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Sequential ethoxycarbonylation, methoximation and *tert*-butyldimethylsilylation for simultaneous determination of amino acids and carboxylic acids by dual-column gas chromatography

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Abstract

Amino acids (AAs) in alkaline solution were first ethoxycarbonylated with subsequent methoximation of keto acids (KAs). After acidification and solid-phase extraction, *tert*-butyldimethylsilylation was performed for direct analysis by gas chromatography (GC) on dual-columns with different polarities, which provided simultaneous separation of multiple amino acids, carboxylic acids (CAs) and keto acids, facilitating accurate peak confirmation based on matching with retention index sets characteristic of each analyte. The present method was linear ($r^2 \geq 0.9955$) with good precision (0.1–9.4%) and accuracy (−8.6 to 9.9%), allowing simultaneous screening for diagnostic amino acids along with carboxylic acids and keto acids in urine from a phenylketonuria patient.

Keywords: Derivatization, GC; Ethoxycarbonylation; Methoximation; *tert*-Butyldimethylsilylation; Retention index matching; Amino acids; Carboxylic acids; Keto acids

1. Introduction

Amino acids (AAs) and carboxylic acids (CAs) including keto acids (KAs) constitute two major chemical groups among the structurally diverse bioactive analytes of current interest in chemical analyses. More than 600 nonprotein amino acids (NPAAs) were identified from plants, fungi, and animal sources in their free form along with 20 protein amino acids [1]. AAs perform important biological functions such as biocatalysts, nutrients, neurotransmitters, and precursors for biosynthesis of cell constituents in cellular metabolism. Some NPAAs are acutely toxic to living organisms [1,2] and others play vital roles in chronic neurodegenerative disorders such as Parkinson's and Alzheimer's diseases [3,4]. In contrast, more than 200 CAs (including KAs) occur in biosamples as the end products or intermediates synthesized from various metabolic pathways, and provide biochemical information on cellular metabolism [5–10]. Most endogenous CAs are major metabolites of AAs while some AAs occur as intermediates in metabolism. Thus, the compositions of AAs and CAs in biosamples are closely interrelated. Therefore, in recent years, metabolomics for the simultaneous metabolic profiling of multiple AAs and CAs in biosamples has gained interest in clinical monitoring and biochemical diagnosis of abnormal states [11–16].

Multicomponent profiling analysis is best suited for the systematic screening to detect new and unexpected AAs, CAs and KAs, and also to determine changes in the ratios among them. Moreover, it has advantages of sample high-throughput analysis at lower cost compared with separate analyses of the two groups. Profiling analyses have mostly been conducted by high-resolution capillary gas chromatography (GC) combined with mass spectrometry (MS) because of their inherent high resolving power, high sensitivity and positive peak confirmation. The GC method alone, employing dual-columns of different polarities, can provide positive peak identification based on retention index (*I*) matching [17–19]. Accurate GC and GC-MS analysis, however, requires one or more appropriate derivatization procedures to block all active protons and labile keto groups present in both AAs and CAs [20]. Alkylsilylation [11,12,14–16,21] and alkoxycarbonyl (AOC)

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esterification [13,22,23] methods have been frequently used for conversion of AAs to volatile derivatives. However, it was preferable to convert non-extractable zwitterionic AAs to solvent-extractable CA forms directly in aqueous sample matrices, followed by volatile derivatization of carboxylic acid functions. As the closest approach to this goal, the aqueous AOC reaction with subsequent esterification [24–26] or alkylsilylation [17] have been conveniently used. The *N*(*O*,*S*)-isobutoxycarbonyl (isoBOC) reaction combined with *tert*-butyldimethylsilyl (TBDMS) reaction was useful for our AA profiling analyses of plant seeds [27,28] and wine [29] as reported elsewhere. This direct isoBOC reaction in alkaline aqueous phase, however, required large excess amounts of isobutyl chloroformate due to instability in alkaline solution. And, the isoBOC group was too bulky for the multifunctional AAs. These problems could be readily overcome by employing solvent extractive two-phase AOC reaction with a smaller alkyl chloroformate such as ethyl chloroformate (ECF) in a dense organic solvent phase as reported elsewhere [30].

Multifunctional CAs are preferentially derivatized through alkylsilylation, which is applicable to virtually all the active protons [31]. In recent years, formation of TBDMS derivatives has been widely used because of their superior GC and MS properties and relatively high stability toward hydrolysis [19,32–34] Most KAs [30] are unstable, and easily decomposed, dimerized or decarboxylated during alkylsilylation [20]. Therefore, keto groups in KAs are normally protected to avoid enolization prior to silylation by stabilization, mainly as oxime- [10], methoxime (MO)- [19,35], ethoxime- [10] or benzyloxime-derivatives [9]. The combined MO-TBDMS reactions were suitable for profiling analysis of urinary CAs and KAs in our previous uterine cervical cancer study [19].

There was an attempt to convert AAs and CAs simultaneously to *N*(*O*,*S*)-ethoxycarbonyl (EOC) ethyl esters and ethyl esters, respectively in a single treatment with ECF [13]. This rapid one-step method, however, could not derivatize alcoholic hydroxyl- and keto-groups, resulting in peak tailing and loss of KAs, respectively. Moreover, in aqueous phase, the ethylation of carboxylic acid groups is disfavored, and the stability of ethyl esters is low. These problems could be circumvented by conducting methoximation of keto groups, either before or after the solvent extractive two-phase EOC reaction, and by converting both carboxylic acid- and hydroxyl-groups to TBDMS derivatives. This might be more suitable for a wider range of AAs, CAs and KAs. However, attempts have been rarely made to apply this approach to the simultaneous profiling analysis of the three groups to date.

As a new attempt in this study, ethoxycarbonylation, methoximation, and *tert*-butyldimethylsilylation were optimally combined and validated for the simultaneous assay of the clinically important 36 AAs (including NPAAs), 36 CAs and nine KAs from aqueous samples. The EOC reaction was conducted in two-phase extractive mode, followed by MO reaction in the same alkaline aqueous medium. The effects of the reaction conditions and their orders on the recovery rates were examined. After acidification, the aqueous phase was subjected to solid-phase extraction (SPE), using Chromosorb P as adsorbent in normal phase partition mode [17–19,27–29,33]. On the recovered EOC derivatives of AAs, and MO derivatives of KAs along with intact CAs, final TBDMS reaction was conducted for the direct analysis by dual-columns with different polarities for the accurate peak quantitation and for the peak confirmation based on GC-*I* matching. The structures of 44 AAs as their EOC/TBDMS derivatives that are new to the literature were confirmed by GC-MS.

2. Experimental

2.1. Reagents

The 44 AAs (including 25 NPAAs), 36 CAs and nine 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_{10}–C_{36}, 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 SPE column in normal phase partition mode.

