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Simultaneous retention index analysis of urinary amino acids and carboxylic acids for graphic recognition of abnormal state

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Abstract

Simultaneous profiling analysis of urinary amino acids (AAs) and carboxylic acids (CAs) was combined with retention index (*I*) analysis for graphic recognition of abnormal metabolic state. The temperature-programmed *I* values of the AA and CA standards measured as ethoxycarbonyl (EOC)/methoxime (MO)/*tert*-butyldimethylsilyl (TBDMS) derivatives were used as the reference *I* values. Urine samples were subjected to the sequential EOC, MO and TBDMS reactions for the analysis by gas chromatography (GC) and GC–mass spectrometry. The complex GC profiles were then transformed into their respective *I* patterns in bar graphic forms by plotting the normalized peak area ratios (%) of the identified AAs and CAs against their reference *I* values as the identification numbers. When the present method was applied to infant urine specimens from normal controls and patients with inherited metabolic diseases such as phenylketonuria, maple syrup urine disease, methylmalonic aciduria or isovaleric aciduria, each *I* pattern of bar graph more distinctly displayed quantitative abundances of urinary AAs and CAs in qualitative *I* scale, thus allowing graphic discrimination between normal and abnormal states.

Keywords: Retention index analysis; Urinary amino acids and carboxylic acids; Ethoxycarbonylation; Methoximation; *tert*-Butyldimethylsilylation; Inherited metabolic disorders

1. Introduction

Amino acids (AAs) and carboxylic acids (CAs) constitute two major chemical groups among the structurally diverse biometabolites of current interest in metabolomics. Most endogenous CAs are major metabolites of AAs while some AAs occur as intermediates in metabolism. The compositions of AAs in biosamples are thus closely interrelated with CAs. In recent years, the simultaneous detection of diverse AAs and CAs in a single run has become an important task in clinical monitoring and biochemical diagnosis of inherited metabolic disorders (IMDs) to detect diagnostic or new and unexpected AAs and CAs, and also to determine changes in the ratios among them [1–6]. Moreover, it has advantages of sample high-throughput analysis at lower cost compared with separate analyses of the two groups.

Profiling analyses are most commonly conducted by high-resolution capillary gas chromatography (GC) combined with mass spectrometry (MS) because of its inherent high resolving power, high sensitivity and positive peak confirmation. Prior to GC and GC–MS analysis, all active protons present in both AAs and CAs are converted to volatile derivatives, mainly by alkylsilylation [1,2,4–8], alkoxycarbonyl (AOC) esterification [3,9–12] and AOC alkylsilylation [13,14]. In our recent report [14], ethoxycarbonylation of amino- and sulfhydryl-groups, methoximation of labile keto groups, and *tert*-butyldimethylsilylation of hydroxyl- and carboxyl-groups were effectively combined in sequence for assay of the clinically important 36 AAs and 45 CAs including nine keto acids (KAs) from aqueous samples in a single run.

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Most of the metabolic profiles are very complex, thus requiring one or more proper pattern recognition analyses for accurate discrimination of abnormal states from normal states [15,16]. For this purpose, diverse statistical methods such as principal component analysis, partial least square models, stepwise discriminant analysis, canonical discriminant analysis, linear discriminant analysis and cluster analysis are widely used. In our previous pattern recognition studies on the urinary profiles of CAs, retention index (*I*) analysis prior to resorting to these statistical tools was found to be potentially useful for visual distinction between smokers and non-smokers [17], and for detection of quantitative differences among uterine myoma- and uterine cervical cancer-patients [18]. For the *I* analysis, the peak area ratios of individual CAs positively identified in GC profiles were normalized to the peak area ratio of the largest peak and were plotted as a function of their reference *I* values in the form of a bar graph. Compared with retention time scale, the *I* scale was more substantial because it provides qualitative information on peak identities [13–21]. Moreover, the relative abundance of each peak allowed direct quantitative comparison between peaks and between samples. However, attempts have been rarely made to apply the simple *I* analysis to simultaneous GC profiles of urinary AAs and CAs in clinical monitoring and biochemical diagnosis of abnormal state to date.

The present study was conducted to investigate the simultaneous profiling analysis of urinary AAs and CAs combined with *I* analysis for graphic comparison between infant urine specimens from normal controls and patients with IMDs such as phenylketonuria (PKU), maple syrup urine disease (MSUD), methylmalonic aciduria (MMA) or isovaleric aciduria (IVA). In this study, the previous database of temperature-programmed *I* sets of 36 AAs and 45 CAs as EOC/MO/TBDMS derivatives measured on dual-columns of different polarities [14] was expanded to comprise a total of 60 AAs and 207 CAs. The reference *I* values were assigned to the corresponding urinary AAs and CAs as the identification (ID) numbers to transform urinary GC profiles into *I* patterns in line graphic forms.

2. Experimental

2.1. Reagents

The 24 AAs and 162 CAs including six KAs examined in this study, methoxyamine hydrochloride, ECF and triethylamine were purchased from various vendors such as Sigma-Aldrich (St. Louis, MO, USA). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). Toluene, diethyl ether, ethyl acetate and dichloromethane of pesticide grade were obtained from Kanto Chemical (Tokyo, Japan). Sodium chloride was purchased from Junsei (Tokyo, Japan), and washed successively with methanol, acetone, dichloromethane and diethyl ether, followed by drying under vacuum (100 °C, 1 h). Sulfuric acid and sodium hydroxide were from Duksan (Seoul, South Korea), and *n*-hydrocarbon standards (C_8–C_{40}, 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) was obtained from Supelco (Bellefonte, PA, USA). A glass column (6 mm I.D.) packed with Chromosorb P (2.0 g) was washed successively with 0.1 M sulfuric acid, methanol, acetone, dichloromethane and diethyl ether. The Chromosorb P column was then activated under vacuum (150 °C, 3 h) prior to being used as a solid-phase extraction (SPE) column in normal phase partition mode.

2.2. Preparation of standard solutions

The standard stock solutions of AAs, CAs and KAs were individually made up at 10 μg/μl in 0.1 M HCl, methanol and distilled water, respectively. The working solutions at varied concentrations were then prepared diluting each stock solution with 0.1 M HCl for AAs, with methanol for CAs, and with distilled water for KAs. Internal standard (IS) stock solution was prepared by dissolving 3,4-dimethoxybenzoic acid at 10.0 μg/μl in methanol and was used to prepare IS working solutions at 0.5 μg/μl in methanol. A total of 186 standards were divided to prepare 20 group mixture samples so that the analytes in each group were completely resolved on the dual-columns in a single run. Each group sample was prepared at different concentration ranges from 0.1 to 5.0 μg/ml depending on analytes by mixing appropriate aliquots of each working solution and diluting in water. Hydrocarbon solution containing *n*-hydrocarbons (C_8–C_{40}, even numbers only), each at 1.0 μg/μl in isooctane, was used as the IS solution for *I* measurement. All standard solutions prepared were stored at 4 °C.

2.3. Instrumentation

The GC analyses for the *I* measurement were performed with an Agilent 6890 gas chromatograph, equipped with electronic pneumatic control system, a split/splitless inlet system, an automatic liquid sampler, two flame ionization detectors (FIDs) and GC Chemstation (Agilent Technologies, Atlanta, GA, USA). The injector was installed with a dual-column system made of DB-5 (SE-54 bonded) and DB-17 (OV-17 bonded) fused-silica capillary columns (30 m × 0.25 mm I.D., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA), which were connected to a deactivated fused-silica tubing (1 m × 0.25 mm I.D.) as retention gap via Y splitter. The injector and detector temperatures were 260 and 290 °C, respectively. The GC Chemstation processed the two FID signals simultaneously in dual-channel mode. Helium was used as carrier gas at a flow rate of 1.0 ml/min with constant flow mode. Samples (ca. 1.0 μl) were injected in the splitless mode with purge delay time of 42 s. The

oven temperature was maintained at 60 °C for 2 min and then programmed to 290 °C at a rate of 4 °C/min. A standard solution of *n*-hydrocarbons (C_8–C_{40}, even numbers only) in toluene was co-injected with samples to compute temperature-programmed *I* values based on the following equation:

$$I = 100z + 100\frac{[t_R(x) - t_R(z)]}{[t_R(y) - t_R(z)]}(y - z)$$

where $t_R(x)$ is the retention time of analyte *x*, $t_R(z)$ and $t_R(y)$ are the retention times of *n*-hydrocarbon containing *z* carbons eluted before *x* and *n*-hydrocarbon containing y carbons eluted after *x*, respectively. The reference *I* database constructed in our previous study [14] was expanded with the present *I* sets measured on the dual-columns. The simultaneous profiling analyses of AAs and CAs in urine samples were conducted employing a single DB-5 column under the same operating conditions. All GC analyses were performed in triplicate.

