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AUTOMATED HIGH-RESOLUTION GAS CHROMATOGRAPHIC SYSTEM FOR RECORDING AND EVALUATION OF METABOLIC PROFILES

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SUMMARY

A fully automated high-resolution gas chromatographic system is described that incorporates repetitive sampling of volatile biological samples onto a glass capillary column, and a simultaneous flame ionization and nitrogen-sensitive detection prior to data acquisition and computer handling. Reliable headspace sampling procedure and reproducible column characteristics that are essential to automation have been studied. The application of this system to the reproducible chromatographic recording of human urinary volatile constituents is shown.

INTRODUCTION

Gas-chromatographic (GC) analyses of derivatized constituents of physiological fluids have promoted notable progress in the biomedical field during the last decade. More recently, high-resolution (capillary) gas–liquid chromatography has been quite successfully applied as a research tool to resolve the complex volatile (underivatized) fraction of physiological fluids.

Although characteristic odors of human urine, breath, and perspiration have long been known as attributes of certain diseases, determination of the volatile constituents received little previous attention. This area of research, pioneered by Zlatkis and Liebich[1] and Teranishi *et al.*[2] who originally utilized the methodology common in aroma research, was further advanced by development of a headspace concentration method[3]. Based on this sampling procedure, various researchers have since concentrated the volatiles of human urine[3–5], breath[3], blood[5,6], and cerebrospinal fluid[5] with subsequent separation utilizing high-resolution GC columns.

The volatile constituents of body fluids include metabolic by-products, intermediates, and terminal products of enzymatic degradations. A primary goal of the recent studies of these metabolites has been to correlate characteristic chromatographic profiles to human normal and disease states. While the simplified versions of such methods may indeed find some diagnostic application, objective biochemical elucidation of physiological and pathological processes is at least equally important. Consequently, high-resolution analytical methods that are capable of the resolution of up to several hundred constituents from a single sample are very attractive for use in studies of metabolic details in both humans and model laboratory animals, pro-

vided data are reliably obtained and meaningfully interpreted. Furthermore, distinction of "normal" and "abnormal" samples of body fluids is complicated by the range of variation in the concentration of metabolites that is considered normal in biological terms[7]. It is important that this range not be extended by imprecise measurement techniques.

Automated GC analysis which incorporates precision sampling, high-precision chromatographic analysis, and reliable computer-aided data evaluation may eventually become a key to comparative biochemical and biomedical investigations. Although the level of reliability existing in today's clinical autoanalyzers based on far simpler principles has yet to arrive for multicomponent chromatographic analyses, the present technology makes it feasible to cope successfully with the real complexity of biological mixtures at a satisfactory level of precision.

To date, only a limited amount of work has been reported on automated GC systems designed for biomedical measurements. Robinson *et al.*[8] have described in detail an apparatus for the quantitative analysis of volatile urinary constituents which utilizes a cryogenic trap for volatiles with subsequent chromatography on a long metal capillary column, utilizing water-saturated carrier gas to overcome problems of column technology. The system is fully automated. A computerized gas chromatograph–mass spectrometer, combining the information from retention indices with the detection of selected ions, has been applied by Sweeley *et al.*[9] for an automated recording of certain derivatized urinary metabolites. Although ordinary packed columns are used in this system, the analytical specificity is obtained by selective monitoring of characteristic ions in repetitive spectral scans.

In this report, we wish to describe a fully-automated GC system based on high-resolution GC and designed primarily for the analysis of volatile constituents of physiological fluids. Unlike in the previous studies, due attention is paid here to the aspects of reproducible automated sample handling and column technology that are essential for the application of pattern recognition techniques. A micro-sampling technique has been developed to be adaptable for a septum-free capsule injection system[10]. Glass capillary columns are utilized in our system for two fundamental reasons: (a) As we reported in a previous study[5], such thin-film columns combine the advantages of inertness, high resolving power and a considerably earlier elution of heavier mixture constituents; and (b) due to a better understanding of the surface chemistry of glass, these capillary columns can now be prepared in our laboratory with highly reproducible film thickness, column efficiency and sorption characteristics.

The system described in this article allows for a reproducible parallel use of a non-specific flame ionization detector (FID) and a specific (nitrogen-sensitive) detector. The two signals are recorded and stored by a commercial data acquisition system and the analytical information is obtained on paper tape for off-line processing by a large computer. Threshold logic units and cluster analysis methods are presently under investigation for evaluation of chromatographic data.

