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review

Quantitative Metabolic Profiling Based on Gas Chromatography

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The quantitative metabolic profiles of volatilizable components of human biological fluids, particularly urinary organic acids, is reviewed, with emphasis on the use of gas-chromatography/mass spectrometer/computer systems. Various definitions of metabolic profiling are considered and techniques for obtaining such profiles are discussed. The role of computer processing of such data is examined, and statistical techniques for treating quantitative metabolic profiles are suggested.

The use of metabolic profiles has its beginnings in the late 1940s; however, *quantitative* metabolic profiles have only just barely become a reality. It may therefore seem premature to review a field that, as yet, has almost no history, especially since two excellent reviews of qualitative metabolic profiling have recently appeared (*1, 2*). The justification for this review is twofold: first, we have attempted to draw together concepts from several fields relevant to quantitative metabolic profiling and, second, it serves to introduce those not yet involved in the field to the potential uses and problems of this technique. The review is intended to be selective rather than exhaustive, and emphasizes gas chromatographic/mass spectrometric (GC-MS) techniques over other methods. More general uses of GC-MS techniques in the clinical laboratory have already been thoroughly discussed by Lawson (*3*) and Burlingame et al. (*4*).

Development of the Concept of Metabolic Profiling

The concept that individuals might have a "metabolic pattern" that would be reflected in the constitutents of their biological fluids was first developed and tested by Roger Williams and his associates during the late 1940s and early 1950s (*5*). 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 taste thresholds and the excretion patterns for a variety of substances varied greatly from individual to individual (Figure 1), but that these patterns were relatively constant for a given individual. He summarized his findings in 1951 as follows (*5*):

> 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 (*5*).

The work of Williams and his group, however, was apparently not duplicated by others, to whom his task must have seem rather herculean, with but few promises of tangible results. Hence, his ideas about the utility of metabolic pattern analysis remained essentially dormant until the late 1960s, when gas chromatography and liquid chromatography was advanced sufficiently to permit such studies to be carried out with considerably less effort. Once these techniques became available, the rate of progress became extremely rapid. In 1970, for example, 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" (*6*), "personal blood 'profiles' " (*7*), and a "characteristic excretion profile" of organic acids in urine of patients with phenylketonuria (*8*).

The phrase most often used to describe the chromatographic patterns observed in biological fluids has been "metabolic profile." This term was introduced by the Hornings in 1971 (*9, 10*); as originally defined, it meant "multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites" (*10*). Commenting on the potential usefulness of this type of technique, the Hornings suggested that "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 (*11*). Other workers have preferred just the term "profile" to mean the same thing (*12*). Johnson (*13*) has taken a more quantitative 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 subject or comparing the profiles of a different group.

Several hospital laboratories have experimented with a related technique, "multiphasic screening" (reviewed in *14*), 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: in multiphasic testing there are single tests for each of the components,

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in profiling a single chromatographic run is used 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 (*15*). Reece (*16*) has used an additional term, "uniphasic synthesis," for multiphasic screening when the test results are analyzed by use of multivariate statistical techniques.

In general, interest in this type of approach is recent enough that the terminology is in a state of flux. In the material that follows, a combination of the Horning and Johnson definitions will be used: metabolic profiling is a means of obtaining, by chromatography, 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, this definition will arbitrarily be limited to exclude studies of small numbers of components (e.g., less than 10 compounds), components of high relative molecular mass (greater than 1000 daltons), and all inorganic compounds.

Techniques for Separating Components of Low Relative Molecular Mass in Biological Fluids

Although the terminology used to describe multi-component analysis of biological mixtures is new, the techniques used are old: 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 by use of paper-chromatographic techniques was that of Williams et al., as described previously. While a great many other groups were successful in devising means of separating various fractions of urine and other biological fluids [e.g., separation of phenolic acids by Armstrong and Shaw (*17*) and of indoles by Dalgliesh (*18*)], these groups generally did not use these techniques to produce *quantitative* human health profiles because of the difficulty of eluting and quantitating individual components. Paper chromatography 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 et al. (*19*) on screening procedures for aminoacidopathies].

Column Chromatography

In contrast, column chromatography, particularly high-pressure liquid chromatography, is an effective means for obtaining profiles for assessing human health. Jolley and Freeman (*20*) and Young (*6*) described early attempts to use a high-resolution chromatographic apparatus to evaluate the health status of several types of individuals by examination of the profile of urinary carbohydrates. Young showed that considerable differences in the carbohydrate profile were apparent for several disease states, while the pattern for a given individual was reasonably constant from day to day. In a classic paper on the ion-exchange chromatography of amino acids, Hamilton (*21*) indicated that about 95 ninhydrin-positive components of urine could be resolved by an automated procedure with a high-resolution column.