GC–MS analyses were performed with an Agilent 6890 gas chromatograph interfaced to an Agilent 5973 mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 (SE-54 bonded phase; 25 m × 0.20 mm I.D., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA, USA). The temperatures of injector, interface and ion source were 260, 300 and 230 °C, respectively. Helium was used as carrier gas at a flow rate of 0.5 ml/min with constant flow mode. Samples were introduced in the split-injection mode (10:1) and the oven temperature was initially at 100 °C for 2 min and programmed to 260 °C at a rate of 3 °C/min and finally to 300 °C at a rate of 20 °C/min with holding time for 10 min. The mass range scanned was 50–650 u at a rate of 0.99 scan/s.

2.4. Sequential EOC/MO/TBDMS derivatization

Each mixed standard solution was subjected to the sequential EOC/MO/TBDMS reactions in the same manner as described elsewhere [14]. Briefly, an aliquot (1.0 ml) of mixed solutions (pH adjusted to ≥12 with 5.0 M sodium hydroxide) was added to dichloromethane (1.0 ml) containing ECF (20 μl) and the mixture was vortexed. The pH was readjusted to pH ca. 13 and was reacted (60 °C for 1 h) with methoxyamine hydrochloride (10.0 mg), followed by washing with diethyl ether (3 ml × 2). The aqueous layer was then acidified (pH ≤ 2.0) (with 10.0% sulfuric acid) and saturated with sodium chloride with subsequent solvent extraction sequentially with diethyl ether (2 ml × 2) and ethyl acetate (2 ml × 2). The combined extracts were evaporated to dryness under a gentle stream of nitrogen (40 °C). Toluene (30 μl) as solvent and MTBSTFA (20 μl) as silylation reagent were added to the residue and the mixture was heated (60 °C for 1 h) to form TBDMS derivatives for GC and GC–MS analysis.

Table 1
Temperature-programmed *I* sets for 61 amino acids and 207 carboxylic acids as *N*(*O*,*S*)-ethoxycarbonlylated methoximated *tert*-butyldimethylsilyl derivatives

No.	Analyte	*I* value[a]	
		DB-5	DB-17
1	Formic acid	889.9 ± <0.1	903.8 ± 0.1
2	Acetic acid	934.1 ± 0.1	952.2 ± 0.1
3	Propionic acid	1014.6 ± 0.1	1046.2 ± 0.1
4	Isobutyric acid	1050.5 ± 0.1	1077.1 ± 0.2
5	Butyric acid	1097.4 ± 0.1	1146.1 ± 0.1
6	Isovaleric acid	1146.9 ± 0.1	1196.3 ± <0.1
7	Valeric acid	1194.8 ± <0.1	1251.9 ± <0.1
8	Tiglic acid	1233.3 ± 0.2	1319.6 ± 0.2
9	3-Hydroxybutyric acid-1[b]	1241.3 ± 0.1	1340.6 ± 0.1
10	3-Hydroxyisovaleric acid-1[b]	1255.0 ± 0.1	1333.9 ± 0.1
11	Pyruvic acid	1259.0 ± <0.1	1316.1 ± <0.1
12	2-Ketobutyric acid-1[c]	1279.7 ± <0.1	1404.3 ± <0.1
13	Caproic acid	1290.5 ± 0.1	1343.5 ± 0.1
14	2-Ketobutyric acid-2[c]	1305.8 ± <0.1	1428.2 ± <0.1
15	2-Ketoisovaleric acid-1[c]	1319.2 ± <0.1	1431.7 ± <0.1
16	Acetoacetic acid-1[c]	1329.8 ± <0.1	1452.7 ± 0.1
17	2-Ketoisovaleric acid-2[c]	1332.3 ± <0.1	1446.8 ± <0.1
18	Acetoacetic acid-2[c]	1344.5 ± <0.1	1459.2 ± <0.1
19	2-Ketovaleric acid-1[c]	1353.1 ± <0.1	1468.6 ± <0.1
20	4-Hydroxybutyric acid-1[b]	1365.3 ± <0.1	1595.7 ± <0.1
21	2-Ketovaleric acid-2[c]	1376.6 ± 0.1	1499.5 ± <0.1
22	Heptanoic acid	1387.5 ± <0.1	1442.9 ± <0.1
23	2-Ketoisocaproic acid-1[c]	1393.1 ± <0.1	1493.0 ± <0.1
24	2-Keto-3-methylvaleric acid-1[c]	1396.1 ± <0.1	1501.4 ± 0.1
25	2-Keto-3-methylvaleric acid-2[c]	1402.6 ± <0.1	1519.2 ± <0.1
26	2-Ketoisocaproic acid-2[c]	1420.6 ± <0.1	1530.8 ± <0.1
27	2-Ketocaproic acid-1[c]	1440.0 ± <0.1	1555.3 ± <0.1
28	Picolinic acid	1445.6 ± <0.1	1775.7 ± 0.1
29	Caprylic acid	1445.7 ± <0.1	1540.2 ± 0.1
30	Glyoxylic acid-1[c]	1450.6 ± <0.1	1580.4 ± <0.1
31	Citraconic acid-1[c]	1460.5 ± <0.1	1647.2 ± <0.1
32	2-Ketocaproic acid-2[c]	1462.6 ± <0.1	1583.7 ± <0.1
33	Glyoxylic acid-2[c]	1469.0 ± <0.1	1604.7 ± 0.9
34	Benzoic acid	1488.3 ± <0.1	1642.6 ± <0.1
35	Lactic acid	1491.1 ± 0.1	1516.1 ± 0.1
36	Glycolic acid	1508.6 ± 0.2	1556.4 ± 0.2
37	Phenylacetic acid	1524.8 ± 0.1	1644.8 ± 0.1
38	Cyclohexaneacetic acid	1527.9 ± <0.1	1624.3 ± 0.1
39	Picalic acid	1534.4 ± <0.1	1775.9 ± 0.1
40	Oxalic acid	1547.7 ± <0.1	1642.6 ± 0.1
41	2-Hydroxybutyric acid	1558.9 ± 0.1	1581.1 ± 0.2
42	2-Methylbenzoic acid	1559.9 ± 0.1	1715.9 ± <0.1
43	Alanine	1560.9 ± <0.1	1719.1 ± <0.1
44	Sarcosine	1567.7 ± 0.1	1743.3 ± <0.1
45	3-Hydroxypropionic acid	1578.3 ± <0.1	1595.7 ± 0.1
46	Glycine	1578.7 ± 0.1	1759.0 ± <0.1
47	Nonanoic acid	1584.4 ± <0.1	1637.5 ± 0.1
48	Propionylglycine	1591.7 ± <0.1	1806.2 ± 0.1
49	3-Hydroxybutyric acid-2[d]	1592.5 ± <0.1	1615.3 ± 1.2
50	2-Hydroxy-2-methylbutyric acid	1594.9 ± 0.1	1594.1 ± 0.3
51	3-Methylphenylacetic acid	1600.0 ± <0.1	1769.9 ± <0.1
52	2-Phenylbutyric acid	1602.3 ± <0.1	1748.8 ± 0.1
53	2-Hydroxyisovaleric acid	1603.8 ± 0.1	1617.6 ± 0.2
54	2-Hydroxy-3-methylbutyric acid	1604.0 ± <0.1	1618.1 ± <0.1
55	4-Methylphenyl acetic acid	1613.2 ± 0.1	1783.1 ± <0.1
56	2-Phenoxypropionic acid	1618.5 ± <0.1	1791.9 ± <0.1
57	α-Ketooctanoic acid-1[c]	1618.8 ± 0.1	1744.7 ± 0.1
58	2-Hydroxyvaleric acid	1620.6 ± 0.1	1641.0 ± 0.1
59	Isobutylglycine	1622.5 ± <0.1	1818.1 ± <0.1

Table 1 (*Continued*)