With the exception of the initial sampling of volatiles, all parts of analysis are fully automated. The sequence of events necessary to handle samples on a repetitive basis is precisely timed and synchronized with a hardwired, digital controller.

EXPERIMENTAL

Automated GC system

A block diagram of the chromatographic system is shown in Fig. 1. The gas chromatograph, Model 3920, the autosampler, AS-41, and the data system, PEP-2, are from Perkin-Elmer.

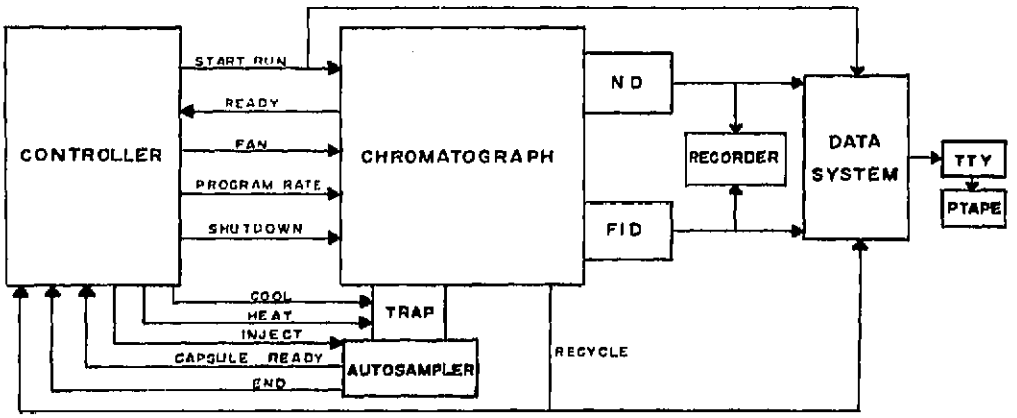

Fig. 1. Block diagram of the automated GC system. ND, nitrogen detector; TTY = teletype.

Due to the prolonged injection employed (as described below) a cryogenic trap is used to concentrate the sample onto the column. This trap consists of a 15-cm double-walled stainless-steel tube which surrounds a straightened portion of the capillary column at the column head. Thus, while trapped, the sample contacts only glass surface. Forcing the chilled nitrogen between the walls maintains the trap at approximately −100°. At the conclusion of trapping, the trap is rapidly heated by an attached 400-W cartridge heater. To increase cooling efficiency, the oven fan is off during trapping.

A digital-logic hardwired controller synchronizes and controls the system for repetitive, unattended operation. The various control lines are also indicated in Fig. 1 which include control of the cryogenic trap and column oven fan, an over-ride on the normal autosampler injection sequence, an over-ride of the normal oven temperature control to provide rate changes as desired, initiation of the temperature program and data acquisition, and system shut-down when all samples have been run. Sense lines signal that the oven and trap temperatures are correct prior to injection, that a capsule is ready for injection, that no sample remains in the autosampler, and that the oven temperature program is complete, causing the system to recycle. Most control and sense lines incorporate optically coupled isolators. Inductive loads are regulated with zero voltage switching circuits. Time intervals are set on binary coded decimal thumbwheel switches. For non-routine operation, each function may be manually controlled.

To provide parallel selective and non-selective detection, the instrument is equipped with a glass-lined stainless-steel column effluent splitter (prepared for us by Scientific Glass Engineering, Melbourne, Australia) and dual detector block. The

splitter evenly divides the effluent between the FID and a nitrogen–phosphorus detector (Perkin-Elmer). Helium make-up gas (30 ml/min) is added at the capillary column-splitter junction to reduce dead volume problems. The splitter outlet to the FID is swept with hydrogen flame gas (50 ml/min); the outlet to the nitrogen detector is swept with hydrogen (5 ml/min) and helium (45 ml/min), to obtain equivalent outlet pressures, while satisfying the differing detector requirements for individual gases. The FID air flow-rate is 250 ml/min, while the nitrogen detector utilizes only 100 ml/min.

The helium carrier gas is regulated at 2.0 ml/min with a Brooks 5840-1 mass flow controller, except during trapping when the flow-rate is increased to 6.0 ml/min. The carrier gas is purified by passing through a Molecular Sieve 13X trap immersed in liquid nitrogen. All other combustion and purge gases are purified accordingly.

Sampling and automated injection of volatiles

The sampling of volatiles by adsorption onto a porous polymer (Tenax GC, 35–60 mesh; Applied Science Labs., State College, Pa., U.S.A.) for subsequent manual injection has previously been described[3,11]. The method has been modified here to comply with the system automation, thus providing reproducible, closed-system injection, and unattended operation.