Similarly, Pitt et al. (*22*) and Scott et al. (*8*) have described a system developed at the Oak Ridge National Laboratory for the analysis of as many as 150 ultraviolet-absorbing substances in body fluids. A minicomputer was used to resolve peaks and store data (*23*). Using gas chromatography–mass spectrometry, they found many of the compounds detected by this system to be aromatic organic acids and amino acids (*22*). Later work expanded this system to multiple-column operation (*24*), sequential use of different column types to achieve more rapid separations (*25*), and more sensitive detection systems (*26*), including fluorescence monitoring of organic acids (*27*).

Other laboratories have utilized systems of this type (*28*), but with computerized data processing (*29*) and other types of detectors (*30, 31*). A highly sophisticated system for computerized analysis of clinical amino acid data (*32, 33*) will be discussed in more detail in the section on statistical analysis of data.

Other workers have concentrated upon improved packing materials. Molnar and Horvath (*34*), for example, have demonstrated the separation of over 100 aromatic organic acids, and predict that improved techniques will allow the separation of as many as 500 components in human urine by high-performance liquid chromatography (HPLC). However, no quantitation has yet been reported with this system.

Gas Chromatography

Although proposed in theory in 1941, in a pioneering paper on liquid–liquid partition chromatography (*35*), gas chromatography (GC) was not successfully applied to complex biological mixtures on a practical basis until the introduction of lightly packed columns loaded with thermostable liquid phases (*36*), of high-sensitivity flame ionization (*37*) and electron capture detectors (*38*), and of suitable derivatization methods (see reference *39* for a history of GC). The number of uses of GC for the analysis of complex organic mixtures rapidly surpassed those of liquid chromatography in the 1960s, because no comparable "universal" detector was available for liquid chromatography and the speed of analysis was then much slower than that of GC.

Unfortunately, as numerous workers were quick to discover, the gas chromatograph by itself provided neither sufficient specificity of detector response nor adequate chromatographic resolution (packed columns) 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 have been few reported attempts on the scale used by Roger Williams in which packed-column GC alone is utilized to find the more subtle metabolic patterns present in biological fluids.

Gas Chromatograph/Mass Spectrometer/ Computer Systems

One solution to the problems associated with using liquid chromatography or GC for profiling purposes was provided by the use of a gas chromatograph coupled to a mass spectrometer. 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 (*40*) and in 1961 by Henneberg (*41*), the introduction of the molecular separator by Ryhage (*42*) and Watson and Biemann (*43*), 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 applied the molecular separator for combined GC-MS to obtain a profile of the fatty acid methyl ester composition of butterfat (*42*). This was quickly followed by a study of the neutral fecal steroids in human subjects (*44*), and then by a virtual flood of papers utilizing GC-MS systems for the