60	α-Aminobutyric acid	1627.5 ± <0.1	1784.9 ± 0.1
61	3-Hydroxycaprylic acid-1[d]	1631.3 ± 0.2	1728.8 ± 0.2
62	α-Ketooctanoic acid-2[c]	1631.7 ± 0.1	1771.2 ± 0.1
63	Malonic acid	1641.2 ± 0.1	1728.6 ± 0.1
64	α-Keto-γ-methiolbutyric acid-1[c]	1650.6 ± <0.1	1846.7 ± 0.1
65	β-Alanine	1651.8 ± <0.1	1847.6 ± <0.1
66	2-Hydroxyisocaproic acid	1652.0 ± 0.1	1661.5 ± 0.2
67	3-Phenylpropionic acid	1652.8 ± <0.1	1817.5 ± 0.1
68	3-Hydroxyisovaleric acid-2[d]	1654.0 ± 0.5	1663.1 ± 0.4
69	Methylmalonic	1657.2 ± 0.1	1729.0 ± 0.1
70	Valine	1660.9 ± <0.1	1813.5 ± <0.1
71	Dimethylmalonic	1662.1 ± 0.1	1715.6 ± 0.2
72	β-Aminoisobutyric acid	1667.6 ± 0.1	1848.5 ± 0.2
73	α-Keto-γ-methiolbutyric acid-2[c]	1670.7 ± <0.1	1866.3 ± 0.1
74	Decenoic acid	1670.9 ± 0.1	1743.2 ± 0.1
75	4-Hydroxybutyric acid-2[d]	1671.2 ± <0.1	1711.1 ± <0.1
76	3-Phenylbutyric acid	1677.4 ± <0.1	1833.1 ± 0.1
77	2-Hydroxy-3-methylvaleric acid	1679.6 ± 0.2	1692.2 ± 0.4
78	Capric acid	1684.6 ± 0.1	1736.3 ± 0.1
79	2-Ethyl-2-hydroxybutyric acid	1685.2 ± <0.1	1685.7 ± <0.1
80	Norvaline	1694.6 ± 0.1	1848.6 ± <0.1
81	2-Hydroxycaproic acid	1699.1 ± 0.1	1716.4 ± 0.2
82	4-Ethylbenzoic acid	1703.5 ± <0.1	1854.5 ± <0.1
83	Isovalerylglycine	1719.3 ± <0.1	1913.3 ± 0.1
84	Ethylmalonic	1721.7 ± 0.1	1789.2 ± 0.2
85	Leucine	1724.6 ± <0.1	1872.1 ± <0.1
86	*allo*-Isoleucine	1732.2 ± <0.1	1885.8 ± <0.1
87	Tropic acid-1[b]	1742.8 ± 0.3	1967.4 ± 0.2
88	Isoleucine	1743.3 ± <0.1	1898.8 ± <0.1
89	Maleic acid	1745.0 ± <0.1	1870.4 ± <0.1
90	Threonine-1[e]	1745.3 ± <0.1	1968.2 ± <0.1
91	Serine-1[e]	1756.8 ± <0.1	1986.5 ± 0.1
92	Succinic acid	1757.6 ± 0.1	1849.4 ± 0.2
93	4-Methoxyphenylacetic acid	1766.4 ± <0.1	1993.2 ± 0.2
94	Methylsuccinic acid	1766.5 ± <0.1	1840.1 ± 0.1
95	Phenylpropiolic acid	1772.0 ± <0.1	2009.5 ± 0.1
96	2,2-Dimethylsuccinic acid	1773.1 ± <0.1	1837.1 ± <0.1
97	3-Phenoxypropionic acid	1774.1 ± <0.1	1981.4 ± <0.1
98	Proline	1776.5 ± <0.1	2015.7 ± 0.1
99	Norleucine	1776.9 ± 0.1	1932.0 ± 0.1
100	γ-Aminobutyric acid-1[e]	1779.4 ± <0.1	1979.4 ± 0.1
101	Citraconic acid-2[d]	1780.2 ± <0.1	1905.7 ± <0.1
102	Undecanoic acid	1782.5 ± <0.1	1834.6 ± 0.1
103	Fumaric acid	1786.8 ± 0.1	1846.3 ± 0.4
104	*trans*-Cinnamic acid	1786.9 ± <0.1	1980.3 ± 0.1
105	Itaconic acid	1787.9 ± 0.2	1898.4 ± 0.3
106	Succinylacetone-1[c]	1790.6 ± <0.1	1988.2 ± <0.1
107	Phenylpyruvic acid	1796.6 ± 0.3	2060.2 ± 0.1
108	Tiglylglycine	1806.2 ± 0.1	2042.1 ± 0.1
109	Succinylacetone-2[c]	1808.1 ± 0.1	2000.0 ± <0.1
110	Cycloleucine	1812.0 ± 0.1	2024.5 ± 0.1
111	Succinylacetone-3[c]	1817.0 ± 0.1	2012.3 ± 0.1
112	Succinylacetone-4[c]	1825.2 ± 0.1	2206.7 ± 0.1
113	3-Hydroxycapric acid-1[b]	1830.9 ± 0.1	1929.4 ± 0.1
114	2-Methylfumaric acid	1831.1 ± <0.1	1905.7 ± <0.1
115	Pipecolic acid	1835.1 ± 0.1	2060.6 ± <0.1
116	3-(2-Methoxyphenyl)-propionic acid	1849.1 ± <0.1	2075.1 ± <0.1
117	Oxaloacetic acid-1[c]	1854.7 ± 0.0	2037.2 ± 0.2
118	Glutaric acid	1855.1 ± <0.1	1948.2 ± 0.1
119	5-Phenylvaleric acid	1869.8 ± <0.1	2045.9 ± 0.4
120	Diglycolic acid	1870.3 ± <0.1	1993.0 ± 0.1
121	3-Methylglutaric acid	1870.3 ± <0.1	1974.4 ± 0.1
122	2-Hydroxycaprylic acid	1872.9 ± <0.1	1885.8 ± 0.1
123	Hexanoylglycine	1873.5 ± 0.2	2085.4 ± <0.1
124	Methylcysteine	1881.9 ± 0.1	2119.9 ± 0.1

Table 1 (*Continued*)

125	Lauric acid	1884.0 ± 0.2	1935.5 ± 0.1
126	Glutaconic acid	1894.7 ± 0.1	2013.3 ± 0.1
127	2,2-Dimethoxyglutaric acid	1896.4 ± 0.1	1963.9 ± 0.1
128	Oxaloacetic acid-2[c]	1896.6 ± 0.1	2042.6 ± 0.1
129	Glyceric acid-1[d]	1896.8 ± 0.1	1950.5 ± 0.1
130	Mandelic acid	1897.9 ± <0.1	2019.8 ± 0.1
131	3-Hydroxycaprylic acid-2[d]	1899.3 ± <0.1	1908.2 ± <0.1
132	β-(4-Methoxy)phenylpropionic acid	1899.5 ± 0.1	2127.8 ± 0.2
133	1-Aminocyclohexanecarboxylic acid	1905.7 ± 0.2	2140.4 ± <0.1
134	Phosphoric acid	1923.5 ± 0.1	1970.3 ± 0.2
135	3-Hydroxy-3-methylglutaric acid	1937.4 ± 0.1	2162.2 ± <0.1
136	Pyroglutamic acid-1[d]	1949.2 ± <0.1	2117.3 ± <0.1
137	2,2-Dimethylglutaric acid	1956.0 ± 0.1	1963.9 ± 0.1
138	3,4-Dimethylbenzoic acid	1957.3 ± 0.1	2240.4 ± <0.1
139	Adipic acid	1963.9 ± 0.1	2061.5 ± 0.2
140	Salicylic acid	1965.3 ± 0.2	2109.5 ± 0.3
141	Atrolactic acid	1970.5 ± 0.1	2080.0 ± <0.1
142	Tridecanoic acid	1983.1 ± 0.2	2035.3 ± 0.3
143	3-Methyladipic acid	1988.7 ± 0.2	2078.6 ± 0.3
144	2-Ketoglutaric acid-1[c]	1989.0 ± 0.1	2134.6 ± <0.1
145	Ethyladipic acid	1989.3 ± 0.1	2078.5 ± <0.1
146	Methionine	1989.6 ± 0.1	2240.1 ± 0.1
147	6-Aminohexanoic acid	2000.0 ± <0.1	2245.5 ± 0.2
148	2-Ketoglutaric acid-2[c]	2007.7 ± 0.1	2156.8 ± 0.1
149	Serine-2[f]	2009.2 ± <0.1	2128.8 ± 0.1
150	Threonine-2[f]	2016.1 ± <0.1	2139.3 ± 0.1
151	2-Phenylglycine	2021.7 ± 0.1	2291.8 ± 0.1
152	Tropic acid-2[d]	2024.4 ± 0.1	2147.7 ± 0.1
153	2-Indolecarboxylic acid	2024.7 ± 0.1	2293.9 ± <0.1
154	2-Hydroxyphenylacetic acid	2026.2 ± 0.1	2169.5 ± 0.2
155	3-Hydroxylauric acid-1[b]	2034.7 ± 0.1	2134.7 ± 0.1
156	3-Methoxycinnamic acid	2035.7 ± 0.1	2276.9 ± 0.1
157	3-Phenyllactic acid	2041.3 ± 0.1	2148.8 ± 0.2
158	3-Cresotinic acid	2041.7 ± 0.1	2170.3 ± <0.1
159	Ethionine	2055.8 ± 0.1	2298.3 ± 0.2
160	3-(2,5-Dimethoxy)-phenylpropionic acid	2057.2 ± 0.1	2330.7 ± 0.1
161	3-Hydroxyphenylacetic acid	2064.2 ± 0.1	2203.2 ± 0.3
162	Pimelic acid	2066.3 ± 0.2	2166.5 ± 0.2
163	2-Aminobenzoic acid	2071.5 ± 0.1	2333.5 ± 0.1
164	Myristoleic acid	2075.0 ± 0.3	2155.3 ± 0.2
165	3-Hydroxycapric acid-2[d]	2081.1 ± 0.1	2086.7 ± 0.1
166	Myristic acid	2085.2 ± 0.3	2137.0 ± 0.1
167	4-Methoxycinnamic acid	2085.2 ± 0.1	2352.6 ± 0.3
168	Hippuric acid	2093.6 ± <0.1	2418.0 ± 0.2
169	*trans–trans*-Muconic acid	2094.6 ± 0.1	2163.0 ± 0.1
170	2,3-Diaminopropionic acid	2100.0 ± 0.1	2385.7 ± <0.1
171	2-Ketoadipic acid	2102.7 ± <0.1	2257.3 ± 0.2
172	*N*-Acetylaspartic acid	2104.3 ± 0.2	2297.4 ± 0.3
173	4-Hydroxyphenylacetic acid	2108.4 ± 0.1	2247.3 ± 0.2
174	γ-Aminobutyric acid-2[f]	2109.5 ± <0.1	2236.2 ± <0.1
175	Phenylalanine	2110.8 ± 0.1	2384.1 ± 0.1
176	Malic acid	2115.2 ± 0.1	2152.6 ± 0.2
177	4-Hydroxybenzoic acid	2117.8 ± 0.2	2229.0 ± 0.2
178	Homoserine	2118.7 ± 0.1	2251.4 ± <0.1
179	Citramalic acid	2127.0 ± 0.1	2143.8 ± 0.1
180	Phthalic acid	2137.2 ± 0.1	2347.2 ± <0.1
181	Cysteine	2142.5 ± 0.1	2432.5 ± 0.1
182	3-Indoleacetic acid	2155.1 ± <0.1	2559.5 ± 0.1
183	Pyridine-2,3-dicarboxylic acid	2165.7 ± 0.2	2435.7 ± 0.1
184	Aspartic acid	2165.8 ± 0.1	2334.6 ± 0.1
185	Suberic acid	2167.9 ± <0.1	2271.9 ± 0.2
186	Pentadecanoic acid	2184.3 ± 0.2	2235.4 ± 0.2
187	4-Hydroxyproline-1[f]	2194.6 ± 0.1	2347.1 ± 0.2

