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Gas chromatographic profiling analysis of urinary organic acids from nonsmokers and smokers

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Abstract

A rapid profiling and screening procedure is described for the comparative analysis of urinary organic acids among the groups of nonsmokers and smokers. The procedure involves solid-phase extraction of organic acids using Chromosorb P in normal-phase partition mode, with subsequent single-step conversion to *tert.*-butyldimethylsilyl derivatives, followed by direct gas chromatographic (GC) analysis on dual-capillary columns. A total of forty-two organic acids were positively identified by retention index (*I*) matching in urine samples (0.25 ml) from eleven nonsmokers and fifteen smokers studied. When the GC profiles were simplified to their corresponding organic acid *I* spectra in bar graphical form, characteristic patterns were obtained for each individual as well as for each average of nonsmoking and smoking groups. When stepwise discriminant analysis was performed on GC data after omitting hippuric acid, seven acids were selected as the variables most discriminating between smokers and nonsmokers. The star symbol plots drawn based on these discriminants were characteristic of each individual and group average, enabling to distinguish smokers from nonsmokers. And canonical plot produced by canonical discriminant analysis using the same variables as the data vectors displayed two separate clusters representing each group. © 1997 Elsevier Science B.V.

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1. Introduction

Cigarette smoking known as the largest preventable cause of death affects a complex network of metabolic inter-relationships, leading to significant changes in concentrations of a large number of constituents of body fluids [1–7]. An assay of several chemical markers including nicotine, cotinine and thiocyanate for exposure to cigarette smoke has been investigated in preventive medicine [3–7]. However, no extensive changes in the levels of other normal body constituents such as organic acids have been demonstrated to date.

Among the various metabolites present in biological fluids, organic acids are known to be important biochemical indicators of a wide variety of

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physiological processes occurring in the living organisms [8–12]. In the literature, organic acid compositions of urine specimens have been well documented [9–12], but attempts were rarely made to correlate the organic acid profiles to smoking-induced physiological changes. It seems, therefore, of interest to investigate the effects of cumulative exposure to cigarette smoke on urinary organic acid levels.

We have previously reported a simple profiling method which can simultaneously screen for various organic acids [13,14]. The method involves solid-phase extraction (SPE) of organic acids using Chromosorb P in normal-phase partition mode with subsequent *tert.*-butyldimethylsilyl (TBDMS) derivatization. The resulting TBDMS derivatives were analyzed by dual-capillary column gas chromatography (GC) in dual-channel mode [14,15]. Retention index (I) values on DB-5 and DB-17 columns of different polarity were then measured, followed by peak identification through computer library matching based on the two I sets and area ratio comparison. This dual-capillary column GC system has been routinely used to screen for organic acids [16–18] in various samples, and to simplify GC profiles to their corresponding I spectra in bar graphical form for the visual comparison between samples. For the winery amino acid profiles, star symbol plot [19] were found to be superior for the visual pattern recognition as reported previously [20].

This work was undertaken to examine the usefulness of our organic acid profiling and I spectral and star symbol plotting as simple pattern recognition methods in monitoring physiological changes associated with cigarette smoking. The canonical discriminant analysis was investigated for classification of urine specimens into two groups according to cumulative exposure to cigarette smoke or not.

2. Experimental

2.1. Materials

Mid-portions of the first morning urine specimens were only used for this study. They were freshly collected from eleven nonsmokers and fifteen smokers and stored frozen before use. All subjects were seemingly healthy males in their 30s. The smokers were those who have been smoking ten or more cigarettes per day for ten years or more. Silylating reagent, *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was purchased from Pierce (Rockford, IL, USA) and triethylamine (TEA) from Aldrich (Milwaukee, WI, USA), *trans*-cinnamic acid from Sigma (St. Louis, MO, USA) and *n*-hydrocarbon standards (C_{12}–C_{34}, even numbers only) from Polyscience (Niles, IL, USA). All other chemicals were of analytical grade and used as received. Chromosorb P (acid-washed, 80–100 mesh) were purchased from Supelco (Bellefonte, PA, USA). A luer-tipped glass tube (5 mm I.D.) packed with Chromosorb P (400 mg) was washed successively with 0.1 M sulfuric acid, methanol, acetone, dichloromethane and diethyl ether and activated under vacuum (150°C, 3h) prior to being used as a SPE tube.