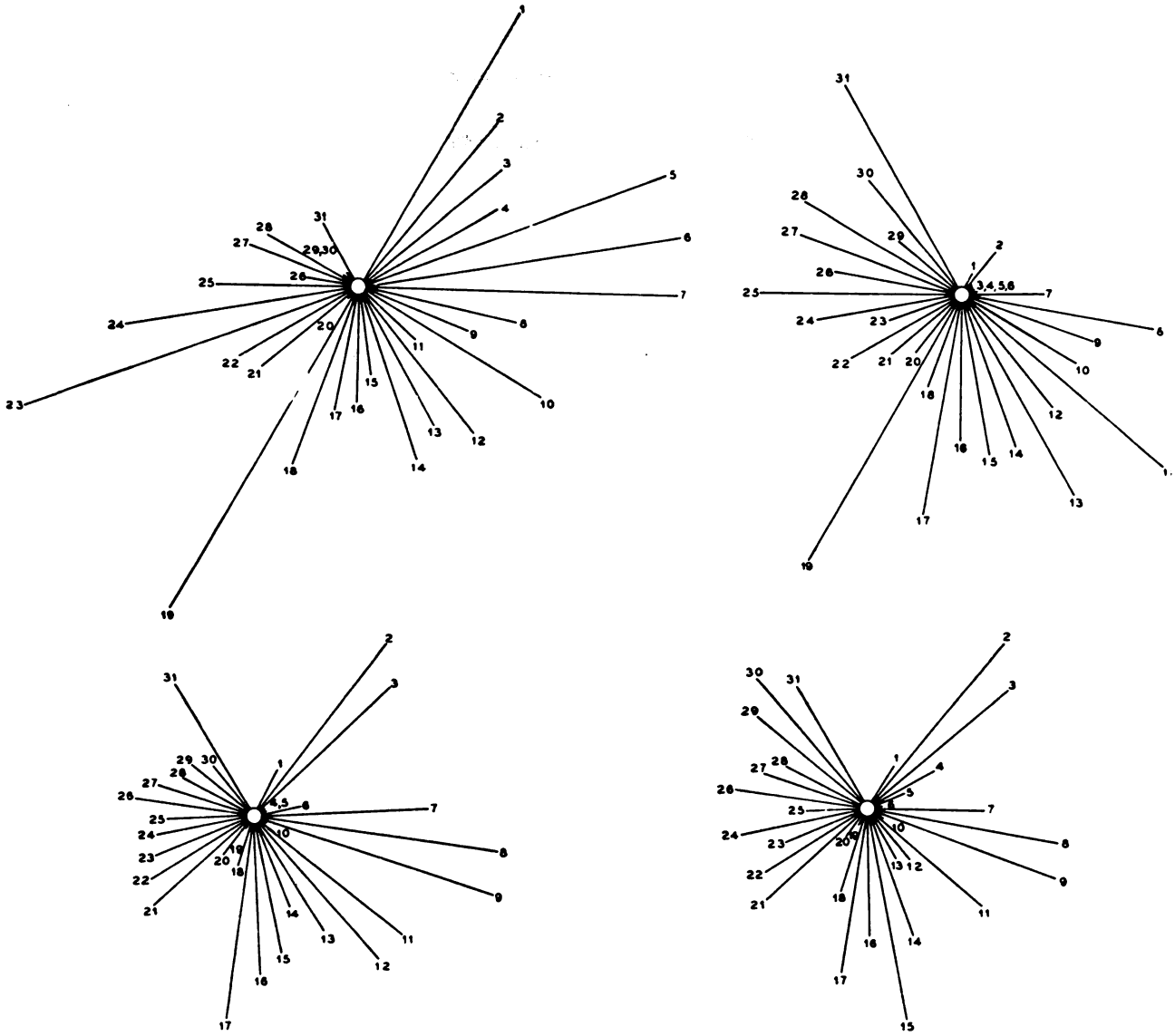

Fig. 1. Early demonstration of biochemical individuality by metabolic profiling

The metabolic patterns of 4 subjects were obtained by measurements of taste thresholds (numbers *1–17*) and urinary metabolites (numbers *18–31*). The lengths of the lines are indicative of the amounts of compounds measured. Reproduced through the courtesy of R. J. Williams, University of Texas (see ref. *5* for details)

analysis of a wide variety of biological, environmental, and geophysical samples. In most of these early papers GC-MS was used primarily for qualitative analysis of a few peaks in a very few samples; researchers were limited principally by the lack of automated data-processing equipment.

An alternative approach, emphasizing quantitative analysis of a very small number of peaks, was first applied to the analysis of GC effluents by Henneberg in 1961 (*41*) and later, independently, in Ryhage's laboratory by Sweeley et al. (*45*). "Selected ion monitoring," originally used for monitoring one or two ions, provides a means of obtaining highly precise measurements on a few 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, *46–50*) this general technique has been limited to the measurement of a very small number of components in any one mixture. While extremely useful for many kinds of studies, it has not had a significant impact on metabolic profiling. Maume et al. (*51, 52*) and Summons et al.(*53*) at Stanford have been notable exceptions; they have successfully examined mixtures of closely related steroids, catecholamines, and amino acids by monitoring ions common to whole classes of these compounds.

Much more useful for metabolic profiling purposes is the computer-based technique called "mass chromatography," first described by Hites and Biemann in 1970 (*54*). In this technique, complete mass spectra are taken at frequent intervals and the entire data set is 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 plots of intensity vs. time ("mass chromatograms") can be displayed for any ion of interest within the entire mass range of the mass spectrometer. Thus, mass chromatograms are equivalent to the traces generated during selected ion monitoring, except that the sampling frequency is much lower for each ion in mass chromatography. This results in lower quantitative precision and reduced sensitivity (*55*), but a much more generalized quantitative ability as 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

Baczynsky et al. (56) and Axelson et al. (57). 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 methods. In instruments with electrostatic mass filters (quadrupoles, dodecapoles), limited-range mass scanning, selected ion monitoring, and repetitive scanning are accomplished with essentially the same data algorithms and hardware; 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 spectra or mass chromatograms, appears to be the most generally useful for analysis of mixtures containing many unidentified components. For this reason, a number of laboratories have developed GC-MS-computer systems utilizing repetitive scanning and mass chromatographic techniques for use in metabolic profiling.