2.2. Preparation of standard solutions

Each standard stock solution of AAs, CAs and KAs were 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. The 81 analytes (36 AAs, 36 CAs and nine KAs) examined in this study were divided to prepare four Group calibration samples. Each Group

calibration samples were prepared at three, four, or five different concentration ranges from 0.1 to 36.0 μg/ml depending on analytes by mixing appropriate aliquots of each working solution and diluting in water. Hydrocarbon solution containing *n*-hydrocarbons (C_{10}–C_{36}, 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 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 80 °C for 2 min and then programmed to 290 °C at a rate of 4 °C/min. A standard solution of *n*-hydrocarbons (C_{10}–C_{36}, even numbers only) in toluene was co-injected with samples to compute temperature-programmed *I* values by linear interpolation between the retention times of adjacent hydrocarbon standards. A database of reference *I* library using *I* sets of 36 AAs, 36 CAs and nine KAs measured on the dual-columns was built in the GC computer system. The GC analyses for the method optimization and validation were conducted employing a single DB-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness) under the same GC 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 reaction mode

A mixed standard solution containing 13 AAs, seven CAs and three KAs at varied amounts (1.0−8.0 μg/ml) depending on analytes, and 3,4-dimethoxybenzoic acid as IS (2.0 μg/ml) was used for the reaction optimization tests. Two-phase EOC reaction of AAs was conducted in one-step by vortex mixing 1.0 ml of alkaline mixed standard solution (adjusted to pH ≥ 12 with 5.0 M sodium hydroxide) with ECF (20 μl) present in dichloromethane phase (1.0 ml). After EOC reaction, the aqueous phase was adjusted to pH ca. 13 and then MO reaction of keto groups was performed with methoxyamine hydrochloride (10.0 mg) at 60 °C for 1 h, followed by washing with diethyl ether (3 ml × 2). The aqueous solution was then acidified (pH ≤ 2.0) with 10.0% sulfuric acid, and saturated with sodium chloride. It was then subjected to 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 containing EOC derivatives of AAs and intact CAs including MO derivatives of KAs, and the mixture was heated at 60 °C for 1 h to form TBDMS derivatives for the direct GC and GC-MS analysis.

2.5. Sequential MO/EOC/TBDMS reactions mode

The MO reaction of KAs was conducted prior to EOC reaction of AAs, followed by washing and solvent extraction with subsequent TBDMS derivatization in the same manner as above.

2.6. Validation of EOC/MO/SPE/TBDMS method for assay of amino acids, carboxylic acids and keto acids

Each calibration sample was added with IS at 1.0 μg/ml and then subjected to the sequential EOC/MO reaction with subsequent washing, acidification and saturation according to the preceding procedures. The acidified aqueous solution was subjected to SPE by loading onto an activated Chromosorb P column [27–29,34], 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. The quantitative calculations of all analytes were based on the peak area ratios relative to that of IS. And the peak area ratios of *syn* and *anti* geometric isomers for each KA were summed up. Linearity was tested by least-squares regression analysis on the peak area ratios against increasing concentration ratios to plot calibration curves. The limit of detection (LOD) of each analyte was estimated based on the lowest concentration giving a signal taken as the sum of the mean blank signal plus three times the standard deviation of the blank signal obtained via three blank measurements. Precision expressed as percentage of relative standard

deviation (%R.S.D.) and accuracy as percentage of relative error (RE%) of the method were determined from calibration samples at two different concentrations (0.5–8.0 μg/ml) varied depending on analytes in triplicate. Recovery tests were performed using CAs only because they could be analyzed as TBDMS derivatives prepared directly without performing the EOC and MO reactions. The recovery rates were assessed by comparing the percentages of peak area ratios of extracted samples prepared by the EOC/MO/SPE/TBDMS mode to those of non-extracted counterparts (representing 100% recovery) prepared by direct TBDMS derivatization in dry neat form at the same nominal concentrations.

2.7. Sample preparation for urinary amino acids, carboxylic acids and keto acids

Aliquots (1.56 ml) of spot urine sample (corresponding to 0.25 mg creatinine) collected from a phenylketonuria (PKU) female patient (aged 2.2 years) were adjusted to pH ≥ 12 (with 5.0 M sodium hydroxide) after addition of IS (10.0 μg). The samples were then subjected to the sequential EOC/MO/SPE/TBDMS procedures as described in the validation section, followed by dual-column GC analyses.

3. Results and discussion

3.1. Sequential EOC/MO/TBDMS reactions

From a number of preliminary experiments, dense dichloromethane (1 ml) was chosen as the optimal solvent phase, which contained 20 μl volume of ECF reagent as the optimal amount for the two-phase extractive EOC reaction of more than 33 AAs (0.10–32 μg/ml) present in alkaline aqueous phase (pH ≥ 12) containing CAs and KAs. The EOC reaction proceeded rapidly to completion at room temperature in a few min by vortex mixing (5 min). All amino-, thiol- and imidazole-groups in AAs were blocked and converted into their *N*(*S*)-EOC derivatives, thus transforming zwitterioinic AAs into solvent-extractable CA forms. Phenolic hydroxyl groups in tyrosine and in all CAs were not transformed under this pH condition, but about 95 and 60% of the hydroxyl functions in 5-hydroxytrypthophan and 4-hydroxyproline were converted into *O*-EOC derivatives, respectively.

The subsequent MO reaction conducted in the same aqueous phase at optimal conditions (pH ≥ 13; 10.0 mg methoxyamine·HCl; 60 °C for 1 h) transformed all carbonyl groups in labile KAs studied into stable MO derivatives. The effect of EOC and MO reaction order on the overall

Table 1
Effect of reaction order on recovery rates of 13 amino acids, seven carboxylic acids and three keto acids

Analyte (μg/ml)	Mean peak area ratio ± S.D. (%R.S.D., $n = 4$)[a]		Comparison, Q[b]
	MO/EOC mode	EOC/MO mode	
3-Hydroxybutyric acid (3.0)[c]	0.543 ± 0.020 (3.8)	1.057 ± 0.007 (0.7)	1.9
Pyruvic acid (2.0)	0.436 ± 0.038 (8.7)	0.833 ± 0.060 (7.1)	1.9
Acetoacetic acid (3.0)[d]	0.055 ± 0.002 (2.7)	0.123 ± 0.002 (1.8)	2.2
Lactic acid (1.0)	0.339 ± 0.029 (8.6)	0.546 ± 0.025 (4.6)	1.6
Alanine (1.0)	0.568 ± 0.022 (3.9)	0.759 ± 0.023 (3.0)	1.3
Propionylglycine (6.0)	0.164 ± 0.013 (7.8)	0.699 ± 0.011 (1.6)	4.3
Norvaline (1.0)	0.748 ± 0.025 (3.4)	0.926 ± 0.026 (2.8)	1.2
Leucine (1.0)	0.682 ± 0.023 (3.4)	0.842 ± 0.024 (2.9)	1.2
Isoleucine (1.0)	0.811 ± 0.027 (3.4)	0.983 ± 0.030 (3.1)	1.2
Proline (1.0)	1.416 ± 0.011 (0.8)	1.425 ± 0.003 (0.2)	1.0
Fumaric acid (1.0)	0.896 ± 0.004 (0.4)	1.632 ± 0.003 (0.2)	1.8
Pipecolic acid (1.0)	1.315 ± 0.024 (1.9)	1.452 ± 0.017 (1.2)	1.1
2-Ketoglutaric acid (3.0)[d]	1.308 ± 0.009 (0.7)	1.311 ± 0.010 (0.7)	1.0
3-Indoleacetic acid (1.0)	0.633 ± 0.015 (2.4)	1.311 ± 0.014 (1.1)	2.1
Aspartic acid (1.0)	0.478 ± 0.022 (4.6)	0.593 ± 0.027 (4.6)	1.2
Glutamic acid (1.0)	0.129 ± 0.012 (9.0)	0.186 ± 0.015 (7.9)	1.4
Asparagine (6.0)	0.042 ± 0.002 (4.6)	0.047 ± 0.002 (3.5)	1.1
3-Indolebutyric acid (3.0)	1.412 ± 0.037 (2.6)	2.711 ± 0.027 (1.0)	1.9
Lysine (2.0)	0.049 ± 0.005 (9.4)	0.122 ± 0.008 (6.8)	2.5
4-Hydroxyphenyllactic acid (2.0)	0.478 ± 0.040 (8.5)	1.089 ± 0.057 (5.2)	2.3
Tyrosine (2.0)	0.197 ± 0.018 (9.3)	0.265 ± 0.020 (7.7)	1.3
Tryptophan (2.0)	0.336 ± 0.027 (8.0)	0.414 ± 0.031 (7.6)	1.2
5-Methoxytryptophan (8.0)	1.057 ± 0.090 (8.5)	1.155 ± 0.096 (8.3)	1.1

