D-アミノ酸を新たな生物系素材とする新規機能性食品開発拠点の形成

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Application of L-methionine γ-lyase in chiral amino acid analysis

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Abbreviations: MGL, L-methionine γ-lyase; PLP, pyridoxal 5′-phosphate; HPLC, high-performance liquid chromatography; OPA, *o*-phthalaldehyde; NAC, *N*-acetyl-L-cysteine

ABSTRACT

Here, a conventional chiral amino acid analysis method using high-performance liquid chromatography was coupled with a sample pretreatment using L-methionine γ-lyase from *Pseudomonas putida* ICR 3460 for the selective analysis of L-methionine and L-tryptophan. The sample was analyzed after the degradation of L-methionine with L-methionine γ-lyase, as L-methionine coelutes with L-tryptophan under the standard chiral amino acid analytical conditions used for precolumn derivatization with *o*-phthalaldehyde and *N*-acetyl-L-cysteine. The L-tryptophan in the sample was then eluted as a clearly separated peak and analyzed further. Since the L-methionine γ-lyase did not act on L-tryptophan, we were able to calculate the L-methionine or L-tryptophan concentration based on the data obtained from 2 individual runs: the sample with and without L-methionine γ-lyase pretreatment. The concentration of L-tryptophan was calculated from the data obtained from the sample with L-methionine γ-lyase pretreatment, while the concentration of L-methionine was calculated using the following equation: L-methionine concentration = {the data from the sample without L-methionine γ-lyase pretreatment}-{the data from the sample with L-methionine γ-lyase pretreatment}. Model samples containing authentic amino acids and a fermented food sample were analyzed by our method, and the calculated concentrations of L-methionine and L-tryptophan were consistently in agreement with the theoretical values.

Keywords:
L-Methionine γ-lyase
Chiral amino acid analysis
L-Amino acid
D-Amino acid
Food analysis
Medical analysis

Introduction

Amino acids are not only an energy source and component of macromolecules such as proteins but also have recently been found to be potent markers for various mammalian diseases. Plasma-free amino acid profiles have been investigated in relation to diseases including chronic gastrointestinal diseases, diabetes, dyslipidemia, and metabolic syndrome [1-3], and serum amino acid levels have been suggested to be related to Parkinson's disease [4, 5]. Urinary amino acid levels have shown positive or negative correlations with metabolic syndrome [6].

D-Amino acids, enantiomers of the corresponding L-amino acids, are also recognized as important in some respects. For example, D-serine exists in the mammalian brain [7] and contributes to excitatory neurotransmission [8]. D-Serine dynamics have been shown to be related to diseases including schizophrenia [9, 10] and amyotrophic lateral sclerosis [11], and D-serine is therefore regarded as a promising biomarker. Batalla et al. [12] and Martín et al. [13] developed enantiomeric detection methods for methionine, leucine, and tyrosine, which are involved in bacterial diseases, to enable the detection of pathogenic bacteria. These recent findings and approaches raise the need to quantify both D- and L-amino acids in many research fields.

One of the conventional methods for D- and L-amino acid quantification is chromatographic separation after derivatization to diastereomers, and many methods for this derivatization have been developed [14-16]. However, problems remain: 1) it is difficult to identify and quantify amino acids whose content is substantially lower than that of other amino acids, such as D-amino acids; 2) some amino acid peaks are detected as partially overlapping or nearly stacking over each other; and 3) expensive equipment is needed for the analysis.