Principal advocates of this approach have been the Hornings at Baylor University, Eldjarn, Jellum, and Stokke at the University of Oslo, and Biemann's group at the Massachusetts Institute of Technology. The Hornings first described metabolic profiling in a pair of now-classic papers published in 1971 (9, 10). These papers not only defined metabolic profiling but also advocated the use of methylene units (a measure of GC retention time that is virtually identical to retention indices, discussed below) for peak identification and proposed a series of techniques for sample isolation that are still followed in many laboratories.

In a similar fashion Jellum and his coworkers utilized a GC-MS-computer system in both the single-scanning and the repetitive scanning modes for metabolic profiling studies in a large-scale screening program for metabolic disorders (58, 60). They have primarily relied on their system to identify major unknown peaks, rather than to detect and evaluate abnormal profiles; only samples that have passed through the entire screening procedure and are still found to be medically interesting are submitted for GC-MS analysis.

Biemann's group at MIT has also developed a system with repetitive scanning techniques, used primarily to identify drugs and drug metabolites in body fluids of drug-overdose victims (61, 62). The system involves both automated and manual identification of individual spectra and manual search of mass chromatograms. A specialized library of over 300 spectra is used to identify known compounds (62), and retention indices are used to help identify new metabolites (62, 63).

Common to the Baylor, Oslo, and MIT systems is an interest in identifying abnormal compounds or compounds present in abnormal amounts. Little or no quantitative data have been published by these groups of workers. This same pattern of interest in qualitative, rather than quantitative, results has been evident in the publications from other laboratories (11, 64–73).

Other groups have, however, reported semi-quantitative results using GC-MS systems with quantitation by GC peak area and identifications, where necessary, by mass spectrometry. In an excellent study, Thompson and Markey described such a system (74), and used it for comparing three methods of extracting organic acids. About 60 substances were quantitated in a scale of 0 to ++++. Many of the substances were grouped together because they were unresolved by the GC. More recently, Thompson et al. measured peak areas of 24 peaks in urines of 92 "healthy" children and four adults and compared excretion values among the six age groups represented (75). Björkman et al. (76) published data from a similar system for 28 substances, about half of which were data for unresolved groups of two or more organic acids. Lindstedt et al. (77) also quantitated several dicarboxylic acids in the urine of an infant with congenital lactic acidosis. Muskie et al. (78) reported quantitative data for several urinary acidic and alcoholic catecholamine metabolites. Lawson et al. (79–82) have made a major contribution with a similar system, and have reported quantitative data for more than 20 large peaks in the GC, some of which were peak clusters containing several components.

High-resolution GC and GC-MS

One of the principal impediments to quantitation of profiling data by GC-MS has been the limited resolution of packed GC columns. Hence, two complementary methods for achieving more nearly complete separation of mixture components have been investigated: high-resolution (capillary) GC column technology and mechanized (automated) data-processing techniques.

With capillary GC columns, resolution is significantly better than with packed GC columns, and they are especially appropriate for separating underivatized volatile fractions of biological fluids and other complex mixtures. Zlatkis and his coworkers have emphasized this approach in a series of papers describing the analysis of ether extracts of urine (83), urine headspace samples (84), organic volatiles in air (85), and serum and plasma headspace volatiles (86). Politzer et al. (87) have even expanded this approach to include the volatile fractions of lung, brain, and liver tissues and have reviewed GC and GC-MS analysis of underivatized volatile compounds in biological fluids (1). Maume and Luyten (52) analyzed derivatized and underivatized steroids down to 10 ng of injected analyte (picomole amounts when selected ion-monitoring techniques were used). Horning et al. (88) developed a method for the preparation of thermostable capillary columns which provide extremely high resolution (100 000 theoretical plates) and are suitable for the analysis of a variety of biological fluid extracts. Luyten and Rutten (89) have used retention indices on the capillary columns to aid in compound identification. Novotny et al. (90) compared stationary phases of different polarity for their applicability in profiling studies and developed a method for concentrating samples prior to analysis (91). More recently, Knights et al. (92) reported use of a direct-coupled capillary GC–low-resolution MS–computer system for the qualitative analysis of acidic urinary metabolites in a diethyl ether extract. Hedfjäll and Ryhage (93) used much more rapid scans (1.4-s cycle time) of a magnetic-sector mass spectrometer to accommodate the need for higher data-collection rates with capillary columns. An even more sophisticated system is that developed in Burlingame's laboratory (94), utilizing high-resolution GC–high-resolution MS; this system and a previous version designed for low-resolution GC–high-resolution MS (95) have been used to identify components in metabolic profiles of organic acids and other materials.[3] Jakobs et al. (96) also reported coupling a capillary GC to a mass spectrometer for the analysis of organic acids, and used this system to quantitate 16 metabolites associated with the presence of maple-syrup urine disease.