Table 1 (*Continued*)

188	2,4-Diaminobutyric acid	2212.1 ± 0.1	2521.8 ± 0.1
189	4-Hydroxyproline-2[g]	2213.2 ± 0.1	2493.4 ± 0.1
190	3-Aminobenzoic acid	2215.6 ± 0.1	2533.6 ± 0.2
191	2-Hydroxyglutaric acid	2216.5 ± <0.1	2261.5 ± 0.2
192	Benzylmalic acid	2216.6 ± <0.1	2384.4 ± 0.1
193	Homophenylalanine	2239.0 ± <0.1	2518.6 ± 0.1
194	2,5-Dimethoxycinnamic acid	2241.2 ± 0.1	2552.9 ± <0.1
195	3-(4-Hydroxyphenyl)propionic acid	2245.9 ± <0.1	2385.3 ± 0.1
196	Homovanillic acid	2255.9 ± 0.2	2431.0 ± 0.1
197	Homocysteine	2259.7 ± 0.1	2558.9 ± 0.2
198	3-Phenylpropionylglycine	2263.2 ± 0.1	2617.7 ± 0.1
199	Vanillic acid	2264.5 ± 0.3	2427.2 ± 0.2
200	Palmitoelic acid	2267.9 ± <0.1	2349.0 ± 0.1
201	γ-Methylglutamic acid	2270.8 ± 0.2	2439.1 ± 0.2
202	Azelaic acid	2271.2 ± 0.1	2373.3 ± 0.1
203	3-Hydroxylauric acid-2[d]	2272.0 ± <0.1	2275.7 ± <0.1
204	Dihydroxyfumaric acid	2272.3 ± <0.1	2508.8 ± <0.1
205	3-Indolepropionic acid	2282.3 ± 0.1	2690.6 ± 0.1
206	Palmitic acid	2287.6 ± 0.1	2339.5 ± 0.2
207	Glyceric acid-2[h]	2287.7 ± 0.1	2338.8 ± <0.1
208	Glutamic acid	2291.6 ± 0.3	2472.0 ± 0.2
209	4-Aminobenzoic acid	2295.2 ± <0.1	2613.0 ± 0.1
210	Docosahexaneoic acid	2306.0 ± 0.1	2454.7 ± 0.1
211	Asparagine	2318.2 ± 0.1	2552.1 ± 0.2
212	Nifulmic acid	2328.0 ± 0.1	2542.8 ± <0.1
213	Ornithine	2346.3 ± <0.1	2679.5 ± 0.1
214	3-Coumaric acid	2349.3 ± 0.2	2492.5 ± 0.4
215	15-Methylpalmitic acid	2352.0 ± 0.1	2394.5 ± <0.1
216	Sebacic acid	2372.1 ± 0.2	2476.6 ± 0.1
217	*trans*-Aconitic acid	2384.6 ± 0.1	2482.9 ± 0.1
218	*cis*-Aconitic acid	2385.6 ± 0.2	2484.7 ± 0.1
219	Heptadecanoic acid	2386.8 ± 0.1	2438.9 ± 0.1
220	Orotic acid	2389.5 ± <0.1	2476.9 ± 0.1
221	Syringic acid	2392.4 ± 0.1	2605.3 ± 0.3
222	α-Aminoadipic acid	2403.1 ± 0.3	2591.3 ± 0.1
223	Threophenylserine	2408.6 ± 0.1	2626.6 ± 0.1
224	3-Indolebutyric acid	2418.7 ± 0.1	2848.5 ± 0.2
225	4-Hydroxyphenylpyruvic acid	2421.9 ± 0.1	2527.3 ± 0.1
226	4-Hydroxymandelic acid	2423.3 ± 0.2	2506.0 ± 0.2
227	Tartaric acid	2430.0 ± 0.1	2415.8 ± 0.2
228	Phytanic acid	2437.3 ± 0.3	2453.4 ± 0.1
229	Glutamine	2440.8 ± <0.1	2691.8 ± 0.1
230	2-Hydroxymyristic acid	2451.3 ± 0.2	2456.2 ± 0.8
231	Lysine	2453.3 ± 0.3	2795.6 ± 0.1
232	Petroselinic acid	2460.0 ± 0.1	2578.8 ± 0.2
233	Linoleic acid	2460.3 ± 0.1	2567.9 ± 0.1
234	3-Indolecarboxylic acid	2461.6 ± 0.2	2756.7 ± 0.2
235	4-Hydroxycinnamic acid	2461.9 ± <0.1	2609.2 ± 0.2
236	Oleic acid	2463.8 ± 0.1	2544.9 ± 0.1
237	3-Indoleglyoxylic acid[i]	2464.8 ± 0.2	2760.0 ± 0.2
238	Linolenic acid	2468.0 ± 0.1	2600.0 ± <0.1
239	Eladic acid	2471.3 ± 0.2	2549.0 ± 0.6
240	Baclofen	2474.1 ± 0.1	2815.8 ± 0.1
241	3-Hydroxyphenylglycine	2474.7 ± 0.1	2836.1 ± 0.1
242	12-Hydroxydodecanoic acid	2481.2 ± 0.1	2524.0 ± 0.1
243	β-Resorcylic acid	2483.2 ± 0.1	2606.9 ± 0.2
244	α-Resorcylic acid	2484.7 ± 0.2	2579.2 ± 0.3
245	Stearic acid	2489.1 ± 0.1	2541.5 ± 0.1
246	Histidine	2504.5 ± <0.1	2883.8 ± 0.2
247	Homogentisic acid	2507.7 ± 0.3	2606.5 ± 0.3
248	α-Aminopimelic acid	2510.5 ± 0.2	2705.0 ± 0.1
249	Vanilmandelic acid	2518.8 ± 0.2	2638.5 ± 0.2
250	3-Aminosalicylic acid	2527.5 ± <0.1	2749.6 ± <0.1
251	Protocatechuic acid	2529.2 ± 0.1	2640.7 ± 0.1
252	4-Hydroxyphenylglycine	2535.2 ± <0.1	2914.0 ± 0.1

Table 1 (*Continued*)