The autosampler utilizes a capsule-injection system[10] suitable for introducing solids (in this case Tenax porous polymer) into the heated injector. An unsealed alu-

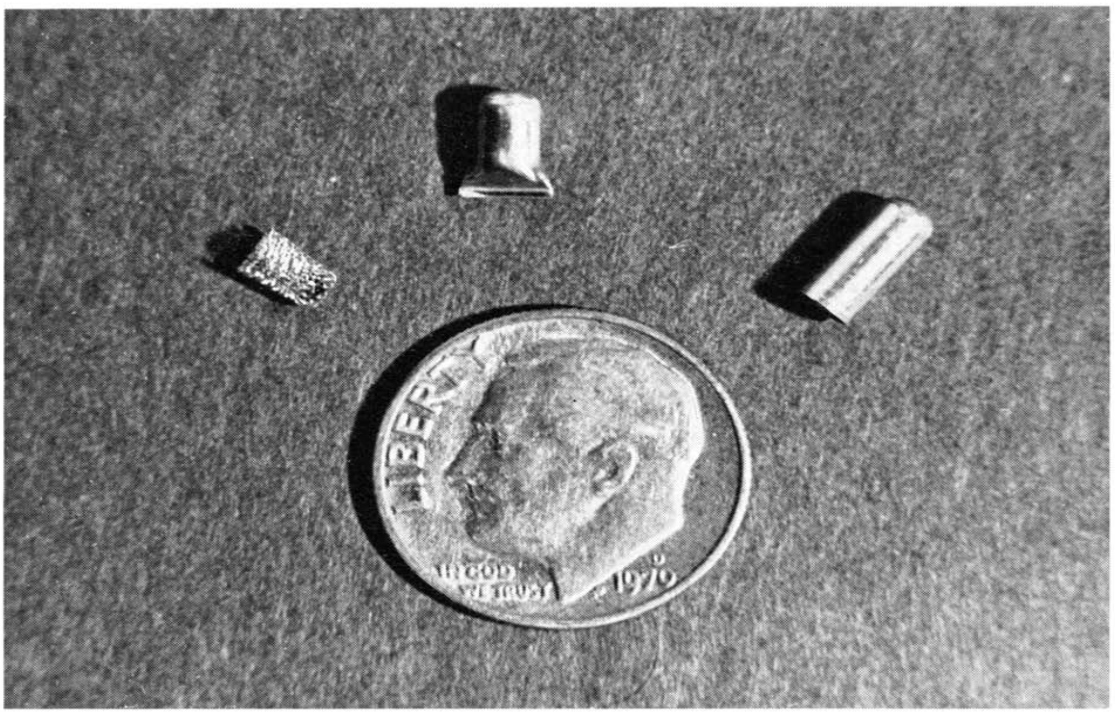

Fig. 2. Components of the capsule sampling injection system. Left, platinum gauze microbasket; right, un-used aluminum autosampler capsule; center, cold-welded capsule with microbasket inside.

minum capsule is shown in Fig. 2, right. As it is necessary to prevent the Tenax beads from blocking mechanically the column inlet, platinum gauze (100 mesh) microbaskets (Fig. 2, left) were fabricated to fit inside the aluminum capsules. Two milligrams of Tenax (about 20 μl, and approx. two-thirds of the total microbasket volume) is weighed into the microbasket prior to sampling. After sampling, the filled microbasket is placed into a capsule, which is then cold-welded shut (Fig. 2, center). The autosampler probe, which carries the capsule into the injector, is adjusted so that the capsule base is punctured, but the microbasket is not damaged. At the injector temperature, the volatiles desorb from the adsorbent and diffuse to the capsule base, where they are swept by carrier gas into the cryogenically cooled column. For urinary profiles, a 45 min trapping step is employed (injector temperature, 270°).

The platinum microbasket is recovered by dissolving the aluminum capsule in acid solution; the Tenax adsorbent is not re-used, thus avoiding memory effects. The use of gold capsules was investigated to determine whether any losses occurred on the aluminum surface. We found no such evidence for urinary profiles.