Despite the high resolution that can be achieved with capillary GC columns, they have thus far only infrequently been used for direct quantitation of profiles. There appear to be at least two reasons for this: the profiles thus obtained are exceedingly complex, making identification and quantitative analysis of individual peaks correspondingly more difficult; and, even with the highest available resolution, capillary GC columns do not completely separate all components of physiological fluids.

[3] Burlingame, A. L., private communication.

One approach to this pair of problems that has achieved some success is that of Robinson and Pauling (*97, 98*), in which capillary GC data have been analyzed with sophisticated algorithms for comparing runs and subtracting baseline contributions. No evidence has yet been presented about the ability of this system to deal with partly resolved peaks.

Computer Processing of GC-MS Data for Metabolic Profiling

A more common approach involves the application of data-processing techniques to achieve deconvolution of the chromatographic peaks from low-resolution GC-MS data.

Forward Library Search Methods

The traditional method for identification of profile data has been the "forward library search," which involves manual or computerized comparison of each sample spectrum of interest to a library of reference spectra to find the best match (see, for example, *11, 64, 80, 99*). These forward searches have often utilized mammoth data bases and sophisticated pattern-analysis algorithms (*100, 101*). Although pre-ordering the library file according to particular structural moieties or compound classes can decrease the amount of time needed for such library searches—Dromey (*102*), for example, has proposed a method for identifying functional groups as an aid to such a search—forward library search methods have been time-consuming and costly.

A major difficulty with forward searches is that they are ill-suited to the analysis of minor components and severely overlapped GC peaks. One approach to the solution of this problem, which still retains the forward search, has been proposed by Biller and Biemann (*103*). To obtain mass spectra that are 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. Smith et al. (*113*) and Blaisdell (*114*) have developed even more sophisticated forward-search routines (described above).

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 (*104*), has also been developed by McLafferty (*105*) and Sweeley et al. (*106*). 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.

There are several published variations of the reverse library search. Abramson's procedure (*104*) 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%. The criterion used to select masses to be compared is generally peak intensity; usually, the 10 most intense masses of the library spectrum are selected for comparison (*104*).

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" (*105*). This procedure, which is now available in time-share systems, uses "probability based matching of mass spectra." It involves examination of the following factors: the uniqueness of a particular ion (m/z) relative to all of the m/z 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 as 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 (*105*). McLafferty has also published a study of the uniqueness of various masses (*107*). Recently, deJong et al. (*108*) 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 Indexes

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 (*63*). In their approach, the GC retention index [developed originally by Kovats (*109*)] is 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, 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 (*62*). Other workers have used retention indices in combination with mass spectral correlations to compute a combined match score, an approach that we have used for reverse library searches (*110–112*) and that has been used for forward library searches by Smith et al. (*113*) and Blaisdell (*114*). Butts (*115*) published a very useful table of retention indices of organic acids that can be used in profiling studies of these substances.

Quantitative Profiling of GC-MS

Four closely related approaches have been developed for obtaining quantitative data from repetitive-scanning, low-resolution GC-MS data. The first of these is a system developed by Sjövall et al. (*57, 116*), 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 (the type of steroid skeleton, in their usual case) is identified where possible. A spectrum may then be compared to library spectra by a forward search procedure, if desired. In addition, a measure of the amount of a compound 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 similar system is described by Gates et al. (*112*), wherein is used a reverse library search of pre-selected mass chromatograms in the area of the GC-MS run corresponding to the expected elution time (retention index) of each component of interest. Quantitation is based on mass chromatogram areas relative to that of an internal standard. Identification and quantitation of more than 100 organic acid components in a single metabolic profile of human urine have been reported by use of this system (*117*) (Figure 2). Quantitative precision of better than 10% has been reported (*112*), with a linear response over a 500 to 1000-fold range (*55*). Limited data have been reported for reference population of adults, juveniles,

and neuroblastoma patients by use of this system (*118*), and the results statistically studied.[4]