[a] Mean peak area ratio relative to that of IS (3,4-dimethoxybenzoic acid at 2.0 μg/ml) measured on DB-5 column; S.D.: standard deviation; R.S.D.: relative standard deviation.
[b] *Q*, reaction quotient corresponding to mean peak area ratio in EOC/MO mode relative to that in MO/EOC mode.
[c] Calculation based on mono-TBDMS peak in triplicate.
[d] Sum of two isomer peaks.

yields was tested employing a test mixture of 13 AAs, seven CAs and three KAs at varied amounts (1.0–8.0 μg/ml), and expressed as mean peak area ratios (Table 1). The EOC/MO mode yielded higher responses (reaction quotient ≥1.0) with better precision (%R.S.D. ≤ 8.3) for all analytes examined than the MO/EOC mode. A huge interfering reagent peak derived from the EOC reaction between methoxyamine and ECF was observed when the MO reaction was performed prior to the EOC reaction. Moreover, the EOC/MO mode was easier to perform than the MO/EOC mode in practice. Therefore, the EOC/MO mode was selected as the optimal reaction order in this study. During the MO reaction in strongly alkaline condition, sulfur-containing AAs such as methionine, cysteine and homocysteine, were partially decomposed, and thus, accurate analysis of these three AAs required omitting the MO step.

The next SPE procedure in normal phase partition mode, using hydrophilic Chromosorb P as the adsorbent, and volatile diethyl ether and ethyl acetate (2 ml each) as the optimal sequential eluting solvents, permitted more rapid recovery of the structurally diverse *N*(*O*,*S*)-EOC derivatives of AAs and the MO derivatives of KAs along with intact CAs within two steps, compared to conventional solvent extraction. The two-step solid-phase extraction providing solvent eluates in very dried forms (moisture free) took only 40 min including solvent evaporation. This SPE procedure had been already proved to be efficient for the separate analyses of AAs [17,27–29] and CAs [18,19,33] in our previous studies.

Upon the final TBDMS reaction, the remaining active protons in carboxylic acid-, phenolic hydroxyl- and alcoholic hydroxyl-groups were all converted into respective

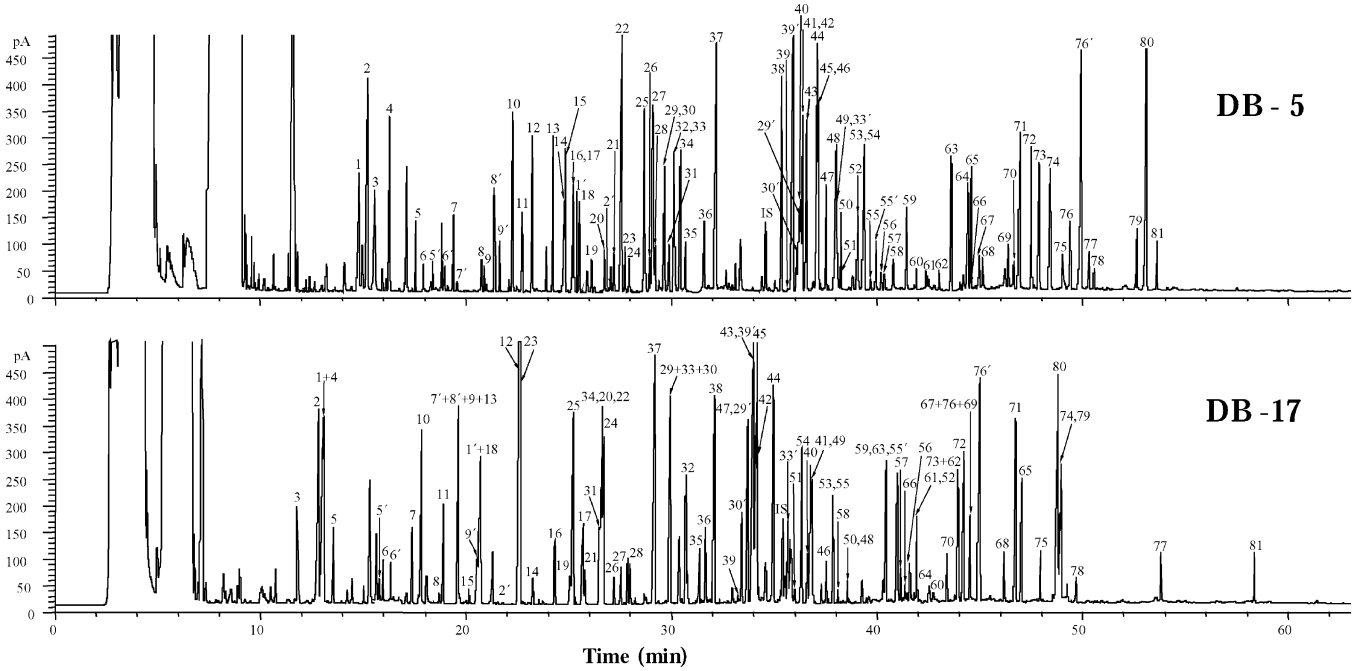

Fig. 1. Dual-profiles of 36 amino acids and 45 carboxylic acids (including nine keto acids) as EOC/MO/TBDMS derivatives separated on DB-5 and DB-17 dual-column (both 30 m × 0.25 mm i.d., 0.25 μm film thickness) system. Helium flow rate was set to 1.0 ml/min with constant flow mode and samples (ca 1.0 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: (1) 3-hydroxybutyric acid (mono-TBDMS); (2) 3-hydroxyisovaleric acid (mono-TBDMS); (3) pyruvic acid; (4) caproic acid; (5) 2-ketoisovaleric acid-1; (6) acetoacetic acid-1; (5′) 2-ketoisovaleric acid-2; (6′) acetoacetic acid-2; (7) 2-keto-3-methylvaleric acid-1; (7′) 2-keto-3-methylvaleric acid-1; (8) 2-ketocaproic acid-1; (9) glyoxylic acid-1; (8′) 2-ketocaproic acid-2; (9′) glyoxylic acid-2; (10) lactic acid; (11) glycolic acid; (12) phenylacetic acid; (13) 2-hydroxybutyric acid; (14) alanine; (15) 3-hydroxypropionic acid; (16) glycine; (17) propionylglycine; (1′) 3-hydroxybutyric acid (di-TBDMS); (18) 2-hydroxyisovaleric acid; (19) α-aminobutyric acid; (20) β-alanine; (2′) 3-hydroxyisovaleric acid (di-TBDMS); (21) valine; (22) β-aminoisobutyric acid; (23) 2-hydroxy-3-methylvaleric acid; (24) norvaline; (25) ethylmalonic acid; (26) leucine; (27) alloisoleucine; (28) isoleucine; (29) threonine (mono-EOC/mono-TBDMS); (30) serine (mono-EOC/mono-TBDMS); (31) methylsuccinic acid; (32) proline; (33) γ-aminobutyric acid (mono-EOC/mono-TBDMS); (34) fumaric acid; (35) phenylpyruvic acid; (36) pipecolic acid; (37) glutaric acid; (38) 3-methyladipic acid; (39) 2-ketoglutaric acid-1; (39′) 2-ketoglutaric acid-2; (30′) serine (mono-EOC/di-TBDMS); (29′) threonine (mono-EOC/di-TBDMS); (40) 2-phenylglycine; (41) 2-indolecarboxylic acid; (42) 2-hydroxyphenylacetic acid; (43) phenyllactic acid; (44) 3-hydroxypheylacetic acid; (45) pimelic acid; (46) *o*-aminobenzoic acid; (47) myristic acid; (48) 2,3-diaminopropionic acid; (49) *N*-acetylaspartic acid; (33′) γ-aminobutyric acid (mono-EOC/di-TBDMS); (50) phenylalanine; (51) homoserine; (52) 3-indoleacetic acid; (53) aspartic acid; (54) suberic acid; (55) 4-hydroxyproline (mono-EOC/di-TBDMS); (55′) 4-hydroxyproline (di-EOC/mono-TBDMS); (56) *m*-aminobenzoic acid; (57) homophenylalanine; (58) palmitoleic acid; (59) glutamic acid; (60) *p*-aminobenzoic acid; (61) asparagine; (62) ornithine; (63) sebacic acid; (64) α-aminoadipic acid; (65) 3-indolebutyric acid; (66) 4-hydroxyphenylpyruvic acid; (67) glutamine; (68) lysine; (69) α-aminopimelic acid; (70) vanillylmandelic acid; (71) 2-hydroxyhippuric acid; (72) dodecanedioic acid; (73) 4-hydroxyphenyllactic acid; (74) 3-indolelactic acid; (75) tyrosine; (76) methylcitric acid-1; (76′) methylcitric acid-2; (77) tryptophan; (78) 4-carboxyphenylglycine; (79) erucic acid; (80) behenic acid; (81) 5-methoxytryptophan; (IS) 3,4-dimethoxybenzoic acid.