The headspace sampling apparatus previously described[3] in which a sweep gas passes over the heated sample (100°) and through a cooled condenser (4°) was modified to accomodate the use of the microbaskets. Fig. 3 shows an axial cross-section of a PTFE sampling unit, in which (point B) the Tenax-filled microbasket is held during

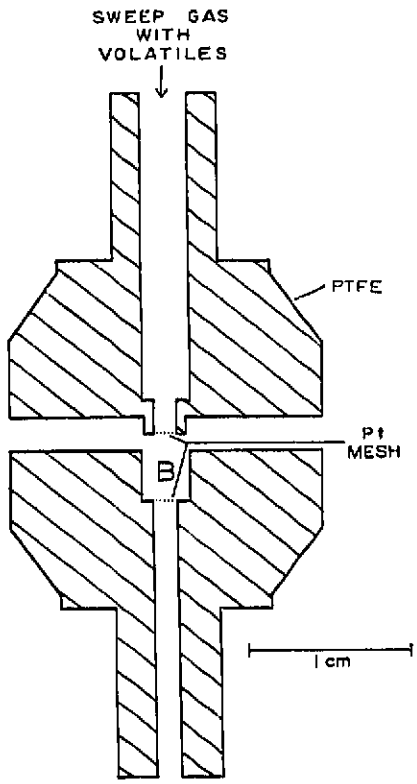

Fig. 3. Axial cross-section of top and bottom sections of the sampling unit. Platinum microbasket is placed at B and the sections clamped together during sampling.

the sampling period. The top and bottom of the sampler unit are held together with a clamp. The platinum screens help hold the microbasket in place and prevent loss of Tenax during the manipulation. The sweep gas and volatiles exit the sampling system condenser into a U-shaped tube attached to the PTFE sampler unit via an Ultra-Torr fitting (Cajon, Cleveland, Ohio, U.S.A.). The sampling time for urinary volatiles is 30 min.

Preparation of reproducible glass capillary columns

Pyrex glass capillary columns were drawn to 0.29 mm (I.D.) × 60 m length. All columns were silylated with hexamethyldisilazane–trimethylchlorosilane (5:1). Upon filling, the mixture was rapidly flushed from the column, leaving a thick film of the silylation liquid behind. Both ends were immediately sealed and the column heated at 200° for 12 h. The silylating mixture was then flushed from the column with pure nitrogen for 1 h at 200°.

A modified method of Van Dalen[12] was utilized for highly reproducible dynamic coating. "Buffer" capillaries of identical diameter were connected to both ends of the column by means of shrinkable PTFE. The tail capillary is connected to a 10-ml tight syringe held in a Sage Model 352 syringe pump, and the entire length of tubing filled with a 10% v/v solution of GE SF-96 in toluene. The other end is then connected to the gas tank through a parallel arrangement of a needle valve and a flow regulator. Coating then proceeds by forcing the solution back into the syringe operated in the withdrawal mode. The syringe pump serves as a brake, preventing a change in coating speed as the plug length diminishes. The variations in film thickness values obtained with this coating method and estimated chromatographically[13] were found to be within 2%. For any given combination of a column length, internal diameter, and coating speed, the experimental conditions must be empirically optimized.

After the solvent removal (12 h at room temperature) by purging dry helium, the columns were conditioned with carrier-gas flow from 35–210° at 0.5°/min and held at 210° for 24 h. All columns were tested for efficiency, film thickness, and retention of standard compounds. The mixtures of selected alkanes, ketones, and aldehydes, were introduced without solvent into the automated system through the capsule injection. While the retention measurements were carried out under the conditions of temperature programming (4°/min), the efficiency measurements were made at 130° for 100-ng samples of dodecanal, tridecanone and pentadecane (determined k-values for these solutes were 2.6, 3.5 and 4.6, respectively).

Computation methods

Peak areas and relative retention times as calculated by the data system from both detectors are obtained on a punched paper tape which is subsequently copied to a magnetic tape for off-line processing on Xerox Sigma 2 and Control Data 6600 computers.

RESULTS AND DISCUSSION

High resolving power of capillary columns appears essential to cope with the complexity of the volatile fraction of urinary metabolites. Although it is usually desirable that as many volatile metabolites as possible be simultaneously recorded, it may

not necessarily apply that nearly all constituents are diagnostically important. On the contrary, only characteristic constituents have to be sought from a given metabolic profile. However, they still must be separated, identified and quantitated in the presence of "less interesting" metabolites.