A derivatization and analytical method with *o*-phthalaldehyde (OPA) and *N*-acetyl-L-cysteine (NAC) is simple, useful, and applicable to various samples, including fermented foods [14] and animal cells [17]. The method can derivatize all α-amino acids except cysteine and proline, and many D- and L-amino acids can be separated, as shown in Fig. 1A. However, some amino acids are detected as overlapping or stacking peaks; for example, peaks 5 (D-Asn and L-Asn), 12 (L-Arg and D-Arg), 17 (D-His and L-His), 20 (L-Met and L-Trp), and 29 (D-Lys and L-Lys) consist of two different amino acids. Among them, peaks 5, 12, 17 and 29 are comprised of both enantiomers of a

particular amino acid. These amino acids can be quantified by degrading one enantiomer using stereospecific enzymes such as L-amino acid oxidase (EC 1.4.3.2) and D-amino acid oxidase (EC 1.4.3.3). Peak 20 consists of two L-amino acids, L-methionine and L-tryptophan, and therefore, a different approach is needed to precisely quantify the two L-amino acids.

L-Methionine γ-lyase (MGL, EC 4.4.1.11) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the cleavage of the carbon-sulfur bond in sulfur-containing L-amino acids. MGL prefers L-methionine as a substrate and converts it to methanethiol, ammonia, and α-ketobutyrate. The enzyme has been identified in various microorganisms, such as *Pseudomonas putida* [18], *Aeromonas* sp. [19], *Entamoeba histolytica* [20], *Citrobacter freundii* [21], and *Streptomyces avermitilis* [22]. MGL from *P. putida* is one of the most investigated forms and shows high activity toward L-methionine among proteinogenic amino acids and almost no activity toward the corresponding D-amino acids [23]. In the present study, we focused on the substrate specificity of *P. putida* MGL and attempted to apply the enzyme to selectively determine L-tryptophan and L-methionine in chiral amino acid analysis using high-performance liquid chromatography (HPLC).

Materials and methods

Reagents

D- and L-Amino acids were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich Co. (St. Louis, MO), or Watanabe Chemical Industries (Hiroshima, Japan). Other reagents were of analytical grade or the best grade available and were from FUJIFILM Wako Pure Chemical Industries, Sigma-Aldrich Co. or Kanto Kagaku Co. (Tokyo, Japan) unless otherwise stated.

Preparation of MGL from P. putida ICR 3460

MGL from *P. putida* ICR 3460 was prepared as described previously [24, 25]. *Escherichia coli* HB101 harboring the plasmid pMGL1204 for MGL gene expression was grown in 5 mL of Luria-Bertani medium (pH 7.0) containing 10 mg/mL

tetracycline hydrochloride at 37°C for 14 h with shaking. The culture broth was transferred to 1.6 L of fermentation medium (pH 7.4) containing 1.2% (w/v) bacto tryptone, 2.4% (w/v) bacto yeast extract, 2.0% (w/v) glycerol, 1.25% (w/v) dipotassium hydrogen phosphate, 0.23% (w/v) potassium dihydrogen phosphate, 0.05% (w/v) polypropylene glycol No. 2000, and 10 mg/mL tetracycline hydrochloride and cultivated at 28°C for 18 h with shaking. Then, gene expression was induced by the addition of isopropyl-*β*-D-thiogalactopyranoside (final concentration of 0.5 mM) after cultivation for 4 h. The harvested *E. coli* cells were washed and resuspended in 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM ethylenediaminetetraacetate, 0.5 mM PLP, 0.01% (w/v) dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and were disrupted with an Insonator 201M homogenizer (Kubota, Tokyo).

The enzyme in the lysate was purified via DEAE-Toyopearl 650M column (Tosoh, Tokyo) chromatography [26] and Sephacryl S-300 HR gel filtration column (GE Health Care, Tokyo) chromatography [25], and the enzyme purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli [27]. The enzyme activity of MGL was determined using the 3-methyl-2-benzothiazolone hydrazone hydrochloride method [28] as described previously [29], and one unit was defined as the amount of MGL that produces one μmol of α-ketobutyrate per min. The purified MGL was stored at -80°C until use.