2.2. Sample preparation

Urine samples were individually processed for organic acid analysis as described previously [13,14]. Briefly, after addition of *trans*-cinnamic acid as internal standard (I.S.) at 10 ppm, an aliquot (0.25 ml) of urine was saturated with sodium bicarbonate, followed by washing with diethyl ether (2×0.5 ml). The aqueous layer was acidified (pH<1) with concentrated sulfuric acid and saturated with sodium chloride. The mixture was loaded onto an activated Chromosorb P tube, followed by elution with diethyl ether (1.5 ml). The eluate was collected in TEA (10 μl) and excess ether was removed under gentle stream of nitrogen, followed by silylation with MTBSTFA (20 μl) in isooctane at 60°C for 2 h. All samples were individually prepared and analyzed by GC in triplicate.

2.3. Gas chromatography and gas chromatography–mass spectrometry

GC analyses were performed with a Hewlett-Packard HP Model 5890A gas chromatograph equipped with a split/splitless capillary inlet system and two flame ionization detection (FID) systems and interfaced to an HP 5985A GC ChemStation (Hewlett-Packard, Avondale, PA, USA). DB-5 (SE-54 bonded

phase) and DB-17 (OV-17 bonded phase) fused-silica capillary columns (J&W Scientific, Rancho Corodova, CA, USA) for the dual-capillary column system were of dimensions 30 m×0.25 mm I.D., 0.25 μm film thickness, which were connected to a deactivated fused-silica tubing (1 mm×0.25 mm I.D.) as retention gap via Y-splitter [14]. Samples (ca. 0.5 μl) were injected in the splitless mode with a purge delay time of 42 s and using the hot needle fast injection technique. The oven temperature was held at 60°C for 2 min, then programmed to 280°C at a rate of 4°C/min. The injector and detector temperatures were 260 and 300°C, respectively. The inlet pressure of nitrogen as the carrier gas was set to