253	2-Hydroxyhippuric acid	2544.7 ± 0.2	2828.1 ± 0.3
254	Kainic acid	2561.7 ± 0.2	2780.5 ± 0.3
255	Dodecanedioic acid	2576.5 ± 0.1	2681.3 ± 0.1
256	Nonadecanoic acid	2588.9 ± 0.1	2641.2 ± 0.1
257	4-Hydroxyphenyllactic acid	2596.1 ± 0.1	2668.3 ± 0.2
258	Ferulic acid	2612.0 ± 0.1	2814.0 ± 0.1
259	Arachidonic acid	2617.9 ± <0.1	2771.8 ± <0.1
260	Citric acid	2625.3 ± 0.3	2655.6 ± 0.2
261	3-Indolelactic acid	2626.5 ± 0.1	2959.6 ± 0.1
262	Eicosapentaenoic acid	2627.7 ± 0.1	2807.2 ± 0.1
263	Pyridoxic acid	2630.0 ± 0.1	2771.7 ± 0.1
264	5-Aminosalicylic acid	2642.9 ± 0.3	2937.9 ± 0.2
265	Tyrosine	2661.7 ± 0.2	2899.0 ± 0.1
266	Eicosenoic acid	2670.3 ± 0.2	2754.0 ± 0.3
267	4-Aminosalicylic acid	2673.1 ± <0.1	2968.1 ± 0.1
268	12-Hydroxystearic acid-1[b]	2678.4 ± 0.6	2840.6 ± 0.1
269	1,11-Undecanedicarboxylic acid	2680.0 ± 0.1	2783.7 ± <0.1
270	Methylcitric acid-1[j]	2680.9 ± 0.1	2698.5 ± 0.1
271	5-Hydroxyindoleacetic acid	2685.0 ± 0.4	3082.7 ± 0.4
272	2,3-Naphthalenedicarboxylic acid	2687.0 ± 0.1	2985.4 ± 0.2
273	Arachidic acid	2691.7 ± 0.1	2747.7 ± 0.5
274	Methylcitric acid-2[j]	2709.0 ± 0.2	2722.8 ± 0.2
275	3-Indoleglyoxylic acid-2[d]	2712.8 ± 0.1	3073.8 ± 0.6
276	Tryptophan	2735.1 ± 0.1	3273.2 ± <0.1
277	4-Carboxyphenylglycine	2751.3 ± <0.1	3007.1 ± <0.1
278	δ-Hydroxylysine-1[j]	2759.2 ± 0.1	3000.0 ± <0.1
279	*trans*-3,5-Dimethoxy-4-hydroxycinnamic acid	2759.8 ± 0.1	3019.4 ± 0.2
280	δ-Hydroxylysine-2[j]	2769.1 ± 0.1	3013.3 ± <0.1
281	3,4-Dihydroxymandelic acid	2772.2 ± 0.1	2825.6 ± <0.1
282	Heneicosanoic acid	2791.2 ± 0.1	2843.6 ± 0.1
283	Gallic acid	2822.8 ± 0.1	2927.2 ± 0.1
284	Docosapentaenoic acid	2833.8 ± 0.1	3019.0 ± 0.1
285	Caffeic acid	2865.4 ± 0.1	2976.3 ± 0.1
286	Erucic acid	2870.9 ± 0.1	2953.9 ± 0.2
287	Behenic acid	2893.7 ± 0.1	2946.0 ± 0.3
288	12-Hydroxystearic acid-2[d]	2911.0 ± 0.2	2922.6 ± 0.1
289	5-Methoxytryptophan	2928.6 ± 0.2	3520.4 ± 0.1
290	Tricosanoic acid	2992.9 ± 0.1	3046.1 ± 0.1
291	Nervonic acid	3066.5 ± 0.1	3158.5 ± 0.1
292	Lignoceric acid	3088.0 ± 0.2	3149.2 ± <0.1
293	Pentacosanoic acid	3194.4 ± 0.1	3247.7 ± <0.1
294	5-Hydroxytryptophan-1[f]	3206.3 ± 0.2	3711.2 ± <0.1
295	5-Hydroxytryptophan-2[g]	3249.9 ± 0.1	3937.4 ± 0.2
296	Cerotic acid	3286.3 ± 0.1	3345.3 ± 0.1
297	Hexacosanoic acid	3286.8 ± 0.1	3346.4 ± 0.1
298	Heptacosanoic acid	3396.4 ± 0.1	3444.4 ± 0.1
299	Octacosanoic acid	3486.4 ± 0.2	3543.9 ± 0.1
300	Nonacosanoic acid	3600.0 ± <0.1	3650.4 ± 0.2

[a] Retention index value; mean ± S.D. ($n = 3$).
[b] As mono-TBDMS derivative.
[c] *Syn* and *anti* isomers.
[d] As di-TBDMS derivative.
[e] As mono-EOC/mono-TBDMS derivative.
[f] As mono-EOC/di-TBDMS derivative.
[g] As di-EOC/mono-TBDMS derivative.
[h] As tri-TBDMS derivative.
[i] As methoxime/TBDMS derivative.
[j] Diastereomeric isomers.

2.5. Sample preparation for urinary amino acids and carboxylic acids

Spot urine samples from three normal controls (aged from 0.25 to 1.25 years) and four patients (aged from 0.01 to 8 years) suffering from PKU, MSUD, MMA and IVA, respectively were provided by Hallym University Hospital (Chunchon, South Korea). They were immediately stored at −20 °C until analyzed. Aliquots of each urine sample (equivalent to 0.25 mg of creatinine) were individually adjusted to pH ≥ 12 after addition of IS (10.0 μg). The samples were then subjected to the sequential EOC/MO reactions with subsequent washing, acidification and saturation according to the preceding procedures. Then SPE was performed to the acidified aqueous layer by loading onto an activated Chromosorb P column [14,17,18] and eluting with diethyl ether (5 ml) and ethyl acetate (3 ml) in sequence. The combined eluates were evaporated and finally converted into TBDMS derivatives as aforementioned in the previous section for GC and GC–MS analyses.

2.6. Retention index analysis

After the peak identification by GC–MS analysis of urine samples, each concentration of the AAs and CAs measured on DB-5 column from triplicate runs was computed in peak area ratio (relative to IS) per 0.25 mg of creatinine and then normalized to the peak area ratio of the largest peak as the base peak in each sample. And each average of the three normal values was normalized to compute the normal average. To each peak was assigned its reference *I* value as the ID number, the normalized peak area ratios (%) were then plotted as a function of increasing *I* values in the form of a bar graph to obtain *I* pattern for each sample using Microsoft™ Excel program.

3. Results and discussion

3.1. Retention index values of amino acids and carboxylic acids as EOC/MO/TBDMS derivatives

In an extension of the previous method [14] to expand *I* database, a total of 24 AAs and 162 CAs in aqueous solutions were subjected to sequential EOC/MO/TBDMS reactions, followed by GC analysis on the dual-columns. Most of the analytes examined displayed a single peak with good peak shape within 60 min. The temperature-programmed *I* sets for AAs as *N*(*O*,*S*)-EOC/*O*-TBDMS derivatives and CAs as *O*-TBDMS derivatives including KAs as MO/*O*-TBDMS derivatives were measured in triplicate. The *I* sets of 60 AAs and 207 CAs including the previous 36 AAs and 45 CAs were summarized in the order of increasing values on nonpolar DB-5 column (Table 1). The precision of each *I* set measured on the dual-columns was excellent (%R.S.D. ≤ 0.03), indicating that it could serve as the reference *I* values in *I* graphic analysis.

3.2. Retention index analysis of urinary amino acids and carboxylic acids

Upon GC–MS analyses of infant urine specimens from three normal controls and four patients, a total of 22 AAs and 62 CAs including six KA were positively identified from minimal volumes (corresponding to 0.25 mg creatinine). The urinary normal excretion levels measured on nonpolar DB-5 column were expressed as the percentages of normalized peak area ratios (Table 2). When a conventional GC profile (Fig. 1A) was transformed into its *I* graphic pattern (Fig. 1B), all the interfering peaks such as solvent, reagents, co-extracted and unidentified components were eliminated. The *I* graphic plot appeared to be more effective in showing quantitative distribution patterns of urinary AAs and CAs in qualitative *I* scale. All three normal urine specimens examined in this study displayed very normal urinary excretion patterns of AAs and CAs (Fig. 2). They looked similar qualitatively, but the differences between them in quantities of several prominent and minor peaks were readily detected by visual inspection. Glycine (peak 15) was the base peak and the second most prominent peak was hippuric acid (peak 49) in N-1 and N-3 while this order was reversed in N-2. When the quantitative data of the normal average (Table 2) were plotted in *I* scale of intermediately polar DB-17 column, the average *I* pattern in DB-5 column mode (Fig. 3A) was transformed into a visually different *I* pattern in DB-17 column mode (Fig. 3B). This suggested that *I* graphic analysis either in the two column modes might be conveniently applied for the graphic discrimination between samples.

The present simultaneous profiling analysis of urinary AAs and CAs was particularly useful for the biochemical diagnosis of amino acidurias such as PKU and MSUD. As depicted by the *I* pattern of a PKU patient examined in this study (Fig. 4-PKU), lactic acid (peak 8) was the base peak and the second most abundant peak was glycine (peak 15). The diagnostic phenylacetic acid (peak 10), phenyllactic acid (peak 46) and phenylalanine (peak 51) along with phenylpyruvic acid (peak 34) and 2-hydroxyphenylacetic acid (peak 45) were more recognizable compared with their respective peaks in the normal pattern. In the MSUD pattern (Fig. 4-MSUD), the diagnostic 2-hydroxyisovaleric acid (peak 16) was the most abundant, followed by leucine (peak 27) and valine (peak 22). 2-Ketoisocaproic acid (peak 5), 2-keto-3-methylvaleric acid (peak 6) and 2-hydroxy-3-methylvaleric acid (peak 24) were prominent. 3-Hydroxyisovaleric acid (peak 2) and 2-hydroxyisocaproic acid (peak 20) were readily recognizable as minor diagnostic CAs.