Treatment of the amino acid solution with MGL and HPLC analysis

The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 8.0), 10 μM PLP, 5 or 50 μM amino acids (D- and L-forms of aspartate, glutamate, asparagine, serine, glutamine, threonine, arginine, alanine, tyrosine, histidine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, and lysine), and 0.005, 0.05, or 0.5 U/mL MGL. After preheating at 37°C for 3 min, the reaction was initiated by the addition of MGL. The reaction mixture was incubated at 37°C for 60 min and sampled at 30-min intervals. The reaction was stopped by the addition of 20% (w/v) trichloroacetic acid (50 μL) to the mixture (100 μL). The mixture was neutralized by the addition of 5 M NaOH (12 μL) and H_2O (38 μL). The neutralized sample was subjected to HPLC analysis.

The D- and L-amino acid concentrations were determined using an HPLC system

from Shimadzu Co. (Kyoto, Japan) consisting of a degasser (DGU-20A5), a high-pressure gradient solvent delivery unit (LC-20AB), a communication bus module (CBM-20A), an autosampler (SIL-20AC), a column oven (CTO-20AC) and a fluorescence detector (RF-10AXL) as described previously [14, 28]. After derivatization of the amino acids with OPA and NAC, they were quantitatively separated using a Develosil ODS-UG-5 column (250 × 6.0 mm i.d., the particle diameter: 5 μm, Nomura Chemical Co., Seto, Japan) equilibrated with 50 mM sodium acetate (mobile phase A) by eluting with a linear gradient of methanol (mobile phase B; 0%, 0 min; 24%, 16–24 min; 40%, 29–50 min; 67%, 69 min; 80%, 69.01–74 min). The flow rate was 1.2 mL/min, and the elution was monitored by fluorescence (excitation at 340 nm, emission at 450 nm).

Application of the proposed pretreatment method for the analysis of a fermented food sample

After centrifugation (12,000 ×*g*, 15 min, 4°C), the supernatant of a commercial rice vinegar was used as a food sample. The food sample (10 μL) was mixed with an equal volume of H_2O, L-methionine (50 μM), L-tryptophan (50 μM), or L-methionine plus L-tryptophan (50 μM each). The mixture was added to the premix solution consisting of 50 μL of 200 mM potassium phosphate buffer (pH 8.0), 10 μL of 100 μM PLP, and 20 μL of H_2O and was preheated at 37°C for 3 min. The enzyme treatment was started by the addition of 10 μL of MGL (5 U/mL). After 30 min at 37°C, the reaction was stopped. Then, the sample was neutralized and subjected to HPLC analysis as described above.

Results and discussion

MGL from *P. putida* ICR 3460 was overexpressed in *E. coli* HB101 cells and purified to homogeneity, and the enzyme activity was assayed as described previously [23]. The recombinant MGL showed high degrading activity toward L-methionine and did not exert any effect on L-tryptophan (data not shown). First, using model samples consisting of authentic D- and L-amino acids, the reaction conditions for MGL pretreatment, such as the MGL content (0.005-0.5 U/mL), reaction time (30 or 60 min)

and amino acid concentration (5 or 50 μM), were validated. The area of peak 20 (L-methionine and L-tryptophan) declined with MGL treatment, and no significant effect on the other L-amino acid and D-amino acid peaks was observed (Fig. 1). Enlarged views around the L-methionine/L-tryptophan peak are shown in Fig. 2. The concentrations of L-methionine and L-tryptophan were calculated from the following two equations: L-methionine concentration = {the data from the sample without MGL pretreatment}-{the data from the sample with MGL pretreatment} and L-tryptophan concentration = the data from the sample with MGL pretreatment, and the calculated concentrations are summarized in Table 1. Both amino acid concentrations could be quantified with good recovery values under all treatment conditions assessed, suggesting that a reaction time over 30 min and an MGL content over 0.005 U/mL are sufficient for the complete removal of L-methionine from the model samples.

Then, the concentrations of L-methionine and L-tryptophan in commercial rice vinegar were analyzed by the proposed pretreatment method, and the results are summarized in Table 2. The calculated concentrations in rice vinegar containing exogenously added authentic L-methionine and/or L-tryptophan showed good agreement with each theoretical value. These results suggest that combining MGL pretreatment with HPLC increases the accuracy of the quantification of L-methionine and L-tryptophan when the amino acids are derivatized with OPA and NAC and that the proposed pretreatment method could be applicable at least for food sample analysis.