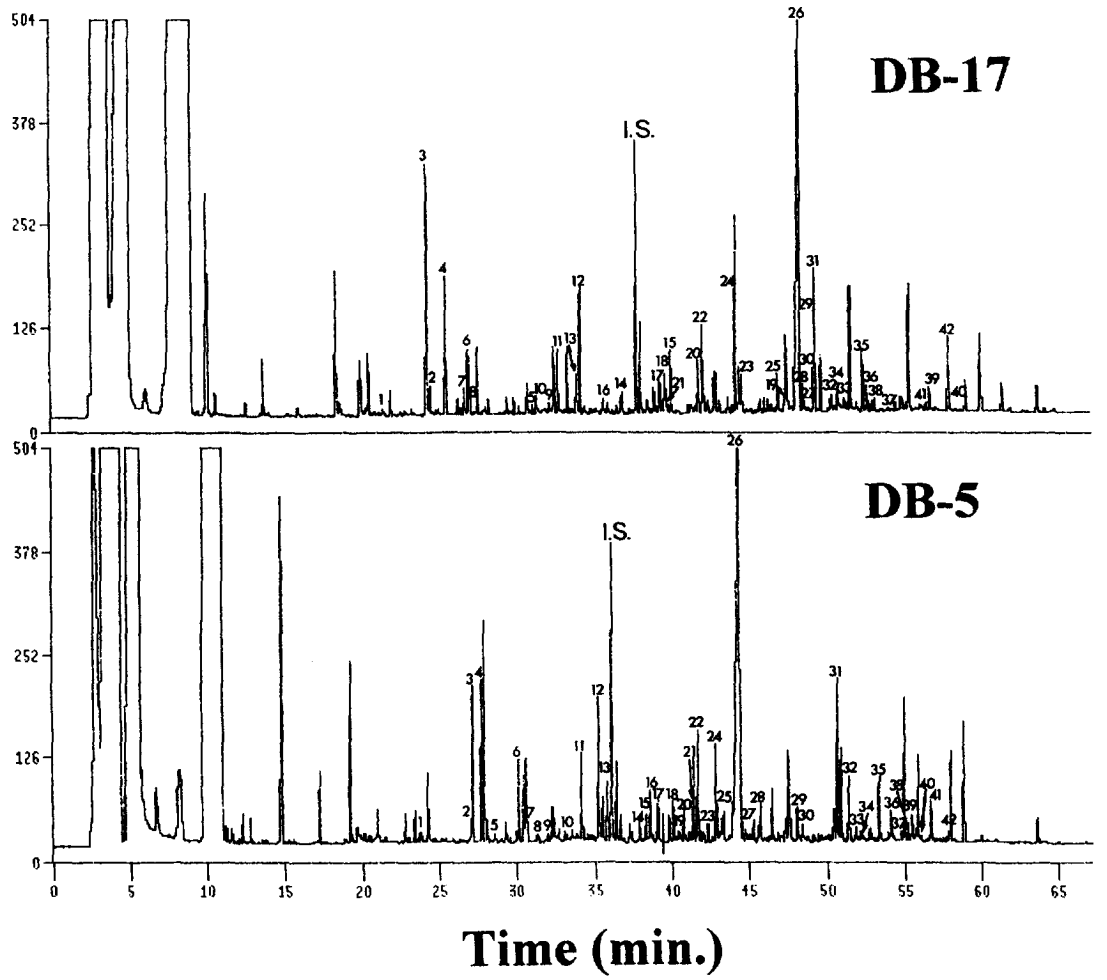

Fig. 1. Dual chromatograms of urinary organic acids in urine of a smoker separated on DB-17 and DB-5 (both 30 m×0.25 mm I.D., 0.25 μm film thickness) dual-capillary column system. GC conditions are described in Section 2.3. Peaks: 1=enanthic acid; 2=caprylic acid; 3=lactic acid; 4=glycolic acid; 5=phenylacetic acid; 6=nonanoic acid; 7=α-hydroxyisovaleric acid; 8=α-hydroxyvaleric acid; 9=malonic acid; 10=capric acid; 11=ethylmalonic acid; 12=succinic acid; 13=methylsuccinic acid; 14=glutaric acid; 15=5-phenylvaleric acid; 16=lauric acid; 17=α-hydroxyphenylacetic acid; 18=3-hydroxy-3-methylglutaric acid; 19=3,4-dimethoxybenzoic acid; 20=adipic acid; 21=tridecanoic acid; 22=3-methyladipic acid; 23=*m*-hydroxyphenylacetic acid; 24=phenylacetic acid; 25=*o*-hydroxyphenylacetic acid; 26=hippuric acid; 27=phthalic acid; 28=suberic acid; 29=homovanillic acid; 30=azelaic acid; 31=*trans*-aconitic acid; 32=*p*-hydroxymandelic acid; 33=linoleic acid; 34=oleic acid; 35=homogentisic acid; 36=17-methylstearic acid; 37=dodecanedioic acid; 38=*p*-hydroxyphenyllactic acid; 39=arachidonic acid; 40=*c*-8,11,14-eicosatrienoic acid; 41=11-eicosaenoic acid; 42=19-methylarachidic acid; I.S.=*trans*-cinnamic acid.

0.07 mPa. The two FID signals were processed simultaneously in dual-channel mode by GC ChemStation. A standard solution of *n*-hydrocarbons in isooctane was co-injected with samples for *I* measurements. For peak identification [14], the *I* value for each peak was calculated and compared with those in the previously compiled *I* reference library containing 160 organic acid standards as their TBDMS derivatives using ChemStation Basic Programs. The peak area ratio of each identified organic acid with respect to I.S. was then calculated using DB-5 chromatographic data for the subsequent pattern recognition analysis.

The confirmation of peak identities was done on a HP 5890A series II gas chromatograph, interfaced to an HP 5970B mass spectrometer (70 eV, electron impact mode), which was on-line to an HP 59940A MS ChemStation. An Ultra-2 (SE-54 bonded phase) capillary column (25 m×0.20 mm I.D., 0.33 μm film thickness) was used in the split injection mode (10:1). The oven temperature was initially 80°C for 2 min and then raised to 290°C at 4°C/min. The injector and interface temperatures were 270 and 290°C, respectively. Each peak was identified by library match using our MS library file containing 160 organic acid standards as their TBDMS derivatives.

