18
 1625
 261,1.000,
 143, 800,261,1000
* 107 2-HYDROXYPENTANEDIOIC  (A-HYDROXYGLUTARIC)
 1635
 349,1.000,
 203, 600,247,2200,349,1000
* 253 U61
 1635
 217,1.000,
 191, 407,204, 636,217,1000
* 110 3-HYDROXY-3-METHYLPENTANEDIOIC  (B-HYDROXY-B-METHYLGLUTARIC)
 1647
 363,1.000,
 183, 236,199,1000,273,1380,363,1530
* 313 U21
 1660
 306,1.000,
 205,1000,306, 526,335, 545

* 354 U62
 1660
 233,1.000,
 233,1000,306, 699,333, 369,335, 749,423, 114
* 374 U82
 1668
 261,1.000,
 171, 254,188, 373,261,1000
* 111 M-HYDROXYBENZOIC
 1669
 267,1.000,
 223, 412,267,1000,282, 490
* 112 2-PYRROLIDONE-5-CARBOXYLIC  (PYROGLUTAMIC)
 1673
 156,1.000,
 156,1000,258,  98
* 375 U83 (5-HYDROXYMETHYL-2-FUROIC)
 1677
 271,1.000,
 122, 332,271,1000,286,  94
* 314 U22
 1680
 245,1.000,
 205, 193,231, 308,245,1000
* 114 O-HYDROXYPHENYLACETIC
 1684
 253,1.000,
 164, 900,252,1000
* 376 U84
 1684
 287,1.000,
 170, 995,185, 424,287,1000,302,  94
* 123 TARTARIC
 1693
 292,1.000,
 219, 745,292,1000,305, 222,423, 280
* 118 HEPTANEDIOIC  (PIMELIC)
 1693
 289,1.000,
 173, 656,186, 190,199, 198,289,1000
* 120 3-HYDROXY-2-PHENYLPROPANOIC  (TROPIC)
 1700
 280,1.000,
 280,1000,295, 132
* 124 ARABONOLACTONE-PEAK 1
 1765
 246,1.000,
 231,1000,246, 810,259, 684,349, 276,364, 988
* 125 2-OXOPENTANEDIOIC OXIME  (A-KETOGLUTARIC OXIME)
 1710
 362,1.000,
 362,1000,377, 647
```

FIGURE B1. (Cont'd.)

```
* 377 U85
 1732
 292,1.000,
 175, 247,275, 142,292, 888,307, 377,333, 533,437,  88
* 127 P-HYDROXYBENZOIC
 1724
 267,1.000,
 126, 151,193, 607,223, 503,267,1000,282, 367
* 129 M-HYDROXYPHENYLACETIC
 1728
 164,1.000,
 164,1000,252, 440,281, 990,296, 990
* 316 U24
 1742
 292,1.000,
 292,1000,333, 319,363, 238
* 126 P-HYDROXYPHENYLACETIC
 1756
 296,1.000,
 252, 857,281, 863,296,1000
* 378 U86
 1766
 292,1.000,
 189, 239,217, 749,292,1000
* 135 RIBONOLACTONE
 1761
 364,1.000,
 259, 872,349,1000,364, 677
* 139 ARABONIC
 1769
 277,1.000,
 277,1000,292,7400,333,1000
* 128 2-AMINOHEXANEDIOIC  (A-AMINOADIPIC)
 1770
 260,1.000,
 128, 324,188, 150,244, 112,260,1000,272,  70,362,  75
* 141 OCTANEDIOIC  (SUBERIC)
 1788
 303,1.000,
 187, 726,303,1000
* 145 2-OXOHEXANEDIOIC OXIME  (A-KETOADIPIC OXIME)
 1792
 302,1.000,
 170,1000,184,  75,302, 124,376,  60
* 144 B-GLYCEROPHOSPHORIC
 1796
 299,1.000,
 227, 152,243,1440,299, 1000,315, 260,389, 220,445, 350
* 356 U64
 1804
 290,1.000,
 172, 219,246, 301,290,1000

* 379 U87
 1817
 292,1.000,
 219, 346,292,1000,333, 564,345,  69,423,  80
* 357 U65
 1830
 158,1.000,
 158,1000,202, 435,304, 318
* 152 A-GLYCEROPHOSPHORIC
 1834
 299,1.000,
 227,  65,299,1000,328, 110,357,1320,370, 352,387, 160,445, 410
* 318 U26
 1841
 345,1.000,
 255, 243,345,1000,435,  94
* 155 CIS-1,2,3-PROPENETRICARBOXYLIC  (CIS-ACONITIC)
 1842
 229,1.000,
 229, 989,285, 630,375,1000
* 358 U66
 1846
 273,1.000,
 220, 259,231, 535,257, 515,273, 495,378, 166
* 319 U27
 1852
 245,1.000,
 245,1000,335, 181,525,  22
* 359 U67
 1856
 277,1.000,
 262, 115,277,1000,319, 266
* 360 U68
 1864
 129,1.000,
 103, 590,117, 515,129,1000,246, 384
* 168 CITRIC
 1874
 375,1.000,
 363,1200,375,1000,465, 900
* 361 U69
 1879
 274,1.000,
 274,1000,348, 241,364, 259,376, 267
* 320 U28
 1889
 226,1.000,
  95,1000,169, 400,226, 546
* 174 NONANEDIOIC  (AZELAIC)
 1891
 317,1.000,
 152, 224,317,1000
```

FIGURE B1. (Cont'd.)

```
* 176 1-HYDROXY-1,2,3-PROPANETRICARBOXYLIC  (ISOCITRIC)
 1891
 375,1.000,
 375,1000,465,  900
* 172 1,4-BENZENEDICARBOXYLIC  (TEREPHTHALIC)
 1892
 295,1.000,
 140,  98,251,  78,295,1000
* 177 4-HYDROXY-3-METHOXYBENZOIC  (VANILLIC)
 1900
 312,1.000,
 126,  340,193,  900,253,  528,312,1000
* 181 U89
 1906
 191,1.000,
 191,1000,348,  626
* 182 U90
 1908
 297,1.000,
 297,1000,370,  230
* 321 U29
 1911
 333,1.000,
 333,1000,423,  257,432,  163,435,  151
* 184 4-HYDROXY-3-METHOXYPHENYLACETIC  (HOMOVANILLIC)
 1923
 326,1.000,
 296,  187,211,  542,326,1000
* 185 GALACTONO-1,4-LACTONE
 1924
 466,1.000,
 305,1000,361,  512,466,  428
* 183 3,4-DIHYDROXYPHENYLACETIC
 1929
 384,1.000,
 104,  111,340,  50,369,  94,384,1000
* 187 2,5-DIHYDROXYPHENYLACETIC  (HOMOGENTISIC)
 1929
 384,1.000,
 341,  264,369,  230,384,1000
* 298 3-HYDROXY-3-(3-HYDROXYPHENYL)-PROPANOIC (M-OHOHYDRACRYLIC)
 1930
 267,1.000,
 267,1000,280,  178,341,  42,383,  35,398,  170
* 181 ISOCITRIC LACTONE
 1918
 211,1.000,
 211,  212,302,1000
* 186 3,4-DIMETHOXYBENZOIC (VERATRIC)
 1930
 165,1.000,
 165,1000,195,  989,239,  951,254,  755
```

FIGURE B1. (Cont'd.)

```
* 322 U30
 1936
 345,1.000,
 255,  283,345,1000,357,  130
* 189 GALACTONOLACTONE-PEAK 2
 1940
 466,1.000,
 305,1000,334,  990,466,1500
* 198 UNK-NE5
 1952
 319,1.000,
 229,  100,319,1000,331,  100,361,  92
* 324 U32
 1955
 292,1.000,
 205,  984,292,1000,305,  448,333,  980
* 194 O-HYDROXYCINNAMIC  (O-COUMARIC)
 1955
 308,1.000,
 161,  228,308,1000
* 197 U-GALACTURONIC-PEAK 2
 1956
 292,1.000,
 292,1000,305,  428,346,  83,359,  122
* 200 GLUCONIC
 1958
 423,1.000,
 359,  320,423,  330,435,  220
* 363 U71
 1982
 287,1.000,
 287,1000,377,  189
* 207 2-HYDROXY-3-(4-HYDROXYPHENYL)-PROPANOIC  (P-HYDROXYPHENYLLACTIC)
 1983
 179,1.000,
 179,1000,281,  78,293,  190,308,  420,355,  120,383,  38
* 209 DECANEDIOIC  (SEBACIC)
 1984
 331,1.000,
 215,  745,331,1000
* 364 U72
 1990
 274,1.000,
 274,1000,301,  433,391,  109
* 216 2-HYDROXY-(4-HYDROXY-3-METHOXYPHENYL)-ACETIC  (VANILMANDELIC)
 2004
 297,1.000,
 297,1000,371,  43,399,  50
* 215 U-GALACTURONIC-PEAK 1
 2007
 305,1.000,
 292,1000,305,1000,449,  297
```

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* 218 ASCORBIC
 2009
 374,1.000,
 332,6500,345, 900,361, 600,374,1000,449,3000
* 383 U91
 2011
 333,1.000,
 333,1000,423, 149
* 225 U33 (GALACTURONIC)
 2012
 217,1.000,
 204, 800,217,1000,292, 352,305, 316
* 365 U73
 2026
 191,1.000,
 191, 892,292, 859,305,1000
* 226 U34 (GALACTURONIC)
 2032
 217,1.000,
 191, 239,204, 721,217,1000,305, 262
* 225 U-GALACTURONIC-PEAK 2
 2033
 292,1.000,
 292, 939,305,1000,449, 278
* 223 3-(3,4-DIHYDROXYPHENYL)-PROPANOIC  (HYDROCAFFEIC)
 2040
 267,1.000,
 267,1000,381, 217,398,1500
* 366 U74
 2041
 310,1.000,
 179,1000,310,  55
* 229 2-OXO-3-(4-HYDROXYPHENYL)-PROPANOIC OXIME  (P-OHPHENYLPYRUVIC)
 2066
 396,1.000,
 277,1000,396, 184
* 291 UNK 2071
 2066
 398,1.000,
 281,1000,293, 560,313,737,398, 713
* 212 HEXADECANOIC  (PALMITIC)
 2085
 313,1.000,
 145, 338,313,1000,328, 120
* 367 U75
 2088
 464,1.000,
 436, 162,464,1000,479, 682
* 235 4-HYDROXY-3-METHOXYCINNAMIC-PEAK 1  (FERULIC)
 2096
 338,1.000,
 308, 800,323, 700,338,1000

* 237 BENZAMIDOACETIC  (HIPPURIC)
 2102
 206,1.000,
 206,1000,236, 181
* 236 3,4-DIHYDROXYCINNAMIC-PEAK 1  (CAFFEIC)
 2109
 396,1.000,
 307, 130,381, 226,396,1000
* 328 U36
 2110
 428,1.000,
 297,1000,413,  31,428, 100
* 368 U76 (HYDROXYDECRNEDIOIC?)
 2128
 419,1.000,
 233,1000,287, 364,303, 483,419, 585
* 369 U77
 2150
 284,1.000,
 173, 281,284,1000
* 329 U37
 2156
 327,1.000,
 327,1000,328, 315,329, 105
* 244 3-INDOLYLACETIC
 2189
 202,1.000,
 202,1000,304,  80,319, 300
* 246 UNK-NE8
 2224
 130,1.000,
 130,1000,232,  78,247, 318
* 251 3,4-DIHYDROXYCINNAMIC-PEAK 2  (CAFFEIC)
 2238
 396,1.000,
 307,  99,381, 234,396,1000
* 254 3-(4-IMIDAZOLYL)-PROPENOIC  (UROCANIC)
 2240
 195,1.000,
 121, 727,151, 343,195,1000,210, 276
* 257 2,6,8-TRIHYDROXYPURINE  (URIC)
 2260
 456,1.000,
 353,  31,367,  67,382, 169,427,  29,441, 803,456,1000
* 258 4-HYDROXY-3-METHOXYCINNAMIC-PEAK 2  (FERULIC)
 2268
 338,1.000,
 279, 118,293, 213,308, 471,323, 477,338,1000
* 332 U40
 2345
 247,1.000,
 157, 320,247,1000,499,  80,514,  50
```

FIGURE B1. (Cont'd.)

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* 323 U41
 2355
 217,1.000,
 204, 966.217,1000,305, 146,375, 163
* 266 M-HYDROXYBENZAMIDOACETIC  (M-HYDROXYHIPPURIC)
 2371
 294,1.000,
 165,  74,193, 863,234,  51,294,1000,324, 220,339, 267
* 334 U42
 2397
 169,1.000,
 169,1000,204, 260,305, 134,375, 104
* 274 3,4,5-TRIMETHOXYCINNAMIC-PEAK 2
 2482
 318,1.000,
 177, 150,221, 160,318,1000
* 272 (5-HYDROXY-3-INDOLYL)-ACETIC
 2418
 290,1.000,
 202,  46,290,1000,364,  26,392,  70,407, 500
* 277 3,4,5-TRIHYDROXYCYLOHEXENOIC  (SHIKIMIC)
 2461
 372,1.000,
 357,  29,372,  32
```

FIGURE B1. (Cont'd.)

APPENDIX C

Compounds excluded from statistical calculations

The compounds listed in Figure C1 were excluded when using the t-test to measure the significance of differences in mean concentrations between subject groups. These are compounds whose concentration values are suspected to be unreliable, based on internal evidence in the MSSMET data.

Figure C1. Compounds excluded from statistical calculations.*

Number	Name	Reason**
33	γ -hydroxybutyric	2
34	unknown RA 183	4
35	methylmalonic	4
36	malonic	1
65	maleic	1
66	phenylacetic	4
67	nicotinic	4
79	3, 3-dimethylglutaric	2
82	citramalic	4
96	mandelic	1
138	α -aminoadipic	1
145	α -ketoadipic oxime	1
181	isocitric lactone	1
187	homogentisic	2
209	sebacic	4
229	p-hydroxyphenylpyruvic oxime	1
236	caffeic-peak 1	2
254	urocanic	2
274	3,4,5-trimethoxycinnamic-peak 2	2

Figure C1. (Cont'd.)

Number	Name	Reason**
277	shikimic	1
297	unknown U5 (cresol)	3
299	unknown U7	2
320	unknown U28	1
332	unknown U40	2
344	unknown U52	2
345	unknown U53	3
348	unknown U56 (deoxythreonic)	1
354	unknown U62	2
356	unknown U64	3
358	unknown U66	2
363	unknown U71	3
366	unknown U74	2
368	unknown U76 (hydroxydecanedioic)	2
373	unknown U81	1
377	unknown U85	2

Figure C1. (Cont'd.)

* Compounds listed were excluded from consideration when comparing subject groups using the t-test. Compounds were selected by manual examination of MSSMET and statistical test data.

** 1 Found in less than 25% of urine samples.

2 Mean match coefficient too low.

3 Mean retention index too far from library retention index.

4 Mean area only slightly above detection limit.

APPENDIX D

Summary of urine samples analyzed

The data listed in Table D1 were collected from the questionnaires (Appendix A) completed by the BCIU subjects and from the attending physician (Dr. Krivit) for the juvenile and neuroblastoma subjects.

Table D1. Summary of urine samples analyzed

GC-MS run number	Sample number	Patient ID	Age or age group (years)	Sex	Creatinine (mg/ml)	Group	Comments
072505	07076N4	115	51-60	F	0.98	B	Receiving estrogens
072506	07086N1	074	41-50	M	1.88	B	Drug-free
072601	07086N2	087	21-30	F	1.40	B	Asthmatic; consumed asprin; smokes cigarettes
072802	06306N7	056	21-30	M	1.42	B	Smokes pipe
072902	06303N8	052	51-60	M	1.32	B	Gout in large toe
091802	09076N3	MG	4 1/2	M	2.13	N	*, T 8 mo., relapsed
091901	07076N6	120	31-40	F	1.10	B	Drug-free
091902	09076N1	MM	2	F	1.20	N	*, T 1 yr., "cured"
102903	09076N7	RG	2	M	1.04	N	*, T 2 mo., deceased
103101	07086N7	080	41-50	F	1.28	B	Hysterectomy, estrogens

Table D1. (Cont'd.)

GC-MS run number	Sample number	Patient ID	Age or age group (years)	Sex	Creatinine (mg/ml)	Group	Comments
103102	09076N5	MM	2	F	0.18	N	*, T 1 yr., "cured"
103103	09076N8	JJ	1 1/2	M	0.15	N	*, T 3 mo., deceased
103106	06306N4	060	31-40	M	3.00	B	Drug-free
111001	07076N2	113	31-40	F	1.20	B	Sterilized
031603	K0451	SW	1	M	-	J	Retinoblastoma
031604	K0381	PO	13	F	-	J	Osteomyelitis, staph.; Gentamycin, Oracillin IV
033004	M001	Foy	1 mo	F	-	F	Uncontrolled seizures
050201	K0411	SJ	2	M	-	J	Otitis media; ampicillin
050202	K0431	JS	6	F	-	J	Acute leukemia; cortisone,

Table D1. (Cont'd.)

GC-MS run number	Sample number	Patient ID	Age or age group (years)	Sex	Creatinine (mg/ml)	Group	Comments
050203	K0401	DM	1	M	-	J	Perforated duodenum, hypercalimentation; ampicillin, gentomycin
050205	K0391	JE	4	F	-	J	Cystic fibrosis, liver, lung disease; vitamins A,E, Gantrisin, Tegopen, Cutyzyse, Vistaril

Key: M Male
F Female
B BCIU
N Neuroblastoma
J Juvenile control
F Foy
* Last dose of drugs received 3 weeks prior to urine collection. Drugs are Vincristine, Cytoxan and DTIC
T Length of drug therapy
- Not measured

APPENDIX E

Relative areas calculated by MSSMET

Twenty-one urine samples were analyzed by MSSMET using BESTLIB. The relative areas reported by MSSMET are included as Table E1. The compound number appears on the left-hand side of the table (refer to Appendix B for the names of the compounds). The run number of each sample is printed across the top of the table; the first six digits refer to the specific sample (see Appendix D), while the seventh digit denotes the type of subject (2 for BCIU, 3 for neuroblastoma, 4 for juvenile control, 6 for Foy urine and 7 for replicate GC-MS analyses of the same sample). Note that one urine (031603) is entered twice in the table, once in class 4 and once in class 7. All values are designate ion areas relative to the area of the designate ion of the tropic acid internal standard, and have been normalized to include creatinine concentration for the BCIU and neuroblastoma groups and urine volume for the juvenile control and Foy urines. The table was produced from MSSMET outputs using the programs RAMAST and RASECD.

TABLE E1. Relative areas calculated by MSSMET

	0725052	0725062	0726012	0728022	0729022
6	0.500E 01	0.421E 01	0.565E 01	0.413E 01	0.869E 01
8	0.929E 01	0.295E 01	0.150E 01	0.208E 02	0.996E 01
10	0.957E 01	0.118E 02	0.416E 01	0.692E 01	0.788E 01
17	0.246E 00	0.000E 00	0.108E 00	0.000E 00	0.000E 00
18	0.311E 01	0.000E 00	0.654E 00	0.749E 02	0.350E 02
23	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
28	0.227E 01	0.142E 01	0.945E 00	0.254E 01	0.276E 01
29	0.000E 00	0.000E 00	0.560E-01	0.191E-01	0.000E 00
33	0.000E 00	0.000E 00	0.220E 00	0.110E 00	0.000E 00
34	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
35	0.209E-01	0.000E 00	0.101E 00	0.412E-01	0.000E 00
36	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
42	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
50	0.502E 02	0.525E 02	0.508E 02	0.696E 02	0.248E 02
51	0.879E 00	0.489E 00	0.559E 00	0.915E 00	0.108E 01
59	0.259E 00	0.328E 00	0.321E 00	0.561E 00	0.127E 01
60	0.184E 01	0.000E 00	0.265E 01	0.136E 01	0.357E 01
61	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
65	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
66	0.888E-02	0.000E 00	0.000E 00	0.000E 00	0.000E 00
67	0.000E 00	0.000E 00	0.134E 00	0.000E 00	0.000E 00
77	0.599E 00	0.000E 00	0.362E 00	0.646E 00	0.948E 00
79	0.326E-01	0.305E-01	0.113E 00	0.329E-01	0.101E 00
82	0.000E 00	0.000E 00	0.340E 00	0.000E 00	0.129E 00
86	0.565E 00	0.117E 01	0.868E 00	0.123E 01	0.179E 01
96	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
98	0.143E 00	0.000E 00	0.000E 00	0.137E 01	0.123E 01
104	0.000E 00	0.000E 00	0.167E 00	0.000E 00	0.000E 00
105	0.585E 00	0.512E 00	0.678E 00	0.880E 00	0.132E 01
107	0.000E 00	0.000E 00	0.412E 01	0.312E 01	0.511E 01
110	0.576E 00	0.328E 00	0.137E 01	0.129E 01	0.136E 01
111	0.789E 00	0.330E 00	0.793E 00	0.218E 00	0.146E 01
112	0.000E 00	0.000E 00	0.000E 00	0.205E 00	0.129E 01
114	0.434E 00	0.273E 00	0.465E 00	0.563E 00	0.333E 00
118	0.887E 00	0.358E 00	0.000E 00	0.564E 00	0.000E 00
120	0.350E 02	0.348E 02	0.348E 02	0.351E 02	0.350E 02
123	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
124	0.184E 01	0.328E 00	0.341E 00	0.155E 01	0.197E 01
125	0.000E 00	0.000E 00	0.656E-01	0.360E-01	0.239E 00
127	0.215E 01	0.151E 01	0.540E 01	0.525E 01	0.249E 01
129	0.951E 00	0.583E 00	0.108E 01	0.143E 01	0.474E 01
135	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.487E 00
136	0.776E 01	0.529E 01	0.650E 01	0.458E 01	0.835E 01
138	0.000E 00	0.121E 00	0.928E-01	0.000E 00	0.200E 00
139	0.260E 01	0.204E 01	0.269E 01	0.258E 01	0.567E 01
141	0.116E 01	0.223E 00	0.440E 00	0.127E 01	0.176E 01
144	0.187E 01	0.000E 00	0.258E 00	0.218E 01	0.121E 01
152	0.693E 01	0.103E 02	0.495E 01	0.809E 01	0.983E 01
155	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
168	0.000E 00	0.000E 00	0.195E 02	0.000E 00	0.748E 01

	0919012	1031012	1031062	1110012	0918023
6	0.266E 01	0.402E 01	0.594E 01	0.201E 01	0.265E 01
8	0.999E 01	0.818E 01	0.974E 01	0.116E 02	0.235E 01
10	0.710E 01	0.149E 02	0.102E 02	0.755E 01	0.631E 01
17	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.811E 00
18	0.541E 02	0.523E 02	0.484E 02	0.550E 02	0.692E 01
23	0.203E 01	0.000E 00	0.000E 00	0.000E 00	0.191E 01
28	0.201E 01	0.267E 01	0.152E 01	0.122E 01	0.121E 01
29	0.526E 00	0.000E 00	0.000E 00	0.200E 00	0.628E-01
33	0.000E 00	0.581E 00	0.167E 00	0.000E 00	0.398E 00
34	0.237E 00	0.000E 00	0.000E 00	0.000E 00	0.146E 00
35	0.226E 00	0.725E 00	0.161E 00	0.000E 00	0.286E 00
36	0.355E-01	0.125E 00	0.000E 00	0.000E 00	0.198E 00
42	0.237E 01	0.000E 00	0.000E 00	0.000E 00	0.211E 00
50	0.183E 02	0.391E 03	0.925E 02	0.704E 02	0.794E 02
51	0.172E 01	0.122E 01	0.000E 00	0.000E 00	0.366E 00
59	0.112E 01	0.185E 01	0.891E 00	0.785E 00	0.147E 01
60	0.106E 02	0.918E 01	0.333E 01	0.566E 01	0.644E 01
61	0.147E 01	0.399E 01	0.120E 01	0.113E 01	0.192E 01
65	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
66	0.152E 00	0.950E-01	0.000E 00	0.197E 00	0.654E-01
67	0.295E 00	0.241E 00	0.000E 00	0.211E 00	0.294E 00
77	0.109E 01	0.789E 00	0.819E 00	0.842E 00	0.151E 01
79	0.260E-01	0.111E 00	0.000E 00	0.000E 00	0.765E-01
82	0.251E 00	0.255E 00	0.000E 00	0.000E 00	0.265E 00
86	0.380E 01	0.422E 01	0.179E 01	0.845E 00	0.283E 01
96	0.196E 00	0.000E 00	0.000E 00	0.000E 00	0.274E-01
98	0.886E 00	0.510E 01	0.000E 00	0.102E 02	0.515E 01
104	0.184E 01	0.129E 00	0.209E 00	0.423E 00	0.151E 00
105	0.151E 01	0.186E 01	0.104E 01	0.000E 00	0.191E 01
107	0.349E 01	0.735E 01	0.000E 00	0.000E 00	0.587E 01
110	0.165E 01	0.252E 01	0.144E 01	0.887E 00	0.456E 01
111	0.654E 00	0.256E 00	0.000E 00	0.102E 01	0.468E 00
112	0.449E 02	0.476E 00	0.000E 00	0.937E 01	0.126E 02
114	0.569E 00	0.354E 00	0.349E 00	0.245E 00	0.145E 00
118	0.346E 00	0.181E 01	0.000E 00	0.489E 00	0.218E 01
120	0.348E 02	0.347E 02	0.347E 02	0.351E 02	0.226E 02
123	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.450E 00
124	0.538E 01	0.138E 01	0.000E 00	0.291E 01	0.249E 01
125	0.214E 00	0.765E 01	0.878E 00	0.000E 00	0.685E 01
127	0.215E 01	0.783E 01	0.271E 01	0.162E 01	0.325E 01
129	0.496E 01	0.222E 01	0.159E 01	0.322E 00	0.142E 01
135	0.118E 01	0.000E 00	0.000E 00	0.979E 00	0.122E 01
136	0.520E 01	0.838E 01	0.900E 01	0.424E 01	0.201E 01
138	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
139	0.466E 00	0.380E 01	0.729E 01	0.000E 00	0.100E 01
141	0.108E 01	0.381E 01	0.101E 01	0.512E 00	0.284E 01
144	0.188E 01	0.373E 01	0.159E 01	0.542E 00	0.385E 01
152	0.882E 01	0.937E 01	0.848E 01	0.450E 01	0.226E 02
155	0.461E 01	0.849E 01	0.247E 01	0.271E 01	0.101E 02
168	0.394E 02	0.332E 02	0.469E 01	0.237E 02	0.102E 02

TABLE E1. (Cont'd.)