Some D- and L-amino acids are regarded as promising biomarkers for the diagnosis of various diseases [1-6, 9-13], and in some cases, the D/L ratio of a particular amino acid varies [10]. Therefore, the amino acid profiles of both D- and L-amino acids are important targets, including those for methionine and tryptophan. Various plasma amino acids, including L-methionine and L-tryptophan, were significantly decreased in cats with chronic gastrointestinal diseases [1], and aromatic amino acids, such as L-tryptophan and branched-chain amino acids, were correlated with obesity [2]. In metabolic syndrome patients, urine L-lysine and L-methionine levels were lower than in healthy subjects [6]. D-Methionine is also a potential marker for the detection of pathogenic bacteria such as *Vibrio cholerae* [12, 13]. In addition to its use as a potent anticancer agent against some methionine-dependent cancer cell lines and primary tumors [30-32], MGL might be applicable for diagnostic approaches through the analysis of amino acid profiles.

Interestingly, we found that when pretreatment was performed with 0.5 U/ml MGL, the peak area derived from L-serine gradually decreased through the reaction with MGL, but no alteration in D-serine was observed (Fig. 1, 3). This result suggests that *P. putida* MGL stereoselectively degrades L-serine, although the activity seems to be quite low. The substrate specificity of MGL from various microorganisms, including *P. putida,* has already been reported. MGL from *P. putida* [33] and *C. freundii* [21, 34] act on L- and DL-homoserine, respectively, and MGL from *P. putida* [35], *C. freundii* [36] and *E. histolytica* [37] react with L-cysteine. The *O*-acetylated form of L-homoserine and L-serine, *O*-succinyl-L-homoserine, and various modified forms of L-cysteine can also act as a substrate for MGL from various microorganisms [35-38]. The reaction for L-cysteine and modified cysteine/serine is considered a β-replacement reaction; however, no report has elucidated the degrading activity of MGL against free L-serine. A PLP-dependent amino acid γ-lyase, cystathionine γ-lyase from *Lactobacillus reuteri*, has been shown to exert weak activity toward L-serine [39], although the reaction mechanism remains unclear. *P. putida* MGL may degrade L-serine in a similar manner. Current work is aimed at a detailed analysis of *P. putida* MGL reactivity against serine and clarification of its physiological meanings.

Conflicts of interest

The authors declare no conflicts of interest associated with this manuscript.

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Author contributions

T. O. designed the studies. T. O., K. I., and S. K. wrote the paper. K. I. and S. K.

conducted the experiments.

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Table 1

Quantification of L-methionine and L-tryptophan in the model sample.

	5 μM Amino acids		50 μM Amino acids	
	Reaction time		Reaction time	
	30 min	60 min	30 min	60 min
	0.005 U/mL MGL			
Calculated amino acid concentration (μM)				
L-Met	5.05 ± 0.22	4.84 ± 0.09	50.6 ± 1.2	50.6 ± 1.3
L-Trp	4.96 ± 0.19	5.13 ± 0.07	49.7 ± 1.0	49.4 ± 1.1
Recovery (%)				
L-Met	101.0	96.8	101.2	101.2
L-Trp	99.2	102.6	99.4	98.8
	0.05 U/mL MGL			
Calculated amino acid concentration (μM)				
L-Met	5.25 ± 0.24*	5.10 ± 0.12	48.8 ± 1.3	53.6 ± 4.1
L-Trp	5.23 ± 0.30*	4.91 ± 0.10	51.0 ± 0.9	47.0 ± 3.5
Recovery (%)				
L-Met	105.0*	102.0	97.6	107.2
L-Trp	104.6*	98.2	102.0	94.0
	0.5 U/mL MGL			
Calculated amino acid concentration (μM)				
L-Met	4.82 ± 0.20	4.81 ± 0.22	46.8 ± 0.8	48.2 ± 1.8
L-Trp	5.15 ± 0.17	5.16 ± 0.19	52.7 ± 0.7	51.5 ± 1.5
Recovery (%)				
L-Met	96.4	96.2	93.6	96.4
L-Trp	103.0	103.2	105.4	103.0