Table 2
Mass spectral data of amino acids as *N*(*O*,*S*)-ethoxycarbonlyated *tert*-butyldimethylsilyl derivatives

Amino acid	Mass spectral data set[a]									
	M_r	$[M-15]$	$[M-57]$	$[M-73]$	$[M-85]$	$[M-103]$	$[M-131]$	$[M-146]$	$[M-159]$	Other prominent ions
Alanine	275	260 (3)	218 (100)	202 (3)	190 (54)	172 (13)	144 (22)	129 (8)	116 (42)	
Glycine	261	246 (3)	204 (100)	188 (1)	176 (81)	158 (24)	130 (11)	115 (15)	102 (10)	
α-Aminobutyric acid	289	274 (2)	232 (100)	216 (2)	204 (46)	186 (3)	158 (2)	143 (6)	130 (60)	
β-Alanine	275	260 (2)	218 (100)	202 (0)	190 (10)	172 (18)	146 (7)	129 (37)	116 (8)	
Valine	303	288 (3)	246 (100)	230 (3)	218 (33)	200 (8)	174 (3)	157 (7)	144 (61)	
β-Aminoisobutyric acid	289	274 (2)	232 (100)	216 (0)	204 (4)	186 (9)	158 (6)	143 (37)	130 (6)	
Norvaline	303	288 (2)	246 (100)	230 (3)	218 (39)	200 (4)	172 (0)	157 (10)	144 (72)	
Leucine	317	302 (3)	260 (100)	244 (2)	232 (31)	214 (11)	186 (1)	171 (15)	158 (62)	
Alloisoleucine	317	302 (2)	260 (100)	244 (3)	232 (25)	214 (2)	186 (0)	171 (6)	158 (72)	
Isoleucine	317	302 (3)	260 (100)	244 (3)	232 (25)	214 (11)	186 (1)	171 (10)	158 (66)	
Threonine-1	305	290 (0)	248 (39)	232 (3)	220 (4)	202 (38)	174 (3)	159 (3)	146 (13)	261 (34), 230 (63), 129 (52), 75 (100)
Serine-1	291	261 (7)	234 (61)	218 (8)	206 (9)	188 (70)	160 (10)	145 (7)	132 (23)	216 (100), 75 (96), 116 (52)
Proline	301	286 (3)	244 (88)	228 (3)	216 (36)	198 (0)	172 (6)	155 (0)	142 (100)	
Norleucine	317	302 (2)	260 (100)	244 (2)	232 (33)	214 (7)	186 (0)	171 (14)	158 (77)	
γ-Aminobutyric acid-1	289	274 (3)	232 (100)	216 (1)	204 (0)	186 (22)	158 (4)	143 (78)	130 (4)	142 (48)
Pipecolic acid	315	300 (2)	258 (35)	242 (2)	230 (7)	212 (0)	184 (2)	169 (0)	156 (100)	
Pyroglutamic acid[b]	357	342 (8)	300 (100)	284 (0)	272 (89)	254 (0)	226 (0)	211 (0)	198 (24)	147 (53)
Methionine	335	320 (2)	278 (100)	262 (16)	250 (10)	232 (53)	204 (4)	189 (9)	176 (29)	261 (57), 129 (46), 75 (74), 61 (92)
Serine-2[c]	405	390 (2)	348 (54)	332 (0)	320 (1)	302 (50)	274 (11)	259 (0)	246 (8)	216 (46), 73 (100)
Threonine-2[c]	419	404 (1)	362 (36)	344 (2)	334 (1)	316 (28)	288 (9)	273 (2)	260 (7)	230 (33), 73 (100), 159 (75)
2-Phenylglycine	337	322 (4)	280 (100)	264 (18)	252 (14)	234 (8)	206 (9)	191 (2)	178 (62)	220 (36), 73 (60)
o-Aminobenzoic acid	323	308 (2)	266 (100)	250 (0)	238 (7)	220 (41)	192 (3)	177 (3)	164 (2)	194 (47), 146 (78)
2,3-Diaminopropionic acid	362	347 (0)	305 (39)	289 (0)	277 (0)	259 (0)	231 (15)	216 (100)	203 (12)	129 (71)
γ-Aminobutyric acid-2[c]	403	388 (2)	346 (52)	330 (1)	318 (0)	300 (0)	272 (1)	257 (0)	244 (1)	201 (100)
Phenylalanine	351	336 (3)	294 (100)	278 (2)	266 (5)	248 (17)	220 (0)	205 (66)	192 (35)	100 (30)
Homoserine	419	404 (3)	362 (100)	346 (0)	334 (0)	316 (28)	288 (27)	273 (0)	260 (12)	
Cysteine	379	364 (1)	322 (100)	306 (4)	294 (3)	276 (19)	248 (10)	233 (0)	220 (49)	204 (30), 188 (42), 204 (30)
Aspartic acid	433	418 (2)	376 (100)	360 (1)	348 (1)	330 (16)	302 (1)	287 (39)	274 (11)	
4-Hydroxyproline-1[c]	431	416 (3)	374 (100)	358 (2)	346 (0)	328 (0)	300 (0)	285 (0)	272 (46)	
4-Hydroxyproline-2[d]	389	374 (19)	332 (41)	316 (0)	304 (0)	286 (0)	258 (0)	243 (0)	230 (1)	242 (30), 170 (38), 140 (100)
m-Aminobenzoic acid	323	308 (2)	266 (100)	250 (1)	238 (2)	220 (45)	192 (11)	177 (2)	164 (6)	
Homophenylalanine	365	350 (3)	308 (100)	292 (2)	280 (2)	262 (17)	234 (0)	219 (6)	206 (8)	261 (56), 117 (38), 91 (79), 75 (35)
Homocysteine	393	378 (2)	336 (60)	320 (2)	308 (0)	290 (7)	262 (5)	247 (0)	234 (15)	128 (100)
Glutamic acid	447	432 (3)	390 (100)	374 (1)	362 (3)	344 (16)	316 (0)	301 (0)	288 (33)	186 (45)
p-Aminobenzoic acid	323	308 (2)	266 (100)	250 (0)	238 (2)	220 (24)	192 (16)	177 (0)	164 (6)	146 (31)
Asparagine	432	417 (4)	375 (100)	359 (0)	347 (0)	329 (39)	301 (0)	286 (30)	273 (2)	
Ornithine	390	375 (0)	333 (12)	317 (0)	305 (0)	287 (26)	259 (0)	244 (0)	231 (0)	142 (100)
α-Aminoadipic acid	461	446 (3)	404 (100)	388 (0)	376 (4)	358 (28)	330 (7)	315 (0)	302 (47)	200 (33), 129 (30)
Glutamine	446	431 (4)	389 (100)	373 (0)	361 (0)	343 (56)	315 (0)	300 (0)	287 (23)	
Lysine	404	389 (0)	347 (17)	331 (0)	319 (0)	301 (31)	273 (0)	258 (0)	245 (0)	156 (100)
Baclofen	399	384 (2)	342 (100)	326 (0)	314 (0)	296 (10)	268 (3)	253 (52)	240 (4)	241 (59), 102 (38), 75 (81)
Histidine	413	398 (0)	356 (64)	340 (0)	328 (0)	310 (80)	282 (0)	267 (60)	254 (100)	238 (43), 154 (36), 130 (41)
α-Aminopimelic acid	475	460 (3)	418 (100)	402 (0)	390 (2)	372 (36)	344 (12)	329 (0)	316 (33)	184 (50)
Tyrosine	481	466 (0)	424 (10)	408 (0)	396 (0)	378 (10)	350 (0)	335 (40)	322 (2)	221 (100)
Tryptophan	390	375 (0)	333 (5)	317 (0)	305 (0)	287 (2)	259 (0)	244 (9)	231 (2)	130 (100)
4-Carboxyphenylglycine	495	480 (4)	438 (100)	422 (1)	410 (0)	392 (32)	364 (0)	349 (0)	336 (14)	73 (44)
5-Methoxytryptophan	420	405 (0)	363 (2)	347 (0)	335 (0)	317 (1)	289 (0)	274 (3)	261 (1)	160 (100)
5-Hydroxytryptophan-1[c]	520	505 (0)	463 (2)	447 (0)	435 (0)	417 (3)	389 (0)	374 (2)	361 (0)	260 (100)
5-Hydroxytryptophan-2[d]	478	463 (0)	421 (5)	405 (0)	393 (0)	375 (2)	347 (0)	332 (14)	272 (0)	218 (100)