	0519023	1029033	1031023	1031033	0216024
6	0.328E 00	0.377E 00	0.235E 01	0.397E 01	0.342E 01
8	0.701E 00	0.196E 01	0.224E 02	0.129E 02	0.555E 01
10	0.584E 00	0.108E 01	0.170E 02	0.816E 01	0.109E 02
17	0.160E 00	0.240E 00	0.164E 01	0.211E 01	0.642E 00
18	0.244E 00	0.267E 00	0.919E 01	0.122E 01	0.000E 00
23	0.928E 00	0.123E 01	0.130E 02	0.784E 01	0.260E 01
28	0.238E 00	0.620E 00	0.546E 01	0.324E 01	0.272E 01
29	0.000E 00	0.557E-01	0.000E 00	0.000E 00	0.000E 00
33	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
34	0.965E-01	0.136E 00	0.655E 00	0.000E 00	0.394E 00
35	0.539E-01	0.000E 00	0.446E 00	0.000E 00	0.000E 00
36	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
42	0.379E-01	0.632E 00	0.273E 01	0.157E 01	0.210E 01
50	0.750E 02	0.102E 03	0.121E 04	0.589E 02	0.962E 02
51	0.171E 00	0.151E 00	0.379E 01	0.000E 00	0.693E 00
59	0.169E 00	0.388E 00	0.178E 01	0.201E 01	0.983E 00
60	0.226E 01	0.232E 01	0.389E 02	0.105E 02	0.467E 01
61	0.724E 00	0.952E 00	0.717E 01	0.701E 01	0.346E 00
65	0.146E-01	0.393E-01	0.720E 00	0.782E 00	0.000E 00
66	0.247E-01	0.000E 00	0.252E 00	0.000E 00	0.000E 00
67	0.121E 00	0.138E 00	0.000E 00	0.000E 00	0.278E 00
77	0.393E 00	0.518E 00	0.322E 01	0.236E 01	0.989E 00
79	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
82	0.644E-01	0.232E 00	0.110E 01	0.000E 00	0.235E 00
86	0.979E 00	0.154E 01	0.173E 02	0.690E 01	0.162E 01
96	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
98	0.449E 00	0.912E 00	0.261E 01	0.286E 01	0.107E 01
104	0.000E 00	0.449E 00	0.213E 02	0.692E 00	0.000E 00
105	0.171E 00	0.352E 00	0.185E 01	0.203E 01	0.638E 00
107	0.116E 01	0.252E 01	0.228E 02	0.168E 02	0.256E 01
110	0.784E 00	0.183E 01	0.968E 01	0.834E 01	0.234E 01
111	0.486E 00	0.548E 00	0.388E 01	0.466E 01	0.000E 00
112	0.864E 01	0.247E 02	0.669E 02	0.685E 02	0.196E 02
114	0.619E-01	0.109E 00	0.701E 00	0.869E 00	0.297E 00
118	0.949E-01	0.662E 00	0.000E 00	0.123E 01	0.711E 00
120	0.347E 02	0.481E 02	0.278E 03	0.334E 03	0.502E 02
123	0.226E 01	0.401E 01	0.000E 00	0.125E 02	0.229E 00
124	0.107E 01	0.116E 01	0.230E 02	0.408E 01	0.247E 01
125	0.751E 00	0.713E 01	0.204E 02	0.235E 02	0.214E 01
127	0.389E 01	0.264E 01	0.219E 02	0.387E 02	0.175E 01
129	0.708E 00	0.326E 00	0.459E 01	0.465E 01	0.578E 00
135	0.268E 00	0.332E 00	0.151E 02	0.136E 01	0.104E 01
136	0.180E 01	0.189E 01	0.173E 02	0.197E 02	0.657E 01
138	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
139	0.379E 00	0.969E 00	0.822E 01	0.552E 01	0.496E 00
141	0.202E 00	0.868E 00	0.208E 02	0.281E 01	0.124E 01
144	0.641E 00	0.153E 01	0.448E 01	0.695E 01	0.267E 01
152	0.492E 01	0.112E 02	0.415E 02	0.375E 02	0.137E 02
155	0.203E 01	0.221E 01	0.000E 00	0.169E 01	0.000E 00
168	0.145E 02	0.462E 02	0.286E 03	0.655E 02	0.122E 02

	0316044	0502014	0502034	0502054	0230046
6	0.169E 02	0.963E 01	0.394E 01	0.336E 01	0.193E 01
8	0.128E 02	0.482E 01	0.859E 01	0.109E 02	0.834E 01
10	0.165E 02	0.116E 02	0.570E 01	0.798E 01	0.416E 01
17	0.000E 00	0.731E 00	0.334E 00	0.653E 00	0.973E 00
18	0.178E 02	0.000E 00	0.000E 00	0.000E 00	0.136E 01
23	0.386E 01	0.188E 01	0.189E 02	0.247E 02	0.165E 02
28	0.289E 01	0.182E 01	0.128E 01	0.418E 01	0.359E 01
29	0.000E 00	0.000E 00	0.626E 00	0.000E 00	0.000E 00
33	0.000E 00	0.000E 00	0.000E 00	0.420E 00	0.113E 01
34	0.170E 00	0.000E 00	0.000E 00	0.269E 00	0.000E 00
35	0.000E 00	0.000E 00	0.000E 00	0.335E 00	0.000E 00
36	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
42	0.261E 01	0.517E 00	0.371E 01	0.120E 01	0.239E 01
50	0.127E 03	0.852E 02	0.121E 02	0.128E 03	0.372E 03
51	0.352E 01	0.900E 00	0.000E 00	0.204E 01	0.372E 00
59	0.175E 01	0.131E 01	0.000E 00	0.217E 01	0.824E 00
60	0.817E 01	0.753E 01	0.163E 02	0.274E 02	0.104E 02
61	0.327E 01	0.114E 01	0.274E 01	0.112E 02	0.291E 01
65	0.000E 00	0.000E 00	0.211E 00	0.000E 00	0.000E 00
66	0.267E 00	0.000E 00	0.142E 00	0.312E 00	0.422E 00
67	0.552E 00	0.000E 00	0.716E 00	0.168E 00	0.000E 00
77	0.768E 00	0.106E 01	0.160E 01	0.144E 02	0.946E 00
79	0.133E 00	0.000E 00	0.000E 00	0.000E 00	0.412E 00
82	0.328E 00	0.203E 00	0.113E 00	0.102E 01	0.000E 00
86	0.387E 01	0.162E 01	0.632E 01	0.131E 02	0.000E 00
96	0.147E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
98	0.202E 01	0.269E 01	0.146E 01	0.374E 01	0.195E 01
104	0.628E 00	0.142E 00	0.777E-01	0.227E 00	0.878E 00
105	0.158E 01	0.921E 00	0.109E 00	0.825E 00	0.588E 00
107	0.436E 01	0.322E 01	0.417E 01	0.285E 02	0.000E 00
110	0.277E 01	0.237E 01	0.149E 01	0.351E 01	0.180E 02
111	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.578E 00
112	0.174E 03	0.418E 01	0.540E 02	0.104E 03	0.564E 01
114	0.466E 00	0.351E 00	0.117E 00	0.629E 00	0.269E 00
118	0.192E 01	0.682E 00	0.000E 00	0.228E 01	0.189E 01
120	0.501E 02	0.500E 02	0.500E 02	0.500E 02	0.500E 02
123	0.000E 00	0.000E 00	0.425E 00	0.000E 00	0.000E 00
124	0.102E 01	0.361E 01	0.143E 01	0.000E 00	0.000E 00
125	0.754E 01	0.131E 01	0.197E 00	0.398E 02	0.000E 00
127	0.179E 02	0.231E 01	0.533E 00	0.217E 02	0.218E 01
129	0.475E 00	0.123E 01	0.363E 00	0.115E 01	0.119E 01
135	0.000E 00	0.106E 01	0.000E 00	0.000E 00	0.647E 00
136	0.626E 01	0.238E 01	0.215E 01	0.567E 02	0.186E 02
138	0.278E 01	0.000E 00	0.000E 00	0.149E 01	0.000E 00
139	0.679E 01	0.000E 00	0.936E 00	0.660E 01	0.599E 01
141	0.237E 01	0.157E 01	0.000E 00	0.405E 01	0.324E 01
144	0.191E 01	0.216E 01	0.257E 01	0.561E 01	0.876E 01
152	0.195E 02	0.138E 02	0.564E 01	0.258E 02	0.474E 02
155	0.697E 01	0.000E 00	0.000E 00	0.116E 02	0.000E 00
168	0.000E 00	0.138E 02	0.111E 02	0.308E 02	0.102E 02

TABLE E1. (Cont'd.)

	0316037	0323027
6	0.342E 01	0.356E 01
8	0.555E 01	0.609E 01
10	0.109E 02	0.112E 02
17	0.642E 00	0.570E 00
18	0.000E 00	0.000E 00
23	0.260E 01	0.264E 01
28	0.272E 01	0.299E 01
29	0.000E 00	0.138E 00
33	0.000E 00	0.000E 00
34	0.394E 00	0.000E 00
35	0.000E 00	0.000E 00
36	0.000E 00	0.000E 00
42	0.210E 01	0.281E 01
50	0.963E 02	0.102E 03
51	0.693E 00	0.115E 01
59	0.903E 00	0.103E 01
60	0.467E 01	0.521E 01
61	0.846E 00	0.103E 01
65	0.000E 00	0.000E 00
66	0.000E 00	0.000E 00
67	0.278E 00	0.395E 00
77	0.989E 00	0.118E 01
79	0.000E 00	0.103E 00
82	0.235E 00	0.323E 00
86	0.162E 01	0.172E 01
96	0.000E 00	0.120E 00
98	0.107E 01	0.125E 01
104	0.000E 00	0.185E 00
105	0.688E 00	0.841E 00
107	0.356E 01	0.373E 01
110	0.234E 01	0.254E 01
111	0.000E 00	0.000E 00
112	0.196E 02	0.413E 02
114	0.297E 00	0.315E 00
118	0.711E 00	0.668E 00
120	0.502E 02	0.500E 02
123	0.229E 00	0.218E 00
124	0.247E 01	0.270E 01
125	0.214E 01	0.259E 01
127	0.175E 01	0.206E 01
129	0.578E 00	0.678E 00
135	0.104E 01	0.902E 00
136	0.657E 01	0.696E 01
138	0.000E 00	0.000E 00
139	0.496E 00	0.000E 00
141	0.124E 01	0.151E 01
144	0.267E 01	0.348E 01
152	0.137E 02	0.157E 02
155	0.000E 00	0.000E 00
168	0.122E 02	0.131E 02

	0725052	0725062	0726012	0729022	0729022
172	0.184E 00	0.298E 00	0.317E 01	0.614E 00	0.000E 00
174	0.161E 01	0.982E 00	0.272E 00	0.446E 00	0.116E 01
176	0.000E 00	0.263E-01	0.000E 00	0.533E-01	0.172E 01
177	0.141E 01	0.155E 01	0.000E 00	0.160E 01	0.176E 01
181	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
183	0.431E 01	0.178E 01	0.317E 01	0.180E 01	0.313E 01
184	0.348E 01	0.309E 01	0.298E 01	0.363E 01	0.385E 01
185	0.981E 00	0.407E 00	0.000E 00	0.953E 00	0.175E 01
186	0.157E 01	0.420E 00	0.131E 01	0.152E 01	0.222E 01
187	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
189	0.000E 00	0.105E 01	0.149E 01	0.000E 00	0.000E 00
194	0.000E 00	0.000E 00	0.671E 00	0.838E 00	0.198E 01
197	0.105E 02	0.000E 00	0.983E 01	0.117E 02	0.155E 02
198	0.720E 01	0.000E 00	0.728E 01	0.888E 01	0.127E 02
200	0.207E 01	0.000E 00	0.345E 01	0.167E 01	0.000E 00
207	0.848E 01	0.297E 01	0.387E 01	0.106E 02	0.887E 01
209	0.000E 00	0.000E 00	0.000E 00	0.153E 00	0.248E 00
212	0.000E 00	0.140E 01	0.119E 01	0.000E 00	0.000E 00
215	0.124E 02	0.103E 02	0.103E 02	0.000E 00	0.343E 02
216	0.126E 02	0.981E 01	0.487E 01	0.577E 01	0.139E 02
218	0.189E 01	0.167E 01	0.123E 01	0.177E 01	0.000E 00
223	0.244E 01	0.185E 01	0.552E 01	0.145E 01	0.286E 01
225	0.568E 01	0.000E 00	0.118E 02	0.129E 02	0.273E 02
229	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
235	0.256E 00	0.226E 00	0.370E 00	0.281E 00	0.639E 00
236	0.458E-01	0.721E-01	0.648E-01	0.230E 00	0.135E 00
237	0.345E 03	0.139E 00	0.000E 00	0.120E 03	0.712E 03
244	0.221E 00	0.328E-01	0.303E 01	0.112E 01	0.331E 01
246	0.449E-01	0.603E-01	0.584E 01	0.807E 01	0.120E 02
251	0.000E 00	0.000E 00	0.933E 00	0.104E 00	0.512E-01
254	0.341E-01	0.000E 00	0.309E-01	0.114E 00	0.202E 00
257	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.127E 00
258	0.113E 01	0.000E 00	0.123E 01	0.952E 00	0.258E 01
266	0.000E 00	0.000E 00	0.403E 02	0.000E 00	0.248E 02
272	0.000E 00	0.000E 00	0.271E 01	0.636E 00	0.164E 01
274	0.248E 00	0.335E 00	0.308E 00	0.318E 00	0.000E 00
277	0.000E 00	0.763E-01	0.111E 00	0.000E 00	0.000E 00
290	0.182E 03	0.713E 02	0.157E 03	0.589E 02	0.166E 03
291	0.000E 00	0.822E 00	0.000E 00	0.140E 01	0.192E 01
293	0.197E 01	0.000E 00	0.102E 00	0.788E 00	0.000E 00
294	0.446E 01	0.134E 01	0.549E 00	0.191E 01	0.670E 00
296	0.113E 01	0.000E 00	0.107E 01	0.196E 01	0.131E 01
297	0.457E 02	0.659E 02	0.000E 00	0.135E 00	0.306E 02
298	0.169E 01	0.148E 01	0.132E 01	0.214E 01	0.161E 01
299	0.000E 00	0.000E 00	0.000E 00	0.164E 01	0.804E 00
301	0.733E 00	0.742E 00	0.142E 00	0.246E 01	0.321E 00
302	0.418E 02	0.238E 02	0.121E 02	0.212E 02	0.334E 02
303	0.438E 01	0.313E 02	0.172E 02	0.404E 02	0.364E 02
305	0.000E 00	0.798E 01	0.735E 01	0.733E 01	0.782E 01
306	0.158E 02	0.179E 02	0.101E 02	0.133E 02	0.171E 02

TABLE E1. (Cont'd.)

	0919012	1031012	1021062	1110012	0918023
172	0 691E 00	0 132E 01	0 565E 00	0 611E 00	0 563E 00
174	0 106E 01	0 227E 01	0 907E 00	0 813E 00	0 225E 01
176	0 000E 00	0 237E 02	0 136E 01	0 000E 00	0 624E 01
177	0 876E 00	0 113E 01	0 168E 01	0 264E 01	0 123E 01
181	0 000E 00	0 000E 00	0 000E 00	0 000E 00	0 000E 00
183	0 000E 00	0 449E 01	0 455E 01	0 905E 01	0 000E 00
184	0 365E 01	0 569E 01	0 481E 01	0 215E 01	0 134E 02
185	0 251E 01	0 125E 01	0 434E 00	0 000E 00	0 000E 00
186	0 597E 00	0 108E 01	0 962E 00	0 322E 01	0 000E 00
187	0 000E 00	0 449E 01	0 000E 00	0 000E 00	0 000E 00
189	0 000E 00	0 219E 01	0 132E 01	0 000E 00	0 000E 00
194	0 935E 00	0 113E 01	0 136E 01	0 000E 00	0 103E 01
197	0 915E 01	0 111E 02	0 118E 02	0 000E 00	0 844E 01
198	0 740E 01	0 536E 01	0 111E 02	0 000E 00	0 525E 01
200	0 110E 01	0 297E 01	0 709E 01	0 000E 00	0 000E 00
207	0 607E 01	0 484E 01	0 269E 01	0 000E 00	0 620E 01
209	0 000E 00	0 484E 00	0 000E 00	0 000E 00	0 000E 00
212	0 124E 01	0 330E 01	0 152E 01	0 179E 01	0 518E 00
215	0 155E 02	0 270E 02	0 247E 02	0 111E 02	0 774E 01
216	0 108E 02	0 206E 02	0 138E 02	0 864E 01	0 285E 02
218	0 187E 01	0 241E 01	0 225E 01	0 151E 01	0 727E 00
223	0 515E 00	0 966E 00	0 898E 01	0 269E 02	0 857E 00
225	0 152E 02	0 188E 02	0 127E 02	0 209E 02	0 110E 02
229	0 000E 00	0 000E 00	0 000E 00	0 000E 00	0 526E-01
235	0 000E 00	0 808E 00	0 915E 00	0 461E 01	0 817E-01
236	0 162E 00	0 413E 00	0 000E 00	0 421E 00	0 148E 00
237	0 473E 03	0 477E 03	0 305E 03	0 139E 04	0 195E 03
244	0 207E 02	0 342E 01	0 251E 01	0 398E 02	0 785E 01
246	0 469E 01	0 276E 01	0 162E 01	0 511E 00	0 831E 01
251	0 894E 00	0 000E 00	0 000E 00	0 153E 01	0 117E 01
254	0 778E 00	0 000E 00	0 000E 00	0 000E 00	0 000E 00
257	0 143E 03	0 000E 00	0 000E 00	0 529E 03	0 777E 02
258	0 000E 00	0 145E 01	0 256E 01	0 319E 02	0 000E 00
266	0 207E 02	0 000E 00	0 279E 02	0 444E 02	0 350E 02
272	0 697E 01	0 289E 01	0 157E 01	0 299E 01	0 000E 00
274	0 276E 00	0 000E 00	0 000E 00	0 957E 00	0 814E-01
277	0 000E 00	0 000E 00	0 000E 00	0 000E 00	0 000E 00
290	0 298E 02	0 650E 02	0 152E 03	0 575E 03	0 448E 02
291	0 962E 00	0 327E 01	0 000E 00	0 000E 00	0 840E 00
293	0 205E 01	0 997E 00	0 234E 01	0 240E 01	0 536E-01
294	0 786E 00	0 142E 01	0 292E 01	0 167E 01	0 420E 00
296	0 222E 01	0 266E 01	0 112E 01	0 000E 00	0 192E 01
297	0 912E 02	0 251E 02	0 512E 02	0 356E 02	0 695E 02
298	0 852E 00	0 172E 01	0 174E 01	0 933E 00	0 601E 00
299	0 141E 01	0 153E 01	0 000E 00	0 187E 01	0 000E 00
301	0 249E 01	0 141E 01	0 295E 01	0 704E 00	0 101E 01
302	0 295E 02	0 250E 02	0 163E 02	0 201E 02	0 164E 02
303	0 258E 02	0 172E 02	0 315E 02	0 324E 02	0 145E 02
305	0 207E 01	0 124E 02	0 118E 02	0 267E 00	0 228E 01
306	0 144E 02	0 223E 02	0 136E 02	0 689E 01	0 246E 02

	0919023	1029033	1031023	1031033	0216034
172	0 122E 00	0 497E 00	0 301E 01	0 112E 01	0 208E 00
174	0 208E 00	0 152E 01	0 468E 01	0 222E 01	0 155E 01
176	0 224E 01	0 408E 01	0 000E 00	0 131E 02	0 302E 01
177	0 138E 01	0 205E 01	0 241E 02	0 000E 00	0 122E 01
181	0 000E 00	0 000E 00	0 000E 00	0 000E 00	0 000E 00
183	0 788E 01	0 247E 01	0 266E 02	0 108E 02	0 000E 00
184	0 336E 02	0 871E 01	0 411E 02	0 995E 02	0 629E 01
185	0 000E 00	0 122E 01	0 837E 01	0 677E 01	0 581E 01
186	0 568E 00	0 782E 00	0 765E 01	0 000E 00	0 270E 00
187	0 708E 01	0 247E 01	0 268E 02	0 108E 02	0 156E 01
189	0 000E 00	0 157E 01	0 188E 02	0 795E 01	0 000E 00
194	0 423E 00	0 858E 00	0 451E 01	0 603E 01	0 124E 01
197	0 512E 01	0 126E 02	0 627E 02	0 842E 02	0 171E 02
198	0 255E 01	0 102E 02	0 447E 02	0 672E 02	0 122E 02
200	0 958E 00	0 388E 01	0 240E 02	0 262E 02	0 223E 01
207	0 828E 00	0 126E 01	0 632E 01	0 740E 01	0 494E 01
209	0 000E 00	0 161E 00	0 000E 00	0 000E 00	0 000E 00
212	0 467E 00	0 842E 00	0 952E 01	0 861E 01	0 105E 01
215	0 377E 01	0 118E 02	0 846E 02	0 511E 02	0 140E 02
216	0 680E 02	0 212E 02	0 100E 04	0 722E 02	0 158E 02
218	0 115E 00	0 000E 00	0 000E 00	0 415E 01	0 137E 01
223	0 151E 00	0 259E 00	0 000E 00	0 314E 01	0 814E 00
225	0 427E 01	0 119E 02	0 865E 02	0 490E 02	0 120E 02
229	0 294E-01	0 000E 00	0 000E 00	0 000E 00	0 000E 00
235	0 181E 00	0 118E 00	0 000E 00	0 219E 01	0 240E 00
236	0 127E 00	0 610E 00	0 121E 01	0 612E 00	0 000E 00
237	0 282E 02	0 703E 02	0 126E 03	0 600E 03	0 140E 03
244	0 116E 01	0 201E 01	0 208E 02	0 861E 01	0 242E 02
246	0 704E 00	0 188E 00	0 570E 01	0 000E 00	0 451E 00
251	0 808E 00	0 164E 01	0 112E 02	0 121E 02	0 000E 00
254	0 000E 00	0 000E 00	0 000E 00	0 000E 00	0 221E 00
257	0 000E 00	0 183E 03	0 000E 00	0 896E 03	0 000E 00
258	0 168E 00	0 000E 00	0 162E 01	0 000E 00	0 000E 00
266	0 249E 01	0 570E 01	0 828E 02	0 147E 02	0 566E 01
272	0 572E 00	0 691E 00	0 848E 01	0 222E 01	0 580E 01
274	0 173E 00	0 399E 00	0 168E 01	0 115E 01	0 203E 00
277	0 000E 00	0 000E 00	0 000E 00	0 000E 00	0 000E 00
290	0 209E 02	0 186E 02	0 242E 03	0 834E 02	0 152E 02
291	0 000E 00	0 000E 00	0 000E 00	0 418E 01	0 000E 00
293	0 263E-01	0 000E 00	0 106E 02	0 244E 02	0 319E 01
294	0 964E 00	0 190E 01	0 638E 01	0 118E 02	0 140E 01
296	0 620E 01	0 140E 01	0 387E 01	0 577E 01	0 302E 01
297	0 226E 01	0 298E 01	0 138E 03	0 145E 03	0 000E 00
298	0 582E-01	0 752E-01	0 121E 01	0 000E 00	0 153E 01
299	0 932E-01	0 194E 00	0 177E 01	0 221E 01	0 668E 00
301	0 244E 00	0 718E 00	0 209E 02	0 198E 01	0 122E 01
302	0 521E 01	0 847E 01	0 932E 02	0 460E 02	0 238E 02
303	0 359E 01	0 416E 01	0 275E 02	0 368E 02	0 115E 02
305	0 421E 00	0 105E 01	0 201E 02	0 102E 02	0 125E 01
306	0 325E 01	0 699E 01	0 933E 02	0 402E 02	0 762E 01

TABLE E1. (Cont'd.)

	0316044	0502014	0502024	0502054	0330046
172	0.460E 00	0.748E 00	0.000E 00	0.167E 00	0.000E 00
174	0.115E 01	0.293E 00	0.583E 00	0.562E 01	0.312E 01
176	0.000E 00	0.464E 01	0.000E 00	0.953E 01	0.000E 00
177	0.130E 01	0.133E 01	0.000E 00	0.682E 01	0.000E 00
181	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.279E 00
183	0.000E 00	0.210E 01	0.000E 00	0.287E 01	0.000E 00
184	0.833E 01	0.789E 01	0.454E 01	0.122E 02	0.169E 02
185	0.000E 00	0.182E 01	0.340E 00	0.282E 01	0.648E 01
186	0.000E 00	0.663E-01	0.000E 00	0.000E 00	0.000E 00
187	0.921E 00	0.210E 01	0.482E 00	0.287E 01	0.146E 01
189	0.000E 00	0.000E 00	0.575E 00	0.000E 00	0.000E 00
194	0.345E 01	0.500E 00	0.121E 00	0.437E 01	0.108E 02
197	0.387E 02	0.681E 01	0.000E 00	0.648E 02	0.145E 03
198	0.714E 02	0.673E 01	0.150E 01	0.596E 02	0.123E 03
200	0.852E 01	0.186E 01	0.000E 00	0.000E 00	0.000E 00
207	0.160E 02	0.278E 01	0.490E 01	0.203E 02	0.239E 02
209	0.447E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
212	0.128E 01	0.155E 01	0.377E 01	0.229E 01	0.260E 01
215	0.231E 02	0.212E 02	0.177E 02	0.000E 00	0.120E 02
216	0.176E 02	0.215E 02	0.122E 02	0.634E 00	0.686E 02
218	0.000E 00	0.232E 01	0.167E 01	0.000E 00	0.122E 02
222	0.000E 00	0.555E 00	0.000E 00	0.000E 00	0.000E 00
225	0.222E 02	0.170E 02	0.128E 02	0.290E 02	0.126E 02
229	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
235	0.000E 00	0.000E 00	0.000E 00	0.104E 01	0.000E 00
236	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
237	0.839E 01	0.000E 00	0.277E 02	0.274E 02	0.000E 00
244	0.233E 02	0.918E 01	0.108E 02	0.712E 02	0.164E 02
246	0.000E 00	0.113E 00	0.135E 00	0.907E 01	0.000E 00
251	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
254	0.000E 00	0.000E 00	0.000E 00	0.122E 01	0.000E 00
257	0.000E 00	0.000E 00	0.000E 00	0.456E 02	0.000E 00
258	0.000E 00	0.000E 00	0.000E 00	0.459E 01	0.000E 00
266	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
272	0.182E 02	0.268E 01	0.781E 01	0.344E 02	0.232E 02
274	0.000E 00	0.602E 00	0.520E 00	0.000E 00	0.165E 01
277	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
290	0.787E 01	0.657E 01	0.176E 01	0.167E 02	0.612E 01
291	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
293	0.248E 01	0.000E 00	0.222E 01	0.123E 01	0.176E 00
294	0.427E 00	0.944E 00	0.566E 00	0.260E 00	0.000E 00
296	0.374E 01	0.163E 01	0.195E 01	0.812E 01	0.519E 01
297	0.000E 00	0.512E 02	0.124E 02	0.000E 00	0.346E 01
298	0.524E 01	0.212E 01	0.862E 01	0.248E 01	0.500E 00
299	0.000E 00	0.527E 00	0.871E 00	0.105E 01	0.000E 00
301	0.173E 01	0.221E 00	0.000E 00	0.703E 01	0.000E 00
302	0.251E 02	0.191E 02	0.420E 02	0.334E 02	0.214E 02
303	0.362E 02	0.267E 02	0.905E 01	0.119E 02	0.159E 02
305	0.119E 02	0.546E 00	0.515E 01	0.275E 02	0.164E 02
306	0.248E 02	0.703E 01	0.121E 02	0.461E 02	0.148E 03

	0316037	0322027
172	0.208E 00	0.623E-01
174	0.155E 01	0.115E 01
176	0.302E 01	0.000E 00
177	0.122E 01	0.115E 01
181	0.000E 00	0.000E 00
183	0.000E 00	0.000E 00
184	0.629E 01	0.594E 01
185	0.581E 01	0.589E 01
186	0.270E 00	0.000E 00
187	0.156E 01	0.125E 01
189	0.000E 00	0.000E 00
194	0.124E 01	0.640E 00
197	0.171E 02	0.157E 02
198	0.123E 02	0.125E 02
200	0.223E 01	0.223E 01
207	0.494E 01	0.496E 01
209	0.000E 00	0.000E 00
212	0.105E 01	0.121E 01
215	0.140E 02	0.121E 02
216	0.158E 02	0.130E 02
218	0.137E 01	0.104E 01
222	0.814E 00	0.000E 00
225	0.120E 02	0.112E 02
229	0.000E 00	0.000E 00
235	0.240E 00	0.201E 00
236	0.000E 00	0.000E 00
237	0.140E 02	0.121E 02
244	0.242E 02	0.223E 02
246	0.451E 00	0.257E 00
251	0.000E 00	0.000E 00
254	0.221E 00	0.000E 00
257	0.000E 00	0.000E 00
258	0.000E 00	0.944E 00
266	0.566E 01	0.000E 00
272	0.580E 01	0.593E 01
274	0.303E 00	0.000E 00
277	0.000E 00	0.000E 00
290	0.152E 02	0.140E 02
291	0.000E 00	0.000E 00
293	0.319E 01	0.619E 01
294	0.140E 01	0.149E 01
296	0.303E 01	0.301E 01
297	0.000E 00	0.000E 00
298	0.153E 01	0.142E 01
299	0.668E 00	0.000E 00
301	0.123E 01	0.147E 01
302	0.238E 02	0.249E 02
303	0.115E 02	0.125E 02
305	0.125E 01	0.150E 01
306	0.763E 01	0.864E 01

TABLE E1. (Cont'd.)