2.4. Pattern recognition analysis

The mean peak area ratios of organic acids identified in each urine were normalized to the largest peak as the base peak. Using MS Excel program, the percentage normalized area ratios were plotted against *I* values in bar graphical form to obtain *I* spectra of each urine sample [16–18]. For group average *I* spectra, normalized median area ratios were used.

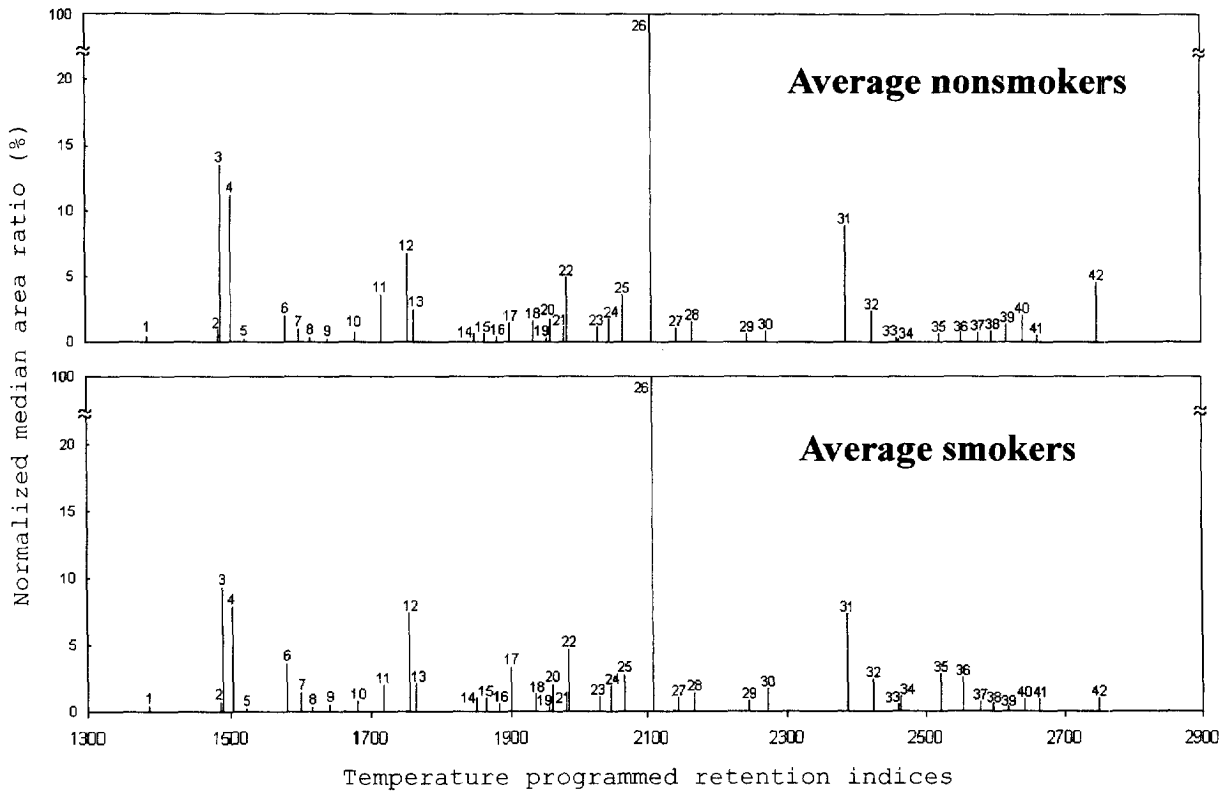

Fig. 2. Average retention index spectra of urinary organic acids from nonsmokers and from smokers. Peak numbers correspond to those in Fig. 1.

The mean peak area ratios were normalized to area ratio sum after omitting hippuric acid from the GC data and subjected to stepwise discriminant analysis by means of the statistical software package SAS. Using MS Excel program, the area ratio of each discriminant variable selected by stepwise discriminant analysis was plotted as a line radiating from a common central point and the far ends of the lines were joined together to produce a star plot. The variables in decreasing order of discriminating power were assigned to rays of the star clockwise. The normalized median area ratios were used for the star plots of the group averages. Canonical discriminant analysis was performed on the area ratios of the same variables of each urine as the data vectors using SAS, followed by plotting the first canonical discriminant function (CAN1) against the second canonical discriminant function (CAN2) for each urine sample to produce a canonical plot.