When the present method was applied to organic acidurias such as MMA and IVA, very characteristic *I* patterns were obtained displaying diagnostic CAs along with AAs (Fig. 5). Compared with the normal average pattern, extremely

Table 2
Amino acids and carboxylic acids found in urine samples from three normal subjects

No.	Compound	% Normalized peak area ratio (Mean ± S.D.)[a]			
		N-1	N-2	N-3	Normal average
1	3-Hydroxybutyric acid[b]	0.03 ± <0.01	0.5 ± <0.1	0.1 ± <0.1	0.3
2	3-Hydroxyisovaleric acid	–[c]	–	–	–
3	Pyruvic acid	–	–	–	–
4	2-Ketoisovaleric acid[d]	–	–	–	–
5	2-Ketoisocaproic acid[d]	–	–	–	–
6	2-Keto-3-methylvaleric acid[d]	–	–	–	–
7	Benzoic acid	2.1 ± 0.1	–	–	2.5
8	Lactic acid	7.2 ± 0.3	1.2 ± 0.1	0.1 ± <0.1	3.3
9	Glycolic acid	24.9 ± 0.2	5.8 ± 0.1	6.4 ± 0.2	14.7
10	Phenylacetic acid	–	–	–	–
11	Oxalic acid	0.6 ± <0.1	0.4 ± <0.1	0.1 ± <0.1	0.4
12	2-Hydroxybutyric acid	–	–	–	–
13	Alanine	44.0 ± 0.2	13.9 ± 0.2	15.1 ± 0.1	28.9
14	3-Hydroxypropionic acid	–	–	–	–
15	Glycine	100.0 ± <0.1	52.3 ± 0.6	100.0 ± <0.1	100.0
16	2-Hydroxyisovaleric acid	–	–	–	–
17	Isovalerylglycine	–	–	–	–
18	α-Aminobutyric acid	2.5 ± 0.1	1.7 ± 0.1	0.9 ± <0.1	2.0
19	Malonic acid	2.4 ± 0.1	1.4 ± 0.1	1.0 ± <0.1	1.9
20	2-Hydroxyisocaproic acid	–	–	–	–
21	Methylmalonic acid	0.4 ± <0.1	0.4 ± <0.1	1.7 ± 0.1	1.0
22	Valine	12.8 ± 0.6	2.5 ± 0.1	3.0 ± 0.1	7.2
23	β-Aminoisobutyric acid	34.2 ± 2.7	36.8 ± 0.4	11.7 ± 0.5	32.8
24	2-Hydroxy-3-methylvaleric acid	–	–	–	–
25	Isovalerylglycine	–	–	–	–
26	Ethylmalonic acid	3.8 ± <0.1	1.5 ± <0.1	1.2 ± 0.1	2.6
27	Leucine	6.6 ± 0.2	4.0 ± 0.1	2.4 ± 0.1	5.2
28	*allo*-Isoleucine	–	–	–	–
29	Isoleucine	3.1 ± 0.1	1.9 ± 0.1	1.0 ± <0.1	2.4
30	Succinic acid	39.2 ± 0.5	18.4 ± 0.4	8.6 ± 0.1	26.2
31	Methylsuccinic acid	4.8 ± 0.2	2.4 ± 0.1	1.3 ± 0.1	3.4
32	Proline	1.7 ± 0.1	1.3 ± 0.1	0.5 ± <0.1	1.4
33	Fumaric acid	4.2 ± 0.3	3.7 ± 0.2	1.3 ± <0.1	3.6
34	Phenylpyruvic acid	–	–	–	–
35	Tiglylglycine	–	–	–	–
36	Glutaric acid	4.6 ± 0.1	3.6 ± 0.1	1.5 ± 0.1	3.8
37	3-Methylglutaric acid	1.1 ± 0.1	1.4 ± 0.1	0.5 ± <0.1	1.2
38	Mandelic acid	–	–	–	–
39	3-Hydroxy-3-methylglutaric acid	2.3 ± 0.2	1.0 ± 0.1	0.8 ± <0.1	1.6
40	Pyroglutamic acid	5.5 ± 0.2	3.1 ± 0.2	1.1 ± <0.1	3.8
41	Adipic acid	5.7 ± 0.3	3.3 ± 0.2	0.9 ± <0.1	3.9
42	3-Methyladipic acid	4.4 ± <0.1	4.2 ± 0.2	1.9 ± 0.1	4.2
43	2-Ketoglutaric acid[d]	12.4 ± 0.7	5.3 ± 0.1	2.6 ± 0.1	8.0
44	Serine[e]	–	–	–	–
45	2-Hydroxyphenylacetic acid	–	–	–	–
46	Phenyllactic acid	–	–	–	–
47	3-Hydroxyphenylacetic acid	0.2 ± <0.1	1.7 ± 0.1	0.3 ± <0.1	0.9
48	Pimelic acid	1.8 ± <0.1	1.3 ± <0.1	0.7 ± <0.1	1.5
49	Hippuric acid	94.1 ± 1.3	100.0 ± <0.1	56.3 ± 0.6	99.2
50	4-Hydroxyphenylacetic acid	9.7 ± 0.3	6.9 ± 0.4	3.4 ± 0.1	7.9
51	Phenylalanine	7.2 ± 0.2	3.1 ± 0.1	1.0 ± <0.1	4.5
52	Malic acid	–	–	–	–
53	4-Hydroxybenzoic acid	0.8 ± <0.1	0.5 ± <0.1	0.1 ± <0.1	0.6
54	Citramalic acid	–	–	–	–
55	Phthalic acid	0.6 ± <0.1	–	2.0 ± 0.2	1.6
56	3-Indoleacetic acid	3.8 ± 0.1	1.6 ± 0.1	0.8 ± 0.1	2.5
57	Aspartic acid	7.6 ± 0.3	10.0 ± 0.2	4.2 ± 0.2	8.6
58	Suberic acid	–	–	–	–
59	4-Hydroxyproline[f]	–	–	–	–
60	2-Hydroxyglutaric acid	4.6 ± 0.1	3.7 ± 0.1	2.0 ± 0.1	4.1
61	Homovanillic acid	1.8 ± 0.1	1.4 ± 0.1	0.4 ± <0.1	1.4

Table 2 (*Continued*)

No.	Compound	% Normalized peak area ratio (Mean ± S.D.)[a]			
		N-1	N-2	N-3	Normal average
62	Vanillic acid	–	–	–	–
63	Azelaic acid	0.8 ± <0.1	0.6 ± <0.1	0.2 ± <0.1	0.7
64	Palmitic acid	7.0 ± 0.2	25.0 ± 0.1	3.1 ± 0.2	13.9
65	Glutamic acid	42.4 ± 0.3	17.2 ± 0.3	19.5 ± 0.5	31.3
66	Asparagine	0.7 ± <0.1	0.7 ± <0.1	0.2 ± <0.1	0.6
67	Ornithine	4.5 ± 0.2	2.8 ± 0.1	2.6 ± <0.1	3.9
68	*trans*-Aconitic acid	48.4 ± 1.0	25.9 ± 0.6	14.0 ± 0.3	35.0
69	Orotic acid	–	–	–	–
70	α-Aminoadipic acid	4.5 ± 0.1	6.0 ± 0.3	3.0 ± 0.2	5.4
71	4-Hydroxymandelic acid	1.3 ± <0.1	1.6 ± 0.1	0.6 ± <0.1	1.4
72	Tartaric acid	–	–	–	–
73	Glutamine	1.8 ± 0.1	3.9 ± 0.2	1.9 ± <0.1	3.0
74	Lysine	18.3 ± 0.4	13.5 ± 0.3	32.2 ± 0.3	25.3
75	Stearic acid	1.7 ± 0.1	1.1 ± <0.1	0.4 ± <0.1	1.2
76	α-Aminopimelic acid	–	–	–	–
77	Vanilmandelic acid	1.7 ± <0.1	1.1 ± 0.1	0.4 ± <0.1	1.3
78	2-Hydroxyhippuric acid	1.0 ± <0.1	0.4 ± <0.1	0.5 ± <0.1	0.7
79	4-Hydroxyphenyllactic acid	1.1 ± <0.1	0.6 ± <0.1	0.2 ± <0.1	0.7
80	Citric acid	19.0 ± 0.4	4.8 ± 0.2	13.1 ± 0.2	14.6
81	3-Indolelactic acid	–	–	–	–
82	Tyrosine	10.5 ± 0.3	10.1 ± 0.4	3.7 ± <0.1	9.6
83	Methylcitric acid[d]	–	–	–	–
84	Tryptophan	7.3 ± 0.2	3.1 ± 0.2	3.9 ± 0.1	5.7

[a] Measured on DB-5 column for normal ($n = 3$).
[b] Calculation based on mono-TBDMS peak.
[c] Not found.
[d] Sum of two isomer peaks.
[e] Calculation based on mono-EOC/di-TBDMS peak.
[f] Sum of mono-EOC/di-TBDMS and di-EOC/mono-TBDMS peaks.