	0725052	0725062	0726012	0728022	0729022
308	0.634E 02	0.736E 02	0.549E 02	0.903E 02	0.882E 02
309	0.175E 02	0.283E 02	0.252E 02	0.468E 02	0.414E 02
310	0.192E 01	0.771E 00	0.751E 00	0.276E 01	0.238E 01
313	0.387E 01	0.917E 01	0.625E 01	0.846E 01	0.670E 01
314	0.316E 01	0.149E 02	0.961E 01	0.799E 01	0.921E 01
316	0.911E 01	0.116E 02	0.149E 02	0.726E 01	0.990E 01
318	0.171E 03	0.115E 03	0.669E 02	0.134E 03	0.115E 03
319	0.303E 02	0.612E 02	0.280E 02	0.294E 02	0.254E 02
320	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
321	0.204E 01	0.498E 01	0.154E 02	0.221E 01	0.146E 02
322	0.729E 01	0.115E 01	0.339E 01	0.525E 01	0.557E 01
324	0.100E 02	0.000E 00	0.983E 01	0.115E 02	0.160E 02
325	0.451E 02	0.300E 02	0.284E 02	0.600E 02	0.150E 03
326	0.234E 02	0.944E 01	0.585E 02	0.626E 02	0.152E 03
328	0.725E 00	0.591E 00	0.848E 00	0.985E 00	0.000E 00
329	0.472E 01	0.581E 01	0.118E 02	0.458E 01	0.961E 01
332	0.000E 00	0.000E 00	0.238E 01	0.230E 01	0.420E 00
333	0.000E 00	0.000E 00	0.187E 01	0.119E 01	0.694E 01
334	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.254E 02
342	0.000E 00	0.000E 00	0.689E 00	0.000E 00	0.000E 00
343	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
344	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
345	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
346	0.523E 01	0.784E 01	0.730E 01	0.729E 01	0.796E 01
347	0.516E 01	0.361E 01	0.321E 01	0.400E 01	0.533E 01
348	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
349	0.299E 00	0.295E 00	0.000E 00	0.467E 00	0.292E 00
350	0.000E 00	0.000E 00	0.371E 01	0.498E 01	0.622E 01
351	0.000E 00	0.285E 01	0.000E 00	0.000E 00	0.000E 00
352	0.319E 01	0.153E 01	0.000E 00	0.430E 01	0.437E 01
353	0.664E 01	0.616E 01	0.752E 01	0.955E 01	0.151E 02
354	0.516E 01	0.974E 01	0.657E 01	0.920E 01	0.829E 01
356	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
357	0.000E 00	0.000E 00	0.000E 00	0.320E 00	0.000E 00
358	0.294E 01	0.276E 01	0.000E 00	0.000E 00	0.000E 00
359	0.192E 01	0.195E 02	0.527E 02	0.283E 01	0.110E 02
360	0.124E 02	0.549E 01	0.000E 00	0.993E 01	0.347E 01
361	0.000E 00	0.000E 00	0.164E 02	0.252E 00	0.553E 01
363	0.000E 00	0.348E 00	0.452E 00	0.428E 00	0.146E 01
364	0.482E 01	0.357E 01	0.472E 01	0.682E 01	0.111E 02
365	0.834E 01	0.000E 00	0.163E 02	0.186E 02	0.395E 02
366	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
367	0.529E 00	0.118E 00	0.000E 00	0.536E 00	0.153E 01
368	0.000E 00	0.000E 00	0.000E 00	0.376E-01	0.943E 00
369	0.000E 00	0.939E-01	0.219E 01	0.360E 00	0.000E 00
371	0.331E 01	0.458E 01	0.184E 01	0.563E 01	0.349E 01
372	0.000E 00	0.106E 02	0.109E 02	0.146E 02	0.156E 02
373	0.000E 00	0.000E 00	0.000E 00	0.230E 01	0.000E 00
374	0.705E 00	0.000E 00	0.227E 01	0.536E 00	0.105E 01
375	0.300E 01	0.000E 00	0.167E 01	0.543E 01	0.119E 02

	0919012	1031012	1031062	1110012	0918023
308	0.829E 02	0.151E 03	0.109E 03	0.366E 02	0.649E 02
309	0.491E 02	0.764E 02	0.492E 02	0.149E 02	0.506E 02
310	0.831E 01	0.195E 01	0.189E 01	0.589E 01	0.438E 01
313	0.143E 01	0.763E 01	0.313E 02	0.685E 00	0.272E 01
314	0.429E 00	0.904E 01	0.984E 01	0.189E 00	0.904E 00
316	0.174E 01	0.107E 02	0.188E 02	0.000E 00	0.000E 00
318	0.117E 03	0.114E 03	0.213E 03	0.331E 03	0.227E 01
319	0.000E 00	0.416E 02	0.943E 02	0.000E 00	0.650E 01
320	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
321	0.449E 00	0.719E 01	0.111E 02	0.000E 00	0.152E 01
322	0.471E 01	0.407E 01	0.259E 01	0.127E 02	0.000E 00
324	0.915E 01	0.107E 02	0.118E 02	0.000E 00	0.844E 01
325	0.697E 02	0.664E 02	0.547E 02	0.409E 02	0.244E 02
326	0.944E 02	0.774E 02	0.459E 02	0.102E 03	0.675E 02
328	0.000E 00	0.412E 00	0.399E 01	0.117E 01	0.213E 00
329	0.833E 01	0.294E 01	0.753E 01	0.212E 02	0.359E 01
332	0.000E 00	0.750E 00	0.119E 02	0.000E 00	0.000E 00
333	0.793E 01	0.340E 01	0.441E 01	0.370E 01	0.110E 02
334	0.159E 02	0.000E 00	0.127E 02	0.000E 00	0.000E 00
342	0.575E 01	0.000E 00	0.000E 00	0.369E 01	0.130E 01
343	0.212E 01	0.000E 00	0.000E 00	0.182E 01	0.648E 00
344	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.232E 00
345	0.000E 00	0.241E 00	0.438E 00	0.110E 00	0.544E 00
346	0.209E 01	0.125E 02	0.120E 02	0.267E 00	0.243E 01
347	0.339E 01	0.803E 01	0.000E 00	0.208E 01	0.632E 01
348	0.277E 00	0.000E 00	0.000E 00	0.000E 00	0.940E 00
349	0.359E 01	0.498E 00	0.000E 00	0.280E 01	0.520E 01
350	0.604E 01	0.441E 01	0.295E 01	0.000E 00	0.421E 01
351	0.996E 01	0.000E 00	0.000E 00	0.896E 01	0.115E 02
352	0.158E 02	0.126E 01	0.214E 01	0.922E 01	0.698E 01
353	0.175E 02	0.929E 01	0.586E 01	0.145E 02	0.187E 02
354	0.320E 01	0.000E 00	0.000E 00	0.000E 00	0.454E 01
356	0.118E 02	0.151E 02	0.231E 01	0.455E 01	0.160E 02
357	0.119E 00	0.224E 01	0.000E 00	0.000E 00	0.134E 02
358	0.288E 01	0.000E 00	0.000E 00	0.000E 00	0.294E 01
359	0.447E 01	0.672E 00	0.000E 00	0.000E 00	0.102E 02
360	0.292E 02	0.000E 00	0.000E 00	0.275E 02	0.115E 02
361	0.430E 02	0.244E 02	0.495E 01	0.196E 02	0.210E 02
363	0.000E 00	0.766E 00	0.000E 00	0.421E 00	0.280E 01
364	0.620E 01	0.891E 01	0.000E 00	0.443E 01	0.122E 02
365	0.232E 02	0.195E 02	0.128E 02	0.249E 02	0.150E 02
366	0.349E 01	0.000E 00	0.000E 00	0.000E 00	0.278E 01
367	0.727E 00	0.110E 01	0.000E 00	0.742E 00	0.169E 01
368	0.151E 00	0.000E 00	0.000E 00	0.257E 00	0.275E 01
369	0.147E 01	0.213E 01	0.919E 00	0.776E 00	0.463E 01
371	0.172E 01	0.187E 01	0.147E 01	0.222E 01	0.184E 01
372	0.547E 01	0.980E 01	0.110E 02	0.000E 00	0.531E 01
373	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
374	0.195E 01	0.475E 01	0.115E 01	0.116E 01	0.374E 01
375	0.151E 01	0.199E 01	0.972E 01	0.214E 01	0.763E 01

TABLE E1. (Cont'd.)

	0919023	1029033	1031023	1031033	0316034
308	0.294E 02	0.623E 02	0.623E 02	0.291E 03	0.569E 02
309	0.875E 01	0.157E 02	0.208E 03	0.135E 03	0.213E 02
310	0.252E 01	0.227E 01	0.436E 02	0.932E 01	0.483E 01
313	0.197E 01	0.209E 01	0.248E 02	0.157E 02	0.181E 01
314	0.301E 00	0.419E 00	0.784E 01	0.536E 01	0.652E 00
316	0.856E 00	0.198E 01	0.340E 02	0.118E 02	0.217E 01
318	0.315E 00	0.115E 01	0.147E 02	0.178E 01	0.358E 01
319	0.406E 01	0.403E 01	0.891E 02	0.370E 02	0.534E 01
320	0.000E 00	0.000E 00	0.312E 01	0.000E 00	0.000E 00
321	0.823E 00	0.762E 00	0.213E 02	0.992E 01	0.000E 00
322	0.311E-01	0.240E-01	0.411E 00	0.000E 00	0.000E 00
324	0.512E 01	0.129E 02	0.623E 02	0.843E 02	0.171E 02
325	0.171E 02	0.410E 02	0.292E 03	0.158E 03	0.542E 02
326	0.267E 02	0.476E 02	0.371E 03	0.191E 03	0.637E 02
328	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
329	0.393E 01	0.443E 01	0.464E 02	0.175E 02	0.293E 01
332	0.614E-01	0.344E 00	0.121E 01	0.317E 01	0.129E 01
333	0.311E 00	0.906E 00	0.694E 02	0.390E 01	0.216E 01
334	0.770E 01	0.886E 01	0.407E 02	0.322E 02	0.110E 02
342	0.310E 01	0.000E 00	0.000E 00	0.313E 02	0.882E 01
343	0.109E 01	0.113E 01	0.748E 01	0.896E 01	0.218E 01
344	0.589E-01	0.397E-01	0.217E 01	0.000E 00	0.152E 00
345	0.167E 00	0.998E-01	0.196E 01	0.849E 00	0.000E 00
346	0.421E 00	0.105E 01	0.201E 02	0.103E 02	0.125E 01
347	0.842E 00	0.274E 01	0.347E 02	0.000E 00	0.236E 01
348	0.995E-01	0.000E 00	0.000E 00	0.000E 00	0.000E 00
349	0.724E 00	0.648E 00	0.937E 01	0.365E 01	0.345E 01
350	0.283E 00	0.465E 00	0.000E 00	0.493E 01	0.000E 00
351	0.319E 01	0.000E 00	0.393E 02	0.000E 00	0.124E 02
352	0.475E 01	0.236E 01	0.490E 02	0.103E 02	0.734E 01
353	0.486E 01	0.602E 01	0.567E 02	0.298E 02	0.197E 02
354	0.247E 01	0.232E 01	0.355E 02	0.170E 02	0.338E 01
356	0.276E 00	0.178E 00	0.939E 00	0.315E 01	0.477E 00
357	0.169E 01	0.269E 01	0.232E 02	0.136E 02	0.131E 01
358	0.990E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
359	0.284E 00	0.000E 00	0.583E 01	0.000E 00	0.000E 00
360	0.132E 02	0.000E 00	0.197E 03	0.342E 02	0.113E 02
361	0.152E 02	0.329E 02	0.000E 00	0.516E 02	0.190E 01
362	0.241E 00	0.209E 00	0.000E 00	0.534E 00	0.650E 00
364	0.173E 01	0.277E 01	0.203E 02	0.000E 00	0.780E 01
365	0.651E 01	0.116E 02	0.881E 02	0.502E 02	0.157E 02
366	0.210E 00	0.135E 00	0.000E 00	0.000E 00	0.105E 01
367	0.179E-01	0.779E-01	0.158E 01	0.000E 00	0.556E 00
368	0.460E-01	0.000E 00	0.000E 00	0.000E 00	0.875E 00
369	0.921E 00	0.637E 00	0.657E 01	0.322E 01	0.000E 00
371	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.302E 01
372	0.185E 01	0.292E 01	0.284E 02	0.150E 02	0.396E 01
373	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
374	0.839E 00	0.143E 01	0.909E 01	0.760E 01	0.306E 01
375	0.127E 01	0.134E 01	0.206E 02	0.589E 01	0.300E 01

	0316044	0502014	0502034	0502054	0330046
308	0.156E 03	0.530E 02	0.977E 02	0.000E 00	0.329E 03
309	0.480E 02	0.275E 02	0.122E 03	0.312E 02	0.975E 02
310	0.140E 01	0.533E 01	0.363E 01	0.348E 01	0.159E 01
313	0.174E 02	0.106E 01	0.427E 01	0.125E 02	0.804E 01
314	0.145E 02	0.307E 00	0.000E 00	0.242E 02	0.155E 02
316	0.158E 02	0.115E 01	0.334E 01	0.269E 02	0.198E 02
318	0.310E 01	0.184E 01	0.000E 00	0.890E 02	0.000E 00
319	0.556E 02	0.145E 01	0.114E 02	0.119E 03	0.887E 01
320	0.000E 00	0.000E 00	0.000E 00	0.502E 01	0.000E 00
321	0.204E 02	0.640E 00	0.164E 01	0.225E 02	0.306E 02
322	0.000E 00	0.000E 00	0.128E 00	0.000E 00	0.000E 00
324	0.387E 02	0.681E 01	0.000E 00	0.648E 02	0.145E 03
325	0.160E 03	0.741E 02	0.610E 02	0.133E 03	0.263E 03
326	0.171E 03	0.779E 02	0.635E 02	0.138E 03	0.102E 03
328	0.639E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
329	0.230E 01	0.687E 01	0.766E 01	0.709E 01	0.110E 02
332	0.957E-01	0.120E 01	0.129E 00	0.424E 01	0.425E 00
333	0.490E 01	0.000E 00	0.000E 00	0.755E 02	0.260E 01
334	0.143E 02	0.143E 02	0.121E 02	0.000E 00	0.364E 02
342	0.714E 01	0.000E 00	0.652E 01	0.605E 01	0.347E 01
343	0.295E 01	0.167E 01	0.170E 01	0.343E 01	0.239E 01
344	0.000E 00	0.000E 00	0.280E 00	0.000E 00	0.000E 00
345	0.249E 00	0.000E 00	0.533E 00	0.000E 00	0.000E 00
346	0.119E 02	0.546E 00	0.514E 01	0.275E 02	0.164E 02
347	0.102E 02	0.243E 01	0.464E 01	0.140E 02	0.447E 02
348	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
349	0.000E 00	0.610E 01	0.151E 02	0.657E 01	0.363E 01
350	0.168E 00	0.997E 00	0.000E 00	0.514E 01	0.000E 00
351	0.000E 00	0.168E 02	0.000E 00	0.406E 01	0.000E 00
352	0.263E 01	0.826E 01	0.528E 01	0.497E 01	0.703E 00
353	0.105E 02	0.212E 02	0.156E 02	0.143E 02	0.504E 01
354	0.174E 02	0.217E 01	0.000E 00	0.151E 02	0.101E 02
356	0.348E 01	0.000E 00	0.000E 00	0.000E 00	0.000E 00
357	0.545E 01	0.000E 00	0.430E 00	0.128E 02	0.626E 00
358	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.227E 01
359	0.197E 02	0.000E 00	0.000E 00	0.793E 02	0.000E 00
360	0.000E 00	0.856E 01	0.493E 01	0.110E 02	0.000E 00
361	0.000E 00	0.123E 02	0.893E 01	0.265E 02	0.289E 01
362	0.389E 01	0.101E 01	0.103E 01	0.456E 01	0.362E 01
364	0.102E 02	0.944E 01	0.101E 02	0.211E 02	0.334E 02
365	0.262E 02	0.233E 02	0.155E 02	0.370E 02	0.296E 02
366	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
367	0.104E 01	0.936E 00	0.000E 00	0.202E 01	0.276E 01
368	0.000E 00	0.000E 00	0.000E 00	0.825E 00	0.000E 00
369	0.000E 00	0.000E 00	0.000E 00	0.581E 01	0.584E 00
371	0.616E 01	0.345E 01	0.780E 01	0.104E 02	0.000E 00
372	0.135E 02	0.290E 01	0.558E 01	0.102E 02	0.277E 02
373	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
374	0.529E 01	0.153E 01	0.555E 01	0.452E 01	0.457E 01
375	0.318E 01	0.393E 01	0.140E 02	0.479E 02	0.615E 00

TABLE E1. (Cont'd.)

	0316037	0323027
308	0.569E 02	0.588E 02
309	0.213E 02	0.231E 02
310	0.483E 01	0.527E 01
313	0.181E 01	0.186E 01
314	0.652E 00	0.796E 00
316	0.217E 01	0.240E 01
318	0.358E 01	0.402E 01
319	0.584E 01	0.617E 01
320	0.000E 00	0.000E 00
321	0.000E 00	0.000E 00
322	0.000E 00	0.000E 00
324	0.171E 02	0.157E 02
325	0.542E 02	0.474E 02
326	0.637E 02	0.573E 02
328	0.000E 00	0.000E 00
329	0.293E 01	0.651E 01
332	0.129E 01	0.112E 01
333	0.216E 01	0.816E 01
334	0.110E 02	0.131E 02
342	0.883E 01	0.979E 01
343	0.218E 01	0.167E 01
344	0.152E 00	0.199E 00
345	0.000E 00	0.000E 00
346	0.125E 01	0.150E 01
347	0.236E 01	0.265E 01
348	0.000E 00	0.000E 00
349	0.345E 01	0.388E 01
350	0.000E 00	0.000E 00
351	0.124E 02	0.131E 02
352	0.724E 01	0.814E 01
353	0.197E 02	0.211E 02
354	0.328E 01	0.329E 01
356	0.477E 00	0.521E 00
357	0.131E 01	0.158E 01
358	0.000E 00	0.000E 00
359	0.000E 00	0.000E 00
360	0.113E 02	0.122E 02
361	0.190E 01	0.110E 02
363	0.650E 00	0.877E 00
364	0.780E 01	0.693E 01
365	0.157E 02	0.148E 02
366	0.105E 01	0.892E 00
367	0.556E 00	0.559E 00
368	0.875E 00	0.849E 00
369	0.000E 00	0.000E 00
371	0.302E 01	0.284E 01
372	0.396E 01	0.412E 01
373	0.000E 00	0.000E 00
374	0.306E 01	0.344E 01
375	0.300E 01	0.340E 01

	0725052	0725062	0726012	0728022	0729022
376	0.221E 01	0.386E 00	0.252E 01	0.301E 01	0.323E 01
377	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
378	0.157E 02	0.150E 02	0.196E 02	0.188E 02	0.357E 02
379	0.000E 00	0.121E 01	0.323E 01	0.000E 00	0.286E 01
381	0.000E 00	0.223E 00	0.741E 00	0.212E 01	0.176E 01
382	0.689E 01	0.667E 01	0.101E 02	0.769E 01	0.106E 02
383	0.540E 02	0.395E 02	0.795E 02	0.653E 02	0.325E 02

	0316037	0323027
376	0.861E 00	0.933E 00
377	0.133E 01	0.000E 00
378	0.339E 01	0.452E 01
379	0.664E 00	0.000E 00
381	0.469E 00	0.434E 00
382	0.199E 01	0.195E 01
383	0.242E 02	0.210E 02

	0919012	1031012	1031062	1110012	0918023
376	0.240E 01	0.342E 01	0.238E 01	0.296E 01	0.730E 01
377	0.000E 00	0.995E 00	0.000E 00	0.000E 00	0.000E 00
378	0.000E 00	0.279E 02	0.539E 02	0.000E 00	0.116E 01
379	0.117E 01	0.568E 01	0.509E 01	0.000E 00	0.000E 00
381	0.123E 01	0.279E 01	0.861E 01	0.000E 00	0.449E 01
382	0.000E 00	0.931E 01	0.000E 00	0.344E 01	0.119E 02
383	0.294E 02	0.189E 03	0.217E 03	0.159E 02	0.476E 02

	0316044	0502014	0502034	0502054	0330046
376	0.385E 01	0.237E 01	0.000E 00	0.000E 00	0.000E 00
377	0.589E 01	0.000E 00	0.000E 00	0.746E 01	0.487E 01
378	0.330E 02	0.262E 01	0.144E 02	0.443E 02	0.401E 02
379	0.933E 01	0.000E 00	0.000E 00	0.103E 02	0.263E 01
381	0.689E 01	0.329E 00	0.214E 02	0.302E 02	0.000E 00
382	0.120E 02	0.300E 01	0.212E 01	0.349E 02	0.216E 02
383	0.465E 03	0.392E 02	0.322E 02	0.172E 03	0.143E 04

	0919023	1029033	1031023	1031033	0316034
376	0.534E 00	0.128E 01	0.000E 00	0.257E 01	0.861E 00
377	0.321E 00	0.000E 00	0.206E 02	0.000E 00	0.133E 01
378	0.323E 01	0.745E 01	0.649E 02	0.477E 02	0.339E 01
379	0.388E 00	0.701E 00	0.000E 00	0.478E 01	0.664E 00
381	0.159E 01	0.352E 01	0.292E 02	0.673E 01	0.469E 00
382	0.582E 01	0.621E 01	0.107E 03	0.000E 00	0.199E 01
383	0.154E 01	0.703E 01	0.831E 02	0.409E 02	0.242E 02

APPENDIX F

Normalized relative areas

Table F1 shows the same data as Table E1, except that the values have been normalized to a partial sum of peak areas. This partial sum was calculated for each urine by summing all of the relative areas listed in Appendix E except for those of the compounds listed in Appendix G. The resulting sum was then multiplied by a factor of 0.001 (to achieve a value between 0.1 and 3), resulting in a "corrected sum." Each relative area for the urine was then divided by the corrected sum for that urine; the resulting values are referred to as normalized relative peak areas.

	0725052	0725062	0726012	0728022	0729022
6	0.283E 02	0.218E 02	0.199E 02	0.124E 02	0.225E 02
8	0.526E 02	0.153E 02	0.529E 01	0.627E 02	0.257E 02
10	0.542E 02	0.612E 02	0.147E 02	0.209E 02	0.204E 02
17	0.196E 01	0.000E 00	0.381E 00	0.000E 00	0.000E 00
18	0.176E 02	0.000E 00	0.230E 01	0.226E 03	0.905E 02
23	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
28	0.129E 02	0.736E 01	0.332E 01	0.765E 01	0.713E 01
29	0.000E 00	0.000E 00	0.197E 00	0.576E-01	0.000E 00
32	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
33	0.000E 00	0.000E 00	0.775E 00	0.231E 00	0.000E 00
34	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
35	0.118E 00	0.000E 00	0.256E 00	0.124E 00	0.000E 00
36	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
42	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
50	0.285E 02	0.272E 02	0.179E 02	0.210E 02	0.641E 02
51	0.498E 01	0.254E 01	0.194E 01	0.276E 01	0.279E 01
59	0.203E 01	0.170E 01	0.117E 01	0.169E 01	0.328E 01
60	0.184E 02	0.000E 00	0.934E 01	0.560E 01	0.923E 01
61	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
65	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
66	0.503E-01	0.000E 00	0.000E 00	0.000E 00	0.000E 00
67	0.000E 00	0.000E 00	0.472E 00	0.000E 00	0.000E 00
77	0.339E 01	0.000E 00	0.128E 01	0.195E 01	0.245E 01
79	0.185E 00	0.158E 00	0.398E 00	0.991E-01	0.261E 00
82	0.000E 00	0.000E 00	0.189E 01	0.000E 00	0.491E 00
86	0.329E 01	0.587E 01	0.206E 01	0.171E 01	0.461E 01
96	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
98	0.810E 00	0.000E 00	0.000E 00	0.412E 01	0.318E 01
104	0.000E 00	0.000E 00	0.588E 00	0.000E 00	0.000E 00
105	0.286E 01	0.265E 01	0.239E 01	0.265E 01	0.241E 01
107	0.000E 00	0.000E 00	0.145E 02	0.940E 01	0.122E 02
110	0.326E 01	0.170E 01	0.483E 01	0.386E 01	0.352E 01
111	0.447E 01	0.156E 01	0.279E 01	0.958E 00	0.377E 01
112	0.000E 00	0.000E 00	0.000E 00	0.618E 00	0.123E 01
114	0.246E 01	0.142E 01	0.164E 01	0.178E 01	0.861E 00
118	0.503E 01	0.181E 01	0.000E 00	0.170E 01	0.000E 00
120	0.198E 03	0.180E 03	0.122E 03	0.186E 03	0.905E 02
123	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
124	0.589E 01	0.175E 01	0.120E 01	0.467E 01	0.509E 01
125	0.000E 00	0.000E 00	0.231E 00	0.108E 00	0.772E 00
127	0.122E 02	0.783E 01	0.198E 02	0.158E 02	0.644E 01
129	0.539E 01	0.302E 01	0.381E 01	0.421E 01	0.123E 02
135	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.126E 01
136	0.440E 02	0.274E 02	0.229E 02	0.136E 02	0.215E 02
138	0.000E 00	0.627E 00	0.327E 00	0.000E 00	0.517E 00
139	0.147E 02	0.106E 02	0.948E 01	0.777E 01	0.147E 02
141	0.657E 01	0.116E 01	0.155E 01	0.383E 01	0.455E 01
144	0.106E 02	0.000E 00	0.126E 01	0.657E 01	0.312E 01
152	0.393E 02	0.534E 02	0.174E 02	0.244E 02	0.223E 02
155	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00

TABLE F1. Normalized relative areas

	0919012	1031012	1031062	1118012	0918023
6	0.714E 01	0.915E 01	0.164E 02	0.549E 01	0.784E 01
8	0.268E 02	0.186E 02	0.270E 02	0.317E 02	0.695E 01
10	0.190E 02	0.129E 02	0.285E 02	0.206E 02	0.187E 02
17	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.240E 01
18	0.145E 03	0.119E 03	0.134E 03	0.150E 03	0.205E 02
23	0.545E 01	0.000E 00	0.000E 00	0.000E 00	0.565E 01
28	0.807E 01	0.698E 01	0.423E 01	0.882E 01	0.358E 01
29	0.141E 01	0.000E 00	0.000E 00	0.546E 00	0.186E 00
32	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
33	0.000E 00	0.132E 01	0.462E 00	0.000E 00	0.119E 01
34	0.636E 00	0.000E 00	0.000E 00	0.000E 00	0.432E 00
35	0.875E 00	0.165E 01	0.446E 00	0.000E 00	0.846E 00
36	0.229E 00	0.285E 00	0.000E 00	0.000E 00	0.319E 00
42	0.636E 01	0.000E 00	0.000E 00	0.000E 00	0.624E 00
50	0.491E 03	0.830E 03	0.256E 03	0.830E 02	0.235E 03
51	0.461E 01	0.278E 01	0.000E 00	0.000E 00	0.108E 01
59	0.300E 01	0.239E 01	0.247E 01	0.214E 01	0.435E 01
60	0.264E 02	0.209E 02	0.922E 01	0.155E 02	0.190E 02
61	0.334E 01	0.908E 01	0.260E 01	0.309E 01	0.568E 01
65	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
66	0.408E 00	0.216E 00	0.000E 00	0.538E 00	0.194E 00
67	0.791E 00	0.549E 00	0.000E 00	0.576E 00	0.870E 00
77	0.292E 01	0.180E 01	0.227E 01	0.230E 01	0.447E 01
79	0.697E-01	0.253E 00	0.000E 00	0.000E 00	0.226E 00
82	0.673E 00	0.581E 00	0.000E 00	0.000E 00	0.784E 00
86	0.102E 02	0.984E 01	0.495E 01	0.231E 01	0.817E 01
96	0.526E 00	0.000E 00	0.000E 00	0.000E 00	0.810E-01
98	0.238E 01	0.116E 02	0.000E 00	0.279E 02	0.152E 02
104	0.279E 01	0.294E 00	0.578E 00	0.120E 01	0.565E 00
105	0.405E 01	0.423E 01	0.288E 01	0.000E 00	0.565E 01
107	0.923E 01	0.167E 02	0.000E 00	0.000E 00	0.274E 02
110	0.443E 01	0.574E 01	0.399E 01	0.220E 01	0.135E 02
111	0.175E 01	0.811E 00	0.000E 00	0.279E 01	0.138E 01
112	0.120E 03	0.108E 01	0.000E 00	0.256E 02	0.373E 02
114	0.153E 01	0.806E 00	0.000E 00	0.969E 00	0.429E 00
118	0.928E 00	0.412E 01	0.000E 00	0.134E 01	0.621E 01
120	0.934E 02	0.798E 02	0.960E 02	0.958E 02	0.698E 02
123	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.113E 01
124	0.144E 02	0.214E 01	0.000E 00	0.795E 01	0.737E 01
125	0.842E 00	0.174E 02	0.243E 01	0.000E 00	0.203E 02
127	0.107E 02	0.178E 02	0.750E 01	0.442E 01	0.108E 02
129	0.123E 02	0.528E 01	0.437E 01	0.907E 00	0.420E 01
135	0.317E 01	0.000E 00	0.000E 00	0.267E 01	0.398E 01
136	0.139E 02	0.191E 02	0.249E 02	0.116E 02	0.595E 01
138	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
139	0.125E 01	0.865E 01	0.202E 02	0.000E 00	0.296E 01
141	0.298E 01	0.685E 01	0.280E 01	0.140E 01	0.840E 01
144	0.483E 01	0.849E 01	0.440E 01	0.148E 01	0.902E 01
152	0.237E 02	0.213E 02	0.235E 02	0.123E 02	0.698E 02
155	0.124E 02	0.193E 02	0.960E 01	0.740E 01	0.299E 02

TABLE F1. (Cont'd.)