3. Results and discussions

In this study, first morning urine specimens from eleven nonsmokers and fifteen smokers were screened for organic acids. Our profiling method produced good GC profiles with 0.25 ml of urine for the comparative studies among smoking and nonsmoking groups. The usefulness of the method for organic acid analysis is well demonstrated in typical dual chromatograms of a smoker's urine (Fig. 1).

By computer comparison of *I* sets with the reference values in our *I* library, a total of forty-two organic acids were positively identified from twenty-six urine samples. The *I* sets measured with dual-capillary columns of different polarity were very useful in cross-checking each organic acid [15], which was further confirmed by GC–MS. At present, our *I* and GC–MS libraries contain 160 organic acid standards as TBDMS derivatives, which are being expanded for a more complete organic acid data base.

When concentrations of acids identified from triplicate runs of each urine sample on the DB-5 column were compared, large variations in the levels of organic acids from subject to subject within each group were observed. Hippuric acid was most abundant except for two nonsmokers. In both mean and median area ratios, the second most abundant acid was lactic acid, followed by glycolic acid, succinic acid and *trans*-aconitic acid, while the orders of succinic acid and *trans*-aconitic acid were switched in smoking group.

When complex GC profiles were transformed to their corresponding *I* spectra in bar graphical form [16–18,20], visual comparison between samples was much easier as exemplified by two group average *I* spectra (Fig. 2). The overall patterns of two spectra look similar qualitatively, but the differences between them in quantities of minor acid peaks are readily detected by visual inspection.

Twenty-six urine samples were classified according to cumulative exposure to cigarette smoke or not.

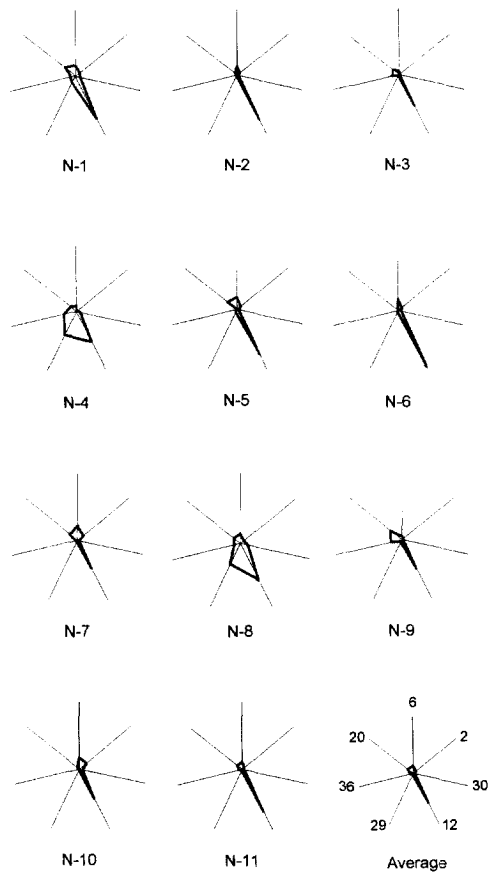

Fig. 3. Star symbol plots of nonsmokers and group average drawn based on seven discriminant variables. Rays: 6=nonanoic acid; 2=caprylic acid; 30=azelaic acid; 12=succinic acid; 29=homovanillic acid; 36=17-methylstearic acid; 20=adipic acid.

Cotinine in serum is widely used as a good marker for evaluating exposure to cigarette smoke, but it is not suitable for the evaluation of the cumulative exposure [7]. Therefore, the GC quantitative data were subjected to the multivariate statistical analysis such as stepwise discriminant and canonical discriminant analyses. When stepwise discriminant analysis was applied after omitting hippuric acid from the GC data, seven acids were selected as the variables which discriminate smokers from nonsmokers. Nonanoic acid (peak No. 6) was selected as the most discriminant variable, followed by caprylic acid (peak No. 2), azelaic acid (peak No. 30), succinic acid (peak No. 12), homovanillic acid (peak No. 29), 17-methylstearic acid (peak No. 36) and adipic acid (peak No. 20). Except for succinic acid, most of the selected variables were minor acids in their area ratios (<2.0%).