While the previously unknown biochemical information concerning the etiology of a particular disease is presently best obtained by combined GC–MS, the number of multicomponent analytical data must be reduced to an amount suitable for biochemical interpretation and diagnostic purposes. The use of trainable pattern classifiers, that have recently been applied to problems of comparable complexity in biomedical[14,15] and chemical[16–19] fields, appears appropriate for evaluation of volatile profiles. While the comparisons of various pattern recognition methods for this application will be reported later[20], this publication is primarily concerned with the design and evaluation of the system that enables the collection of data for further computational handling. Thus, with such a system a large number of reproducible data can be collected. Similar or differing characteristics of such data may not be otherwise apparent due to the complexity and quantity of the total recorded information.

The system described here (Fig. 1) incorporates all the fully automated functions of a gas chromatograph that are essential to record sequentially volatile profiles from a number of samples: automated sample concentration from the sample capsule onto the first (cooled) section of a glass capillary column, subsequent thermal desorption of volatiles, and unattended chromatographic operation with a simultaneous dual detector capability.

Reproducible headspace sampling and sample introduction onto the column and high-precision chromatography are essential for acquiring reproducible profiles for comparative purposes. The headspace sampling procedure in which the trap containing a thermostable porous polymer is eventually inserted into a modified injection port[3,11] has been the first essential step to minimizing the sampling error associated with most extractions of physiological fluids, the usual solvent interference and syringe injection problems. This sampling procedure further miniturized and investigated in greater detail[11] was modified for the automated operation in this study. With the strictly controlled sampling conditions and the sample collection apparatus described above, it is now possible to obtain the necessary sampling reproducibility compatible with the volatile profile studies.

The degree of reproducibility of consecutive complex chromatograms obtained with this system is shown in Fig. 4 for aliquots of the same human urinary samples. While we do not claim absolute repeatibility for all recorded peaks, this degree of sampling reproducibility for the vast majority of recorded peaks appears adequate for the pattern recognition objectives. The two major contributions to minor quantitative discrepancies in the shown results are thought to be resulting primarily from the following sources: (a) minor fluctuations in temperature and flow conditions during the sampling; and (b) incompatibility of certain sample constituents with the used stationary phase. While the former problem can be quite effectively eliminated by addition of appropriate internal standards directly into a studied physiological fluid, the column technology problems will be discussed in a greater detail below.

The high-precision measurements in GC are primarily influenced by the reproducibility of temperature and flow conditions on a run-to-run basis as well as