	0919023	1029033	1031023	1031033	0316034
6	0.308E 01	0.264E 01	0.231E 01	0.551E 01	0.135E 02
8	0.659E 01	0.137E 02	0.154E 02	0.179E 02	0.218E 02
10	0.549E 01	0.758E 01	0.117E 02	0.113E 02	0.429E 02
17	0.150E 01	0.168E 01	0.113E 01	0.293E 01	0.253E 01
18	0.229E 01	0.187E 01	0.633E 01	0.169E 01	0.000E 00
23	0.872E 01	0.863E 01	0.896E 01	0.109E 02	0.102E 02
28	0.310E 01	0.435E 01	0.376E 01	0.449E 01	0.107E 02
29	0.000E 00	0.391E 00	0.000E 00	0.000E 00	0.000E 00
32	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
33	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
34	0.907E 00	0.954E 00	0.451E 00	0.000E 00	0.155E 01
35	0.507E 00	0.000E 00	0.307E 00	0.000E 00	0.000E 00
36	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
42	0.356E 00	0.443E 01	0.188E 01	0.218E 01	0.826E 01
50	0.705E 03	0.716E 03	0.834E 03	0.817E 03	0.379E 03
51	0.161E 01	0.106E 01	0.261E 01	0.000E 00	0.273E 01
59	0.159E 01	0.272E 01	0.123E 01	0.279E 01	0.355E 01
60	0.212E 02	0.163E 02	0.213E 02	0.146E 02	0.184E 02
61	0.681E 01	0.668E 01	0.494E 01	0.972E 01	0.333E 01
65	0.137E 00	0.626E 00	0.496E 00	0.108E 01	0.000E 00
66	0.232E 00	0.000E 00	0.174E 00	0.000E 00	0.000E 00
67	0.114E 01	0.968E 00	0.000E 00	0.000E 00	0.109E 01
77	0.369E 01	0.363E 01	0.222E 01	0.327E 01	0.389E 01
79	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
82	0.605E 00	0.163E 01	0.758E 00	0.000E 00	0.925E 00
86	0.920E 01	0.108E 02	0.119E 02	0.957E 01	0.637E 01
96	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
98	0.422E 01	0.640E 01	0.249E 01	0.535E 01	0.421E 01
104	0.000E 00	0.315E 01	0.147E 02	0.960E 00	0.000E 00
105	0.161E 01	0.248E 01	0.128E 01	0.282E 01	0.271E 01
107	0.109E 02	0.177E 02	0.152E 02	0.233E 02	0.140E 02
110	0.662E 01	0.128E 02	0.662E 01	0.116E 02	0.921E 01
111	0.457E 01	0.384E 01	0.267E 01	0.646E 01	0.000E 00
112	0.812E 02	0.243E 03	0.461E 02	0.950E 02	0.771E 02
114	0.582E 00	0.765E 00	0.483E 00	0.121E 01	0.117E 01
118	0.892E 00	0.464E 01	0.000E 00	0.171E 01	0.280E 01
120	0.326E 03	0.337E 03	0.192E 03	0.463E 03	0.198E 03
123	0.212E 02	0.281E 02	0.000E 00	0.173E 02	0.901E 00
124	0.101E 02	0.814E 01	0.159E 02	0.566E 01	0.972E 01
125	0.706E 01	0.500E 02	0.141E 02	0.326E 02	0.842E 01
127	0.366E 02	0.255E 02	0.220E 02	0.537E 02	0.689E 01
129	0.666E 01	0.229E 01	0.316E 01	0.645E 01	0.227E 01
135	0.252E 01	0.233E 01	0.104E 02	0.189E 01	0.409E 01
136	0.169E 02	0.133E 02	0.119E 02	0.273E 02	0.258E 02
138	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
139	0.356E 01	0.680E 01	0.567E 01	0.766E 01	0.195E 01
141	0.190E 01	0.609E 01	0.143E 01	0.390E 01	0.488E 01
144	0.603E 01	0.107E 02	0.309E 01	0.964E 01	0.105E 02
152	0.462E 02	0.793E 02	0.286E 02	0.520E 02	0.539E 02
155	0.191E 02	0.155E 02	0.000E 00	0.234E 01	0.000E 00

	0316044	0502014	0502034	0502054	0330046
6	0.320E 02	0.409E 02	0.134E 02	0.302E 01	0.349E 01
8	0.243E 02	0.205E 02	0.292E 02	0.980E 01	0.151E 02
10	0.313E 02	0.493E 02	0.194E 02	0.718E 01	0.753E 01
17	0.000E 00	0.311E 01	0.113E 01	0.587E 00	0.176E 01
18	0.338E 02	0.000E 00	0.000E 00	0.000E 00	0.246E 01
23	0.580E 01	0.799E 01	0.642E 02	0.222E 02	0.299E 02
28	0.396E 01	0.774E 01	0.435E 01	0.376E 01	0.649E 01
29	0.000E 00	0.000E 00	0.213E 01	0.000E 00	0.000E 00
32	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
33	0.000E 00	0.000E 00	0.000E 00	0.378E 00	0.204E 01
34	0.322E 00	0.000E 00	0.000E 00	0.242E 00	0.000E 00
35	0.000E 00	0.000E 00	0.000E 00	0.301E 00	0.000E 00
36	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
42	0.495E 01	0.220E 01	0.126E 02	0.108E 01	0.432E 01
50	0.260E 03	0.362E 03	0.411E 02	0.115E 03	0.675E 03
51	0.667E 01	0.383E 01	0.000E 00	0.183E 01	0.675E 00
59	0.332E 01	0.557E 01	0.000E 00	0.195E 01	0.149E 01
60	0.155E 02	0.320E 02	0.553E 02	0.246E 02	0.188E 02
61	0.620E 01	0.485E 01	0.930E 01	0.101E 02	0.526E 01
65	0.000E 00	0.000E 00	0.716E 00	0.000E 00	0.000E 00
66	0.506E 00	0.000E 00	0.482E 00	0.281E 00	0.782E 00
67	0.105E 01	0.000E 00	0.243E 01	0.151E 00	0.000E 00
77	0.146E 01	0.451E 01	0.543E 01	0.130E 02	0.171E 01
79	0.252E 00	0.000E 00	0.000E 00	0.000E 00	0.745E 00
82	0.607E 00	0.863E 00	0.384E 00	0.917E 00	0.000E 00
86	0.734E 01	0.689E 01	0.215E 02	0.118E 02	0.000E 00
96	0.279E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
98	0.493E 01	0.114E 02	0.496E 01	0.336E 01	0.360E 01
104	0.121E 01	0.604E 00	0.264E 00	0.249E 00	0.159E 01
105	0.300E 01	0.392E 01	0.370E 00	0.742E 00	0.106E 01
107	0.827E 01	0.141E 02	0.142E 02	0.256E 02	0.000E 00
110	0.518E 01	0.101E 02	0.506E 01	0.765E 01	0.181E 02
111	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.105E 01
112	0.330E 03	0.174E 02	0.183E 03	0.935E 02	0.102E 02
114	0.884E 00	0.149E 01	0.397E 00	0.566E 00	0.487E 00
118	0.193E 01	0.290E 01	0.000E 00	0.205E 01	0.197E 01
120	0.950E 02	0.213E 03	0.170E 03	0.450E 02	0.905E 02
123	0.000E 00	0.000E 00	0.148E 01	0.000E 00	0.000E 00
124	0.193E 01	0.153E 02	0.486E 01	0.000E 00	0.000E 00
125	0.143E 02	0.557E 01	0.104E 01	0.358E 02	0.000E 00
127	0.339E 02	0.982E 01	0.181E 01	0.195E 02	0.394E 01
129	0.901E 00	0.565E 01	0.123E 01	0.103E 01	0.215E 01
135	0.000E 00	0.451E 01	0.000E 00	0.000E 00	0.117E 01
136	0.119E 02	0.162E 02	0.730E 01	0.510E 02	0.192E 02
138	0.527E 01	0.000E 00	0.000E 00	0.134E 01	0.000E 00
139	0.129E 02	0.000E 00	0.338E 01	0.594E 01	0.108E 02
141	0.449E 01	0.667E 01	0.000E 00	0.364E 01	0.586E 01
144	0.362E 01	0.918E 01	0.873E 01	0.505E 01	0.158E 02
152	0.370E 02	0.587E 02	0.192E 02	0.232E 02	0.858E 02
155	0.132E 02	0.000E 00	0.000E 00	0.104E 02	0.000E 00

TABLE F1. (Cont'd.)

	0316037	0322027
6	0.135E 02	0.131E 02
8	0.218E 02	0.225E 02
10	0.429E 02	0.411E 02
17	0.253E 01	0.210E 01
18	0.000E 00	0.000E 00
23	0.102E 02	0.973E 01
28	0.107E 02	0.110E 02
29	0.000E 00	0.509E 00
32	0.000E 00	0.000E 00
33	0.000E 00	0.000E 00
34	0.155E 01	0.000E 00
35	0.000E 00	0.000E 00
36	0.000E 00	0.000E 00
42	0.826E 01	0.104E 02
50	0.379E 03	0.376E 03
51	0.272E 01	0.424E 01
59	0.255E 01	0.280E 01
60	0.184E 02	0.192E 02
61	0.333E 01	0.280E 01
65	0.000E 00	0.000E 00
66	0.000E 00	0.000E 00
67	0.109E 01	0.146E 01
77	0.389E 01	0.435E 01
79	0.000E 00	0.280E 00
82	0.925E 00	0.119E 01
86	0.627E 01	0.624E 01
96	0.000E 00	0.442E 00
98	0.421E 01	0.461E 01
104	0.000E 00	0.682E 00
105	0.271E 01	0.310E 01
107	0.140E 02	0.138E 02
110	0.921E 01	0.937E 01
111	0.000E 00	0.000E 00
112	0.771E 02	0.152E 03
114	0.117E 01	0.116E 01
118	0.280E 01	0.246E 01
120	0.198E 02	0.184E 02
123	0.901E 00	0.804E 00
124	0.972E 01	0.996E 01
125	0.842E 01	0.955E 01
127	0.669E 01	0.768E 01
129	0.227E 01	0.250E 01
135	0.409E 01	0.333E 01
136	0.258E 02	0.257E 02
138	0.000E 00	0.000E 00
139	0.195E 01	0.000E 00
141	0.488E 01	0.557E 01
144	0.105E 02	0.128E 02
152	0.539E 02	0.579E 02
155	0.000E 00	0.000E 00

	0725052	0725062	0726012	0728022	0729022
168	0.000E 00	0.000E 00	0.687E 02	0.000E 00	0.193E 02
172	0.184E 01	0.154E 01	0.112E 02	0.185E 01	0.000E 00
174	0.912E 01	0.509E 01	0.958E 00	0.134E 01	0.330E 01
176	0.000E 00	0.136E 00	0.000E 00	0.161E 00	0.445E 01
177	0.799E 01	0.804E 01	0.000E 00	0.482E 01	0.455E 01
181	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
183	0.244E 02	0.923E 01	0.112E 02	0.542E 01	0.809E 01
184	0.197E 02	0.160E 02	0.185E 02	0.189E 02	0.995E 01
185	0.556E 01	0.211E 01	0.000E 00	0.287E 01	0.452E 01
186	0.890E 01	0.218E 01	0.462E 01	0.458E 01	0.574E 01
187	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
189	0.000E 00	0.544E 01	0.525E 01	0.000E 00	0.000E 00
194	0.000E 00	0.000E 00	0.236E 01	0.253E 01	0.512E 01
197	0.595E 02	0.000E 00	0.346E 02	0.353E 02	0.401E 02
198	0.408E 02	0.000E 00	0.257E 02	0.268E 02	0.328E 02
200	0.117E 02	0.000E 00	0.122E 02	0.503E 01	0.000E 00
207	0.480E 02	0.154E 02	0.136E 02	0.319E 02	0.229E 02
209	0.000E 00	0.000E 00	0.000E 00	0.461E 00	0.641E 00
212	0.000E 00	0.726E 01	0.416E 01	0.000E 00	0.000E 00
215	0.703E 02	0.534E 02	0.363E 02	0.000E 00	0.887E 02
216	0.714E 02	0.509E 02	0.172E 02	0.174E 02	0.359E 02
218	0.107E 02	0.866E 01	0.433E 01	0.533E 01	0.000E 00
222	0.138E 02	0.959E 01	0.195E 02	0.437E 01	0.739E 01
225	0.122E 02	0.000E 00	0.416E 02	0.289E 02	0.706E 02
229	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
235	0.145E 01	0.117E 01	0.130E 01	0.606E 00	0.165E 01
236	0.259E 00	0.374E 00	0.228E 00	0.693E 00	0.349E 00
237	0.295E 04	0.721E 00	0.000E 00	0.362E 03	0.184E 04
244	0.125E 01	0.170E 00	0.107E 02	0.337E 01	0.856E 01
246	0.254E 00	0.313E 00	0.206E 02	0.243E 02	0.310E 02
251	0.000E 00	0.000E 00	0.329E 01	0.313E 00	0.132E 00
254	0.137E 00	0.000E 00	0.109E 00	0.344E 00	0.522E 00
257	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.328E 00
258	0.640E 01	0.000E 00	0.432E 01	0.287E 01	0.667E 01
266	0.000E 00	0.000E 00	0.142E 03	0.000E 00	0.641E 02
272	0.000E 00	0.000E 00	0.955E 01	0.192E 01	0.424E 01
274	0.141E 01	0.174E 01	0.109E 01	0.958E 00	0.000E 00
277	0.000E 00	0.196E 00	0.191E 00	0.000E 00	0.000E 00
290	0.103E 04	0.370E 03	0.552E 03	0.177E 03	0.429E 03
291	0.000E 00	0.426E 01	0.000E 00	0.422E 01	0.496E 01
293	0.112E 02	0.000E 00	0.359E 00	0.237E 01	0.000E 00
294	0.253E 02	0.695E 01	0.193E 01	0.576E 01	0.173E 01
296	0.640E 01	0.000E 00	0.377E 01	0.591E 01	0.339E 01
297	0.259E 03	0.242E 03	0.000E 00	0.407E 00	0.791E 02
298	0.958E 01	0.767E 01	0.465E 01	0.645E 01	0.416E 01
299	0.000E 00	0.000E 00	0.000E 00	0.494E 01	0.288E 01
301	0.415E 01	0.385E 01	0.508E 00	0.741E 01	0.830E 00
302	0.237E 03	0.123E 03	0.426E 02	0.639E 02	0.863E 02
303	0.248E 02	0.162E 03	0.606E 02	0.122E 03	0.941E 02
305	0.000E 00	0.414E 02	0.259E 02	0.221E 02	0.202E 02

TABLE F1. (Cont'd.)

	0919012	1031012	1031062	1110812	0918023
168	0.106E 03	0.756E 02	0.130E 02	0.647E 02	0.302E 02
172	0.185E 01	0.301E 01	0.156E 01	0.167E 01	0.167E 01
174	0.284E 01	0.540E 01	0.251E 01	0.222E 01	0.666E 01
176	0.000E 00	0.767E 02	0.542E 01	0.000E 00	0.188E 02
177	0.235E 01	0.257E 01	0.465E 01	0.721E 01	0.364E 01
181	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
183	0.000E 00	0.102E 02	0.126E 02	0.247E 02	0.000E 00
184	0.979E 01	0.134E 02	0.111E 02	0.860E 01	0.396E 02
185	0.673E 01	0.285E 01	0.120E 01	0.000E 00	0.000E 00
186	0.160E 01	0.246E 01	0.266E 01	0.907E 01	0.000E 00
187	0.000E 00	0.102E 02	0.000E 00	0.000E 00	0.000E 00
189	0.000E 00	0.726E 01	0.354E 01	0.000E 00	0.000E 00
194	0.251E 01	0.257E 01	0.376E 01	0.000E 00	0.305E 01
197	0.245E 02	0.253E 02	0.327E 02	0.000E 00	0.250E 02
198	0.139E 02	0.122E 02	0.307E 02	0.000E 00	0.155E 02
200	0.295E 01	0.676E 01	0.855E 01	0.000E 00	0.000E 00
207	0.163E 02	0.920E 01	0.745E 01	0.000E 00	0.183E 02
209	0.000E 00	0.110E 01	0.000E 00	0.000E 00	0.000E 00
212	0.333E 01	0.751E 01	0.423E 01	0.489E 01	0.151E 01
215	0.416E 02	0.615E 02	0.684E 02	0.303E 02	0.229E 02
216	0.290E 02	0.469E 02	0.382E 02	0.226E 02	0.843E 02
218	0.582E 01	0.776E 01	0.622E 01	0.412E 01	0.218E 01
223	0.138E 01	0.220E 01	0.249E 02	0.735E 02	0.253E 01
225	0.408E 02	0.428E 02	0.352E 02	0.571E 02	0.325E 02
229	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.156E 00
235	0.000E 00	0.184E 01	0.253E 01	0.126E 02	0.000E 00
236	0.435E 00	0.948E 00	0.000E 00	0.115E 01	0.438E 00
237	0.127E 04	0.109E 04	0.844E 03	0.380E 04	0.378E 03
244	0.555E 02	0.779E 01	0.695E 01	0.189E 03	0.222E 02
246	0.126E 02	0.628E 01	0.448E 01	0.140E 01	0.246E 02
251	0.240E 01	0.000E 00	0.000E 00	0.418E 01	0.346E 01
254	0.209E 01	0.000E 00	0.000E 00	0.000E 00	0.000E 00
257	0.384E 03	0.000E 00	0.000E 00	0.144E 04	0.230E 03
258	0.000E 00	0.320E 01	0.709E 01	0.871E 02	0.000E 00
266	0.555E 02	0.000E 00	0.769E 02	0.121E 04	0.104E 03
272	0.187E 02	0.659E 01	0.435E 01	0.109E 02	0.000E 00
274	0.740E 00	0.000E 00	0.000E 00	0.261E 01	0.241E 00
277	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
290	0.799E 02	0.148E 03	0.421E 03	0.157E 04	0.113E 03
291	0.258E 01	0.213E 02	0.000E 00	0.000E 00	0.248E 01
293	0.558E 01	0.227E 01	0.648E 01	0.655E 01	0.159E 00
294	0.211E 01	0.322E 01	0.805E 01	0.456E 02	0.124E 01
296	0.598E 01	0.606E 01	0.310E 01	0.000E 00	0.568E 01
297	0.245E 03	0.571E 02	0.142E 03	0.972E 02	0.206E 02
298	0.229E 01	0.392E 01	0.482E 01	0.255E 01	0.178E 01
299	0.378E 01	0.348E 01	0.000E 00	0.511E 01	0.000E 00
301	0.668E 01	0.321E 01	0.816E 01	0.830E 00	0.299E 01
302	0.791E 02	0.569E 02	0.451E 02	0.549E 02	0.485E 02
303	0.692E 02	0.392E 02	0.872E 02	0.885E 02	0.429E 02
305	0.555E 01	0.282E 02	0.327E 02	0.729E 00	0.704E 01

	0919023	1029033	1031023	1031033	0316034
168	0.126E 03	0.324E 03	0.197E 03	0.908E 02	0.480E 02
172	0.116E 01	0.349E 01	0.207E 01	0.155E 01	0.818E 00
174	0.188E 01	0.107E 02	0.323E 01	0.309E 01	0.610E 01
176	0.211E 02	0.286E 02	0.000E 00	0.182E 02	0.119E 02
177	0.130E 02	0.214E 02	0.166E 02	0.000E 00	0.480E 01
181	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
183	0.290E 02	0.173E 02	0.185E 02	0.150E 02	0.000E 00
184	0.316E 03	0.611E 02	0.283E 03	0.138E 03	0.247E 02
185	0.000E 00	0.856E 01	0.577E 01	0.939E 01	0.229E 02
186	0.000E 00	0.268E 01	0.527E 01	0.000E 00	0.396E 01
187	0.290E 02	0.173E 02	0.185E 02	0.150E 02	0.614E 01
189	0.000E 00	0.110E 02	0.130E 02	0.110E 02	0.000E 00
194	0.398E 01	0.602E 01	0.311E 01	0.926E 01	0.489E 01
197	0.481E 02	0.884E 02	0.422E 02	0.117E 03	0.679E 02
198	0.334E 02	0.716E 02	0.308E 02	0.932E 02	0.484E 02
200	0.901E 01	0.272E 02	0.165E 02	0.365E 02	0.877E 01
207	0.778E 01	0.884E 01	0.436E 01	0.103E 02	0.194E 02
209	0.000E 00	0.113E 01	0.000E 00	0.000E 00	0.000E 00
212	0.439E 01	0.591E 01	0.657E 01	0.119E 02	0.413E 01
215	0.354E 02	0.828E 02	0.583E 02	0.709E 02	0.551E 02
216	0.639E 03	0.149E 03	0.689E 03	0.100E 03	0.622E 02
218	0.296E 01	0.000E 00	0.000E 00	0.576E 01	0.539E 01
223	0.142E 01	0.182E 01	0.000E 00	0.436E 01	0.320E 01
225	0.481E 02	0.835E 02	0.596E 02	0.680E 02	0.472E 02
229	0.276E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
235	0.179E 01	0.821E 00	0.000E 00	0.000E 00	0.454E 00
236	0.354E 01	0.428E 01	0.903E 00	0.849E 01	0.000E 00
237	0.265E 03	0.213E 03	0.869E 02	0.832E 03	0.551E 03
244	0.103E 02	0.141E 02	0.212E 02	0.119E 02	0.952E 02
246	0.662E 01	0.132E 01	0.395E 01	0.000E 00	0.177E 01
251	0.752E 01	0.115E 02	0.772E 01	0.168E 02	0.000E 00
254	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.870E 00
257	0.000E 00	0.128E 04	0.000E 00	0.124E 04	0.000E 00
258	0.346E 01	0.000E 00	0.112E 01	0.000E 00	0.000E 00
266	0.328E 02	0.408E 02	0.571E 02	0.204E 02	0.223E 02
272	0.538E 01	0.485E 01	0.585E 01	0.308E 01	0.228E 02
274	0.163E 01	0.280E 01	0.116E 01	0.160E 01	0.119E 01
277	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
290	0.196E 03	0.130E 03	0.168E 03	0.116E 03	0.598E 02
291	0.000E 00	0.000E 00	0.000E 00	0.580E 01	0.000E 00
293	0.247E 00	0.000E 00	0.721E 01	0.338E 02	0.126E 02
294	0.906E 01	0.133E 02	0.448E 01	0.164E 02	0.551E 01
296	0.592E 01	0.982E 01	0.680E 01	0.800E 01	0.119E 02
297	0.212E 02	0.279E 02	0.951E 02	0.201E 03	0.000E 00
298	0.547E 00	0.525E 00	0.874E 00	0.000E 00	0.602E 01
299	0.876E 00	0.136E 01	0.122E 01	0.307E 01	0.263E 01
301	0.229E 01	0.223E 01	0.144E 02	0.275E 01	0.484E 01
302	0.498E 02	0.594E 02	0.643E 02	0.628E 02	0.936E 02
303	0.337E 02	0.292E 02	0.198E 02	0.510E 02	0.452E 02
305	0.396E 01	0.737E 01	0.139E 02	0.143E 02	0.492E 01

TABLE F1. (Cont'd.)

	0316044	0502014	0502034	0502054	0330046
168	0.000E 00	0.799E 02	0.377E 02	0.277E 02	0.186E 02
172	0.872E 00	0.318E 01	0.000E 00	0.150E 00	0.000E 00
174	0.210E 01	0.125E 01	0.190E 01	0.505E 01	0.564E 01
176	0.000E 00	0.197E 02	0.000E 00	0.857E 01	0.000E 00
177	0.246E 01	0.565E 01	0.000E 00	0.613E 01	0.000E 00
181	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.505E 00
183	0.000E 00	0.893E 01	0.000E 00	0.258E 01	0.000E 00
184	0.158E 02	0.335E 02	0.154E 02	0.118E 02	0.304E 02
185	0.000E 00	0.774E 01	0.115E 01	0.255E 01	0.117E 02
186	0.000E 00	0.282E 00	0.000E 00	0.000E 00	0.000E 00
187	0.177E 01	0.893E 01	0.177E 01	0.258E 01	0.264E 01
189	0.000E 00	0.000E 00	0.195E 01	0.000E 00	0.000E 00
194	0.654E 01	0.213E 01	0.411E 00	0.393E 01	0.195E 02
197	0.714E 02	0.289E 02	0.000E 00	0.583E 02	0.262E 03
198	0.135E 02	0.286E 02	0.509E 01	0.536E 02	0.223E 03
200	0.162E 02	0.791E 01	0.000E 00	0.000E 00	0.000E 00
207	0.383E 02	0.118E 02	0.166E 02	0.183E 02	0.432E 02
209	0.848E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
212	0.262E 01	0.659E 01	0.128E 02	0.215E 01	0.478E 01
215	0.628E 02	0.901E 02	0.601E 02	0.000E 00	0.217E 03
216	0.334E 02	0.914E 02	0.414E 02	0.570E 00	0.124E 03
218	0.000E 00	0.124E 02	0.567E 01	0.000E 00	0.241E 02
223	0.000E 00	0.236E 01	0.000E 00	0.000E 00	0.000E 00
225	0.421E 02	0.723E 02	0.469E 02	0.351E 02	0.228E 02
229	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
235	0.000E 00	0.000E 00	0.000E 00	0.935E 00	0.000E 00
236	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
237	0.159E 04	0.000E 00	0.128E 04	0.336E 03	0.000E 00
244	0.442E 02	0.390E 02	0.367E 02	0.641E 02	0.297E 02
246	0.000E 00	0.438E 00	0.629E 00	0.816E 01	0.000E 00
251	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
254	0.000E 00	0.000E 00	0.000E 00	0.119E 01	0.000E 00
257	0.000E 00	0.000E 00	0.000E 00	0.410E 02	0.000E 00
258	0.000E 00	0.000E 00	0.000E 00	0.449E 01	0.000E 00
266	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
272	0.345E 02	0.156E 02	0.238E 02	0.309E 02	0.420E 02
274	0.000E 00	0.256E 01	0.177E 01	0.000E 00	0.299E 01
277	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
290	0.149E 02	0.279E 02	0.598E 01	0.150E 02	0.111E 02
291	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
293	0.455E 01	0.000E 00	0.113E 02	0.120E 01	0.318E 00
294	0.810E 00	0.401E 01	0.192E 01	0.224E 00	0.000E 00
296	0.709E 01	0.693E 01	0.662E 01	0.731E 01	0.939E 01
297	0.000E 00	0.218E 03	0.421E 02	0.000E 00	0.626E 01
298	0.994E 01	0.901E 01	0.293E 02	0.223E 01	0.985E 00
299	0.000E 00	0.224E 01	0.296E 01	0.944E 00	0.000E 00
301	0.328E 01	0.136E 01	0.000E 00	0.632E 01	0.000E 00
302	0.476E 02	0.812E 02	0.143E 03	0.345E 02	0.387E 02
303	0.686E 02	0.114E 03	0.307E 02	0.107E 02	0.288E 02
305	0.226E 02	0.232E 01	0.175E 02	0.247E 02	0.297E 02

	0316037	0323027
168	0.480E 02	0.483E 02
172	0.818E 00	0.233E 00
174	0.610E 01	0.424E 01
176	0.119E 02	0.000E 00
177	0.480E 01	0.424E 01
181	0.000E 00	0.000E 00
183	0.000E 00	0.000E 00
184	0.247E 02	0.219E 02
185	0.229E 02	0.217E 02
186	0.106E 01	0.000E 00
187	0.614E 01	0.461E 01
189	0.000E 00	0.000E 00
194	0.488E 01	0.236E 01
197	0.673E 02	0.579E 02
198	0.484E 02	0.498E 02
200	0.877E 01	0.841E 01
207	0.194E 02	0.183E 02
209	0.000E 00	0.000E 00
212	0.413E 01	0.483E 01
215	0.551E 02	0.446E 02
216	0.622E 02	0.479E 02
218	0.539E 01	0.383E 01
223	0.328E 01	0.000E 00
225	0.472E 02	0.413E 02
229	0.000E 00	0.000E 00
235	0.944E 00	0.741E 00
236	0.000E 00	0.000E 00
237	0.551E 03	0.446E 03
244	0.952E 02	0.822E 02
246	0.177E 01	0.948E 00
251	0.000E 00	0.000E 00
254	0.870E 00	0.000E 00
257	0.000E 00	0.000E 00
258	0.000E 00	0.348E 01
266	0.223E 02	0.000E 00
272	0.228E 02	0.219E 02
274	0.119E 01	0.000E 00
277	0.000E 00	0.000E 00
290	0.598E 02	0.516E 02
291	0.000E 00	0.000E 00
293	0.126E 02	0.228E 02
294	0.551E 01	0.549E 01
296	0.119E 02	0.111E 02
297	0.000E 00	0.000E 00
298	0.602E 01	0.527E 01
299	0.263E 01	0.000E 00
301	0.484E 01	0.542E 01
302	0.936E 02	0.918E 02
303	0.452E 02	0.461E 02
305	0.492E 01	0.553E 01

TABLE F1. (Cont'd.)