Another system for quantitation of profiles has been described by a group at Stanford (*113, 119*). Their system involves a "cleanup" program on the GC-MS data to remove background and to separate contributions of overlapping components based on the profiles of well-resolved mass chromatogram peaks. The processed spectra are then compared to a historical library of spectra obtained from previous analyses of similar samples. Quantitation is based on comparing the summed ion intensities for each peak to the area of one or more internal standards. With this system, about 40 peaks are quantitated in a set of organic acids extracted from human urine by anion exchange. For replicated analyses of the same urine the precision has averaged about 15% of the standard deviation (excluding standards and an unusually variable artifact) when a manual extraction procedure is used.

Blaisdell and Sweeley[5] reported a similar method for quantitation, using a technique to resolve overlapping data from unresolved chromatographic peaks that differs from the procedures described by Biller and Biemann (*103*) and Dromey et al. (*119*). Using singular value analysis and linear least squares techniques, Blaisdell and Sweeley could resolve as many as 10 components,[5] which is comparable to the results achieved by Dromey et al. (*119*) by sequential use of a tabular peak modeling procedure.

Disease Diagnosis by Metabolic Profiling

Regardless of the methodology used for metabolic profiling, the same problems must be confronted once the data are collected: development of a method for the analysis of the data, and assignment of clinical significance to the results. 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 the amounts of one or a few compounds in urine or blood. Much of this literature has been listed in four articles (*2, 120–122*). 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.

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 mean values or, at best, means and standard deviations for individual compounds. Thus, for example, Young has reported "normal laboratory values" for more than 200 constitutents of blood, serum, and urine (*123*). A growing body of literature suggests, however, that under some circumstances mean values and standard deviations may be misleading. Burnett (*124*) has suggested the use of means and standard deviations with "outlier" values (values which are 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. (*125, 126*) have suggested using estimates of normality that 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 (*127*). Westgard and Hunt (*128*) 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 (*129*). This review carefully distinguishes between "normal values" and "reference values," favoring the latter term for most uses, but summarizing the types of information that should accompany reference values. He 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 vs. diabetic). Young has published a review of the computerized interpretation of clinical chemical data that discusses this and other problems of data treatment (*130*), and Werner and Marsh (*131*) have reviewed practical considerations when establishing normal values. An excellent book by Martin, Gudzinowicz, and Funger has summarized the whole field of "normal" values in clinical chemistry (*132*).

Harris (*133*) 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 the use of such standards is inappropriate (*134*) and has recommended that, where possible, previous values from the same individual be used instead (*133*). This requires a different type of statistical approach (*135*).

A few workers have begun to apply more complex statistical methods to clinical laboratory 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 (*136*), who (with his coworkers) has suggested that multivariate statistical approaches could be used successfully to relate various test results. They point 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 (*13*) 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 (*137*), and Reece (*16*) has predicted the use of this type of statistic as a principal feature distinguishing the hospital screening process of the 1980s.

Only a few, usually rather preliminary, studies have been completed with quantitative metabolic profiling. Young et al. (*138*) reported a study of the effects on patients of an artificial diet. This study, in which the amounts of approximately 300 ninhydrin-positive, ultraviolet-absorbing or carbohydrate components of urine and serum were measured, led them to conclude that many of the compounds in the volunteers' samples were of dietary, rather than endogenous, origin. They

[4] Gates, S. C., Blaisdell, B. E., and Sweeley, C. C., manuscript in preparation.

[5] Blaisdell, B. E., and Sweeley, C. C., manuscript in preparation.