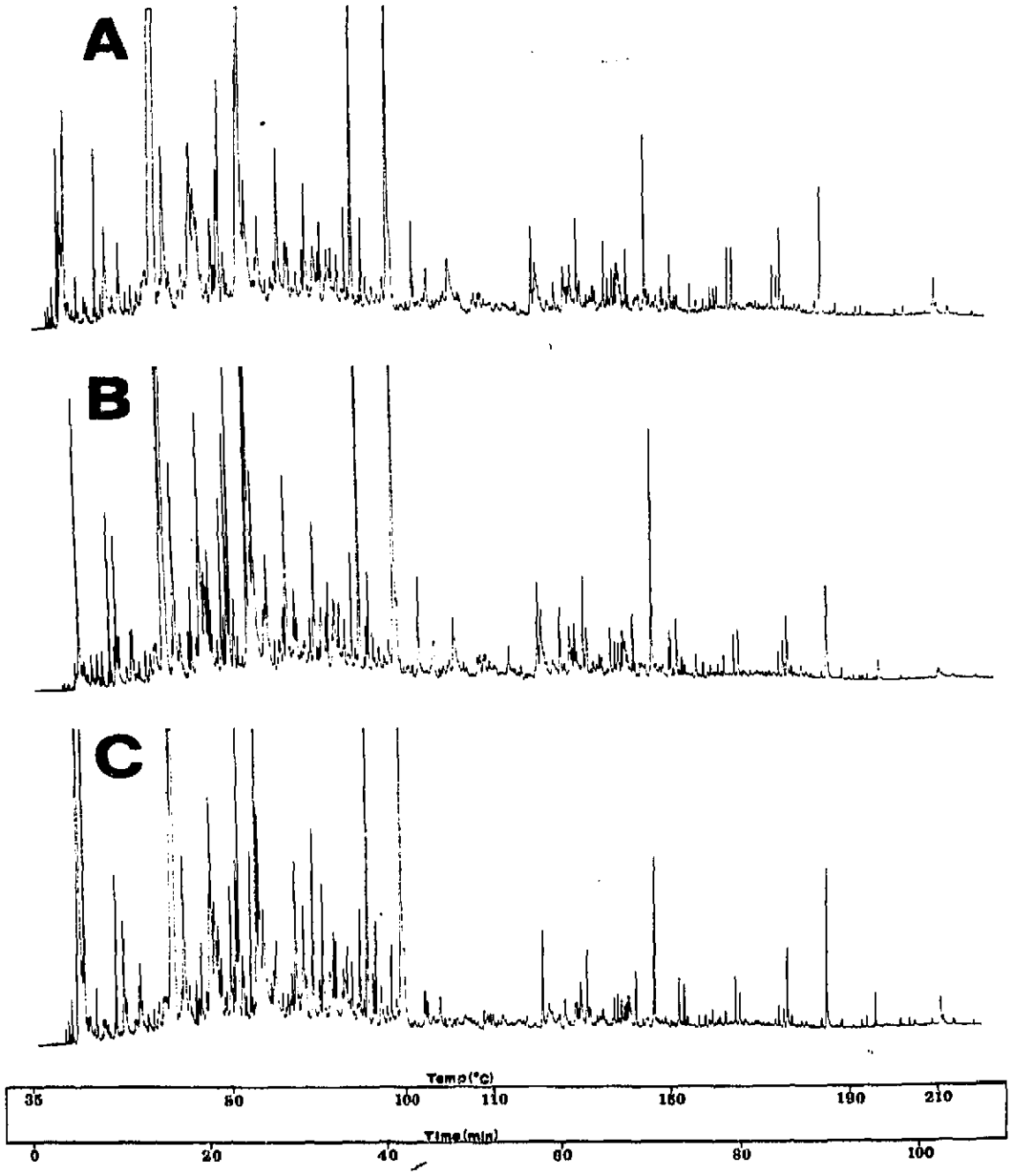

Fig. 4. Successive chromatograms of volatiles from aliquots of a single 24-h urine sample of a normal male. Each aliquot, 100 ml urine with 30 g ammonium sulphate; helium sweep-rate, 160 ml/min; sampling time 30 min at 100°. Glass capillary column number 4. See text for description.

certain column characteristics. While we consider employment of the commercially available as well as home-made temperature and flow control equipment used in our work practical and adequate and the timing precision of the digital controller (better than 1 msec) as having almost negligible effect, considerably more attention has been paid to the chromatographic column.

It should be emphasized that reproducible sorption characteristics of the columns used in our system have been our primary concern. We feel that a run-to-run and day-to-day reproducibility of retention data for a long period of time strongly underlines any successful approach to automation of high-resolution GC methods and computer-aided evaluation. Furthermore, the reproducible technology of column preparation is equally important.

It should be pointed out that the characteristics of these columns incorporated into the automated system have been (at least presently) a moderate compromise on the account of efficiency and deactivation for some polar compounds. Columns of higher quality are available in our laboratory for research purposes; however, their preparation invariably involves a series of surface treatments that make the task of reproducible column-to-column preparation more difficult.

A nonpolar methylsilicone oil has been used as the stationary phase in this work for several reasons. Although most body fluid constituents are of a polar nature and the selection of a polar stationary phase would seem to be more appropriate, many heavier and more polar constituents that are likely to be metabolically significant, could not be eluted on a polar phase at reasonable temperatures. Furthermore, most polar phases are known to change their characteristics after a longer use more rapidly than the nonpolar column substrates. Our experience shows that nonpolar silicone phases coated on glass capillary columns can be used for many months without apparent changes in their analytical characteristics.

Using a single-step silylation procedure as the only treatment of the inner wall of glass capillaries and the reproducible dynamic coating procedure by a modified method of Van Dalen[12], we have been able to reproduce the film thickness of the stationary phase (measured from chromatographic data[13]) within 2% or less. The results of a study pertaining to the reproducible preparation of glass capillary columns on a consecutive basis are shown in Table I, which lists column efficiencies, relative retention times, and retention indices under the conditions of temperature programming, for selected samples of different polarity.

The retention indices in Table I are based on a timing precision of ± 0.005 min; this propagates an uncertainty of ± 0.5 unit in the retention index. As the retention index is quite dependent on the character of the stationary phase and glass surface, the columns are reproducible in this respect within the timing uncertainty.