	0725052	0725062	0726012	0728022	0729022
306	0.895E 02	0.928E 02	0.356E 02	0.401E 02	0.442E 02
308	0.359E 03	0.382E 03	0.193E 03	0.272E 03	0.228E 03
309	0.991E 02	0.147E 03	0.888E 02	0.141E 03	0.107E 03
310	0.109E 02	0.400E 01	0.265E 01	0.832E 01	0.615E 01
313	0.219E 02	0.475E 02	0.220E 02	0.255E 02	0.173E 02
314	0.179E 02	0.772E 02	0.339E 02	0.241E 02	0.238E 02
316	0.516E 02	0.601E 02	0.525E 02	0.219E 02	0.256E 02
318	0.969E 03	0.596E 03	0.236E 03	0.404E 03	0.297E 03
319	0.172E 03	0.317E 03	0.987E 02	0.886E 02	0.657E 02
320	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
321	0.116E 02	0.258E 02	0.543E 02	0.666E 01	0.377E 02
322	0.413E 02	0.596E 01	0.119E 02	0.158E 02	0.144E 02
324	0.567E 02	0.000E 00	0.346E 02	0.347E 02	0.414E 02
325	0.256E 03	0.156E 03	0.100E 03	0.181E 03	0.388E 03
326	0.133E 03	0.489E 02	0.206E 03	0.189E 03	0.393E 03
328	0.411E 01	0.306E 01	0.299E 01	0.297E 01	0.000E 00
329	0.267E 02	0.301E 02	0.416E 02	0.138E 02	0.248E 02
332	0.000E 00	0.000E 00	0.101E 02	0.693E 01	0.109E 01
333	0.000E 00	0.000E 00	0.659E 01	0.359E 01	0.179E 02
334	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.657E 02
342	0.000E 00	0.000E 00	0.243E 01	0.000E 00	0.000E 00
343	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
344	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
345	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
346	0.296E 02	0.406E 02	0.257E 02	0.220E 02	0.206E 02
347	0.292E 02	0.187E 02	0.113E 02	0.121E 02	0.138E 02
348	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
349	0.169E 01	0.153E 01	0.000E 00	0.141E 01	0.755E 00
350	0.000E 00	0.000E 00	0.131E 02	0.150E 02	0.161E 02
351	0.000E 00	0.148E 02	0.000E 00	0.000E 00	0.000E 00
352	0.181E 02	0.793E 01	0.000E 00	0.130E 02	0.113E 02
353	0.376E 02	0.319E 02	0.265E 02	0.288E 02	0.390E 02
354	0.292E 02	0.505E 02	0.232E 02	0.277E 02	0.217E 02
356	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
357	0.000E 00	0.000E 00	0.000E 00	0.964E 00	0.000E 00
358	0.167E 02	0.143E 02	0.000E 00	0.000E 00	0.000E 00
359	0.109E 02	0.101E 03	0.186E 03	0.853E 01	0.284E 02
360	0.759E 02	0.285E 02	0.000E 00	0.299E 02	0.897E 01
361	0.000E 00	0.000E 00	0.578E 02	0.762E 00	0.143E 02
363	0.000E 00	0.180E 01	0.159E 01	0.129E 01	0.377E 01
364	0.273E 02	0.185E 02	0.166E 02	0.206E 02	0.287E 02
365	0.473E 02	0.000E 00	0.574E 02	0.560E 02	0.102E 03
366	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
367	0.300E 01	0.612E 00	0.000E 00	0.162E 01	0.395E 01
368	0.000E 00	0.000E 00	0.000E 00	0.113E 00	0.244E 01
369	0.000E 00	0.487E 00	0.772E 01	0.108E 01	0.000E 00
371	0.188E 02	0.237E 02	0.648E 01	0.170E 02	0.902E 01
372	0.000E 00	0.550E 02	0.384E 02	0.440E 02	0.403E 02
373	0.000E 00	0.000E 00	0.000E 00	0.693E 01	0.000E 00
374	0.399E 01	0.000E 00	0.800E 01	0.162E 01	0.271E 01

	0919012	1031012	1031062	1110012	0918023
306	0.386E 02	0.508E 02	0.376E 02	0.166E 02	0.728E 02
308	0.222E 03	0.344E 03	0.302E 03	0.999E 02	0.192E 03
309	0.132E 03	0.174E 03	0.136E 03	0.407E 02	0.150E 03
310	0.223E 02	0.444E 01	0.523E 01	0.161E 02	0.130E 02
313	0.384E 01	0.174E 02	0.866E 02	0.187E 01	0.805E 01
314	0.115E 01	0.206E 02	0.272E 02	0.513E 00	0.267E 01
316	0.467E 01	0.244E 02	0.520E 02	0.000E 00	0.000E 00
318	0.314E 03	0.260E 03	0.590E 03	0.904E 03	0.671E 01
319	0.000E 00	0.947E 02	0.261E 03	0.000E 00	0.192E 02
320	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
321	0.120E 01	0.164E 02	0.307E 02	0.000E 00	0.453E 01
322	0.126E 02	0.927E 01	0.717E 01	0.347E 02	0.000E 00
324	0.245E 02	0.244E 02	0.327E 02	0.000E 00	0.250E 02
325	0.187E 03	0.151E 03	0.151E 03	0.112E 03	0.722E 02
326	0.253E 03	0.176E 03	0.127E 03	0.279E 03	0.200E 03
328	0.000E 00	0.940E 00	0.110E 02	0.319E 01	0.630E 00
329	0.223E 02	0.669E 01	0.208E 02	0.579E 02	0.106E 02
332	0.000E 00	0.171E 01	0.329E 02	0.000E 00	0.000E 00
333	0.213E 02	0.774E 01	0.122E 02	0.101E 02	0.325E 02
334	0.427E 02	0.000E 00	0.352E 02	0.000E 00	0.000E 00
342	0.154E 02	0.000E 00	0.000E 00	0.101E 02	0.385E 01
343	0.569E 01	0.000E 00	0.000E 00	0.497E 01	0.192E 01
344	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.686E 00
345	0.000E 00	0.549E 00	0.121E 01	0.300E 00	0.161E 01
346	0.561E 01	0.285E 02	0.332E 02	0.729E 00	0.719E 01
347	0.909E 01	0.183E 02	0.000E 00	0.568E 01	0.187E 02
348	0.743E 00	0.000E 00	0.000E 00	0.000E 00	0.278E 01
349	0.963E 01	0.113E 01	0.000E 00	0.765E 01	0.154E 02
350	0.162E 02	0.100E 02	0.816E 01	0.000E 00	0.125E 02
351	0.267E 02	0.000E 00	0.000E 00	0.245E 02	0.340E 02
352	0.424E 02	0.287E 01	0.592E 01	0.252E 02	0.206E 02
353	0.469E 02	0.212E 02	0.162E 02	0.396E 02	0.553E 02
354	0.858E 01	0.000E 00	0.000E 00	0.000E 00	0.134E 02
356	0.317E 02	0.344E 02	0.639E 01	0.124E 02	0.473E 02
357	0.319E 00	0.510E 01	0.000E 00	0.000E 00	0.396E 02
358	0.773E 01	0.000E 00	0.000E 00	0.000E 00	0.870E 01
359	0.120E 02	0.153E 01	0.000E 00	0.000E 00	0.302E 02
360	0.783E 02	0.000E 00	0.000E 00	0.751E 02	0.340E 02
361	0.115E 03	0.556E 02	0.137E 02	0.535E 02	0.621E 02
363	0.000E 00	0.174E 01	0.000E 00	0.115E 01	0.828E 01
364	0.166E 02	0.203E 02	0.000E 00	0.122E 02	0.361E 02
365	0.622E 02	0.444E 02	0.354E 02	0.680E 02	0.444E 02
366	0.936E 01	0.000E 00	0.000E 00	0.000E 00	0.822E 01
367	0.195E 01	0.250E 01	0.000E 00	0.203E 01	0.500E 01
368	0.405E 00	0.000E 00	0.000E 00	0.702E 00	0.813E 01
369	0.394E 01	0.485E 01	0.254E 01	0.212E 01	0.137E 02
371	0.461E 01	0.426E 01	0.407E 01	0.606E 01	0.544E 01
372	0.147E 02	0.223E 02	0.304E 02	0.000E 00	0.157E 02
373	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
374	0.523E 01	0.108E 02	0.318E 01	0.317E 01	0.111E 02

TABLE F1. (Cont'd.)

	0919023	1029033	1031023	1031033	0316034
306	0.306E 02	0.490E 02	0.643E 02	0.558E 02	0.300E 02
308	0.276E 03	0.437E 03	0.429E 03	0.404E 03	0.224E 03
309	0.823E 02	0.110E 03	0.143E 03	0.187E 03	0.838E 02
310	0.237E 02	0.159E 02	0.301E 02	0.129E 02	0.190E 02
313	0.185E 02	0.147E 02	0.171E 02	0.218E 02	0.712E 01
314	0.283E 01	0.294E 01	0.540E 01	0.743E 01	0.257E 01
316	0.805E 01	0.139E 02	0.234E 02	0.164E 02	0.854E 01
318	0.296E 01	0.807E 01	0.101E 02	0.247E 01	0.141E 02
319	0.382E 02	0.283E 02	0.614E 02	0.513E 02	0.230E 02
320	0.000E 00	0.000E 00	0.215E 01	0.000E 00	0.000E 00
321	0.774E 01	0.535E 01	0.147E 02	0.138E 02	0.000E 00
322	0.292E 00	0.168E 00	0.283E 00	0.000E 00	0.000E 00
324	0.481E 02	0.985E 02	0.429E 02	0.117E 03	0.673E 02
325	0.161E 03	0.288E 03	0.201E 03	0.219E 03	0.213E 03
326	0.251E 03	0.334E 03	0.256E 03	0.265E 03	0.251E 03
328	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
329	0.369E 02	0.311E 02	0.320E 02	0.243E 02	0.115E 02
332	0.577E 00	0.241E 01	0.834E 00	0.440E 01	0.508E 01
333	0.762E 01	0.636E 01	0.478E 02	0.541E 01	0.850E 01
334	0.724E 02	0.622E 02	0.281E 02	0.447E 02	0.433E 02
342	0.291E 02	0.000E 00	0.000E 00	0.434E 02	0.347E 02
343	0.102E 02	0.793E 01	0.516E 01	0.124E 02	0.858E 01
344	0.554E 00	0.278E 00	0.150E 01	0.000E 00	0.598E 00
345	0.157E 01	0.700E 00	0.135E 01	0.118E 01	0.000E 00
346	0.396E 01	0.737E 01	0.139E 02	0.143E 02	0.492E 01
347	0.791E 01	0.192E 02	0.239E 02	0.000E 00	0.929E 01
348	0.935E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
349	0.681E 01	0.455E 01	0.646E 01	0.506E 01	0.136E 02
350	0.266E 01	0.326E 01	0.000E 00	0.684E 01	0.000E 00
351	0.299E 02	0.000E 00	0.271E 02	0.000E 00	0.488E 02
352	0.447E 02	0.166E 02	0.228E 02	0.143E 02	0.289E 02
353	0.457E 02	0.422E 02	0.391E 02	0.413E 02	0.775E 02
354	0.232E 02	0.163E 02	0.245E 02	0.236E 02	0.133E 02
356	0.259E 01	0.125E 01	0.647E 00	0.437E 01	0.188E 01
357	0.159E 02	0.189E 02	0.160E 02	0.189E 02	0.515E 01
358	0.931E 01	0.000E 00	0.000E 00	0.000E 00	0.000E 00
359	0.267E 01	0.000E 00	0.405E 01	0.000E 00	0.000E 00
360	0.124E 03	0.000E 00	0.136E 03	0.474E 02	0.445E 02
361	0.143E 03	0.238E 03	0.000E 00	0.716E 02	0.748E 01
363	0.227E 01	0.147E 01	0.000E 00	0.741E 00	0.256E 01
364	0.163E 02	0.194E 02	0.140E 02	0.000E 00	0.307E 02
365	0.612E 02	0.814E 02	0.607E 02	0.696E 02	0.618E 02
366	0.197E 01	0.947E 00	0.000E 00	0.000E 00	0.413E 01
367	0.168E 00	0.546E 00	0.109E 01	0.000E 00	0.219E 01
368	0.432E 00	0.000E 00	0.000E 00	0.000E 00	0.344E 01
369	0.772E 01	0.447E 01	0.453E 01	0.447E 01	0.000E 00
371	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.119E 02
372	0.174E 02	0.205E 02	0.196E 02	0.208E 02	0.156E 02
373	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
374	0.789E 01	0.100E 02	0.627E 01	0.105E 02	0.120E 02

	0316044	0502014	0502034	0502054	0330046
306	0.645E 02	0.299E 02	0.445E 02	0.415E 02	0.268E 03
308	0.296E 03	0.225E 03	0.332E 03	0.000E 00	0.595E 03
309	0.910E 02	0.117E 03	0.414E 03	0.281E 02	0.176E 04
310	0.265E 01	0.227E 02	0.123E 02	0.313E 01	0.288E 01
313	0.330E 02	0.451E 01	0.145E 02	0.112E 02	0.145E 02
314	0.275E 02	0.131E 01	0.000E 00	0.218E 02	0.280E 02
316	0.300E 02	0.489E 01	0.113E 02	0.242E 02	0.358E 02
318	0.588E 01	0.782E 01	0.000E 00	0.800E 02	0.000E 00
319	0.105E 03	0.616E 01	0.387E 02	0.107E 03	0.160E 02
320	0.000E 00	0.000E 00	0.000E 00	0.451E 01	0.000E 00
321	0.387E 02	0.272E 01	0.557E 01	0.202E 02	0.554E 02
322	0.000E 00	0.000E 00	0.435E 00	0.000E 00	0.000E 00
324	0.734E 02	0.289E 02	0.000E 00	0.583E 02	0.262E 03
325	0.303E 03	0.315E 03	0.207E 03	0.120E 03	0.476E 03
326	0.324E 03	0.331E 03	0.216E 03	0.124E 03	0.185E 03
328	0.121E 01	0.000E 00	0.000E 00	0.000E 00	0.000E 00
329	0.436E 01	0.292E 02	0.260E 02	0.638E 01	0.199E 02
332	0.181E 00	0.510E 01	0.438E 00	0.381E 01	0.769E 00
333	0.929E 01	0.000E 00	0.000E 00	0.679E 02	0.470E 01
334	0.271E 02	0.608E 02	0.411E 02	0.000E 00	0.659E 02
342	0.135E 02	0.000E 00	0.221E 02	0.544E 01	0.153E 02
343	0.559E 01	0.710E 01	0.577E 01	0.308E 01	0.432E 01
344	0.000E 00	0.000E 00	0.951E 00	0.000E 00	0.000E 00
345	0.472E 00	0.000E 00	0.181E 01	0.000E 00	0.000E 00
346	0.226E 02	0.232E 01	0.175E 02	0.247E 02	0.297E 02
347	0.193E 02	0.103E 02	0.158E 02	0.126E 02	0.809E 02
348	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
349	0.000E 00	0.259E 02	0.512E 02	0.591E 01	0.657E 01
350	0.319E 00	0.424E 01	0.000E 00	0.462E 01	0.000E 00
351	0.000E 00	0.714E 02	0.000E 00	0.365E 01	0.000E 00
352	0.499E 01	0.351E 02	0.179E 02	0.447E 01	0.127E 01
353	0.199E 02	0.901E 02	0.530E 02	0.129E 02	0.912E 01
354	0.330E 02	0.922E 01	0.000E 00	0.136E 02	0.183E 02
356	0.660E 01	0.000E 00	0.000E 00	0.000E 00	0.000E 00
357	0.103E 02	0.000E 00	0.146E 01	0.115E 02	0.113E 01
358	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.411E 01
359	0.374E 02	0.000E 00	0.000E 00	0.713E 02	0.000E 00
360	0.000E 00	0.364E 02	0.167E 02	0.989E 01	0.000E 00
361	0.000E 00	0.523E 02	0.303E 02	0.238E 02	0.523E 01
363	0.738E 01	0.429E 01	0.350E 01	0.410E 01	0.655E 01
364	0.193E 02	0.401E 02	0.343E 02	0.190E 02	0.604E 02
365	0.497E 02	0.990E 02	0.526E 02	0.333E 02	0.535E 02
366	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
367	0.197E 01	0.398E 01	0.000E 00	0.182E 01	0.499E 01
368	0.000E 00	0.000E 00	0.000E 00	0.742E 00	0.000E 00
369	0.000E 00	0.000E 00	0.000E 00	0.523E 01	0.106E 01
371	0.117E 02	0.147E 02	0.265E 02	0.935E 01	0.000E 00
372	0.256E 02	0.123E 02	0.189E 02	0.917E 01	0.501E 02
373	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
374	0.100E 02	0.650E 01	0.188E 02	0.407E 01	0.827E 01

TABLE F1. (Cont'd.)

	0316037	0323027
306	0.300E 02	0.319E 02
308	0.224E 03	0.217E 03
309	0.838E 02	0.852E 02
310	0.190E 02	0.194E 02
313	0.712E 01	0.686E 01
314	0.257E 01	0.294E 01
316	0.854E 01	0.885E 01
318	0.141E 02	0.148E 02
319	0.230E 02	0.228E 02
320	0.000E 00	0.000E 00
321	0.000E 00	0.000E 00
322	0.000E 00	0.000E 00
324	0.673E 02	0.579E 02
325	0.213E 03	0.175E 03
326	0.251E 03	0.211E 03
328	0.000E 00	0.000E 00
329	0.115E 02	0.240E 02
332	0.508E 01	0.413E 01
333	0.850E 01	0.301E 02
334	0.433E 02	0.483E 02
342	0.347E 02	0.361E 02
343	0.858E 01	0.616E 01
344	0.598E 00	0.734E 00
345	0.000E 00	0.000E 00
346	0.492E 01	0.553E 01
347	0.929E 01	0.977E 01
348	0.000E 00	0.000E 00
349	0.136E 02	0.143E 02
350	0.000E 00	0.000E 00
351	0.488E 02	0.483E 02
352	0.289E 02	0.300E 02
353	0.775E 02	0.778E 02
354	0.133E 02	0.121E 02
356	0.188E 01	0.192E 01
357	0.515E 01	0.583E 01
358	0.000E 00	0.000E 00
359	0.000E 00	0.000E 00
360	0.445E 02	0.454E 02
361	0.748E 01	0.406E 02
363	0.256E 01	0.323E 01
364	0.307E 02	0.256E 02
365	0.618E 02	0.546E 02
366	0.413E 01	0.329E 01
367	0.219E 01	0.206E 01
368	0.344E 01	0.313E 01
369	0.000E 00	0.000E 00
371	0.119E 02	0.105E 02
372	0.156E 02	0.152E 02
373	0.000E 00	0.000E 00
374	0.120E 02	0.127E 02

	0725052	0725062	0726012	0728022	0729022
375	0.170E 02	0.000E 00	0.588E 01	0.164E 02	0.308E 02
376	0.125E 02	0.200E 01	0.888E 01	0.907E 01	0.835E 01
377	0.000E 00	0.000E 00	0.000E 00	0.000E 00	0.000E 00
378	0.890E 02	0.778E 02	0.691E 02	0.567E 02	0.923E 02
379	0.000E 00	0.627E 01	0.114E 02	0.000E 00	0.739E 01
381	0.000E 00	0.116E 01	0.261E 01	0.639E 01	0.455E 01
382	0.390E 02	0.346E 02	0.356E 02	0.232E 02	0.274E 02
383	0.306E 03	0.205E 03	0.280E 03	0.197E 03	0.840E 02

	0919012	1031012	1031062	1110012	0918023
375	0.405E 01	0.453E 01	0.269E 02	0.584E 01	0.226E 02
376	0.644E 01	0.779E 01	0.659E 01	0.808E 01	0.216E 02
377	0.000E 00	0.227E 01	0.000E 00	0.000E 00	0.000E 00
378	0.000E 00	0.635E 02	0.149E 03	0.000E 00	0.343E 01
379	0.314E 01	0.129E 02	0.141E 02	0.000E 00	0.000E 00
381	0.330E 01	0.635E 01	0.238E 02	0.000E 00	0.133E 02
382	0.000E 00	0.212E 02	0.000E 00	0.939E 01	0.352E 02
383	0.789E 02	0.430E 03	0.601E 03	0.434E 02	0.141E 03

	0919023	1029033	1031023	1031033	0316034
375	0.119E 02	0.940E 01	0.142E 02	0.817E 01	0.118E 02
376	0.502E 01	0.898E 01	0.000E 00	0.356E 01	0.339E 01
377	0.302E 01	0.000E 00	0.142E 02	0.000E 00	0.523E 01
378	0.304E 02	0.523E 02	0.447E 02	0.662E 02	0.133E 02
379	0.365E 01	0.492E 01	0.000E 00	0.663E 01	0.261E 01
381	0.149E 02	0.247E 02	0.201E 02	0.933E 01	0.185E 01
382	0.547E 02	0.436E 02	0.738E 02	0.000E 00	0.783E 01
383	0.145E 02	0.493E 02	0.573E 02	0.567E 02	0.952E 02

	0316044	0502014	0502034	0502054	0330046
375	0.603E 01	0.167E 02	0.475E 02	0.431E 02	0.111E 01
376	0.730E 01	0.101E 02	0.000E 00	0.000E 00	0.000E 00
377	0.112E 02	0.000E 00	0.000E 00	0.671E 01	0.881E 01
378	0.626E 02	0.111E 02	0.489E 02	0.398E 02	0.725E 02
379	0.177E 02	0.000E 00	0.000E 00	0.926E 01	0.476E 01
381	0.131E 02	0.140E 01	0.727E 02	0.273E 02	0.000E 00
382	0.228E 02	0.128E 02	0.720E 01	0.314E 02	0.391E 02
383	0.882E 03	0.167E 03	0.109E 03	0.155E 03	0.259E 04

	0316037	0323027
375	0.118E 02	0.125E 02
376	0.339E 01	0.344E 01
377	0.523E 01	0.000E 00
378	0.133E 02	0.167E 02
379	0.261E 01	0.000E 00
381	0.185E 01	0.160E 01
382	0.783E 01	0.719E 01
383	0.952E 02	0.774E 02

APPENDIX G

Compounds omitted when calculating statistical normalization factor

In performing statistical comparisons, the following compounds were omitted when calculating a normalization factor. (See the Methods section for details). Compounds listed in G1 are referred to as the "35 compounds omitted" in the text; those in Table G2 are referred to as the "12 compounds omitted."

Figure G1. 35 Compounds omitted.

Compound number	Compound name
18	β -hydroxybutyric
50	phosphoric
112	pyroglutamic
120	tropic
152	α-glycerophosphoric
168	citiric
184	homovanillic
197	hexuronic-peak 2
198	unknown NE-5
215	hexuronic-peak 3
216	vanilmandelic
225	hexuronic-peak 4

Figure G1 (Cont'd.)

Compound number	Compound name
237	hippuric
257	uric
266	m-hydroxyhippuric
290	m-hydroxyphenylhydracrylic
297	unknown U5 (cresol)
302	unknown U10 (4-deoxyerythronic)
303	unknown U11 (4-deoxythreonic)
306	unknown U14 (2-deoxytetronic)
308	unknown U16 (erythronic)
309	unknown U17 (threonic)
318	unknown U26
319	unknown U27
324	unknown U32
325	unknown U33 (hexuronic-peak 3)
326	unknown U34 (hexuronic-peak 4)
360	unknown U68
361	unknown U69
365	unknown U73
378	unknown U86
383	unknown U91

Figure G2. 12 Compounds omitted.

Compound number	Compound name
50	phosphoric
120	tropic
168	citric
237	hippuric
257	uric
266	m-hydroxyhippuric
308	U16 (erythronic)
309	U17 (threonic)

Figure G2. (Cont'd.)

Compound number	Compound name
318	U26
319	U27
325	U33 (hexuronic-peak 3)

APPENDIX H

Clincal report form

This form is produced from a single MSSMET output by a computer program called MSSRPT. The relative peak areas calculated by MSSMET are converted to concentrations in mg/ml using a file of correction factors, when these factors are known; otherwise, data are reported as relative concentrations. Each concentration is compared to a table of mean values and standard deviations (taken from a table similar to that shown in Appendix J). In the example shown, this is a table of normalized relative peak areas of a group of juvenile control samples. The correction factor listed on the report form is used to convert relative concentrations to normalized relative concentrations. Each datum is plotted as the number of standard deviations it is from the mean. (E.g., if the mean ± the standard deviation is 5 ± 2, a value of 9 would be plotted in the "+2" column of the table.) In addition, the minimum detectable value is estimated by assuming it is zero, and plotting a "greater than" symbol at the corresponding location (e.g., in the above example, a value of zero would correspond to -2.5 standard deviation units).