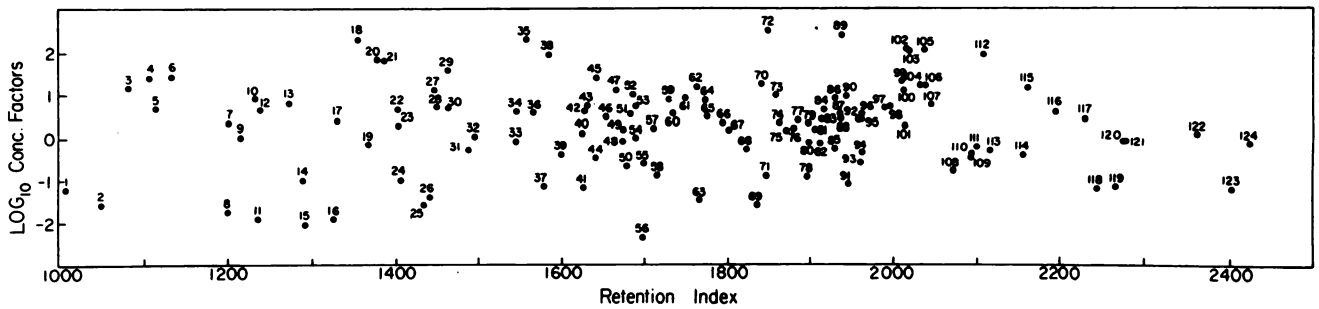

Fig. 2. Quantitative metabolic profiling of organic acids from urine of reference adult subjects

The logarithm of the mean values of concentration factors (*112*) are plotted here versus the retention indices of the compounds on 5% OV-17 (*118*). Data for a total of 124 substances are presented; 46 substances have not yet been identified, or tentative identification has not been confirmed with authentic samples. Identified compounds are (*3*) α-hydroxyisobutyric, (*4*) lactic, (*6*) glycolic, (*7*) β-hydroxybutyric, (*8*) glyoxylic oxime, (*9*) pyruvic oxime, (*11*) oxalic, (*13*) glycerol, (*14*) methylmalonic, (*17*) 2-methylglyceric, (*18*) phosphoric, (*19*) benzoic, (*20*) 4-deoxyerythronic, (*21*) 4-deoxythreonic, (*22*) succinic, (*24*) fumaric, (*25*) phenylacetic, (*26*) nicotinic, (*28*) 3-deoxytetronic, (*29*) 2-deoxytetronic (*32*) glutaric, (*33*) 3-methylglutaconic, (*34*) malic, (*35*) erythronic, (*37*) threono-1,4-lactone, (*38*) threonic, (*39*) adipic, (*40*) 3-methyladipic, (*41*) *o*-hydroxybenzoic, (*44*) α-hydroxyglutaric, (*46*) β-hydroxy-β-methylglutaric, (*48*) *m*-hydroxybenzoic, (*50*) pyroglutamic, (*51*) 5-hydroxymethyl-2-furoic, (*54*) *o*-hydroxyphenylacetic, (*55*) pimelic, (*56*) tartaric, (*57*) arabonolactone, (*58*) α-ketoglutaric oxime, (*59*) *p*-hydroxybenzoic, (*60*) *m*-hydroxyphenylacetic, (*62*) *p*-hydroxyphenylacetic, (*63*) ribonolactone, (*65*) arabonic, (*66*) suberic, (*67*) β-glycerophosphoric, (*70*) α-glycerophosphoric, (*71*) *cis*-aconitic, (*76*) citric, (*78*) isocitric, (*79*) azelaic, (*80*) terephthalic, (*81*) vanillic, (*83*) 3,4-dihydroxyphenylacetic, (*85*) galactono-1,4-lactone, (*86*) homovanillic, (*87*) veratric, (*89*) *m*-hydroxyphenylhydracrylic, (*91*) galactono-1,4-lactone, (*93*) *o*-coumaric, (*94*) gluconic, (*96*) hexuronic, (*97*) *p*-hydroxyphenyllactic, (*99*) vanillmandelic, (*100*) hexuronic, (*101*) ascorbic, (*103*) hexuronic, (*105*) hexuronic, (*106*) hexuronic, (*107*) hydrocaffeic, (*110*) palmitic, (*111*) ferulic, (*112*) hippuric, (*116*) indoleacetic, (*118*) caffeic, (*119*) uric, (*120*) ferulic, and (*124*) 5-hydroxyindoleacetic

also noted that at least four days of the artificial diet were required to reach a stable set of values for many of the compounds. Interestingly, some of the compounds, including creatinine, were excreted at the same rate regardless of the diet. Harris and DeMets (*139*) extensively studied a smaller number of compounds; they found serum ionized calcium to be constant, within their analytical precision, for single individuals during 10 to 12 weeks, even when there were considerable inter-individual variations. Witten et al. (*140*) reported the normal concentrations of organic acids 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, because an ethyl acetate–ether extraction procedure was used and quantitation was by peak area on low-resolution GC. Björkman et al. (*76*) similarly determined the excretion of several major acidic metabolites in the urine of newborn humans; and Chalmers and Watts (*141*) examined unconjugated aromatic acids in phenylketonuria, followed by a study of volatile fatty acids in several metabolic disorders (*142*). Liebich et al. (*143*) used a similar semi-quantitative method to measure low-molecular-mass aliphatic alcohols in normal and diabetic individuals. In a more quantitative study, Yamamoto et al. (*144*) found a seasonal variation in urinary metanephrine and a minor seasonal variation in vanilmandelic acid when these compounds were studied during a five-year period. Useful suggestions for measuring sample stability (*145*) and long-term analytical variations (*146*) could well be applied in these quantitative studies.