Retention indices have proven quite useful in the identification of sample components, as they are the expression of retention least influenced by instrumental parameters. However, in the analysis of highly complex samples, the retention index is less useful, as it is inconvenient to further complicate samples by the use of many internal reference standards. Obtaining standard retention times from runs separate from those of samples requires exceedingly careful control of chromatographic parameters and a considerable loss of time. Computational methods for determining the retention index based on only a few widely spaced standards have been developed[21,22], but are not satisfactory for temperature programmed analyses. A compromise is the

TABLE I

EVALUATION OF THE CHARACTERISTICS OF SUCCESSIVELY PREPARED GLASS CAPILLARY COLUMNS

Sample	*Column number*				
	1	*2*	*3*	*4*	*5*
*Column efficiencies, theoretical plates × 1000**					
N-Dodecanal	180 ± 42	221 ± 12	209 ± 8	181 ± 1	140 ± 30
N-Tridecanone	197 ± 4	228 ± 11	207 ± 14	218 ± 10	182 ± 5
N-Pentadecane	205 ± 1	201 ± 13	200 ± 16	206 ± 1	187 ± 3
*Relative retention referenced to pentadecane***					
Undecane	0.505 ± 0.001	0.504 ± 0.010	0.508 ± 0.002	0.506 ± 0.002	0.500 ± 0.005
Decanal	0.623 ± 0.001	0.620 ± 0.006	0.620 ± 0.004	0.626 ± 0.001	0.617 ± 0.002
Dodecane	0.632 ± 0.001	0.631 ± 0.002	0.631 ± 0.001	0.638 ± 0.001	0.629 ± 0.001
Undecanone	0.700 ± 0.001	0.699 ± 0.002	0.698 ± 0.001	0.705 ± 0.001	0.696 ± 0.002
Tridecane	0.757 ± 0.001	0.757 ± 0.002	0.757 ± 0.001	0.761 ± 0.002	0.756 ± 0.001
Dodecanal	0.871 ± 0.001	0.870 ± 0.002	0.870 ± 0.002	0.872 ± 0.001	0.869 ± 0.001
Tetradecane	0.880 ± 0.001	0.880 ± 0.001	0.880 ± 0.000	0.882 ± 0.001	0.879 ± 0.001
Tridecanone	0.944 ± 0.001	0.944 ± 0.001	0.945 ± 0.001	0.946 ± 0.001	0.944 ± 0.001
Pentadecane	1.000	1.000	1.000	1.000	1.000
Hexadecane	1.114 ± 0.001	1.114 ± 0.001	1.114 ± 0.001	1.112 ± 0.001	1.115 ± 0.001
Pentadecanone	1.175 ± 0.001	1.174 ± 0.001	1.174 ± 0.001	1.172 ± 0.001	1.175 ± 0.001
Heptadecane	1.224 ± 0.001	1.222 ± 0.001	1.223 ± 0.001	1.219 ± 0.001	1.224 ± 0.002
Octadecane	1.328 ± 0.001	1.325 ± 0.001	1.327 ± 0.001	1.321 ± 0.001	1.328 ± 0.002
Heptadecanone	1.383 ± 0.001	1.381 ± 0.002	1.382 ± 0.001	1.376 ± 0.001	1.384 ± 0.002
Nonadecane	1.425 ± 0.002	1.422 ± 0.002	1.424 ± 0.001	1.417 ± 0.001	1.426 ± 0.003
*Retention index***					
Undecanonone	1253.9 ± 0.6	1253.2 ± 0.4	1253.4 ± 0.5	1253.4 ± 0.1	1253.1 ± 1.4
Dodecanal	1392.8 ± 0.3	1392.5 ± 1.3	1391.7 ± 1.5	1392.0 ± 0.6	1391.8 ± 0.8
Tridecanone	1454.1 ± 0.2	1454.1 ± 0.2	1454.2 ± 0.2	1454.4 ± 0.2	1454.0 ± 0.2
Pentadecanone	1655.1 ± 0.4	1655.2 ± 0.2	1655.4 ± 0.4	1655.5 ± 0.3	1655.2 ± 0.3
Heptadecanone	1856.7 ± 0.4	1857.1 ± 0.5	1857.6 ± 0.4	1857.4 ± 0.4	1856.8 ± 0.2

* Mean and standard deviation of a minimum of 3 runs, isothermal at 130°.
** Mean and standard deviation of a minimum of 4 runs, programmed from 70–210° at 4°/min.

method of Sweeley *et al.*[9], but even this requires eight reference standards.

In this application, it is only necessary to locate reliably, a peak in repetitive runs. As peaks that are only poorly resolved in the profiles are generally separated by more than 0.10 min, a precision of 0.003 relative retention units referenced to a centrally located standard (50 min retention) is needed to recognize a peak. The relative retention data in Table I indicate that columns 1–3 in combination with the existing control of instrumental parameters, are sufficiently reproducible to allow the use of a single retention standard. Inclusion of as few as three standards allows column 4 to be used without recognition ambiguity as this column was found to be reproducible with regards to the retention index. The variation in relative retention is probably caused by a slight difference in internal diameter, yielding a different linear velocity.