URINE PROFILE ANALYSIS

MSU/NIH MASS SPECTROMETRY FACILITY
CURRENT DATE: 25-JUL-77

DATE OF MSSMET ANALYSIS: 07-APR-77
NAME OF OPERATOR: S. GATES
ID NUMBER OF URINE SAMPLE:09076N5
ALL VALUES CORRECTED BY FACTOR OF 0.690
SAMPLE DESCRIPTION: NEUROBLASTOMA URINE FROM DR. KRIVIT
ML URINE EXTRACTED: 1.000
MG CREATININE/ML URINE: 0.180
UG INTERNAL STANDARD: 50.000
DATE FILE OF REFERENCE VALUES LAST MODIFIED: 25-JUL-77
DATE FILE OF K-FACTORS LAST MODIFIED: 14-JAN-77

	COMPOUND	UG/MG C	RANGE IN STANDARD DEVIATION UNITS -4 -2 0 +2 +4 VERY LOW LOW NORMAL HIGH VERY HI
293	U1	10.600#	
6	A-HYDROXYISOBUTYRIC	2.647	
8	LACTIC	22.400#	
294	U2	6.380#	
10	GLYCOLIC	17.000#	
17	GLYOXYLIC OXIME	1.640#	
18	B-HYDROXYBUTYRIC	9.190#	
296	U4	9.870#	
297	U5 (CRESOL)	138.000#	
23	OXALIC	13.000#	
298	U6	1.210#	
343	U51	7.480#	
344	U52	2.170#	
299	U7	1.770#	
28	GLYCEROL	5.460#	
35	METHYLMALONIC	0.446#	
34	UNK RA183 (GLYOXYLIC??	0.655# @	
42	UNK-OXB1	2.730#	
301	U9 (2-METHYLGLYCERIC)	20.900#	
50	PHOSPHORIC	1210.000#	
302	U10 (DEOXYERYTHRONIC)	93.300#	
51	BENZOIC	6.443	
303	U11 (DEOXYTHREONIC)	27.500#	
60	SUCCINIC	71.070	
59	UNKNOWN NF2	1.780#	
61	FUMARIC	7.170#	
65	MALEIC	0.720#	
345	U53	1.960#	
66	PHENYLACETIC	0.252#	
346	U54	20.100#	
305	U13 (DEOXYTETRONIC)	20.100#	
306	U14	93.300#	
347	U55	34.700#	
349	U57	9.370#	
77	GLUTARIC	5.796	
82	CITRAMALIC	1.100#	
86	MALIC	36.330	
308	U16 (ERYTHRONIC)	623.000#	
372	U80 (3-ME-GLUTACONIC)	28.400#	

FIGURE H1. Clinical report form

	COMPOUND	UG/MG C	RANGE IN STANDARD DEVIATION UNITS -4 -2 0 +2 +4 VERY LOW LOW NORMAL HIGH VERY HI
351	U59	39.300#	
309	U17 (THREONIC)	208.000#	
98	ADIPIC	17.689	
105	3-METHYLADIPIC	1.850#	
104	O-HYDROXYBENZOIC	21.300#	
352	U60	49.000#	
310	U18	43.600#	
107	A-HYDROXYGLUTARIC	22.000#	
353	U61	56.700#	
110	HYDROXYMETHYLGLUTARIC	21.120	
313	U21	24.800#	
354	U62	35.500#	
374	U82	9.090#	
111	M-HYDROXYBENZOIC	0.621 @	
112	PYROGLUTAMIC	120.420	
375	U83	20.600#	
314	U22	7.840#	
114	O-HYDROXYPHENYLACETIC	0.701#	
120	TROPIC (INTERNAL STD.)	278.000#	
124	ARABONOLACTONE-PEAK 1	23.000#	
125	A-KETOGLUTARIC OXIME	20.400#	
377	U85	20.600#	
127	P-HYDROXYBENZOIC	31.900#	
129	M-HYDROXYPHENYLACETIC	7.803	
316	U24	34.000#	
136	P-HYDROXYPHENYLACETIC	17.300#	
378	U86	64.900#	
135	RIBONOLACTONE	15.100#	
139	ARABONIC	8.220#	
141	SUBERIC	2.080#	
144	B-GLYCEROPHOSPHORIC	4.480#	
356	U64	0.939#	
357	U65	23.200#	
152	A-GLYCEROPHOSPHORIC	31.540	
318	U26	14.700#	
319	U27	89.100#	
359	U67	5.880#	
360	U68	197.000#	
168	CITRIC	514.800	
320	U28	3.120#	
174	AZELAIC	4.680#	
172	TEREPHTHALIC	3.010#	
177	VANILLIC	28.920	
381	U89	29.200#	
382	U90	107.000#	
321	U29	21.300#	
184	HOMOVANILLIC	411.000#	
185	GALACTONO-1,4-LACTONE	8.370#	
183	3,4-DI-OH-PHEN. ACETIC	4.288	
187	HOMOGENTISIC	4.288	
290	M-OHPHENYLHYDRACRYLIC	243.000#	
186	VERATRIC	7.650#	
322	U30	0.411#	
189	GALACTONOLACTONE-PK 2	18.800#	
198	UNKNOWN NE5	44.700#	

FIGURE H1. (Cont'd.)

	COMPOUND	UG/MG C	RANGE IN STANDARD DEVIATION UNITS -4 -2 0 +2 +4 VERY LOW LOW NORMAL HIGH VERY HI
324	U32	62.300#	> *
194	O-COUMARIC	14.883	> *
197	GALACTURONIC-PEAK 2	62.700#	> *
200	GLUCONIC	24.000#	> *
207	P-OH-PHENYLLACTIC	6.320#	>*
364	U72	20.300#	> *
216	VANILMANDELIC	4200.000	> *
215	HEXURONIC	84.600#	> *
383	U91	83.100#	> *
325	U33	292.000#	> *
365	U73	88.100#	> *
326	U34	371.000#	> *
225	GALACTURONIC-PEAK 2	86.500#	> *
212	PALMITIC	9.530#	> *
367	U75	1.580#	> *
237	HIPPURIC	504.000	>*
236	3,4-DIHYDROXYCINNAMIC-	1.310# @	
369	U77	6.570#	> *
329	U37	46.400#	> *
244	INDOLEACETIC	16.324	> *
246	UNKNOWN NE8	5.700#	> *
251	3,4-DIHYDROXYCINNAMIC-	6.832 @	
258	FERULIC-PEAK 2	1.620#	> *
332	U40	1.210#	>*
333	U41	69.400#	> *
266	M-HYDROXYHIPPURIC	82.800#	> *
334	U42	40.700#	> *
274	3,4,5-TRIMETHOXYCINN	1.680#	> *
272	5-HYDROXYINDOLEACETIC	1.442	> *
29	LEVULINIC	0.000#	>*
67	NICOTINIC	0.000#	>*
79	3,3-DIMETHYLGLUTARIC	0.000	>*
96	MANDELIC	0.000#	>*
118	PIMELIC	0.000#	>*
123	TARTARIC	0.000#	>*
155	CIS-ACONITIC	0.000#	>*
176	ISOCITRIC	0.000#	>*
209	SEBACIC	0.000#	>*
218	ASCORBIC	0.000	>*
223	HYDROCAFFEIC	0.000#	>*
235	FERULIC-PEAK 1	0.000#	>*
254	UROCANIC	0.000#	>*
257	URIC	0.000#	>*
328	U36	0.000#	>*
342	U50	0.000#	>*
350	U58 (3-ME-GLUTACONIC)	0.000#	>*
361	U69	0.000#	>*
363	U71	0.000#	>*
366	U74	0.000#	>*
368	U76	0.000#	>*
371	U79	0.000#	>*
376	U84	0.000#	>*
379	U87	0.000#	>*

KEY: > SHOWS MINIMUM VALUE DETECTED IN SYSTEM.
@ IS COMPOUND FOR WHICH REFERENCE VALUE HAS NOT BEEN ESTABLISHED.
# IS COMPOUND FOR WHICH K-FACTOR HAS NOT BEEN ESTABLISHED. HENCE VALUES ARE REPORTED AS RELATIVE AMOUNTS / MG CREATININE.

FIGURE H1. (Cont'd.)

APPENDIX I

Complete MSSMET "found" file

A complete MSSMET "found" file is shown in Table I1. The entries have been explained in the caption for Figure 14. This "found" file was produced using the library illustrated in Appendix B to analyze the organic acids in one sample from the group of subjects with neuroblastoma.

TABLE I1. Complete MSSMET "found" file

```
07-APR-77 *COMPOUNDS FOUND* DK1:BESTLIB.FST
DK1:091802
     1.000 ML
     2.120 MG CREATININE
DK1:091802 19:37:05  8.0 UL 09076N3,KRIVI

    50.000 UG I.S.
TROPIC
  1 +  98  473588.  0.000E 00 31:29 + 0:00 1700    0  364  370  379
    +  98  132616.  0.000E 00
 293 U1
  2 +  94    1082.  0.536E-01  7:29 - 0:10  983   -6    8   10   15
    +  89      99.  0.175E-01
 342 U50
  1 +  95   26324.  0.130E 01  7:45 - 0:19  992  -12    7   14   29
    +  91    6842.  0.121E 01
   6 2-HYDROXY-2-METHYLPROPANOIC  (A-HYDROXYISOBUTYRIC)
  2 +  98   53531.  0.265E 01 10:13 + 0:00 1073    0   45   51   58
    +  98   14003.  0.248E 01
   8 2-HYDROXYPROPANOIC  (LACTIC)
  2 +  89   47343.  0.235E 01 10:53 + 0:00 1101    0   56   61   73
    +  92   12871.  0.228E 01
 294 U2
  1 +  95    8468.  0.420E 00 11:21 + 0:07 1113    3   63   68   73
    +  94    2140.  0.379E 00
  10 HYDROXYACETIC  (GLYCOLIC)
  1 +  94  127204.  0.631E 01 12:01 + 0:05 1130    2   73   78   92
    +  94   34204.  0.605E 01
  17 OXOACETIC OXIME  (GLYOXYLIC OXIME)
  2 +  88   16366.  0.811E 00 14:37 + 0:07 1198    3  112  117  124
    +  89    4137.  0.732E 00
  18 3-HYDROXYBUTANOIC  (B-HYDROXYBUTRYIC)
  1 +  98  139662.  0.692E 01 14:37 + 0:05 1198    2  110  117  130
    +  98   35012.  0.620E 01
 296 U4
  2 +  92   38814.  0.192E 01 15:09 + 0:03 1212    1  121  125  135
    +  93   10842.  0.192E 01
 297 U5
  1 +  97 1401540.  0.695E 02 15:53 + 0:19 1231    8  126  136  146
    +  98  383952.  0.680E 02
 371 U79
  1 +  92   37128.  0.184E 01 15:45 + 0:02 1227    0  129  134  148
    +  94    7264.  0.129E 01
  22 OXALIC
  1 +  94   38617.  0.191E 01 15:57 + 0:05 1233    2  131  137  144
    +  93   11957.  0.212E 01
 298 U6
  3 +  95   12132.  0.601E 00 16:01 + 0:02 1234    0  135  138  149
    +  95    3279.  0.580E 00
 343 U51
  1 +  99   13064.  0.648E 00 16:29 + 0:02 1247    1  141  145  153
    +  99    3152.  0.558E 00
 344 U52
  1 +  97    4688.  0.232E 00 16:29 + 0:02 1247    1  140  145  153
    +  97    1263.  0.224E 00
  28 1,2,3-PROPANETRIOL  (GLYCEROL)
  1 +  95   24318.  0.121E 01 17:17 + 0.00 1268    0  149  157  164
    +  95    6491.  0.115E 01
  29 4-OXOPENTANOIC  (LEVULINIC)
  2 +  91    1266.  0.628E-01 17:33 - 0:06 1275   -4  158  161  165
    +  97     434.  0.768E-01
  36 MALONIC
  2 +  98    2184.  0.108E 00 17:57 + 0:13 1287    6  162  167  174
    +  99     480.  0.850E-01
  33 4-HYDROXYBUTANOIC  (G-HYDROXYBUTRYIC)
  2 +  93    8040.  0.398E 00 18:01 + 0:14 1289    7  160  168  173
    +  89    1684.  0.298E 00
  35 METHYLMALONIC
  2 +  92    5760.  0.286E 00 17:53 + 0:00 1285    0  162  166  171
    +  94    1724.  0.305E 00
  34 UNK RA183 (GLYOXYLIC??)
  2 +  95    2945.  0.146E 00 17:53 - 0:03 1285   -2  162  166  173
    +  95     789.  0.140E 00
  42 UNK-OXB1
  1 +  97    4260.  0.211E 00 19:09 + 0:02 1322    1  180  185  193
    +  98    1147.  0.203E 00
 301 U9
  2 +  91   20344.  0.101E 01 19:21 + 0:03 1327    1  182  188  193
    +  95    5160.  0.913E 00
  50 PHOSPHORIC
  1 +  98 1601380.  0.794E 02 20:05 + 0:00 1349    0  195  199  214
    +  98  374400.  0.663E 02
 302 U10
  2 +  98  330680.  0.164E 02 20:37 + 0:04 1363    1  201  207  213
    +  99   78388.  0.139E 02
  51 BENZOIC
  2 +  98    7384.  0.366E 00 20:37 + 0:04 1363    1  204  207  214
    +  98    1612.  0.285E 00
 303 U11
  2 +  97  292736.  0.145E 02 21:17 + 0:05 1382    2  213  217  224
    +  98   77683.  0.138E 02
  60 SUCCINIC
  3 +  99  129868.  0.644E 01 21:53 + 0:04 1399    2  221  226  240
    +  99   38744.  0.686E 01
  59 UNK-NF2
  1 +  97   29736.  0.147E 01 21:49 + 0:00 1397    0  220  225  234
    +  99    8080.  0.143E 01
  61 FUMARIC
  2 +  99   38656.  0.192E 01 22:01 + 0:04 1403    2  222  228  232
    +  92   13080.  0.232E 01
```

TABLE II. (Cont'd.)

345 U53
1 + 99 10972. 0.544E 00 22:53 - 0:10 1427 -5 236 241 253
+ 97 2424. 0.429E 00
66 PHENYLACETIC
1 + 99 1324. 0.656E-01 22:53 - 0:01 1427 -1 236 241 247
+ 98 374. 0.662E-01
67 3-PYRIDINECARBOXYLIC (NICOTINIC)
1 + 97 5925. 0.294E 00 23:01 - 0:10 1430 -6 238 243 250
+ 98 1550. 0.274E 00
346 U54
2 + 99 49076. 0.243E 01 23:21 + 0:00 1440 0 243 248 253
+ 99 13688. 0.242E 01
205 U12
2 + 89 47925. 0.238E 01 23:21 - 0:08 1440 -4 243 248 253
+ 88 13581. 0.240E 01
306 U14
1 + 95 495524. 0.246E 02 23:57 + 0:00 1457 0 246 257 264
+ 94 133876. 0.237E 02
347 U55
2 + 99 127592. 0.632E 01 22:53 - 0:04 1455 -2 252 256 261
+ 97 32208. 0.570E 01
348 U56
2 + 99 18961. 0.940E 00 24:17 - 0:02 1467 -2 252 262 275
+ 97 5192. 0.919E 00
349 U57
2 + 98 184916. 0.520E 01 24:41 - 0:01 1480 -1 263 268 273
+ 96 31220. 0.554E 01
77 PENTANEDIOIC (GLUTARIC)
2 + 92 30470. 0.151E 01 24:49 - 0:08 1484 -5 263 270 279
+ 93 8536. 0.151E 01
79 3,3-DIMETHYLPENTANEDIOIC (3,3-DIMETHYLGLUTARIC)
2 + 93 1544. 0.765E-01 25:17 - 0:01 1499 -1 274 277 280
+ 92 472. 0.836E-01
82 2-HYDROXY-2-METHYLBUTANEDIOIC (CITRAMALIC)
1 + 95 5344. 0.265E 00 25:37 - 0:00 1509 -1 276 282 287
+ 94 1610. 0.285E 00
86 HYDROXYBUTANEDIOIC (MALIC)
2 + 89 57029. 0.283E 01 26:37 + 0:02 1541 1 288 297 304
+ 94 14077. 0.249E 01
350 U58
2 + 92 84937. 0.421E 01 26:37 + 0:06 1541 3 286 297 300
+ 93 20433. 0.362E 01
308 U16
1 + 94 1308507. 0.649E 02 26:57 + 0:00 1552 0 293 302 311
+ 92 199786. 0.354E 02
372 U80
1 + 87 107220. 0.531E 01 27:13 + 0:01 1560 0 298 306 309
+ 88 12712. 0.243E 01
351 U59
2 + 99 231678. 0.115E 02 27:41 + 0:05 1575 2 304 313 321
+ 91 60402. 0.107E 02

309 U17
2 + 95 1021060. 0.506E 02 27:53 + 0:06 1582 3 311 316 320
+ 95 190581. 0.337E 02
96 PHENYL-2-HYDROXYACETIC (MANDELIC)
2 - 71 552. 0.274E-01 28:09 + 0:16 1591 9 317 320 325
+ 93 184. 0.327E-01
98 HEXANEDIOIC (ADIPIC)
2 + 92 102913. 0.515E 01 28:21 + 0:06 1597 3 316 323 336
+ 94 29819. 0.528E 01
105 3-METHYLHEXANEDIOIC (3-METHYLADIPIC)
3 + 94 38574. 0.191E 01 29:01 + 0:02 1619 1 328 333 342
+ 95 11063. 0.196E 01
104 O-HYDROXYBENZOIC
1 + 85 3844. 0.191E 00 29:09 + 0:05 1623 2 320 325 328
+ 87 1012. 0.179E 00
352 U60
2 + 98 140724. 0.698E 01 29:13 + 0:07 1626 4 331 336 340
+ 97 39848. 0.705E 01
310 U18
1 + 91 88306. 0.438E 01 29:13 + 0:01 1626 1 325 336 345
+ 95 22696. 0.419E 01
107 2-HYDROXYPENTANEDIOIC (A-HYDROXYGLUTARIC)
2 + 96 118524. 0.587E 01 29:37 + 0:07 1639 4 335 342 350
+ 96 28624. 0.507E 01
353 U61
1 + 99 377964. 0.187E 02 29:37 + 0:07 1639 4 328 342 349
+ 95 58456. 0.103E 02
110 3-HYDROXY-2-METHYLPENTANEDIOIC (B-HYDROXY-B-METHYLGLUTARIC)
2 + 87 91906. 0.456E 01 29:57 + 0:05 1649 2 336 347 360
+ 90 24963. 0.442E 01
313 U21
2 + 95 54940. 0.272E 01 30:21 + 0:05 1663 3 348 353 360
+ 95 15060. 0.267E 01
354 U62
3 + 96 91668. 0.454E 01 30:21 + 0:05 1663 3 344 353 359
+ 95 19635. 0.348E 01
374 U82
1 + 95 75408. 0.374E 01 30:25 - 0:05 1665 -3 345 354 364
+ 96 14220. 0.252E 01
111 M-HYDROXYBENZOIC
2 + 97 9448. 0.468E 00 30:33 + 0:00 1669 0 351 356 361
+ 97 2244. 0.415E 00
112 2-PYRROLIDONE-5-CARBOXYLIC (PYROGLUTAMIC)
* 1 + 99 2526068. 0.126E 03 30:41 + 0:01 1672 0 344 358 371
+ 99 484722. 0.858E 02
375 U83
1 + 92 153920. 0.763E 01 30:49 + 0:02 1678 1 355 360 366
+ 98 47032. 0.833E 01
314 U22
3 + 87 18224. 0.904E 00 30:57 + 0:04 1682 2 358 362 370
+ 87 5121. 0.906E 00

TABLE II. (Cont'd.)

114 O-HYDROXYPHENYLACETIC
1 + 99 2916. 0.145E 00 31:01 + 0:01 1684 0 359 363 367
+ 97 840. 0.149E 00
376 U84
2 + 87 147292. 0.730E 01 31:01 + 0:01 1684 0 356 363 370
+ 86 40384. 0.715E 01
123 TARTARIC
2 + 85 9085. 0.450E 00 31:13 - 0:03 1691 -2 363 366 371
+ 94 2493. 0.441E 00
118 HEPTANEDIOIC (PIMELIC)
2 + 96 42307. 0.210E 01 31:17 + 0:00 1693 0 358 367 372
+ 95 8349. 0.148E 01
120 3-HYDROXY-2-PHENYLPROPANOIC (TROPIC)
1 + 99 475828. 0.236E 02 31:29 + 0:00 1700 0 364 370 379
+ 99 132728. 0.235E 02
124 ARABONOLACTONE-PEAK 1
2 + 90 50259. 0.249E 01 31:45 + 0:07 1709 4 367 374 380
+ 90 12540. 0.222E 01
125 2-OXOPENTANEDIOIC OXIME (A-KETOGLUTARIC OXIME)
2 + 99 138140. 0.685E 01 31:49 + 0:02 1711 1 370 375 381
+ 99 39340. 0.696E 01
127 P-HYDROXYBENZOIC
2 + 92 68256. 0.339E 01 32:13 + 0:02 1725 1 376 381 392
+ 98 20274. 0.359E 01
129 M-HYDROXYPHENYLACETIC
2 + 91 28551. 0.142E 01 32:17 - 0:00 1727 -1 378 382 388
+ 89 8633. 0.153E 01
136 P-HYDROXYPHENYLACETIC
2 + 96 40609. 0.201E 01 33:05 - 0:00 1755 -1 388 394 395
+ 96 26591. 0.471E 01
378 U86
2 + 91 22387. 0.116E 01 33:05 - 0:18 1755 -11 392 394 395
+ 81 9255. 0.164E 01
135 RIBONOLACTONE
2 + 94 26672. 0.122E 01 33:21 + 0:06 1764 3 394 398 409
+ 92 5080. 0.899E 00
139 ARABONIC
4 + 95 20273. 0.100E 01 33:33 + 0:04 1771 2 399 401 408
+ 97 4819. 0.853E 00
141 OCTANEDIOIC (SUBERIC)
3 + 99 57200. 0.284E 01 34:09 + 0:07 1792 4 404 410 423
+ 98 12044. 0.213E 01
144 B-GLYCEROPHOSPHORIC
2 + 93 61577. 0.305E 01 34:17 + 0:01 1797 1 407 412 423
+ 95 18451. 0.327E 01
356 U64
2 + 99 323220. 0.160E 02 34:37 + 0:07 1808 3 413 417 427
+ 98 112647. 0.199E 02
357 U65
2 + 98 270961. 0.134E 02 35:09 - 0:05 1827 -3 421 425 434
+ 99 77460. 0.137E 02

152 A-GLYCEROPHOSPHORIC
2 + 90 475738. 0.236E 02 35:21 + 0:00 1834 0 423 428 443
+ 90 118694. 0.210E 02
318 U26
2 + 93 45754. 0.227E 01 35:37 + 0:04 1843 2 425 422 442
+ 92 11875. 0.210E 01
155 CIS-1,2,3-PROPENETRICARBOXYLIC (CIS-ACONITIC)
2 + 91 203252. 0.101E 02 35:33 - 0:00 1841 -1 422 431 441
+ 91 54093. 0.957E 01
358 U66
2 + 99 59403. 0.294E 01 35:45 + 0:04 1848 2 427 434 438
+ 88 12521. 0.222E 01
319 U27
2 + 97 131080. 0.650E 01 35:53 + 0:02 1853 1 429 436 440
+ 96 19478. 0.345E 01
359 U67
2 + 99 205256. 0.102E 02 36:01 + 0:04 1858 2 433 438 449
+ 96 56674. 0.100E 02
360 U68
3 + 99 232760. 0.115E 02 36:17 + 0:07 1868 4 438 442 446
+ 94 56360. 0.998E 01
168 CITRIC
2 + 96 205159. 0.102E 02 36:29 + 0:03 1876 2 439 445 447
+ 91 51895. 0.919E 01
361 U69
2 + 99 423519. 0.210E 02 36:37 + 0:03 1880 1 438 447 449
+ 96 205022. 0.363E 02
174 NONANEDIOIC (AZELAIC)
2 + 98 45402. 0.225E 01 37:09 + 0:15 1900 9 445 455 461
+ 93 6638. 0.117E 01
176 1-HYDROXY-1,2,3-PROPANETRICARBOXYLIC (ISOCITRIC)
3 + 94 127979. 0.634E 01 36:57 + 0:03 1893 2 449 452 465
+ 95 30000. 0.531E 01
172 1,4-BENZENEDICARBOXYLIC (TEREPHTHALIC)
2 + 97 11366. 0.563E 00 36:57 + 0:02 1893 1 445 452 456
+ 97 2496. 0.442E 00
177 4-HYDROXY-3-METHOXYBENZOIC (VANILLIC)
2 + 93 24796. 0.123E 01 37:13 + 0:05 1903 2 448 456 460
+ 92 5616. 0.994E 00
381 U89
3 + 90 90628. 0.449E 01 37:21 + 0:03 1908 2 454 458 462
+ 93 20009. 0.354E 01
382 U90
1 + 95 240120. 0.119E 02 37:25 + 0:04 1910 2 446 459 462
+ 95 57890. 0.102E 02
321 U29
4 + 94 30919. 0.153E 01 37:33 + 0:07 1915 4 455 461 463
+ 95 6736. 0.119E 01
184 4-HYDROXY-3-METHOXYPHENYLACETIC (HOMOVANILLIC)
2 + 98 269337. 0.134E 02 37:45 + 0:00 1923 0 459 464 470
+ 99 74159. 0.131E 02

TABLE II. (Cont'd.)

290 3-HYDROXY-3-(3-HYDROXYPHENYL)-PROPANOIC (M-OH0HYDRACRYLIC)
 2 + 97 904680. 0.448E 02 38:01 + 0:03 1932 1 460 468 482
 + 97 200756. 0.355E 02
198 UNK-NE5
 3 + 94 105853. 0.525E 01 38:45 + 0:10 1957 5 477 479 485
 + 93 29464. 0.522E 01
324 U32
 2 + 93 170204. 0.844E 01 38:45 + 0:04 1957 2 475 479 484
 + 96 36529. 0.647E 01
194 O-HYDROXYCINNAMIC (O-COUMARIC)
 2 + 90 20716. 0.103E 01 38:37 - 0:03 1953 -2 471 477 482
 + 90 6948. 0.123E 01
197 U-GALACTURONIC-PEAK 2
 2 + 93 170204. 0.844E 01 38:45 + 0:03 1957 1 475 479 484
 + 93 36529. 0.647E 01
363 U71
 2 + 99 56507. 0.280E 01 39:25 - 0:01 1981 -1 485 489 498
 + 95 7954. 0.141E 01
207 2-HYDROXY-3-(4-HYDROXYPHENYL)-PROPANOIC (P-HYDROXYPHENYLLACTIC)
 2 + 89 125174. 0.620E 01 39:29 + 0:00 1983 0 483 490 496
 + 87 30455. 0.539E 01
364 U72
 1 + 98 245516. 0.122E 02 39:37 - 0:03 1988 -2 478 492 501
 + 98 57576. 0.102E 02
216 2-HYDROXY-(4-HYDROXY-3-METHOXYPHENYL)-ACETIC (VANILMANDELIC)
 2 + 99 575067. 0.285E 02 40:01 - 0:03 2002 -2 493 498 504
 + 99 174856. 0.310E 02
215 U-GALACTURONIC-PEAK 1
 1 + 95 156176. 0.774E 01 40:17 + 0:07 2011 4 493 502 505
 + 97 32478. 0.575E 01
218 ASCORBIC
 1 + 85 14871. 0.737E 00 40:13 + 0:00 2009 0 495 501 506
 + 85 2559. 0.453E 00
383 U91
 1 + 96 959836. 0.476E 02 40:09 - 0:06 2006 -5 493 500 516
 + 96 161216. 0.285E 02
325 U23 (GALACTURONIC)
 1 + 96 493230. 0.244E 02 40:17 - 0:00 2011 -1 497 502 504
 + 93 150650. 0.267E 02
365 U73
 2 + 89 302706. 0.150E 02 40:45 + 0:08 2032 5 504 509 518
 + 88 66541. 0.118E 02
326 U24 (GALACTURONIC)
 2 + 95 1362058. 0.675E 02 40:45 + 0:00 2032 0 504 509 521
 + 94 287898. 0.510E 02
225 U-GALACTURONIC-PEAK 2
 2 + 98 221885. 0.110E 02 40:45 - 0:01 2032 -1 505 509 517
 + 98 46275. 0.819E 01
223 3-(2,4-DIHYDROXYPHENYL)-PROPANOIC (HYDROCAFFEIC)
 4 + 86 17294. 0.857E 00 40:53 - 0:02 2037 -3 508 511 517
 + 96 2738. 0.485E 00
366 U74
 2 + 99 56084. 0.278E 01 40:53 - 0:04 2037 -4 501 511 516
 + 95 10840. 0.192E 01
229 2-OXO-3-(4-HYDROXYPHENYL)-PROPANOIC OXIME (P-OHPHENYLPYRUVIC)
 1 + 90 1061. 0.526E-01 41:21 - 0:11 2058 -8 514 518 526
 + 99 246. 0.435E-01
291 UNK 2071
 2 - 79 16938. 0.840E 00 41:33 + 0:00 2066 0 517 521 530
 + 90 4262. 0.754E 00
212 HEXADECANOIC (PALMITIC)
 2 + 82 10284. 0.510E 00 42:05 + 0:01 2089 0 525 529 534
 + 87 2028. 0.359E 00
367 U75
 1 + 98 34100. 0.169E 01 42:05 + 0:02 2089 1 523 529 535
 + 98 10036. 0.178E 01
235 4-HYDROXY-3-METHOXYCINNAMIC-PEAK 1 (FERULIC)
 1 - 74 1649. 0.817E-01 42:13 - 0:00 2095 -1 522 531 535
 + 88 393. 0.695E-01
237 BENZAMIDOACETIC (HIPPURIC)
 2 + 99 2530556. 0.125E 03 42:25 + 0:03 2104 2 522 534 548
 + 99 442488. 0.783E 02
236 3,4-DIHYDROXYCINNAMIC-PEAK 1 (CAFFEIC)
 2 + 91 2983. 0.148E 00 42:29 - 0:02 2107 -2 531 535 542
 + 94 1121. 0.200E 00
328 U36
 2 - 71 4289. 0.213E 00 42:33 + 0:00 2110 0 527 536 542
 + 96 728. 0.129E 00
368 U76 (HYDROXYDECANEDIOIC?)
 1 + 95 55476. 0.275E 01 42:57 - 0:00 2127 -1 534 542 552
 + 96 13624. 0.241E 01
369 U77
 1 + 96 93312. 0.463E 01 43:25 - 0:02 2148 -2 544 549 562
 + 96 25269. 0.447E 01
329 U37
 1 + 95 72419. 0.359E 01 43:37 + 0:01 2156 0 544 552 562
 + 96 17836. 0.316E 01
244 3-INDOLYLACETIC
 2 + 94 158420. 0.785E 01 44:21 + 0:00 2189 0 553 563 571
 + 94 42888. 0.759E 01
246 UNK-NE8
 2 + 98 167584. 0.831E 01 45:09 - 0:02 2222 -2 568 575 581
 + 97 43900. 0.777E 01
251 3,4-DIHYDROXYCINNAMIC-PEAK 2 (CAFFEIC)
 2 + 87 23656. 0.117E 01 45:33 + 0:01 2238 0 577 581 590
 + 88 5056. 0.895E 00
257 2,6,8-TRIHYDROXYPURINE (URIC)
 2 + 94 1567351. 0.777E 02 46:01 - 0:02 2258 -2 581 588 597
 + 94 180991. 0.320E 02
333 U41
 2 + 96 221835. 0.110E 02 48:21 + 0:00 2355 0 619 623 627
 + 87 46733. 0.827E 01

TABLE II. (Cont'd.)