[a] *m*/*z* values, with relative abundances of ions (%) in parentheses.
[b] Pyroglutamic acid as di-TBDMS derivative.
[c] Serine-2, threonine-2, γ-aminobutyric acid-2, 4-hydroxyproline-1 and 5-hydroxytryptophan-1 as mono-EOC/di-TBDMS derivatives.
[d] 4-Hydroxyproline-2 and 5-hydroxytryptophan-2 as di-EOC/mono-TBDMS derivatives.

Table 3
Linearity, precision and accuracy for the assay of 36 amino acids, 36 carboxylic acids and nine keto acids investigated

Analyte	Calibration range (μg/ml)	Linearity[a] r^2	LOD[b] (ng/ml)	Added (μg/ml)	Precision (%R.S.D.)	Accuracy (RE%)	Added (μg/ml)	Precision (%R.S.D.)	Accuracy (RE%)
3-Hydroxybutyric acid[c]	0.3–12.0	0.9996	0.1	0.3	9.4	−4.7	12.0	5.1	0.1
3-Hydroxyisovaleric acid[c]	0.3–6.0	0.9998	0.5	0.3	2.1	7.9	6.0	1.6	0.4
Pyruvic acid	0.2–8.0	0.9999	0.2	0.2	8.2	7.0	8.0	4.8	0.2
Caproic acid	0.5–20.0	0.9998	0.5	5.0	1.2	−4.7	20.0	3.2	0.2
2-Ketoisovaleric acid[d]	0.3–6.0	0.9998	0.03	1.5	4.1	−2.9	6.0	1.4	0.2
Acetoacetic acid[d]	0.3–12.0	0.9988	6.2	1.5	2.3	−2.9	12.0	4.3	0.5
2-Keto-3-methylvaleric acid[d]	0.3–12.0	0.9991	0.04	3.0	1.5	−6.6	12.0	4.6	−1.7
2-Ketocaproic acid[d]	0.3–6.0	0.9992	0.1	3.0	0.3	−3.2	6.0	1.4	0.7
Glyoxylic acid[e]	0.2–8.0	0.9995	21.5	2.0	1.3	2.6	8.0	0.9	−0.004
Lactic acid	0.5–4.0	0.9999	6.1	1.0	8.8	−7.1	4.0	3.8	0.3
Glycolic acid	0.1–4.0	0.9992	1.7	1.0	1.6	−4.7	4.0	4.4	−12.5
Phenylacetic acid	0.1–2.0	0.9999	0.2	0.1	1.2	6.3	2.0	1.5	0.1
2-Hydroxybutyric acid	0.1–2.0	0.9999	0.2	0.1	1.5	5.6	2.0	1.7	0.1
Alanine	0.1–4.0	0.9994	<0.01	0.5	2.8	−2.5	4.0	3.5	0.7
3-Hydroxypropionic acid	0.6–24.0	0.9986	1.9	3.0	8.9	−7.5	24.0	4.5	−1.6
Glycine	0.1–4.0	0.9992	0.05	0.5	7.4	−3.5	4.0	1.7	0.4
Propionylglycine	0.6–24.0	0.9987	0.1	3.0	5.3	−6.7	24.0	1.2	1.0
2-Hydroxyisovaleric acid	0.1–2.0	0.9989	1.8	0.5	3.2	7.2	2.0	2.1	−0.4
α-Aminobutyric acid	0.1–4.0	0.9998	0.05	0.5	5.1	−5.0	4.0	6.5	0.2
β-Alanine	0.1–4.0	0.9983	0.01	0.5	8.7	−2.0	4.0	3.3	−1.9
Valine	0.1–4.0	0.9998	0.01	0.5	2.5	−0.6	4.0	0.7	0.4
β-Aminoisobutyric acid	0.1–4.0	0.9992	<0.01	0.1	3.4	−8.6	4.0	4.9	−0.8
2-Hydroxy-3-methylvaleric acid	0.1–4.0	0.9984	0.9	1.0	0.8	4.3	4.0	3.6	−2.1
Norvaline	0.1–4.0	0.9994	0.02	0.5	4.2	−1.1	4.0	6.2	0.7
Ethylmalonic acid	0.1–4.0	0.9997	<0.01	0.5	1.1	−2.6	4.0	1.4	−0.2
Leucine	0.1–4.0	0.9997	0.1	0.5	2.7	−1.6	4.0	0.5	0.5
Alloisoleucine	0.1–2.0	0.9987	0.01	0.5	0.6	4.3	2.0	2.1	−1.1
Isoleucine	0.1–4.0	0.9999	2.5	0.1	2.7	−1.0	4.0	5.7	−0.2
Threonine[f]	0.4–16.0	0.9996	2.5	2.0	1.0	−1.4	16.0	0.8	−0.3
Serine[f]	0.6–12.0	0.9977	0.01	3.0	0.1	3.5	12.0	0.6	−1.6
Methylsuccinic acid	0.1–4.0	0.9999	0.2	0.1	7.9	−8.6	4.0	2.5	−0.1
Proline	0.1–4.0	0.9999	0.01	0.1	3.4	9.1	4.0	1.6	0.3
γ-Aminobutyric acid[f]	0.1–2.0	0.9981	<0.01	0.5	1.6	3.8	2.0	3.9	7.6
Fumaric acid	0.1–4.0	0.9999	1.1	0.1	3.7	1.1	4.0	6.4	0.1
Phenylpyruvic acid	0.1–4.0	0.9999	0.2	0.5	4.1	3.1	4.0	3.2	−0.04
Pipecolic acid	0.1–4.0	0.9999	<0.01	0.1	2.3	8.3	4.0	1.8	0.3
Glutaric acid	0.1–4.0	0.9999	3.3	0.1	3.2	−0.7	4.0	5.5	−0.3
3-Methyladipic acid	0.1–4.0	0.9998	<0.01	0.5	0.3	2.1	4.0	1.5	0.5
2-Ketoglutaric acid[d]	0.3–12.0	0.9999	6.4	1.5	3.9	−1.1	12.0	5.4	−0.1
2-Phenylglycine	0.1–4.0	0.9993	<0.01	0.5	2.0	−4.3	4.0	4.2	−0.8
2-Indolecarboxylic acid	0.1–4.0	0.9997	1.5	0.5	2.5	−0.6	4.0	2.2	0.6
2-Hydroxyphenylacetic acid	0.1–4.0	0.9995	1.1	0.5	8.2	−0.9	4.0	6.8	0.3
Phenyllactic acid	0.1–4.0	0.9992	<0.01	0.5	2.0	−4.2	4.0	1.7	−0.9
3-Hydroxypheylacetic acid	0.1–4.0	0.9998	0.09	0.5	3.9	−3.2	4.0	9.3	−0.2
Pimelic acid	0.1–2.0	0.9986	<0.01	0.5	0.6	2.5	2.0	1.3	−1.2
o-Aminobenzoic acid	0.3–12.0	0.9998	0.1	1.5	0.2	−2.4	12.0	2.3	0.4
Myristic acid	0.3–12.0	0.9998	7.2	3.0	0.5	−3.5	12.0	2.5	0.2
2,3-Diaminopropionic acid	0.3–6.0	0.9974	0.02	1.5	1.2	8.8	6.0	5.1	−1.6
N-Acetylaspartic acid	0.8–32.0	0.9991	0.09	4.0	3.3	−8.6	32.0	5.8	0.7
Phenylalanine	0.1–4.0	0.9988	0.01	0.5	2.9	−5.3	4.0	0.8	0.5
Homoserine	0.6–24.0	0.9996	0.6	0.6	6.0	−1.3	24.0	5.6	−0.4
3-Indoleacetic acid	0.1–4.0	0.9992	2.4	0.5	5.9	−8.5	4.0	5.6	−0.3
Aspartic acid	0.1–4.0	0.9971	2.7	1.0	2.2	−4.8	4.0	3.0	1.5
Suberic acid	0.1–2.0	0.9976	<0.01	0.5	0.8	2.4	2.0	1.2	−1.6
4-Hydroxyproline[g]	0.4–16.0	0.9991	25.0	2.0	3.7	1.5	16.0	2.9	−0.9
m-Aminobenzoic acid	0.3–12.0	0.9999	0.5	0.3	7.0	−3.2	12.0	3.8	−0.1
Homophenylalanine	0.1–2.0	0.9972	<0.01	1.0	4.1	8.5	2.0	3.5	6.1
Palmitoleic acid	0.4–16.0	0.9997	1.8	2.0	5.0	6.5	16.0	4.9	−0.2
Glutamic acid	0.1–4.0	0.9990	0.02	1.0	4.3	−2.8	4.0	7.1	0.3
p-Aminobenzoic acid	0.3–6.0	0.9984	21.4	1.5	1.6	6.7	6.0	1.3	6.0
Asparagine	0.6–24.0	0.9999	0.04	3.0	3.8	−0.4	24.0	1.9	−0.01
Ornithine	0.3–12.0	0.9978	6.4	1.5	4.9	−4.1	12.0	5.0	1.4