All numbers are the average of three or six* independent experiments. The concentrations of L-tryptophan and L-methionine were calculated from the remaining peak area and the decrease in the peak area after MGL pretreatment, respectively.

Table 2

Quantification of L-methionine and L-tryptophan in a fermented food sample.

Sample	Amino acid	Calculated value (μM)	Theoretical value (μM)	Recovery (%)
Rice vinegar	L-Met	4.27 ± 0.90 (=X)	-	-
	L-Trp	12.8 ± 0.6 (=Y)	-	-
Rice vinegar + L-Met	L-Met	52.7 ± 3.1	X + 50	97.1
	L-Trp	13.5 ± 2.1	Y	105.5
Rice vinegar + L-Trp	L-Met	4.90 ± 2.56	X	114.8
	L-Trp	62.5 ± 1.8	Y + 50	99.5
Rice vinegar + L-Met and L-Trp	L-Met	55.1 ± 6.8	X + 50	101.5
	L-Trp	65.1 ± 4.7	Y + 50	103.7

All numbers are the average of triplicate measurements.

Figure legends

Fig. 1. Representative chromatograms of amino acid analyses. The amino acid solution (50 μM) was treated with MGL (0.5 U/mL) for 0 (A), 30 (B), or 60 (C) min and then analyzed using HPLC. Peaks 1-29 correspond to D-Asp, L-Asp, L-Glu, D-Glu, D-Asn + L-Asn, D-Ser, L-Ser, L-Gln, D-Gln, D-Thr, L-Thr, L-Arg + D-Arg, D-Ala, L-Ala, L-Tyr, D-Tyr, D-His + L-His, L-Val, D-Met, L-Met + L-Trp, D-Val, D-Trp, D-Phe, L-Phe, L-Ile, D-Ile, D-Leu, L-Leu, and D-Lys + L-Lys, respectively.

Fig. 2. An enlarged view of the chromatograms around the L-methionine and L-tryptophan peaks. The amino acid solution was treated with MGL for 0, 30, or 60 min and then analyzed using HPLC. The amino acid content was 5 (A, B, C) or 50 (D, E, F) μM, and the MGL content was 0.005 (A, D), 0.05 (B, E) or 0.5 (C, F) U/mL.

Fig. 3. An enlarged view of the chromatograms around the D- and L-serine peaks. The amino acid solution (50 μM) was treated with MGL (0.5 U/mL) for 0, 30, or 60 min and then analyzed using HPLC.