```
266 M-HYDROXYBENZAMIDOACETIC  (M-HYDROXYHIPPURIC)
 1 +  96  705996.  0.150E 02 48:37 - 0:06 2366   -5  614  627  639
   +  95  158226.  0.260E 02
274 3,4,5-TRIMETHOXYCINNAMIC-PEAK 2
 2 +  89    1642.  0.814E-01 49:23 + 0:05 2406    4  638  641  644
   +  81     484.  0.856E-01
```

APPENDIX J

T-test of $\log_{10}$ of normalized data and tabulation of compound means, standard deviations, standard errors and coefficients of variation

MSSMET "found" files from 9 BCIU, 5 neuroblastoma and 5 juvenile subjects were analyzed using MSSTAT. The normalized relative peak areas listed in Appendix F were all converted to their logarithms (base 10) before further calculations were performed.

Compound names correspond to the numbers listed in Appendix B. As an example, the first entry in the table refers to compound 6 (α-hydroxybutyric). The first line of numbers in the entry corresponds to the mean, standard deviation, standard error and coefficient of variation of the compound for the 9 BCIU urines (group 2); each value is expressed in exponential notation. The second, third and fourth lines of numbers give corresponding results for groups 3 (neuroblastoma), 4 (juvenile control) and 10 (sum of groups 2, 3 and 4). The t-test section of the entry compares each pair of subject groups. Thus , for compound 6, the value of t for a comparison of

groups 2 and 3 is 4.37, which is significant at the 0.1% level, as indicated by the four asterisks (1 = 10%, 2 = 5%, 3 = 1%, 4 = 0.1% level of significance). These values are computed using standard techniques found in any elementary text on statistics.

TABLE J1. T-test and mean values

T-TEST ON LOG OF NORMALIZED DATA (SUM-32 CMPDS) 2=BCIU, 3=NEURO, 4=JUVEN

1

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
6	2	0.115E 01	0.247E 00	0.824E-01	0.216E 02		9
6	3	0.582E 00	0.226E 00	0.101E 00	0.389E 02		5
6	4	0.117E 01	0.444E 00	0.198E 00	0.379E 02		5
6	10	0.100E 01	0.387E 00	0.887E-01	0.385E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	4.37	****
	2	4	-0.15	
	3	4	-2.90	**

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
8	2	0.139E 01	0.315E 00	0.105E 00	0.227E 02		9
8	3	0.105E 01	0.203E 00	0.908E-01	0.194E 02		5
8	4	0.130E 01	0.181E 00	0.810E-01	0.140E 02		5
8	10	0.127E 01	0.285E 00	0.653E-01	0.225E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	2.23	**
	2	4	0.53	
	3	4	-2.18	*

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
10	2	0.142E 01	0.212E 00	0.710E-01	0.149E 02		9
10	3	0.100E 01	0.202E 00	0.904E-01	0.202E 02		5
10	4	0.139E 01	0.338E 00	0.151E 00	0.243E 02		5
10	10	0.131E 01	0.301E 00	0.690E-01	0.230E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	3.83	***
	2	4	0.29	
	3	4	-2.35	**

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
17	2	-.180E 01	0.100E 01	0.334E 00	0.000E 00		9
17	3	0.261E 00	0.164E 00	0.734E-01	0.630E 02		5
17	4	-.317E 00	0.115E 01	0.513E 00	0.000E 00		5
17	10	-.869E 00	0.127E 01	0.292E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-4.68	****
	2	4	-2.64	**
	3	4	1.18	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
18	2	0.135E 01	0.151E 01	0.502E 00	0.112E 03		9
18	3	0.595E 00	0.461E 00	0.206E 00	0.774E 02		5
18	4	-.154E 01	0.171E 01	0.766E 00	0.000E 00		5
18	10	0.292E 00	0.173E 01	0.411E 00	0.457E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1.12	
	2	4	2.41	***
	3	4	2.85	**

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
22	2	-.196E 01	0.101E 01	0.337E 00	0.000E 00		9
23	3	0.923E 00	0.104E 00	0.466E-01	0.113E 02		5
23	4	0.117E 01	0.418E 00	0.187E 00	0.359E 02		5
23	10	-.380E 00	0.170E 01	0.390E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-6.58	****
	2	4	-6.78	****
	3	4	-1.33	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
28	2	0.833E 00	0.171E 00	0.577E-01	0.208E 02		9
28	3	0.582E 00	0.655E-01	0.293E-01	0.112E 02		5
28	4	0.746E 00	0.202E 00	0.904E-01	0.271E 02		5
28	10	0.744E 00	0.186E 00	0.427E-01	0.250E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	3.20	***
	2	4	0.89	
	3	4	-1.82	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
32	2	-.230E 01	0.000E 00	0.000E 00	0.000E 00		9
32	3	-.230E 01	0.000E 00	0.000E 00	0.000E 00		5
32	4	-.230E 01	0.000E 00	0.000E 00	0.000E 00		5
32	10	-.230E 01	0.000E 00	0.000E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.00	
	2	4	0.00	
	3	4	0.00	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
34	2	-.207E 01	0.781E 00	0.234E 00	0.000E 00		9
34	3	-.615E 00	0.956E 00	0.428E 00	0.000E 00		5
34	4	-.110E 01	0.114E 01	0.508E 00	0.000E 00		5
34	10	-.143E 01	0.106E 01	0.243E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-3.41	***
	2	4	-2.07	*
	3	4	0.78	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
35	2	-.104E 01	0.101E 01	0.337E 00	0.000E 00		9
35	3	-.110E 01	0.111E 01	0.497E 00	0.000E 00		5
35	4	-.195E 01	0.796E 00	0.356E 00	0.000E 00		5
35	10	-.129E 01	0.101E 01	0.233E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.10	
	2	4	1.73	*
	3	4	1.47	

TABLE J1. (Cont'd.)

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
	42	2	- 396E 01	0 182E 01	0 545E 00	0 000E 00	9
	42	3	0 121E 00	0 441E 00	0 197E 00	0 363E 01	5
	42	4	0 617E 00	0 412E 00	0 192E 00	0 790E 02	5
	42	10	- 732E 00	0 142E 01	0 326E 00	0 000E 00	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-4 39	****
	2	4	-5 45	****
	3	4	-1 91	*

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
	50	2	0 216E 01	0 351E 00	0 117E 00	0 149E 02	9
	50	3	0 278E 01	0 232E 00	0 104E 00	0 834E 01	5
	50	4	0 225E 01	0 418E 00	0 187E 00	0 182E 02	5
	50	10	0 244E 01	0 388E 00	0 889E-01	0 159E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-2 49	**
	2	4	0 57	
	3	4	2 70	**

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
	51	2	- 125E 00	0 122E 01	0 411E 00	0 000E 00	9	
	51	3	- 324E 00	0 112E 01	0 499E 00	0 000E 00	5	
	51	4	- 289E-01	0 128E 01	0 572E 00	0 000E 00	5	
	51	10	- 160E 00	0 115E 01	0 265E 00	0 000E 00	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0 29	
	2	4	+0 14	
	3	4	-0 40	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
	59	2	0 325E 00	0 128E 00	0 460E-01	0 424E 02	9	
	59	3	0 362E 00	0 217E 00	0 972E-01	0 601E 02	5	
	59	4	- 387E-01	0 127E 01	0 570E 00	0 000E 00	5	
	59	10	0 239E 00	0 640E 00	0 147E 00	0 268E 01	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0 40	
	2	4	0 91	
	3	4	0 73	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
	60	2	0 701E 00	0 115E 01	0 382E 00	0 163E 01	9
	60	3	0 126E 01	0 729E-01	0 326E-01	0 579E 01	5
	60	4	0 142E 01	0 218E 00	0 974E-01	0 153E 02	5
	60	10	0 104E 01	0 840E 00	0 193E 00	0 809E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1 11	
	2	4	-1 41	
	3	4	-1 62	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
	61	2	- 990E 00	0 156E 01	0 520E 00	0 000E 00	9	
	61	3	0 819E 00	0 118E 00	0 493E-01	0 135E 02	5	
	61	4	0 794E 00	0 280E 00	0 854E-01	0 252E 02	5	
	61	10	- 442E-01	0 139E 01	0 320E 00	0 000E 00	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-2 64	**
	2	4	-2 68	**
	3	4	0 25	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
	66	2	- 157E 01	0 512E 00	0 164E 00	0 000E 00	9
	66	3	- 134E 01	0 877E 00	0 332E 00	0 000E 00	5
	66	4	- 115E 01	0 105E 01	0 471E 00	0 000E 00	5
	66	10	- 140E 01	0 905E 00	0 208E 00	0 000E 00	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0 47	
	2	4	-0 01	
	3	4	+0 33	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
	67	2	- 138E 01	0 109E 01	0 344E 00	0 000E 00	9	
	67	3	- 924E 00	0 126E 01	0 562E 00	0 000E 00	5	
	67	4	- 535E 00	0 180E 01	0 484E 00	0 000E 00	5	
	67	10	- 104E 01	0 113E 01	0 259E 00	0 000E 00	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	+0 74	
	2	4	-1 45	
	3	4	-0 56	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
	77	2	0 501E-01	0 896E 00	0 297E 00	0 178E 04	9
	77	3	0 520E 00	0 113E 00	0 503E-01	0 213E 02	5
	77	4	0 651E 00	0 340E 00	0 152E 00	0 522E 02	5
	77	10	0 324E 00	0 675E 00	0 155E 00	0 203E 01	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1 22	
	2	4	-1 49	
	3	4	-0 82	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
	86	2	0 674E 00	0 223E 00	0 743E-01	0 331E 02	9	
	86	3	0 994E 00	0 402E-01	0 402E-01	0 269E-01	0 405E 01	5
	86	4	0 982E 00	0 221E 00	0 99GE-01	0 225E 02	5	
	86	10	0 840E 00	0 245E 00	0 561E-01	0 291E 02	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-3 24	***
	2	4	-2 58	**
	3	4	0 14	

TABLE J1. (Cont'd.)

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	98	2	- .332E 00	0.154E 01	0.512E 00	0.000E 00		9
	98	3	0.748E 00	0.208E 00	0.129E 00	0.385E 02		5
	98	4	0.719E 00	0.201E 00	0.901E-01	0.280E 02		5
	98	10	0.229E 00	0.117E 01	0.269E 00	0.512E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.39	
	2	4	-1.56	
	3	4	0.19	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	104	2	- .188E 01	0.119E 01	0.397E 00	0.000E 00		9
	104	3	- .188E 00	0.170E 01	0.583E 00	0.000E 00		5
	104	4	- .724E 00	0.926E 00	0.414E 00	0.000E 00		5
	104	10	- .747E 00	0.116E 01	0.266E 00	0.000E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.36	
	2	4	-0.59	
	3	4	0.81	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	105	2	0.179E 00	0.934E 00	0.331E 00	0.523E 01		9
	105	3	0.181E 00	0.249E 00	0.112E 00	0.654E 02		5
	105	4	0.188E 00	0.445E 00	0.199E 00	0.237E 02		5
	105	10	0.234E 00	0.673E 00	0.154E 00	0.287E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.49	
	2	4	-0.02	
	3	4	0.30	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	107	2	- .417E 00	0.179E 01	0.596E 00	0.000E 00		9
	107	3	0.121E 01	0.128E 00	0.516E-01	0.992E 01		5
	107	4	0.115E 01	0.174E 00	0.778E-01	0.151E 02		5
	107	10	0.426E 00	0.145E 01	0.333E 00	0.341E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-2.08	*
	2	4	-2.00	*
	3	4	0.67	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	110	2	0.545E 00	0.166E 00	0.553E-01	0.304E 02		9
	110	3	0.909E 00	0.155E 00	0.694E-01	0.157E 02		5
	110	4	0.854E 00	0.139E 00	0.622E-01	0.163E 02		5
	110	10	0.743E 00	0.248E 00	0.569E-01	0.334E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-5.09	****
	2	4	-3.65	***
	3	4	1.51	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	111	2	0.159E-01	0.905E 00	0.302E 00	0.569E 04		9
	111	3	0.525E 00	0.255E 00	0.114E 00	0.486E 02		5
	111	4	- .230E 01	0.000E 00	0.000E 00	0.000E 00		5
	111	10	- .468E 00	0.138E 01	0.299E 00	0.000E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.26	
	2	4	5.85	****
	3	4	26.28	****

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	112	2	- .596E 00	0.175E 01	0.585E 00	0.000E 00		9
	112	3	0.218E 01	0.369E 00	0.165E 00	0.376E 02		5
	112	4	0.198E 01	0.481E 00	0.215E 00	0.243E 02		5
	112	10	0.791E 00	0.181E 01	0.415E 00	0.229E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-7.48	***
	2	4	-7.29	***
	3	4	0.49	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	114	2	0.952E-01	0.186E 00	0.628E-01	0.204E 03		9
	114	3	- .191E 00	0.179E 00	0.801E-01	0.938E 00		5
	114	4	- .921E-01	0.223E 00	0.100E 00	0.988E 00		5
	114	10	- .333E-01	0.224E 00	0.514E-01	0.994E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	2.86	**
	2	4	1.69	
	3	4	-0.68	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	118	2	- .556E 00	0.131E 01	0.443E 00	0.000E 00		9
	118	3	- .132E 00	0.126E 01	0.563E 00	0.000E 00		5
	118	4	- .159E 00	0.128E 01	0.537E 00	0.000E 00		5
	118	10	- .348E 00	0.122E 01	0.281E 00	0.000E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.61	
	2	4	-0.58	
	3	4	0.04	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
	120	2	0.205E 01	0.138E 00	0.462E-01	0.675E 01		9
	120	3	0.237E 01	0.123E 00	0.144E 00	0.137E 02		5
	120	4	0.210E 01	0.284E 00	0.127E 00	0.135E 02		5
	120	10	0.215E 01	0.265E 00	0.600E-01	0.122E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-2.71	**
	2	4	-0.43	
	3	4	1.49	

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
123	2	- 220E 01	0.000E 00	0.000E 00	0.000E 00		9
123	3	0.368E 00	0.158E 01	0.708E 00	0.431E 02		5
123	4	- 136E 01	0.138E 01	0.598E 00	0.000E 00		5
123	10	- 135E 01	0.148E 01	0.340E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-5.45	****
	2	4	-2.36	**
	3	4	2.00	*

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
124	2	0.302E 00	0.103E 01	0.342E 00	0.340E 03		9
124	3	0.947E 00	0.168E 00	0.750E-01	0.177E 02		5
124	4	0.169E 00	0.142E 01	0.636E 00	0.841E 03		5
124	10	0.427E 00	0.101E 01	0.233E 00	0.232E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.42	
	2	4	0.23	
	3	4	1.29	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
125	2	-.705E 00	0.129E 01	0.431E 00	0.006E 00		9
125	3	0.128E 01	0.328E 00	0.147E 00	0.252E 02		5
125	4	0.880E 00	0.563E 00	0.254E 00	0.644E 02		5
125	10	0.283E 00	0.134E 01	0.307E 00	0.468E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-3.63	***
	2	4	-2.01	**
	3	4	1.53	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
127	2	0.101E 01	0.217E 00	0.724E-01	0.216E 02		9
127	3	0.141E 01	0.273E 00	0.122E 00	0.194E 02		5
127	4	0.902E 00	0.485E 00	0.217E 00	0.494E 02		5
127	10	0.111E 01	0.353E 00	0.809E-01	0.319E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-3.16	***
	2	4	0.15	
	3	4	1.82	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
129	2	0.662E 00	0.343E 00	0.114E 00	0.518E 02		9
129	3	0.923E 00	0.240E 00	0.893E-01	0.328E 02		5
129	4	0.224E 00	0.328E 00	0.147E 00	0.146E 03		5
129	10	0.539E 00	0.347E 00	0.796E-01	0.644E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.24	
	2	4	2.36	**
	3	4	2.48	**

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
175	2	- 142E 01	0.133E 01	0.442E 00	0.000E 00		9
175	3	0.531E 00	0.295E 00	0.132E 00	0.557E 02		5
175	4	- 161E 01	0.161E 01	0.719E 00	0.000E 00		5
175	10	- 830E 00	0.144E 01	0.331E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-3.32	***
	2	4	-0.30	
	3	4	2.41	**

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
176	2	0.131E 01	0.109E 00	0.599E-01	0.137E 02		9
176	3	0.111E 01	0.241E 00	0.108E 00	0.214E 02		5
176	4	0.125E 01	0.323E 00	0.145E 00	0.258E 02		5
176	10	0.125E 01	0.238E 00	0.545E-01	0.191E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1.68	
	2	4	0.45	
	3	4	-0.74	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
139	2	0.585E 00	0.114E 01	0.379E 00	0.194E 03		9
139	3	0.698E 00	0.179E 00	0.802E-01	0.257E 02		5
139	4	0.802E-01	0.137E 01	0.611E 00	0.170E 04		5
139	10	0.482E 00	0.102E 01	0.236E 00	0.213E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.23	
	2	4	0.77	
	3	4	1.06	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
141	2	0.467E 00	0.205E 00	0.952E-01	0.611E 02		9
141	3	0.547E 00	0.324E 00	0.146E 00	0.594E 02		5
141	4	0.851E-01	0.134E 01	0.598E 00	0.157E 04		5
141	10	0.388E 00	0.702E 00	0.161E 00	0.181E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.50	
	2	4	0.88	
	3	4	0.80	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
144	2	0.285E 00	0.102E 01	0.340E 00	0.358E 03		9
144	3	0.849E 00	0.222E 00	0.991E-01	0.261E 02		5
144	4	0.837E 00	0.197E 00	0.883E-01	0.236E 02		5
144	10	0.578E 00	0.751E 00	0.172E 00	0.130E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.25	
	2	4	-1.23	
	3	4	0.03	

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
152	2	0.139E 01	0.185E 00	0.615E-01	0.133E 02		9
152	3	0.172E 01	0.173E 00	0.774E-01	0.101E 02		5
152	4	0.154E 01	0.216E 00	0.966E-01	0.140E 02		5
152	10	0.151E 01	0.228E 00	0.522E-01	0.150E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-3.40	***
	2	4	-1.49	
	3	4	1.48	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
155	2	-.808E 00	0.177E 01	0.591E 00	0.000E 00		9
155	3	0.403E 00	0.157E 01	0.702E 00	0.389E 03		5
155	4	-.953E 00	0.185E 01	0.826E 00	0.000E 00		5
155	10	-.523E 00	0.174E 01	0.400E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.32	
	2	4	0.15	
	3	4	1.33	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
168	2	0.239E 00	0.200E 01	0.667E 00	0.591E 03		9
168	3	0.208E 01	0.396E 00	0.175E 00	0.138E 02		5
168	4	0.360E 00	0.178E 01	0.794E 00	0.206E 03		5
168	10	0.933E 00	0.175E 01	0.401E 00	0.187E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.97	*
	2	4	-0.50	
	3	4	1.59	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
172	2	0.426E-01	0.927E 00	0.309E 00	0.217E 04		9
172	3	0.267E 00	0.179E 00	0.799E-01	0.669E 02		5
172	4	-.554E 00	0.189E 01	0.485E 00	0.000E 00		5
172	10	-.552E-01	0.868E 00	0.199E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.55	
	2	4	1.13	
	3	4	1.77	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
174	2	0.465E 00	0.304E 00	0.101E 00	0.654E 02		9
174	3	0.625E 00	0.298E 00	0.133E 00	0.478E 02		5
174	4	0.444E 00	0.291E 00	0.130E 00	0.655E 02		5
174	10	0.502E 00	0.292E 00	0.671E-01	0.583E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.99	
	2	4	0.13	
	3	4	1.03	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
176	2	-.844E 00	0.161E 01	0.536E 00	0.000E 00		9
176	3	0.602E 00	0.162E 01	0.727E 00	0.270E 03		5
176	4	-.260E 00	0.137E 01	0.835E 00	0.000E 00		5
176	10	-.310E 00	0.170E 01	0.390E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.67	
	2	4	-0.64	
	3	4	0.83	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
177	2	0.350E 00	0.101E 01	0.338E 00	0.289E 03		9
177	3	0.385E 00	0.153E 01	0.684E 00	0.398E 03		5
177	4	0.624E-01	0.133E 01	0.595E 00	0.213E 04		5
177	10	0.284E 00	0.119E 01	0.270E 00	0.415E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.05	
	2	4	0.46	
	3	4	0.38	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
183	2	0.694E 00	0.114E 01	0.381E 00	0.165E 03		9
183	3	0.568E 00	0.161E 01	0.719E 00	0.283E 03		5
183	4	-.111E 01	0.164E 01	0.735E 00	0.000E 00		5
183	10	0.187E 00	0.155E 01	0.355E 00	0.829E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.18	
	2	4	2.53	**
	3	4	1.73	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
184	2	0.107E 01	0.115E 00	0.383E-01	0.107E 02		9
184	3	0.210E 01	0.199E 00	0.178E 00	0.190E 02		5
184	4	0.127E 01	0.191E 00	0.852E-01	0.150E 02		5
184	10	0.139E 01	0.492E 00	0.113E 00	0.353E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-7.68	****
	2	4	-2.53	**
	3	4	4.43	***

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
185	2	-.117E 00	0.126E 01	0.419E 00	0.000E 00		9
185	3	-.387E 00	0.175E 01	0.782E 00	0.000E 00		5
185	4	0.829E-01	0.142E 01	0.635E 00	0.171E 04		5
185	10	-.136E 00	0.136E 01	0.313E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.35	
	2	4	-0.28	
	3	4	-0.49	

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
186	2	0.594E 00	0.270E 00	0.899E-01	0.454E 02		9
186	3	-.509E 00	0.164E 01	0.736E 00	0.000E 00		5
186	4	-.149E 01	0.114E 01	0.508E 00	0.000E 00		5
186	10	-.243E 00	0.131E 01	0.301E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	2.11	*
	2	4	5.61	****
	3	4	1.16	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
189	2	-.968E 00	0.159E 01	0.531E 00	0.000E 00		9
189	3	-.281E 00	0.184E 01	0.825E 00	0.000E 00		5
189	4	-.178E 01	0.116E 01	0.518E 00	0.000E 00		5
189	10	-.998E 00	0.153E 01	0.362E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.75	
	2	4	1.05	
	3	4	1.64	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
194	2	-.443E 00	0.139E 01	0.465E 00	0.000E 00		9
194	3	0.656E 00	0.191E 00	0.854E-01	0.291E 02		5
194	4	0.408E 00	0.479E 00	0.214E 00	0.117E 03		5
194	10	0.676E-01	0.109E 01	0.250E 00	0.161E 04		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.80	*
	2	4	-1.36	
	3	4	1.14	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
197	2	0.685E 00	0.170E 01	0.566E 00	0.248E 02		9
197	3	0.175E 01	0.265E 00	0.119E 00	0.152E 02		5
197	4	0.924E 00	0.181E 01	0.809E 00	0.196E 03		5
197	10	0.102E 01	0.149E 01	0.342E 00	0.145E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.42	
	2	4	-0.26	
	3	4	1.07	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
198	2	0.581E 00	0.164E 01	0.547E 00	0.282E 03		9
198	3	0.161E 01	0.311E 00	0.139E 00	0.194E 02		5
198	4	0.154E 01	0.526E 00	0.235E 00	0.341E 02		5
198	10	0.110E 01	0.124E 01	0.285E 00	0.112E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.41	
	2	4	-1.30	
	3	4	0.25	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
200	2	-.202E 00	0.159E 01	0.528E 00	0.000E 00		9
200	3	0.574E 00	0.162E 01	0.726E 00	0.283E 03		5
200	4	-.311E 00	0.182E 01	0.814E 00	0.000E 00		5
200	10	-.263E-01	0.161E 01	0.368E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.91	
	2	4	0.12	
	3	4	0.86	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
207	2	0.846E 00	0.121E 01	0.402E 00	0.143E 03		9
207	3	0.950E 00	0.225E 00	0.101E 00	0.237E 02		5
207	4	0.127E 01	0.147E 00	0.658E-01	0.116E 02		5
207	10	0.984E 00	0.834E 00	0.191E 00	0.847E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.20	
	2	4	-0.79	
	3	4	-2.78	**

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
212	2	-.281E 00	0.150E 01	0.501E 00	0.000E 00		9
212	3	0.697E 00	0.330E 00	0.148E 00	0.474E 02		5
212	4	0.658E 00	0.314E 00	0.140E 00	0.476E 02		5
212	10	0.214E 00	0.114E 01	0.262E 00	0.533E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.50	
	2	4	-1.44	
	3	4	0.20	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
215	2	0.128E 01	0.135E 01	0.450E 00	0.106E 03		9
215	3	0.169E 01	0.230E 00	0.103E 00	0.136E 02		5
215	4	0.994E 00	0.184E 01	0.825E 00	0.185E 03		5
215	10	0.121E 01	0.128E 01	0.294E 00	0.978E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.69	
	2	4	0.35	
	3	4	0.89	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
216	2	0.152E 01	0.212E 00	0.706E-01	0.139E 02		9
216	3	0.235E 01	0.441E 00	0.197E 00	0.188E 02		5
216	4	0.133E 01	0.396E 00	0.401E 00	0.673E 02		5
216	10	0.169E 01	0.642E 00	0.147E 00	0.381E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-5.03	****
	2	4	0.65	
	3	4	2.42	**

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
218	2	0.448E 00	0.104E 01	0.347E 00	0.232E 03		9
218	3	-.606E 00	0.155E 01	0.695E 00	0.000E 00		5
218	4	-.405E 00	0.174E 01	0.777E 00	0.000E 00		5
218	10	-.539E-01	0.139E 01	0.319E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1.59	
	2	4	1.21	
	3	4	-0.21	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
223	2	0.963E 00	0.538E 00	0.179E 00	0.559E 02		9
223	3	-.169E 00	0.121E 01	0.539E 00	0.000E 00		5
223	4	-.120E 01	0.156E 01	0.672E 00	0.000E 00		5
223	10	0.944E-01	0.135E 01	0.309E 00	0.143E 04		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	2.57	**
	2	4	4.16	***
	3	4	1.28	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
225	2	0.120E 01	0.132E 01	0.439E 00	0.110E 03		9
225	3	0.173E 01	0.168E 00	0.750E-01	0.970E 01		5
225	4	0.167E 01	0.115E 00	0.516E-01	0.690E 01		5
225	10	0.146E 01	0.920E 00	0.211E 00	0.630E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.91	
	2	4	-0.82	
	3	4	0.63	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