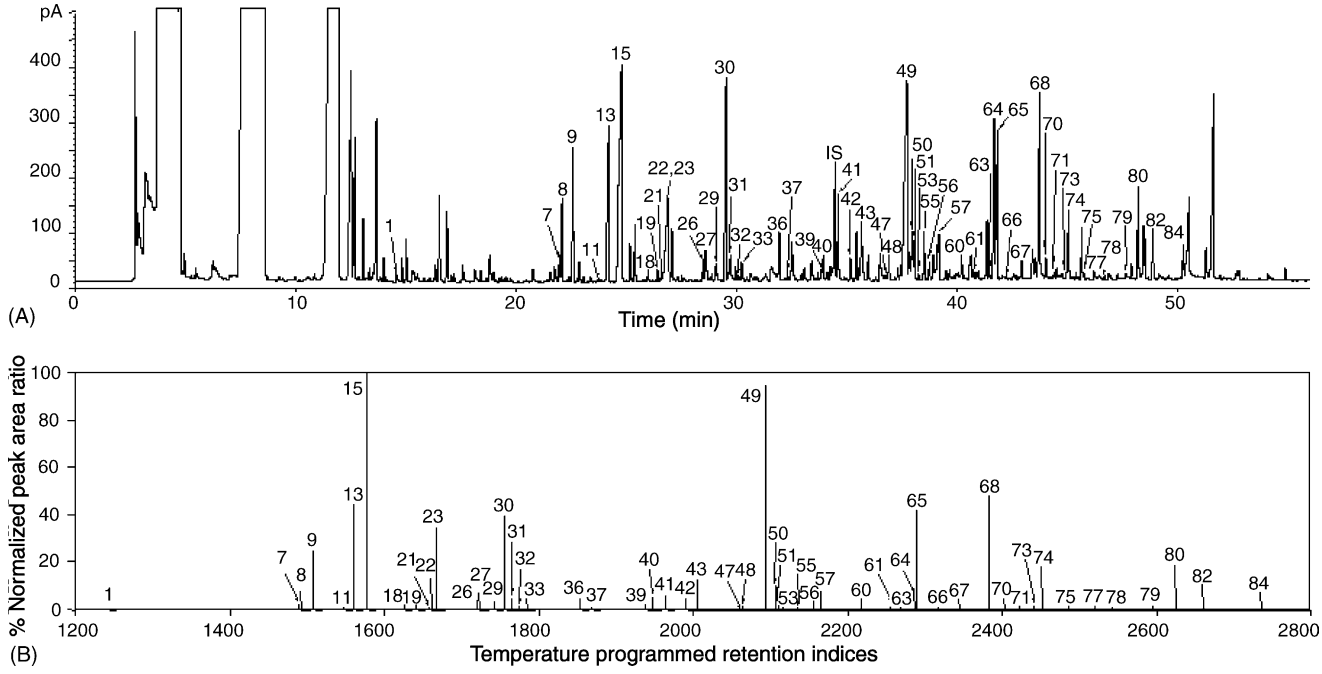

Fig. 1. Urinary (A) GC profile and (B) retention index pattern of amino acids and carboxylic acids in a normal control as EOC/MO/TBDMS derivatives separated on DB-5 column (30 m × 0.25 mm I.D., 0.25 μm film thickness). Helium flow rate was set to 1.0 ml/min with constant flow mode and samples (ca. 1.0 μl) were injected in the splitless mode with purge delay time of 42 s. The oven temperature was held at 80 °C for 2 min, then programmed to 290 °C at a rate of 4 °C/min. Peaks correspond to those in Table 2.

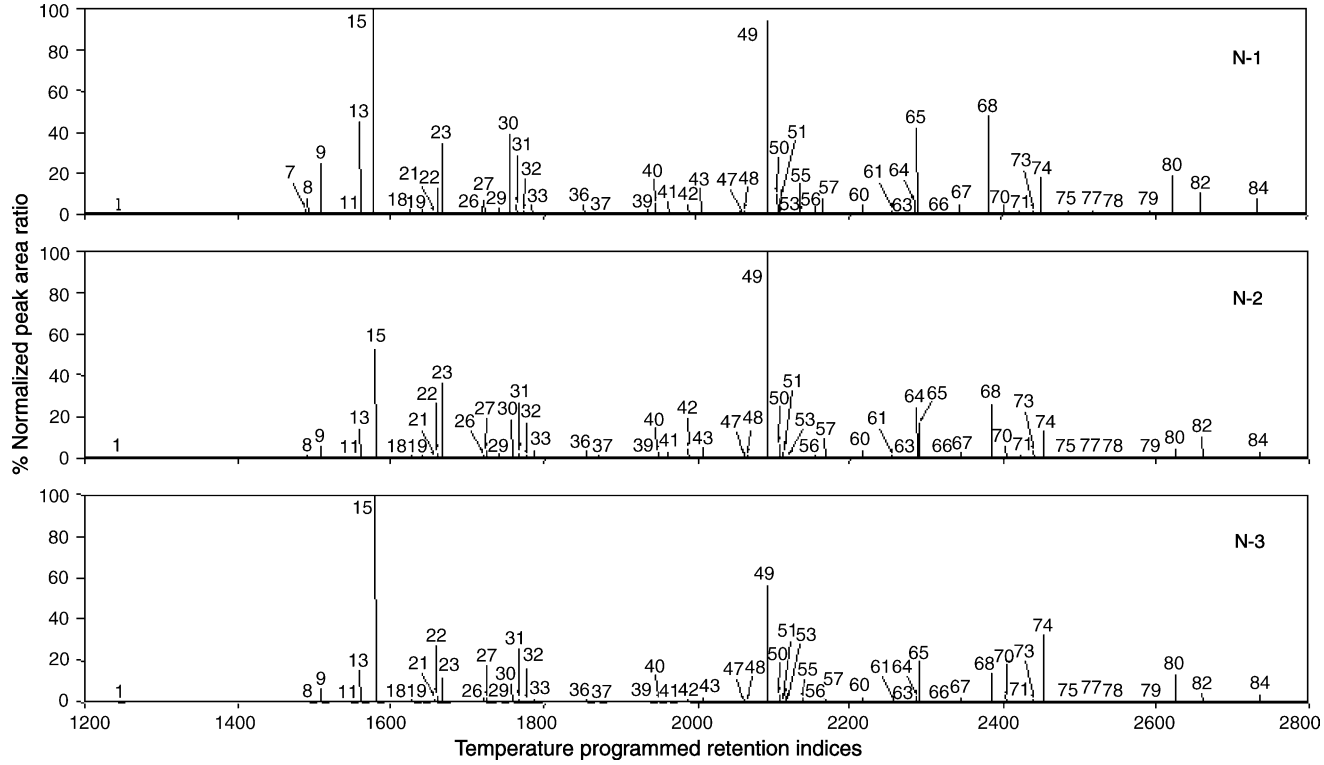

Fig. 2. Retention index spectra of urinary amino acids and carboxylic acids measured on DB-5 column as EOC/MO/TBDMS derivatives from three normal controls (N-1, N-2 and N-3). Peaks correspond to those in Table 2.

elevated level of methylmalonic acid (peak 21), a major marker for MMA, was observed in the MMA patient examined in this study (Fig. 5-MMA). The second most prominent metabolite was glycine (peak 15). From the IVA patient tested, a very simple pattern characteristic of IVA was obtained showing one big peak corresponding to the diagnostic isovalerylglycine (peak 25) with a few minor peaks (Fig. 5-IVA).