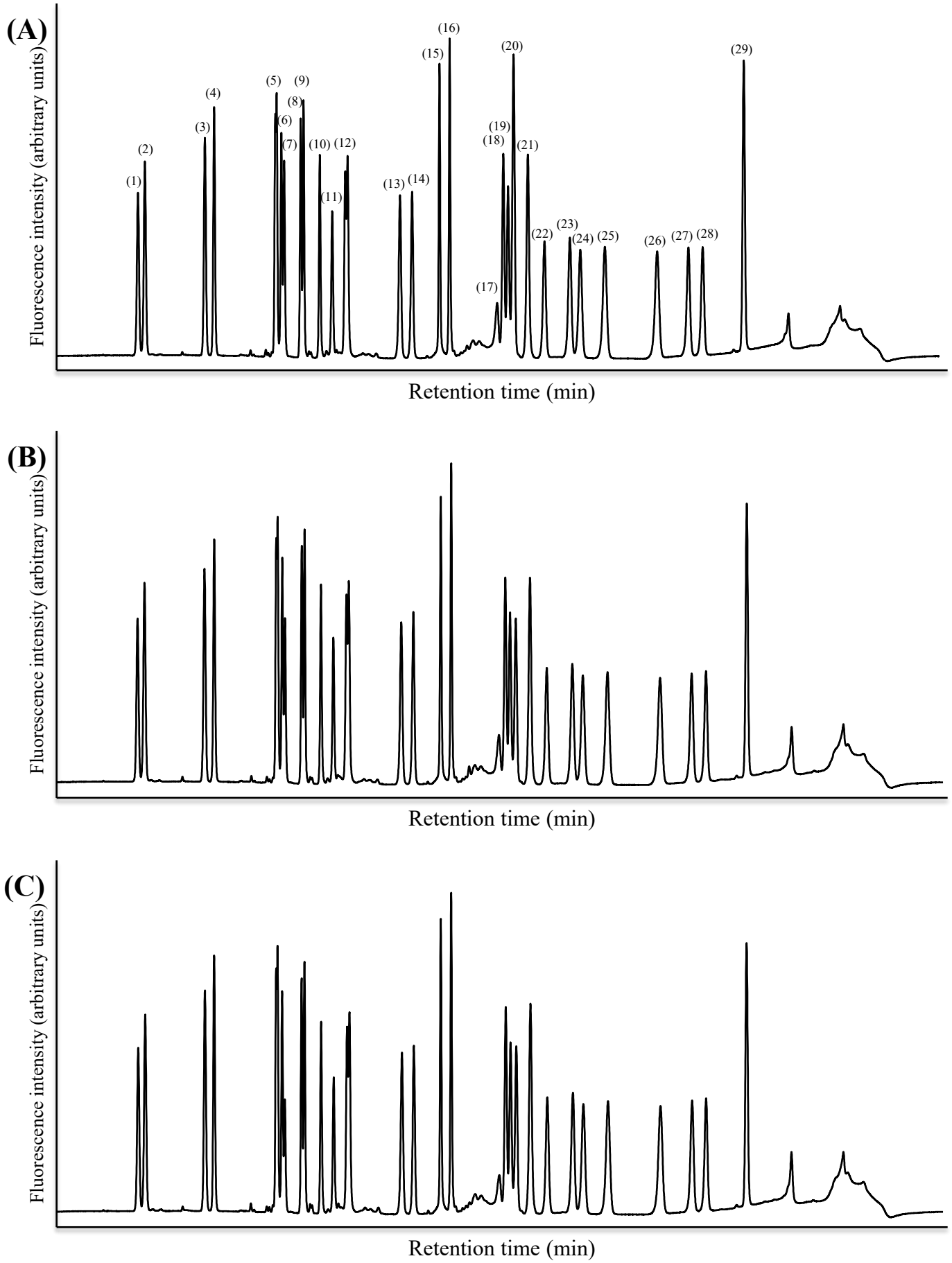

Figure 1, Kato et al.