235	2	-.208E-01	0.927E 00	0.309E 00	0.000E 00		9
235	3	-.457E 00	0.111E 01	0.496E 00	0.000E 00		5
235	4	-.139E 01	0.125E 01	0.557E 00	0.000E 00		5
235	10	-.496E 00	0.116E 01	0.265E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.82	
	2	4	2.45	**
	3	4	1.33	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
237	2	0.215E 01	0.200E 01	0.668E 00	0.934E 02		9
237	3	0.244E 01	0.357E 00	0.160E 00	0.147E 02		5
237	4	0.186E 01	0.234E 01	0.105E 01	0.126E 03		5
237	10	0.215E 01	0.175E 01	0.402E 00	0.817E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.22	
	2	4	0.26	
	3	4	0.58	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
244	2	0.815E 00	0.831E 00	0.277E 00	0.102E 03		9
244	3	0.119E 01	0.148E 00	0.661E-01	0.124E 02		5
244	4	0.172E 01	0.174E 00	0.777E-01	0.101E 02		5
244	10	0.115E 01	0.682E 00	0.156E 00	0.592E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.03	
	2	4	-2.46	**
	3	4	-5.47	****

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
246	2	0.643E 00	0.795E 00	0.265E 00	0.124E 03		9
246	3	0.125E 00	0.143E 01	0.640E 00	0.115E 04		5
246	4	-.332E 00	0.120E 01	0.538E 00	0.000E 00		5
246	10	0.250E 00	0.111E 01	0.255E 00	0.444E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.92	
	2	4	1.92	*
	3	4	0.58	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
251	2	-.101E 01	0.132E 01	0.439E 00	0.000E 00		9
251	3	0.918E 00	0.255E 00	0.114E 00	0.278E 02		5
251	4	-.210E 01	0.000E 00	0.000E 00	0.000E 00		5
251	10	-.841E 00	0.150E 01	0.344E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-3.31	***
	2	4	2.25	**
	3	4	29.98	****

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
257	2	-.950E 00	0.225E 01	0.750E 00	0.000E 00		9
257	3	0.792E 00	0.284E 01	0.127E 01	0.358E 03		5
257	4	-.152E 01	0.175E 01	0.783E 00	0.000E 00		5
257	10	-.641E 00	0.236E 01	0.541E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.32	
	2	4	0.51	
	3	4	1.64	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
258	2	0.159E 00	0.146E 01	0.487E 00	0.918E 03		9
258	3	-.126E 01	0.143E 01	0.640E 00	0.000E 00		5
258	4	-.171E 01	0.132E 01	0.591E 00	0.000E 00		5
258	10	-.787E 00	0.159E 01	0.365E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1.83	*
	2	4	2.46	**
	3	4	0.54	

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
266	2	0.163E 00	0.237E 01	0.790E 00	0.145E 04		9
266	3	0.164E 01	0.265E 00	0.118E 00	0.161E 02		5
266	4	-.157E 01	0.162E 01	0.720E 00	0.000E 00		5
266	10	0.954E-01	0.212E 01	0.489E 00	0.223E 04		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.42	
	2	4	1.50	
	3	4	4.61	***

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
272	2	0.117E 00	0.140E 01	0.467E 00	0.120E 04		9
272	3	0.740E-01	0.131E 01	0.596E 00	0.180E 04		5
272	4	0.139E 01	0.134E 00	0.598E-01	0.960E 01		5
272	10	0.441E 00	0.127E 01	0.291E 00	0.286E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.06	
	2	4	-2.08	*
	3	4	-2.33	**

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
290	2	0.257E 01	0.408E 00	0.136E 00	0.159E 02		9
290	3	0.216E 01	0.929E-01	0.416E-01	0.430E 01		5
290	4	0.127E 01	0.371E 00	0.166E 00	0.292E 02		5
290	10	0.212E 01	0.638E 00	0.146E 00	0.301E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	2.23	**
	2	4	6.11	****
	3	4	5.55	****

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
291	2	-.613E 00	0.162E 01	0.540E 00	0.000E 00		9
291	3	-.115E 01	0.158E 01	0.708E 00	0.000E 00		5
291	4	-.218E 01	0.000E 00	0.000E 00	0.000E 00		5
291	10	-.120E 01	0.149E 01	0.343E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.62	
	2	4	2.38	**
	3	4	1.73	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
293	2	-.999E-01	0.132E 01	0.440E 00	0.000E 00		9
293	3	-.262E 00	0.150E 01	0.672E 00	0.000E 00		5
293	4	0.117E 00	0.141E 01	0.632E 00	0.121E 04		5
293	10	-.857E-01	0.132E 01	0.303E 00	0.000E 00		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.22	
	2	4	-0.30	
	3	4	-0.44	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
294	2	0.659E 00	0.372E 00	0.124E 00	0.565E 02		9
294	3	0.807E 00	0.454E 00	0.203E 00	0.563E 02		5
294	4	0.181E 00	0.556E 00	0.249E 00	0.307E 03		5
294	10	0.572E 00	0.487E 00	0.112E 00	0.852E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.69	
	2	4	2.02	*
	3	4	2.07	*

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
296	2	0.147E-01	0.132E 01	0.439E 00	0.898E 04		9
296	3	0.851E 00	0.981E-01	0.439E-01	0.115E 02		5
296	4	0.891E 00	0.105E 00	0.470E-01	0.118E 02		5
296	10	0.465E 00	0.985E 00	0.226E 00	0.212E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.45	
	2	4	-1.52	
	3	4	-0.65	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
298	2	0.667E 00	0.205E 00	0.685E-01	0.308E 02		9
298	3	-.534E 00	0.101E 01	0.452E 00	0.000E 00		5
298	4	0.989E 00	0.404E 00	0.181E 00	0.444E 02		5
298	10	0.415E 00	0.795E 00	0.182E 00	0.192E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	3.69	***
	2	4	-1.57	
	3	4	-3.15	**

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
301	2	0.428E 00	0.461E 00	0.154E 00	0.108E 03		9
301	3	0.556E 00	0.341E 00	0.152E 00	0.613E 02		5
301	4	-.329E-01	0.129E 01	0.578E 00	0.000E 00		5
301	10	0.341E 00	0.740E 00	0.170E 00	0.217E 03		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.56	
	2	4	1.03	
	3	4	1.05	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
302	2	0.188E 01	0.226E 00	0.798E-01	0.126E 02		9
302	3	0.175E 01	0.605E-01	0.271E-01	0.345E 01		5
302	4	0.185E 01	0.244E 00	0.109E 00	0.132E 02		5
302	10	0.184E 01	0.204E 00	0.468E-01	0.111E 02		19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1.17	
	2	4	0.20	
	3	4	-0.92	

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
102	2	0 186E 01	0 249E 00	0 829E-01	0 133E 02	9
102	3	0 152E 01	0 166E 00	0 741E-01	0 109E 02	5
102	4	0 161E 01	0 388E 00	0 174E 00	0 241E 02	5
102	10	0 171E 01	0 302E 00	0 693E-01	0 177E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	2 83	**
	2	4	1 54	
	3	4	-0 51	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
105	2	0 772E 00	0 128E 01	0 425E 00	0 165E 01	9
105	3	0 922E 00	0 232E 00	0 104E 00	0 252E 02	5
105	4	0 191E 01	0 457E 00	0 204E 00	0 453E 02	5
105	10	0 874E 00	0 890E 00	0 204E 00	0 102E 03	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0 27	
	2	4	-0 41	
	3	4	-0 41	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
106	2	0 165E 01	0 224E 00	0 746E-01	0 136E 02	9
106	3	0 172E 01	0 144E 00	0 451E-01	0 847E 01	5
106	4	0 161E 01	0 139E 00	0 620E-01	0 863E 01	5
106	10	0 165E 01	0 102E 00	0 457E-01	0 618E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0 68	
	2	4	0 27	
	3	4	1 33	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
108	2	0 249E 01	0 181E 00	0 603E-01	0 752E 01	9
108	3	0 252E 01	0 156E 00	0 636E-01	0 617E 01	5
108	4	0 148E 01	0 281E 01	0 396E 00	0 143E 03	5
108	10	0 219E 01	0 114E 01	0 252E 00	0 502E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1 33	
	2	4	1 39	
	3	4	1 17	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
109	2	0 284E 01	0 187E 00	0 622E-01	0 954E 01	9
109	3	0 211E 01	0 127E 00	0 614E-01	0 450E 01	5
109	4	0 208E 01	0 418E 00	0 187E 00	0 283E 02	5
109	10	0 260E 01	0 249E 00	0 563E-01	0 124E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0 74	
	2	4	0 27	
	3	4	0 59	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
110	2	0 954E 00	0 300E 00	0 100E 00	0 351E 02	9
110	3	0 126E 01	0 164E 00	0 734E-01	0 131E 02	5
110	4	0 929E 00	0 448E 00	0 197E 00	0 473E 02	5
110	10	0 980E 00	0 345E 00	0 798E-01	0 352E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-2 05	**
	2	4	-0 39	
	3	4	1 65	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
113	2	0 123E 01	0 511E 00	0 170E 00	0 417E 02	9
113	3	0 118E 01	0 167E 00	0 745E-01	0 141E 02	5
113	4	0 105E 01	0 227E 00	0 146E 00	0 212E 02	5
113	10	0 117E 01	0 298E 00	0 894E-01	0 254E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0 19	
	2	4	0 73	
	3	4	0 87	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
114	2	0 111E 01	0 720E 00	0 240E 00	0 652E 02	9
114	3	0 598E 00	0 208E 00	0 894E-01	0 329E 02	5
114	4	0 204E 00	0 135E 01	0 676E 00	0 755E 03	5
114	10	0 732E 00	0 949E 00	0 218E 00	0 130E 03	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1 60	
	2	4	1 61	
	3	4	0 61	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
116	2	0 105E 01	0 134E 01	0 434E 00	0 124E 03	9
116	3	0 466E 00	0 154E 01	0 690E 00	0 331E 03	5
116	4	0 111E 01	0 325E 00	0 145E 00	0 293E 02	5
116	10	0 918E 00	0 118E 01	0 270E 00	0 129E 03	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0 78	
	2	4	-0 10	
	3	4	-0 96	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO. VALUES
118	2	0 265E 01	0 231E 00	0 769E-01	0 871E 01	9
118	3	0 721E 00	0 272E 00	0 122E 00	0 378E 02	5
118	4	0 483E 00	0 162E 01	0 723E 00	0 335E 03	5
118	10	0 157E 01	0 132E 01	0 302E 00	0 838E 02	19

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	14 66	****
	2	4	4 25	****
	3	4	0 24	

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
319	2	0.114E 01	0.197E 01	0.655E 00	0.172E 02		9
319	3	0.156E 01	0.242E 00	0.903E-01	0.129E 02		5
319	4	0.156E 01	0.517E 00	0.231E 00	0.222E 02		5
319	10	0.136E 01	0.115E 01	0.310E 00	0.893E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.49	
	2	4	-0.47	
	3	4	0.02	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
321	2	0.788E 00	0.126E 01	0.424E 00	0.160E 03		9
321	3	0.316E 00	0.223E 00	0.104E 00	0.254E 02		5
321	4	0.755E 00	0.155E 01	0.694E 00	0.452E 03		5
321	10	0.676E 00	0.111E 01	0.262E 00	0.161E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	+0.23	
	2	4	0.59	
	3	4	0.85	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
222	2	0.114E 01	0.283E 00	0.943E-01	0.247E 02		9
222	3	-.129E 01	0.926E 00	0.414E 00	0.000E 00		5
222	4	-.191E 01	0.867E 00	0.388E 00	0.000E 00		5
222	10	-.362E 00	0.156E 01	0.358E 00	0.000E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	7.88	****
	2	4	10.34	****
	3	4	1.16	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
224	2	0.602E 00	0.163E 01	0.543E 00	0.149E 03		9
224	3	0.175E 01	0.268E 00	0.120E 00	0.153E 02		5
224	4	0.924E 00	0.101E 01	0.883E 00	0.109E 03		5
224	10	0.102E 01	0.149E 01	0.342E 00	0.145E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.43	
	2	4	-0.26	
	3	4	1.07	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
225	2	0.224E 01	0.178E 00	0.595E-01	0.799E 01		9
225	3	0.223E 01	0.228E 00	0.102E 00	0.102E 02		5
225	4	0.234E 01	0.163E 00	0.757E-01	0.723E 01		5
225	10	0.224E 01	0.185E 00	0.425E-01	0.819E 01		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.02	
	2	4	-1.11	
	3	4	-0.89	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
226	2	0.224E 01	0.259E 00	0.863E-01	0.115E 02		9
226	3	0.241E 01	0.794E-01	0.355E-01	0.329E 01		5
226	4	0.237E 01	0.174E 00	0.778E-01	0.733E 01		5
226	10	0.232E 01	0.209E 00	0.480E-01	0.902E 01		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.44	
	2	4	-1.02	
	3	4	0.49	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
328	2	-.115E 00	0.127E 01	0.423E 00	0.000E 00		9
328	3	-.188E 01	0.929E 00	0.420E 00	0.000E 00		5
328	4	-.182E 01	0.187E 01	0.477E 00	0.000E 00		5
328	10	-.193E 01	0.140E 01	0.321E 00	0.000E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	2.82	**
	2	4	2.65	**
	3	4	-0.09	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
329	2	0.137E 01	0.270E 00	0.901E-01	0.197E 02		9
329	3	0.140E 01	0.217E 00	0.968E-01	0.155E 02		5
329	4	0.190E 01	0.764E 00	0.342E 00	0.338E 02		5
329	10	0.139E 01	0.302E 00	0.692E-01	0.217E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.20	
	2	4	1.78	*
	3	4	1.70	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
333	2	0.259E 00	0.147E 01	0.490E 00	0.567E 03		9
333	3	0.112E 01	0.448E 00	0.197E 00	0.392E 02		5
333	4	-.175E 00	0.197E 01	0.883E 00	0.000E 00		5
333	10	0.372E 00	0.145E 01	0.334E 00	0.259E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.71	
	2	4	0.49	
	3	4	1.92	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO	VALUES
334	2	-.979E 00	0.198E 01	0.661E 00	0.000E 00		9
334	3	0.898E 00	0.179E 01	0.801E 00	0.201E 03		5
334	4	0.833E 00	0.176E 01	0.786E 00	0.211E 03		5
334	10	-.104E-01	0.201E 01	0.461E 00	0.000E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.82	*
	2	4	-1.77	
	3	4	0.05	

TABLE J1. (Cont'd.)

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
342	2	-	1.25E 01	0 1.59E 01	0 5.31E 00	0 0.00E 00	9	
342	3		1.93E 00	0 1.97E 01	0 9.85E 00	0 0.00E 00	5	
342	4	0	4.98E 00	0 1.59E 01	0 7.11E 00	0 3.24E 02	5	
342	10	-	5.10E 00	0 1.77E 01	0 4.46E 00	0 0.00E 00	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.15	
	2	4	-2.04	*
	3	4	-0.63	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
343	2	-	1.63E 01	0 1.33E 01	0 4.45E 00	0 0.00E 00	9	
343	3	0	0.00E 00	0 1.32E 00	0 1.14E 00	0 4.02E 02	5	
343	4	0	7.57E 00	0 1.67E 00	0 7.46E-01	0 2.21E 02	5	
343	10	-	3.62E 00	0 1.53E 01	0 3.51E 00	0 0.00E 00	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-4.10	***
	2	4	-4.07	***
	3	4	0.29	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
346	2	0	1.13E 01	0 3.57E 00	0 1.19E 00	0 4.67E 02	9	
346	3	0	9.24E 00	0 2.32E 00	0 1.04E 00	0 2.51E 02	5	
346	4	0	1.81E 01	0 4.57E 00	0 2.04E 00	0 2.53E 02	5	
346	10	0	1.07E 01	0 4.59E 00	0 1.05E 00	0 4.27E 02	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1.06	
	2	4	0.65	
	3	4	-0.40	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
347	2	0	7.43E 00	0 1.15E 01	0 3.83E 00	0 1.56E 02	9	
347	3	0	5.96E 00	0 1.59E 01	0 7.10E 00	0 2.12E 03	5	
347	4	0	1.11E 01	0 1.71E 00	0 7.64E-01	0 1.57E 02	5	
347	10	0	7.73E 00	0 1.14E 01	0 2.62E 00	0 1.41E 03	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.34	
	2	4	-0.03	
	3	4	-0.91	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
349	2	-	2.49E 00	0 1.22E 01	0 4.07E 00	0 4.90E 00	9	
349	3	0	8.28E 00	0 2.08E 00	0 9.30E-01	0 2.48E 02	5	
349	4	0	5.45E 00	0 1.63E 01	0 7.26E 00	0 2.99E 02	5	
349	10	0	2.46E 00	0 1.22E 01	0 2.81E 00	0 4.96E 01	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-2.02	*
	2	4	-1.08	
	3	4	0.42	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
350	2	-	3.12E-01	0 1.71E 01	0 5.68E 00	0 0.00E 00	9	
350	3	0	1.14E 00	0 1.38E 01	0 6.15E 00	0 1.21E 04	5	
350	4	-	7.61E 00	0 1.48E 01	0 6.62E 00	0 0.00E 00	5	
350	10	-	1.05E 00	0 1.52E 01	0 3.50E 00	0 0.00E 00	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.17	
	2	4	0.83	
	3	4	1.03	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
351	2	-	1.09E 01	0 1.82E 01	0 6.89E 00	0 0.00E 00	9	
351	3	-	3.24E-01	0 2.07E 01	0 9.26E 00	0 0.00E 00	5	
351	4	-	9.95E-01	0 2.07E 01	0 9.26E 00	0 0.00E 00	5	
351	10	-	5.52E 00	0 1.91E 01	0 4.38E 00	0 0.00E 00	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.04	
	2	4	-0.97	
	3	4	0.05	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
352	2	0	6.98E 00	0 1.18E 01	0 3.92E 00	0 1.69E 03	9	
352	3	0	1.17E 01	0 1.09E 00	0 4.87E-01	0 1.53E 02	5	
352	4	0	1.12E 01	0 4.22E 00	0 1.89E 00	0 3.77E 02	5	
352	10	0	9.98E 00	0 8.67E 00	0 1.99E 00	0 8.69E 02	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.30	
	2	4	-0.60	
	3	4	1.27	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
353	2	0	1.14E 01	0 1.47E 00	0 4.90E-01	0 9.95E 01	9	
353	3	0	1.65E 01	0 5.07E-01	0 2.62E-01	0 3.56E 01	5	
353	4	0	1.60E 01	0 3.72E 00	0 1.67E 00	0 2.34E 02	5	
353	10	0	1.35E 01	0 2.72E 00	0 4.95E-01	0 1.33E 02	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-2.44	**
	2	4	-0.04	
	3	4	0.33	

CMPD	NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
357	2	-	1.51E 01	0 1.22E 01	0 4.07E 00	0 0.00E 00	9	
357	3	0	1.31E 01	0 1.45E 00	0 7.36E-01	0 1.12E 02	5	
357	4	0	1.30E 00	0 1.41E 01	0 6.28E 00	0 1.08E 04	5	
357	10	-	3.37E 00	0 1.62E 01	0 3.71E 00	0 0.00E 00	19	

TTEST	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-5.26	****
	2	4	-2.38	**
	3	4	1.98	*

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
359	2	0.404E 00	0.169E 01	0.564E 00	0.135E 03		9
359	3	- 418E 00	0.176E 01	0.783E 00	0.000E 00		5
359	4	- 696E 00	0.220E 01	0.984E 00	0.000E 00		5
359	10	- 637E-01	0.183E 01	0.419E 00	0.000E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.98	
	2	4	1.17	
	3	4	0.23	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
360	2	0.292E 00	0.197E 01	0.654E 00	0.673E 03		9
360	3	0.105E 01	0.188E 01	0.840E 00	0.183E 03		5
360	4	0.625E 00	0.166E 01	0.741E 00	0.265E 03		5
360	10	0.571E 00	0.179E 01	0.411E 00	0.313E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.71	
	2	4	-0.33	
	3	4	0.38	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
361	2	0.541E 00	0.173E 01	0.577E 00	0.320E 03		9
361	3	0.113E 01	0.196E 01	0.876E 00	0.167E 03		5
361	4	0.639E 00	0.167E 01	0.746E 00	0.265E 03		5
361	10	0.731E 00	0.179E 01	0.389E 00	0.222E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.65	
	2	4	-0.10	
	3	4	0.50	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
362	2	0.339E 00	0.130E 01	0.434E 00	0.383E 03		9
362	3	0.580E 00	0.162E 01	0.724E 00	0.279E 03		5
362	4	0.144E 01	0.140E 00	0.662E-01	0.103E 02		5
362	10	0.955E 00	0.114E 01	0.265E 00	0.121E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.43	
	2	4	-1.04	
	3	4	-1.25	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
363	2	0.139E 01	0.126E 01	0.452E 00	0.164E 03		9
363	3	0.179E 01	0.971E-01	0.434E-01	0.541E 01		5
363	4	0.175E 01	0.171E 00	0.767E-01	0.983E 01		5
363	10	0.155E 01	0.941E 00	0.216E 00	0.608E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.83	
	2	4	-0.74	
	3	4	0.59	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
367	2	- 282E 00	0.117E 01	0.389E 00	0.414E 03		9
367	3	- 520E 00	0.112E 01	0.503E 00	0.216E 03		5
367	4	- 161E 00	0.128E 01	0.518E 00	0.800E 00		5
367	10	- 313E 00	0.111E 01	0.254E 00	0.808E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.19	
	2	4	-0.19	
	3	4	-0.52	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
369	2	- 228E 00	0.123E 01	0.411E 00	0.808E 00		9
369	3	0.796E 00	0.216E 00	0.966E-01	0.271E 02		5
369	4	- 170E 01	0.134E 01	0.604E 00	0.000E 00		5
369	10	- 341E 00	0.140E 01	0.322E 00	0.408E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.87	*
	2	4	2.17	**
	3	4	4.32	***

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
371	2	0.926E 00	0.297E 00	0.998E-01	0.321E 02		9
371	3	- 169E 01	0.136E 01	0.607E 00	0.000E 00		5
371	4	0.114E 01	0.172E 00	0.771E-01	0.151E 02		5
371	10	0.293E 00	0.144E 01	0.331E 00	0.477E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1.96	****
	2	4	-1.53	
	3	4	-4.01	****

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
372	2	0.563E 00	0.163E 01	0.543E 00	0.255E 03		9
372	3	0.127E 01	0.523E-01	0.233E-01	0.409E 01		5
372	4	0.119E 01	0.171E 00	0.764E-01	0.144E 02		5
372	10	0.941E 00	0.117E 01	0.268E 00	0.123E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.82	
	2	4	-0.71	
	3	4	1.13	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
374	2	0.289E 00	0.108E 01	0.334E 00	0.347E 03		9
374	3	0.952E 00	0.104E 00	0.464E-01	0.109E 02		5
374	4	0.956E 00	0.255E 00	0.114E 00	0.267E 02		5
374	10	0.639E 00	0.762E 00	0.175E 00	0.119E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-1.51	
	2	4	-1.50	
	3	4	-0.03	

TABLE J1. (Cont'd.)

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
375	2	0.651E 00	0.114E 01	0.334E 00	0.175E 01		9
375	3	0.189E 01	0.172E 00	0.770E-01	0.158E 02		5
375	4	0.123E 01	0.181E 00	0.179E 00	0.294E 02		5
375	10	0.932E 00	0.845E 00	0.194E 00	0.906E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.87	
	2	4	-1.20	
	3	4	-1.04	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
376	2	0.858E 00	0.223E 00	0.743E-01	0.262E 02		9
376	3	0.249E 00	0.146E 01	0.651E 00	0.587E 03		5
376	4	-.441E 00	0.171E 01	0.765E 00	0.000E 00		5
376	10	0.352E 00	0.120E 01	0.275E 00	0.341E 01		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	1.31	
	2	4	2.41	**
	3	4	0.73	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
378	2	0.975E 00	0.196E 01	0.624E 00	0.191E 03		9
378	3	0.144E 01	0.521E 00	0.233E 00	0.362E 02		5
378	4	0.145E 01	0.342E 00	0.153E 00	0.236E 02		5
378	10	0.122E 01	0.138E 01	0.298E 00	0.106E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.56	
	2	4	-0.58	
	3	4	-0.04	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
379	2	-.158E 00	0.162E 01	0.540E 00	0.000E 00		9
379	3	-.505E 00	0.164E 01	0.734E 00	0.000E 00		5
379	4	-.394E 00	0.177E 01	0.794E 00	0.000E 00		5
379	10	-.312E 00	0.159E 01	0.361E 00	0.000E 00		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	0.48	
	2	4	0.26	
	3	4	-0.11	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
381	2	0.440E-02	0.135E 01	0.451E 00	0.308E 05		9
381	3	0.119E 01	0.164E 00	0.731E-01	0.137E 02		5
381	4	0.965E 00	0.743E 00	0.332E 00	0.77AE 02		5
381	10	0.570E 00	0.112E 01	0.257E 00	0.196E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-2.00	*
	2	4	-1.51	
	3	4	0.71	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
382	2	0.576E 00	0.164E 01	0.547E 00	0.285E 03		9
382	3	0.898E 00	0.179E 01	0.802E 00	0.200E 03		5
382	4	0.114E 01	0.281E 00	0.126E 00	0.246E 02		5
382	10	0.810E 00	0.141E 01	0.324E 00	0.174E 03		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	-0.36	
	2	4	-0.78	
	3	4	-0.32	

CMPD NO	CLASS	MEAN	STD DEV	STD ERR	COEF VAR	NO.	VALUES
383	2	0.227E 01	0.376E 00	0.125E 00	0.166E 02		9
383	3	0.178E 01	0.353E 00	0.158E 00	0.207E 02		5
383	4	0.227E 01	0.288E 00	0.174E 00	0.171E 02		5
383	10	0.212E 01	0.436E 00	0.100E 00	0.205E 02		19

TTEST:	CLASS X	CLASS Y	T	SIGNIFICANCE
	2	3	2.86	**
	2	4	-0.03	
	3	4	-2.59	**

APPENDIX K

List of publications

C.C. Sweeley, S. Gates and J.F. Holland, "Mass Chromatographic Approach to Quantitation of Compounds in Complex Biological Mixtures." In: O.A. Mamer, W.J. Mitchell and C.R. Scriver (Editors), Application of Gas Chromatography-Mass Spectrometry to the Investigation of Human Disease, McGill University Montreal Childrens Hospital Research Institute, p. 141 (1974).

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S.C. Gates, C.C. Sweeley, N.D. Young and J.F. Holland, "Automated Multicomponent Analysis of Biological Mixtures by Gas Chromatography-Mass Spectrometry." In: A. Frigerio (Editor), Advances in Mass Spectrometry in Biochemistry and Medicine, Vol. II, Spectrum Publications, New York, p. 171, 1976.

APPENDIX K (Cont'd.)

C.C. Sweeley, S.C. Gates, R.H. Thompson, J. Harten, N. Dendramis and J.F. Holland, "Techniques for Quantitative Measurements by Mass Spectrometry." In: Proceedings of the International Symposium on Quantitative Mass Spectrometry in Life Sciences, Elsevier, 1977.

S.C. Gates and C.C. Sweeley, "Library Data for the Computer Identification of Organic Acids by Gas Chromatography-Mass Spectrometry." Submitted to Biomed. Mass Spec.

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