Retention indices are still used to correllate otherwise ambiguous GC and GC–MS data.

Before incorporation into the automated gas chromatograph, our columns are further evaluated for chromatographic efficiency and the symmetry of the dodecanal peak. Thus, for instance, column 5 would not be considered satisfactory on these grounds. A minor dispersion within the efficiency measurements with the other columns does not cause a major problem for most profile constituents.

When using metabolic profile methods to study biochemical aspects of normal and pathological states, it will often be essential that more than one class of metabolically important compounds be recorded from a given sample. In addition, it should be emphasized that a great amount of information may be available already in one profile, provided that suitable detection means are available. For example, the use of mass-chromatographic methods for clinically important samples[9] shows clearly such a trend.

Even with advances in purely GC detection means, similar approaches may prove fruitful. The recently described[23] thermionic (nitrogen-sensitive) detector, previously shown by ourselves to be applicable to the analysis of urinary volatiles[24], has also been incorporated into this automated system in parallel with the FID. Fig. 5 shows the dual chromatogram obtained by splitting the capillary column effluent into the two detectors. In a similar fashion, the sulphur-sensitive detector, that has been used by Zlatkis *et al.*[25] in their studies of diabetic urines, could also be incorporated. With a properly engineered effluent splitter system, only a moderate amount of chromatographic resolution may be sacrificed.

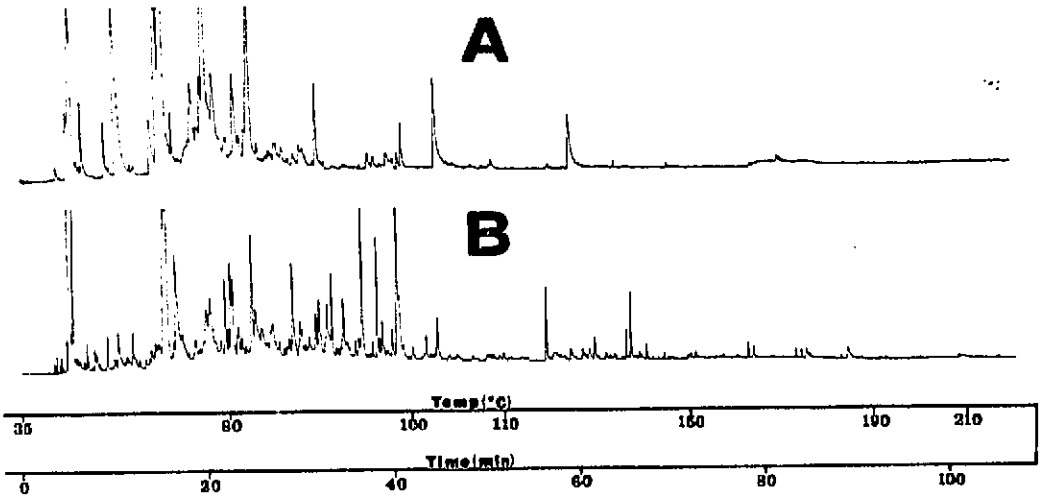

Fig. 5. Chromatograms of volatiles from a 24-h urine sample of a normal male, obtained simultaneously with column effluent splitter and dual detector system. A, nitrogen-detector; B, FID.

While the reproducibility of the successive chromatograms is good for the most profile constituents, the possibility of some artefact formation during essentially all stages of sampling and the automated GC operation cannot be ruled out. Although a reproducible formation of artefacts (most likely, no multicomponent analytical method is free of these phenomena) may not limit the use of metabolic profiles as

"fingerprints" of certain metabolic states, biochemical interpretation of the acquired data should be carried out with more caution.

CONCLUSIONS

Although the automated system described here has been primarily designed for reliable acquisition and evaluation of complex metabolic profiles of the volatile body fluid constituents, its use is not necessarily confined to such applications. For instance, a similar approach to automated sampling could be applicable to routine analyses in the fields of air-pollution control, flavor studies, forensic "fingerprinting" applications, etc. Furthermore, a solvent-free concentration technique[26] can easily be adopted in combination with the capsule sampling and high-resolution chromatography of less volatile samples.

It is foreseeable that automated high-resolution GC methods combined with the powerful computational evaluation techniques will find an increasing number of applications in the biomedical field and other scientific disciplines.

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