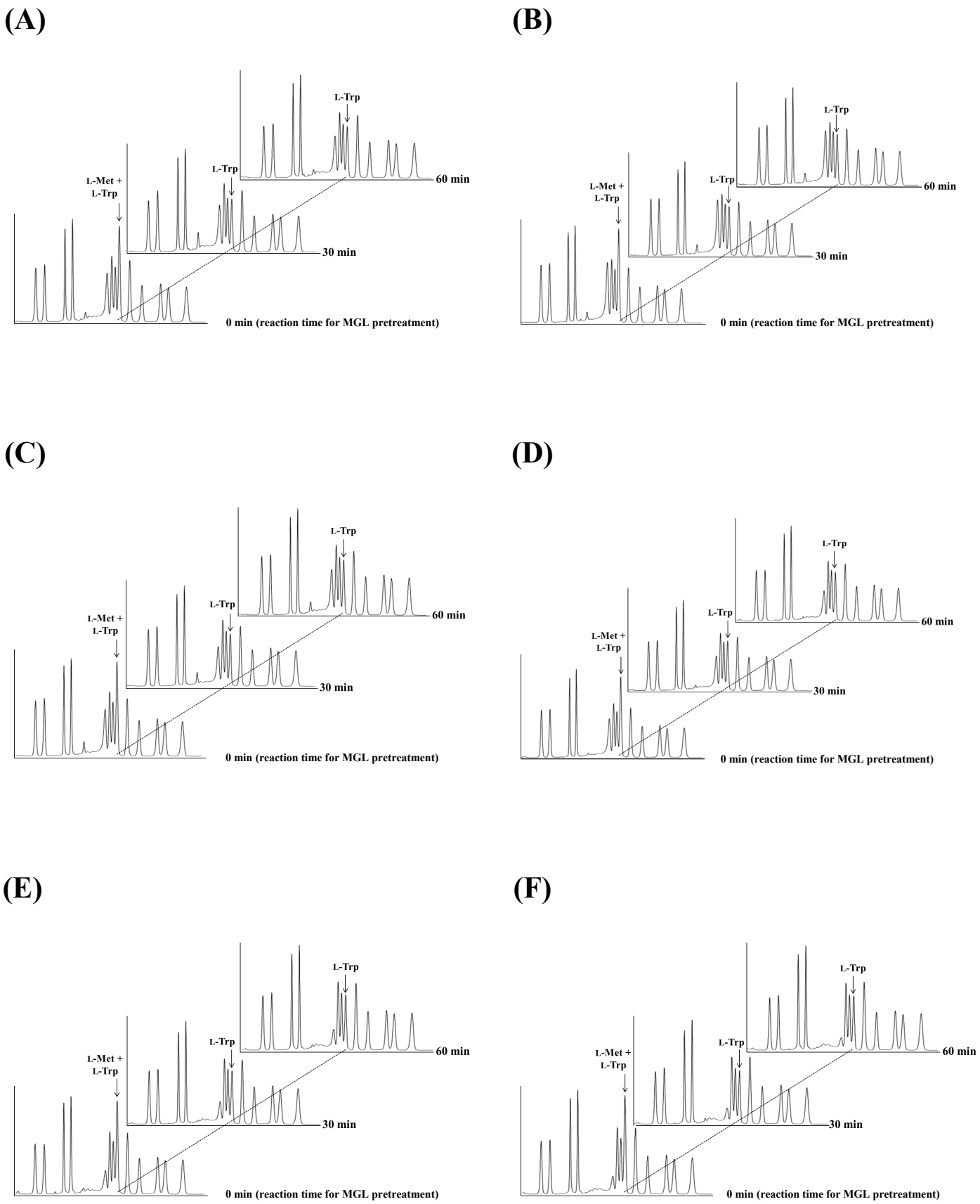

Figure 2, Kato et al.

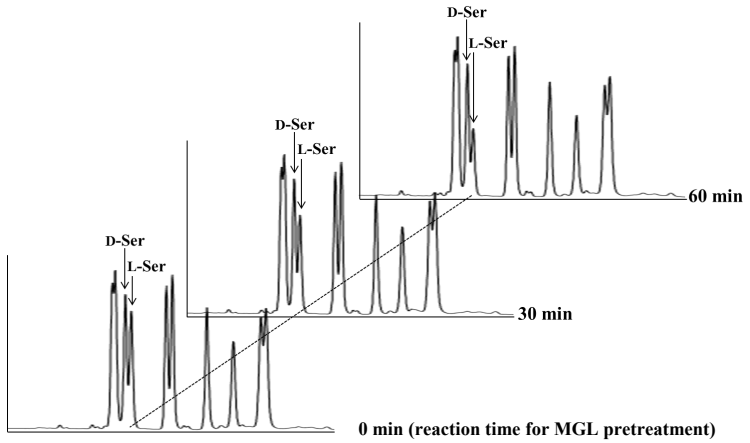

Figure 3, Kato et al.