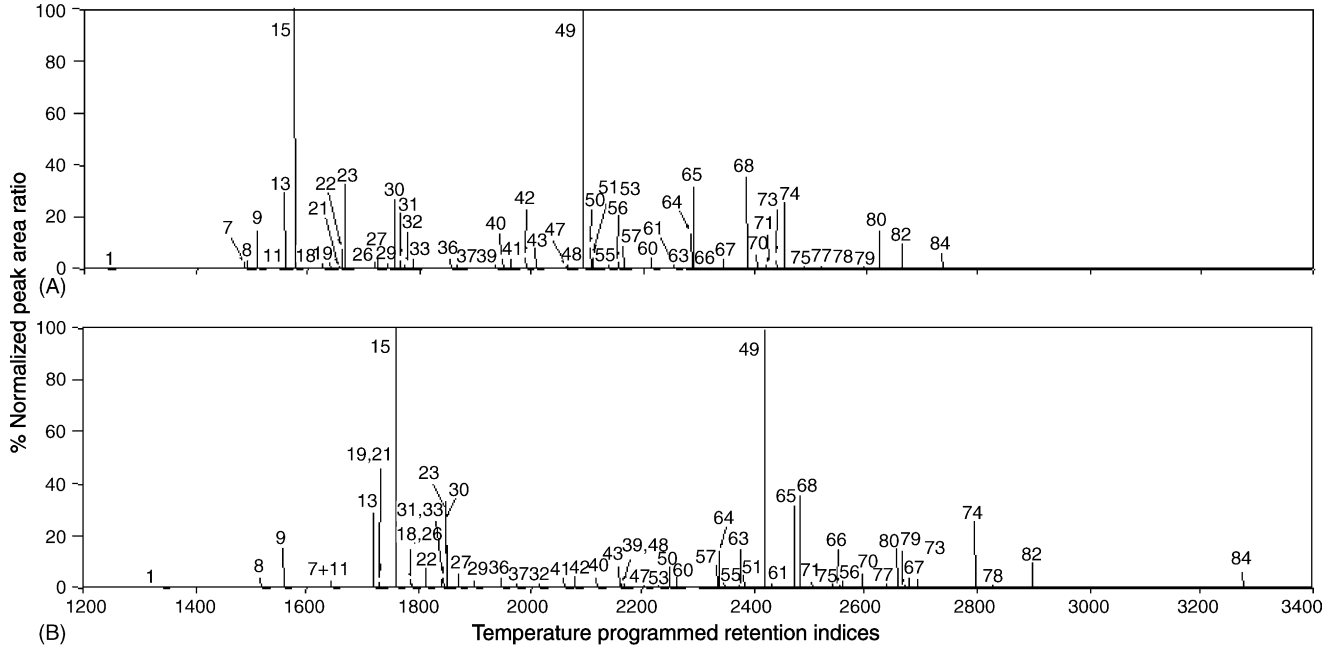

Fig. 3. Retention index spectra of urinary amino acids and carboxylic acids as EOC/MO/TBDMS derivatives from the normal average plotted on (A) retention index scale of DB-5 column and (B) retention index scale of DB-17 column. Peaks correspond to those in Table 2.

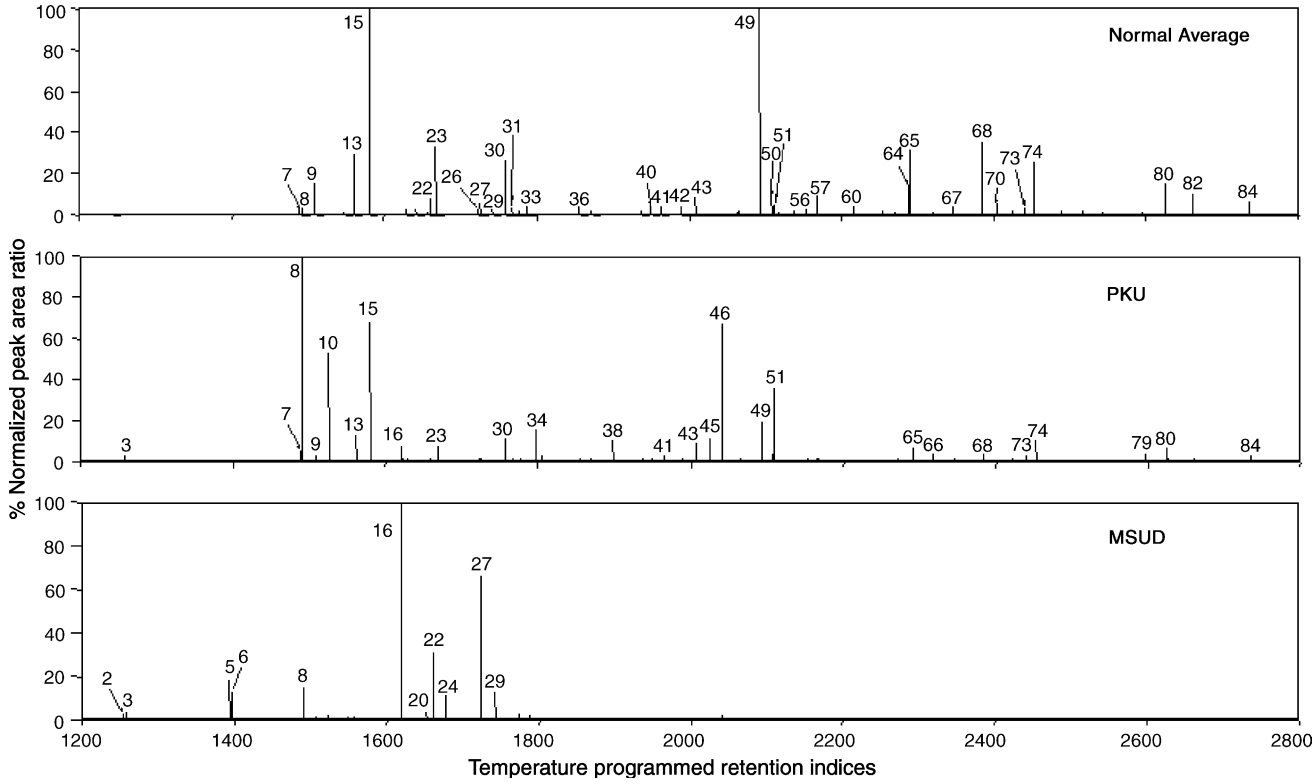

Fig. 4. Retention index spectra of urinary amino acids and carboxylic acids measured on DB-5 column as EOC/MO/TBDMS derivatives from normal average (top), a phenylketonuria patient (PKU) and a maple syrup urine disease patient (MSUD). Peaks correspond to those in Table 2.

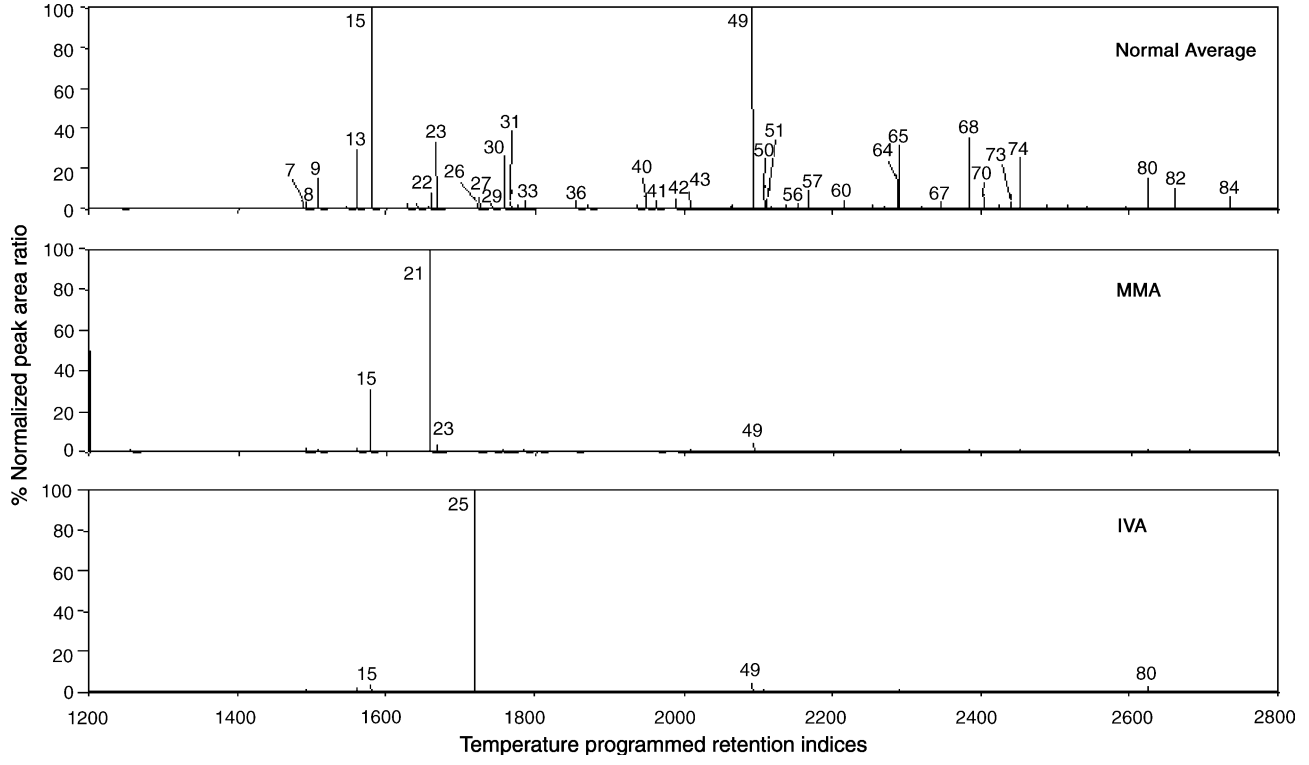

Fig. 5. Retention index spectra of urinary amino acids and carboxylic acids measured on DB-5 column as EOC/MO/TBDMS derivatives from normal average (top), a methylmalonic aciduria patient (MMA) and an isovaleric aciduria patient (IVA). Peaks correspond to those in Table 2.

4. Conclusions

The present *I* analysis of urinary AAs and CAs as EOC/MO/TBDMS derivatives in a single run generated visually distinctive patterns of line graphs to enable rapid detection of quantitative differences among urine samples. It might be useful as a graphic recognition tool in the detection of abnormality in the metabolism. The present GC-*I* and GC–MS databases will continue to be expanded to include other AAs and CAs to be more versatile in routine screening for AAs and CAs in aqueous samples. An extension of the present method is under way for the visual pattern recognition of other diverse IMDs.

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