Routh and Paul (*147*) found that therapy with aspirin affects the concentrations of several serum constituents. Lawson et al. (*80–82*) 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. (*148*) reported preliminary data suggesting general abnormalities among mentally retarded subjects. Blau et al. (*149*), 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 (*98, 150*). These workers have used 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 in a specially-selected subset of the peaks to reduce inter-sample variability. The collected data are compared to one another by use of the Wilcoxon test, a nonparametric statistical ranking procedure (*150*). 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 vs. non-fasting, breast cancer, and Duchenne dystrophy, with significant differences reported in each case except that of grade-point average. However, they pointed out (*150*) 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 labelled. 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."

Another system for the statistical analysis of profiling data was described by Schoengold et al. (*32, 33*), a system developed to allow processing of amino acid data; it is noteworthy in that it allows extensive interindividual comparisons by use of 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 use.

The most recent statistical 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 (*151*) has illustrated the use of such a procedure to distinguish patients suffering from two liver diseases on the basis of the concentrations of eight enzymes in blood. No one has yet published a similar study, with use of pattern recognition on concentrations of substances of low relative molecular mass.

Summary and Conclusions

Quantitative metabolic profiling is so new that most reports thus far have been concerned primarily with techniques used rather than results. Thus, it is difficult to compare analytical features of these systems such as accuracy, precision, linear range, and sensitivity. However, it is probably safe to conclude that metabolic profiling by use of repetitive scanning GC-MS is about one order of magnitude less precise, and two orders of magnitude less sensitive than selected ion monitoring techniques for a single substance. Repetitive scanning GC-MS systems are also considerably less reliable than capillary GC systems. It appears possible to achieve somewhat greater resolution of individual components by GC-MS-computer than by capillary-GC-computer.

Barring a major breakthrough in GC-MS design, it seems likely that repetitive scanning GC-MS will be restricted to the exhaustive quantitative analysis of a relatively small number of samples, and will not be used widely for broad-scale screening programs. It thus provides a complementary, rather than competitive, alternative to other, already well-established techniques. Choice of technique will depend upon the type of metabolic profile desired, including the number of components to be monitored, the acceptability of inaccuracies in identification and quantitation, the number of samples to be analyzed, and similar considerations.

Several improvements in the GC-MS techniques for metabolic profiling seem almost inevitable. These include the use of faster scan speeds, and the design of software that would enable electrostatic sector instruments (e.g., quadrupoles) to follow a constantly varying, pre-selected set of ions. Thus, hundreds of components could be followed with selected-ion-monitoring precision. This process might well use retention indices to switch ion sets (*55*). Improved algorithms for real-time data reduction and off-line resolution of individual peaks seem likely, as do systems for real-time profile analysis and microprocessor-automated profiling.

Even with improved systems, however, other difficult problems remain to be solved. Few of the extraction procedures typically used for metabolic profiling are completely satisfactory; most will need considerable modification before they can be considered routine, easy-to-use procedures. Likewise, for substances needing derivatization, there is as yet no "ideal" derivative that yields spectra that are optimum for the data-processing procedures described. Even with relatively sophisticated statistical and pattern-recognition techniques, it can be expected that interpretation of metabolic profiling data will continue to be complicated because of the effects of diet, drug ingestion, bacterial action, undetected diseases, degenerative processes, aging, and so forth (e.g., *152–154*). The most difficult and yet the most important problem faced by the metabolic profilist may well be the careful selection of questions that can be answered with some certainty. Certainly this new technology can deal effectively with simpler systems than the human biological fluids. For example, relatively homogeneous laboratory-animal populations, cell cultures, and agricultural crops will provide more consistent profiles, and hence perturbations in these systems will be easier to measure. Regardless of the material examined, however, it can be expected that metabolic profiling will enable researchers to seek a whole new series of answers—and to be confronted by a whole new series of exciting questions.

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