Star symbol plots drawn based on these seven variables were very useful for the visual pattern recognition of each urine. Eleven nonsmokers (Fig. 3) are individually different but they look similar to their average star pattern except for four subjects. Star patterns of fifteen smokers (Fig. 4) resemble to one another with the exception of four subjects. The average star plots representing for smoking and nonsmoking groups are clearly distinguishable from one another.

When canonical discriminant analysis was performed using the seven variables selected by stepwise discriminant analysis, twenty-six urine specimens were separated into two distinct clusters, one

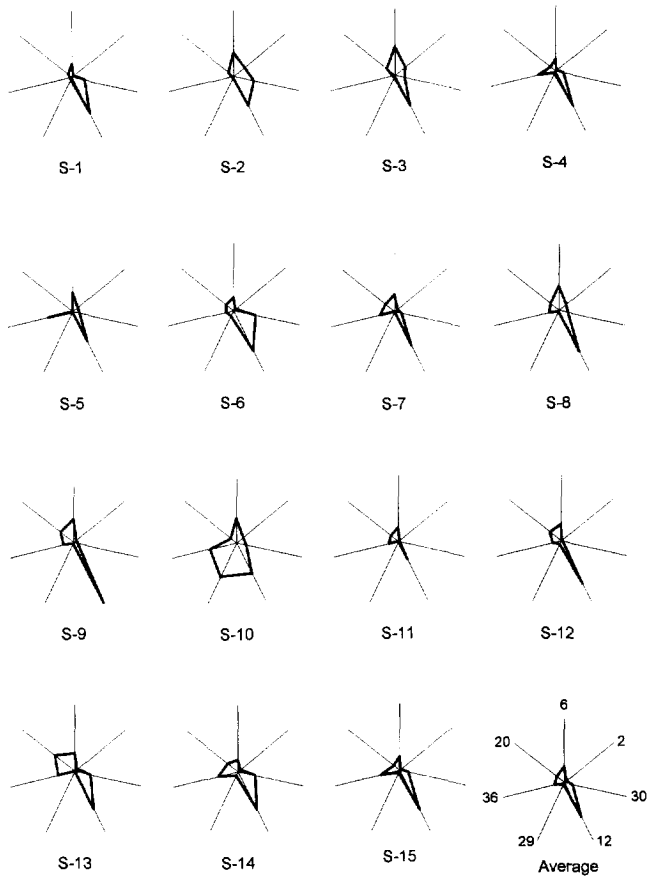

Fig. 4. Star symbol plots of smokers and group average drawn based on seven discriminant variables. Rays: 6=nonanoic acid; 2=caprylic acid; 30=azelaic acid; 12=succinic acid; 29=homovanillic acid; 36=17-methylstearic acid; 20=adipic acid.

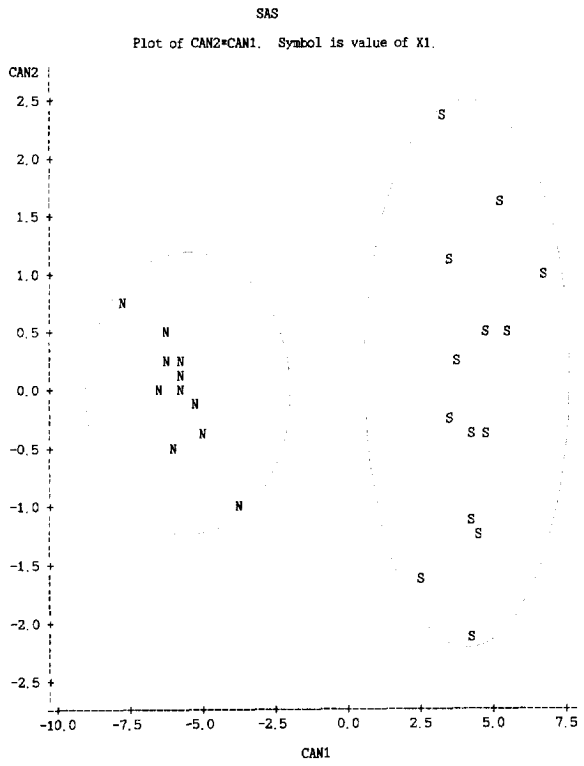

Fig. 5. Canonical plot of (N) nonsmokers and (S) smokers based on seven organic acid variables.

corresponding to each group (Fig. 5). Urine samples were correctly classified into two groups according to cigarette smoking or not. Smoking group shows more scattered clustering than that of nonsmoking group, suggesting that smokers need to be grouped further according to daily consumption of cigarette.