Table 3 (*Continued*)

Analyte	Calibration range (μg/ml)	Linearity[a] r^2	LOD[b] (ng/ml)	Added (μg/ml)	Precision (%R.S.D.)	Accuracy (RE%)	Added (μg/ml)	Precision (%R.S.D.)	Accuracy (RE%)
Sebacic acid	0.1-4.0	0.9999	0.6	0.1	5.5	0.4	4.0	4.6	−0.1
α-Aminoadipic acid	0.1–4.0	0.9983	0.03	1.0	4.5	−4.0	4.0	3.7	0.4
3-Indolebutyric acid	0.3–12.0	0.9999	0.02	0.3	3.2	−3.2	12.0	1.8	−0.01
4-Hydroxyphenylpyruvic acid	0.9–36.0	0.9994	8.4	4.5	3.8	4.9	36.0	2.1	−2.4
Glutamine	0.8–32.0	0.9996	55.4	4.0	2.8	5.8	32.0	1.5	−1.1
Lysine	0.2–8.0	0.9999	22.4	1.0	2.4	−2.8	8.0	4.7	0.1
α-Aminopimelic acid	0.1–4.0	0.9997	0.01	0.5	5.5	5.8	4.0	1.0	0.1
Vanillylmandelic acid	0.3–12.0	0.9999	0.1	1.5	1.9	4.1	12.0	5.7	−0.1
2-Hydroxyhippuric acid	0.1–4.0	0.9996	<0.01	0.5	4.7	−5.8	4.0	4.1	0.2
Dodecanedioic acid	0.1–4.0	0.9997	<0.01	0.5	1.3	0.1	4.0	2.3	0.5
4-Hydroxyphenyllactic acid	0.1–2.0	0.9957	<0.01	0.5	1.2	4.6	2.0	4.3	−2.3
3-Indolelactic acid	0.1–4.0	0.9998	<0.01	0.1	2.7	4.2	4.0	1.8	0.1
Tyrosine	0.2–8.0	0.9995	2.5	1.0	6.7	−7.3	8.0	9.2	0.1
Methylcitric acid[h]	0.8–16.0	0.9955	1.6	4.0	0.8	5.5	16.0	1.3	−2.1
Tryptophan	1.0–8.0	0.9988	0.5	1.0	3.4	−8.3	8.0	2.2	0.1
4-Carboxyphenylglycine	0.2–8.0	0.9997	0.02	2.0	4.3	−3.3	8.0	2.0	0.4
Erucic acid	0.8–32.0	0.9999	0.7	4.0	2.4	0.3	32.0	4.4	0.1
Behenic acid	0.8–32.0	0.9999	0.03	0.8	7.7	3.3	32.0	5.0	0.09
5-Methoxytryptophan	4.0–32.0	0.9992	0.01	4.0	4.0	9.9	32.0	6.2	0.2

[a] Correlation coefficient.
[b] LOD, limit of detection.
[c] 3-Hydroxybutyric- and 3-hydroxyisovaleric-acids, calculated on mono-TBDMS derivatives.
[d] 2-Ketoisovaleric-, acetoacetic-, 2-keto-3-methylvaleric-, 2-ketocaproic- and 2-ketoglutaric-acids, calculated on sum of syn and anti isomers.
[e] Glyoxylic acid, calculated on one isomer.
[f] Threonine, serine and γ-aminobutyric acid, calculated on sum of mono-EOC/mono-TBDMS and mono-EOC/di-TBDMS derivatives.
[g] 4-Hydroxyproline, calculated on sum of mono-EOC/di-TBDMS and di-EOC/mono-TBDMS derivatives.
[h] Methylcitric acid, calculated on sum of two isomers. All quantitative calculations were based on peak area ratios relative to that of IS (1.0 μg/ml) measured on DB-5 column.

O-TBDMS derivatives possessing excellent chromatographic and mass spectrometric properties [20], except for the hydroxyl groups with steric hindrance due to the bulky TBDMS moiety present in the carboxyl site. Therefore, about 25 and 95% of the hydroxyl groups in threonine and serine were derivatized into *O*-TBDMS derivatives, respectively, but less than 5% was transformed for 3-hydroxybutyric acid and 3-hydroxyisovaleric acid even after extended heating at 60 °C. In contrast, about 30% of the remaining proton in the *N*-EOC group of γ-aminobutyric acid was converted into *N*-TBDMS, yielding two derivatives corresponding to *N*-EOC/*O*-TBDMS and *N*-EOC/*N*-TBDMS/*O*-TBDMS derivatives. About 5 and 40% of hydroxyl groups in 5-hydroxytryptophan and 4-hydroxyproline that remained intact during the aforementioned EOC step were converted to *O*-TBDMS derivatives, respectively.

3.2. GC analysis of amino acids, carboxylic acids and keto acids

Among the clinically important urinary metabolites reported in the literature [5–16], the 81 compounds composed of 36 AAs, 36 CAs, and nine KAs were selected as the analytes to examine the applicability of the present EOC/MO/SPE/TBDMS method. Upon analysis on the dual-columns with different polarities, most of the analytes displayed a single peak with good peak shape within 60 min (Fig. 1). For each keto acid, two peaks (*syn* and *anti* geometric isomers) were observed [9,10,19,35]. The aforementioned 3-hydroxybutyric acid, 3-hydroxyisovaleric acid, threonine, serine, γ-aminobutyric acid and 4-hydroxyproline showed two peaks. Methylcitric acid, a mixture of two diastereomeric isomers, yielded two peaks. Most of the unresolved peaks on nonpolar DB-5 column were resolved on DB-17 column of intermediate polarity, and vice versa. The well-resolved 3,4-dimethoxybenzoic acid [17] on both columns was found to be suitable as the IS. The dual-columns were thus complements each of the other to enable accurate peak quantitation. Moreover, it was noticed that the elution orders of most of the analytes on the two columns were very different. The temperature-programmed *I* set was thus characteristic of each analyte and useful as a crosscheck for each compound by simple *I* matching.

3.3. Mass spectral properties of amino acids as EOC/TBDMS derivatives

All derivatives were subjected to GC-MS analysis and the electron impact MS data of 44 AAs as *N*(*O*,*S*)-EOC/*O*-TBDMS derivatives were summarized in Table 2. Like the

previous *N*(*O*,*S*)-isoBOC/*O*-TBDMS derivatives [17], the molecular ion peaks of most AAs were either unobservable or very weak (<10%). Most of the mass spectral patterns exhibited characteristic $[M-57]^+$, $[M-85]^+$, $[M-103]^+$, $[M-146]^+$, and $[M-159]^+$ fragment ions, thus facilitating their rapid structural confirmation. The peaks at $[M-15]^+$, $[M-57]^+$, $[M-73]^+$, $[M-131]^+$, and $[M-159]^+$ were formed by the loss of CH_3, $C(CH_3)_3$, EOC, OTBDMS, and COOTBDMS from molecular ions, respectively. For most AAs, $[M-57]^+$ ions were either prominent or constituted the base peaks. The relatively intense peaks at $[M-85]^+$ and $[M-103]^+$ were assumed to be formed by the loss of $CHCH_3$ and $HOCH_2CH_3$ from $[M-57]^+$ ions, respectively. Prominent $[M-146]^+$ ions, most likely formed by the consecutive elimination of OTBDMS and CH_3 from molecular ions, were prominent in most AAs, while it was the base peak of 2,3-diaminopropionic acid. Threonine-2, serine-2, γ-aminobutyric acid-2, 5-hydroxytryptophan-1 and 4-hydroxyproline-1 were identified as respective mono-EOC/di-TBDMS derivatives, while 5-hydroxytryptophan-2 and 4-hydroxyproline-2 were as di-EOC/mono-TBDMS derivatives.

3.4. Method validation for simultaneous assay of amino acids, carboxylic acids and keto acids

The calibration curves of 36 AAs, 36 CAs and nine KAs measured at different ranges (0.1–36.0 μg/ml) under each optimal condition of the four sequential procedures were linear ($r^2 = 0.9955$–0.9999), and the LODs were varied from values lower than 0.01–55.4 ng/ml (Table 3). The excellent overall linearity proved suitability of the present method for quantitative measurements of AAs, CAs and KAs in unknown samples. The ranges of precision (%R.S.D.) and accuracy (RE%) of the overall procedure for each analyte measured at two different added amounts varied from 0.1 to 9.4 and from −8.6 to 9.9, respectively (Table 3). These values of the present method indicated that the levels of AAs, CAs and KAs could be measured with acceptable precision and accuracy. The average recovery rates examined on the 29 CAs at two varied concentrations (0.5–8.0 μg/ml) were ranged from 41.2 to 116.9% (Table 4). Most of the acids had recovery rates higher than 70% with good precision (%R.S.D. ≤ 9.1). 3-Hydroxyisovaleric-, 2-hydroxyphenylacetic-, 3-hydroxy-

Table 4
Recovery of the overall EOC/MO/SPE/TBDMS procedures for assay of 29 carboxylic acids

Analyte	Added (μg/ml)	Recovery (%)[a] (%R.S.D., $n = 3$)	Added (μg/ml)	Recovery (%)[a] (%R.S.D., $n = 3$)
3-Hydroxybutyric acid[b]	1.5	80.9 ± 3.6 (4.4)	3.0	96.5 ± 4.8 (5.0)
3-Hydroxyisovaleric acid[b]	1.5	50.1 ± 0.9 (1.8)	3.0	49.4 ± 2.2 (4.4)
Caproic acid	2.5	84.6 ± 3.0 (3.6)	5.0	71.3 ± 2.2 (3.0)
Lactic acid	0.5	101.9 ± 5.8 (5.7)	1.0	93.6 ± 4.8 (5.1)
Glycolic acid	0.5	76.3 ± 1.3 (1.6)	1.0	55.9 ± 2.0 (3.5)
Phenylacetic acid	0.5	87.7 ± 2.2 (2.5)	1.0	82.6 ± 1.6 (1.9)
2-Hydroxybutyric acid	0.5	100.6 ± 3.7 (3.7)	1.0	82.5 ± 5.2 (6.3)
3-Hydroxypropionic acid	3.0	93.8 ± 2.5 (2.7)	6.0	76.0 ± 3.2 (4.2)
Propionylglycine	3.0	64.7 ± 4.3 (6.7)	6.0	82.0 ± 5.6 (6.8)
Ethylmalonic acid	0.5	103.4 ± 1.8 (1.8)	1.0	116.9 ± 3.5 (3.0)
Fumaric acid	0.5	80.5 ± 2.3 (2.8)	1.0	102.5 ± 3.2 (3.1)
Glutaric acid	0.5	84.3 ± 6.8 (8.1)	1.0	101.4 ± 2.4 (2.4)
3-Methyladipic acid	0.5	105.4 ± 1.6 (1.5)	1.0	105.4 ± 2.1 (2.0)
2-Hydroxyphenylacetic acid	0.5	47.6 ± 3.8 (8.0)	1.0	51.3 ± 2.3 (4.5)
2-Indolecarboxylic acid	0.5	83.0 ± 2.8 (3.4)	1.0	97.0 ± 3.5 (3.6)
Phenyllactic acid	0.5	96.5 ± 0.6 (0.6)	1.0	94.7 ± 3.0 (3.2)
3-Hydroxypheylacetic acid	0.5	43.1 ± 2.0 (4.7)	1.0	46.7 ± 4.2 (9.1)
Myristic acid	1.5	113.3 ± 1.3 (1.1)	3.0	102.6 ± 3.3 (3.3)
N-Acetylaspartic acid	4.0	55.8 ± 1.2 (2.1)	8.0	64.2 ± 3.6 (5.7)
3-Indoleacetic acid	0.5	42.9 ± 2.4 (5.7)	1.0	44.7 ± 3.0 (6.8)
Palmitoleic acid	2.0	72.6 ± 1.5 (2.0)	4.0	61.5 ± 1.0 (1.7)
Sebacic acid	0.5	106.5 ± 4.0 (3.8)	1.0	106.6 ± 4.3 (4.1)
3-Indolebutyric acid	1.5	62.0 ± 2.8 (4.5)	3.0	73.1 ± 2.0 (2.8)
2-Hydroxyhippuric acid	0.5	102.2 ± 5.3 (5.2)	1.0	104.9 ± 2.3 (2.2)
Dodecanedioic acid	0.5	102.6 ± 5.2 (5.1)	1.0	109.3 ± 3.1 (2.8)
4-Hydroxyphenyllactic acid	0.5	41.2 ± 0.9 (2.2)	1.0	51.5 ± 2.6 (5.1)
Methylcitric acid[c]	4.0	51.3 ± 2.4 (4.7)	8.0	50.8 ± 2.4 (4.7)
Erucic acid	4.0	56.7 ± 1.4 (2.5)	8.0	54.4 ± 2.2 (4.1)
Behenic acid	4.0	79.9 ± 2.3 (2.9)	8.0	86.8 ± 3.1 (3.6)

[a] Recovery rate, calculated at different concentrations for each carboxylic acid, according to (extracted peak area ratio/non-extracted peak area ratio) × 100; R.S.D., relative standard deviation for triplicate runs.