4. Conclusions

The present SPE and silylation with subsequent dual-capillary column GC analysis were suitable for the routine profiling and screening for organic acids in urine. Simplification of GC profiles to their corresponding *I* spectra enabled readily to detect qualitative and quantitative differences among samples. Star symbol plots based on the area ratios of nonanoic acid, caprylic acid, azelaic acid, succinic acid, homovanillic acid, 17-methylstearic acid and adipic acid as the seven discriminant variables selected by stepwise discriminant analysis were useful for visual pattern recognition between individual as well as groups. Canonical plot based on the same variables correctly grouped twenty-six urine samples into two separate clusters according to smoking or not. From our results, it can be stated that there might be some correlation between urinary organic acid levels and cigarette smoking for the twenty-six urine specimens studied.

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References

[1] P.E. Cryer, M.W. Haymond, J.V. Santiago, S.D. Shah, New Engl. J. Med. 295 (1976) 573.
[2] N.W. Tietz (Editor), Textbook of Clinical Chemistry, W.B. Saunders, 1986, pp. 504–505.
[3] W.C.Y. Wu, S.L. Wang, C. Lim, M. Millers, A. Slomiany, B. Slomiany, Prostaglandins Leukot. Essent. Fatty Acids 47 (1992) 101.
[4] S.L. Wang, M. Millers, W.C.Y. Wu, G. Mardirossian, C. Leung, A. Slomiany, B.L. Slomiany, Toxicology 75 (1992) 145.
[5] P. Kintz, J. Chromatogr. 580 (1992) 347.
[6] C. Kirschbaum, S. Wust, C.J. Strasburger, Life Sci. 50 (1992) 435.
[7] J. Pre, Pathol. Biol. Paris 40 (1992) 1015.
[8] R.A. Chalmers and A.M. Lawson, Organic Acids in Man, Chapman and Hall, New York, 1982.
[9] K. Tanaka, A. West-Dull, D.G. Hine, T.H. Lynn, T. Lowe, Clin. Chem. 26 (1980) 1847.
[10] E. Jellum, E.A. Kvittingen, O. Stokke, Biomed. Environ. Mass Spectrom. 16 (1988) 57.
[11] M.F. Leferere, B.J. Verhaeghe, D.M. Declerck, A.P. De Leenheer, Biomed. Environ. Mass Spectrom. 16 (1988) 311.
[12] H.M. Liebich, C. Foerst, J. Chromatogr. 525 (1990) 1.
[13] K.R. Kim, M.K. Hahn, A. Zlatkis, E.C. Horning, B.S. Middleditch, J. Chromatogr. 468 (1989) 289.
[14] K.R. Kim, J.H. Kim, H.K. Park, J. Korean Chem. Soc. 34 (1990) 352.
[15] K.R. Kim, J.H. Kim, H.K. Park, C.H. Oh, Bull. Korean Chem. Soc. 14 (1993) 250.
[16] J.H. Kim, K.R. Kim, J.Y. Choi, H.K. Park, K.S. Choi, Korean J. Food Sci. Technol. 26 (1994) 162.

[17] J.H. Kim, K.R. Kim, J.Y. Choi, C.H. Oh, H.K. Park, K.S. Choi, Korean J. Food Sci. Technol. 26 (1994) 665.
[18] K.R. Kim, J.H. Kim, Y.J. Park, J.O. Kim, Yakhak Hoeji 39 (1995) 283.
[19] A.M.P. Vasconcelos, H.J. Chaves das Neves, J. High Resolut. Chromatogr. 13 (1990) 494.
[20] K.R. Kim, J.H. Kim, E.J. Cheong, C.M. Jeong, J. Chromatogr. A 722 (1996) 303.