[b] 3-Hydroxybutyric- and 3-hydroxyisovaleric-acids, calculated on mono-TBDMS derivatives.

[c] Methylcitric acid, calculated on sum of two isomers. All quantitative calculations were based on peak area ratio relative to that of IS (2.0 μg/ml) measured on DB-5 column.

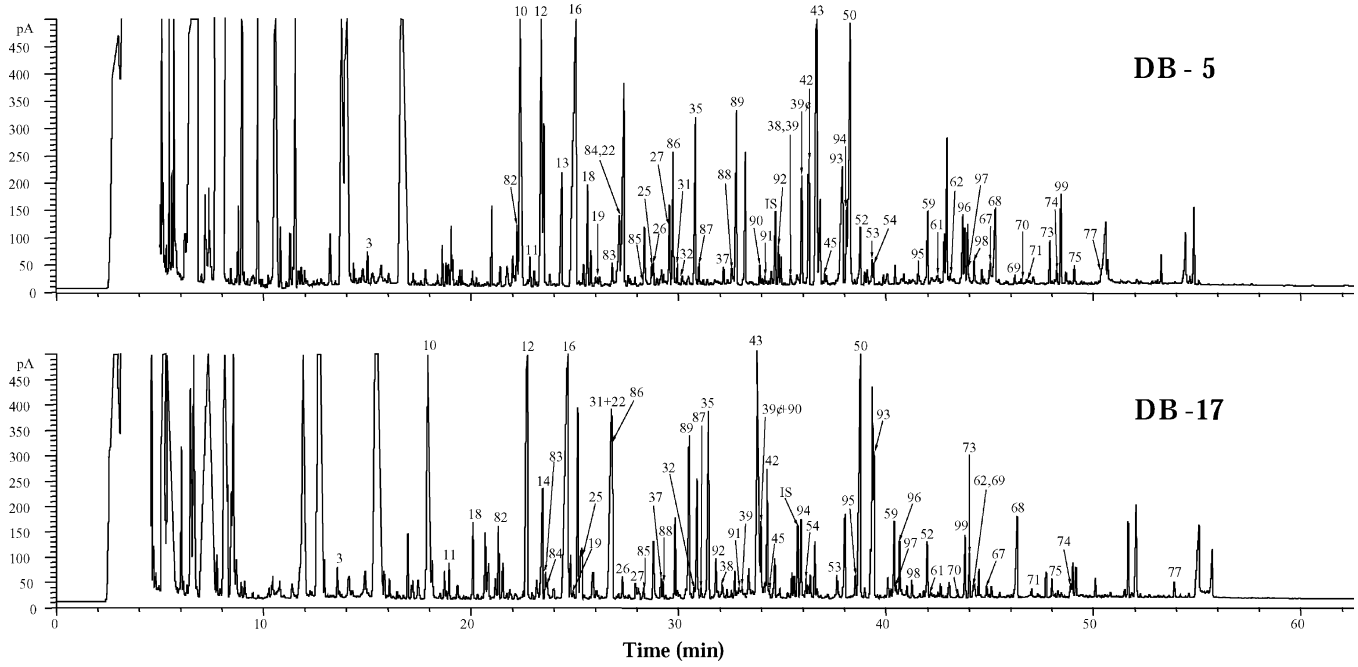

Fig. 2. Dual-profiles of urinary amino acids and carboxylic acids (including keto acids) as EOC/MO/TBDMS derivatives from a patient with phenylketonuria. Peaks: (3) pyruvic acid; (10) lactic acid; (11) glycolic acid; (12) phenylacetic acid; (14) alanine; (16) glycine; (18) 2-hydroxyisovaleric acid; (19) α-aminobutyric acid; (22) β-aminoisobutyric acid; (25) ethylmalonic acid; (26) leucine; (27) alloisoleucine; (31) methylsuccinic acid; (32) proline; (35) phenylpyruvic acid; (37) glutaric acid; (38) 3-methyladipic acid; (39) 2-ketoglutaric acid-1; (39′) 2-ketoglutaric acid-2; (42) 2-hydroxyphenylacetic acid; (43) phenyllactic acid; (45) pimelic acid; (50) phenylalanine; (52) 3-indoleacetic acid; (53) aspartic acid; (54) suberic acid; (59) glutamic acid; (61) asparagine; (62) ornithine; (67) glutamine; (68) lysine; (69) α-aminopimelic acid; (70) vanillylmandelic acid; (71) 2-hydroxyhippuric acid; (73) 4-hydroxyphenyllactic acid; (74) 3-indolelactic acid; (75) tyrosine; (77) tryptophan, (82) benzoic acid; (83) malonic acid; (84) methylmalonic acid; (85) isovalerylglycine; (86) succinic acid; (87) tiglylglycine; (88) 3-methylglutaric acid; (89) mandelic acid; (90) 3-hydroxy-3-methylglutaric acid; (91) pyroglutamic acid; (92) adipic acid; (93) hippuric acid; (94) 4-hydroxyphenylacetic acid; (95) azelaic acid; (96) *trans*-aconitic acid; (97) orotic acid; (98) 4-hydroxymandelic acid; (99) citric acid; (IS) 3,4-dimethoxybenzoic acid. GC conditions are in the Fig. 1.

phenylacetic-, *N*-acetylaspartic-, 3-indoleacetic-, and 4-hydroxyphenyllactic-, methylcitric- and erucic-acids yielded rates lower than 60%, but the high calibration linearity ($r^2 \geq 0.9955$) with good precision and accuracy (Table 3) ensured the correct quantitations. From the storage stability test (data were not shown), the derivatives of most analytes were found to be stable for at least ten days when refrigerated securely capped in vials.

3.5. Profiling analysis of urinary amino acids and carboxylic acids

When the present method was applied to urine specimen from a PKU female patient (aged 2.2 years), good dual profiles composed of urinary AAs, CAs and KAs were obtained in one analytical run from minimal volumes (corresponding to 0.25 mg creatinine) (Fig. 2). A total of 18 AAs, 34 CAs and three KAs were positively identified upon GC-MS analysis and further confirmed by crosschecking of each *I* set measured on the two columns. As expected, elevated levels of diagnostic phenylacetic acid (peak 12), phenylpyruvic acid (peak 35), 2-hydroxyphenylacetic acid (peak 42), phenyllactic acid (peak 43), and phenylalanine (peak 50) were observed.

4. Conclusions

The present method has the following three main advantages: first, it allowed simultaneous recovery of structurally diverse AAs, CAs and KAs from aqueous samples. Second, the overall linearity ($r^2 \geq 0.9955$), precision %R.S.D. = 0.1–9.4) and accuracy (RE% = −8.6 to 9.9) were satisfactory for precise and accurate quantitation of AAs, CAs and KAs in a single run. Third, the dual-columns with different polarities provided complete separation of AAs and CAs together with characteristic *I* sets that can be used for further confirmation of peaks identified by GC-MS analysis. An extension of the present method to the other analytes is under way to expand the present *I* library to be more versatile in routine screening of diverse samples for AAs, CAs and KAs.

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