Electronic Supplementary Material (ESI) for Green Chemistry

Supporting Information

ω-Transaminase-catalyzed kinetic resolution of chiral amines using L-threonine as an amino acceptor precursor

M. Shaheer Malik, Eul-Soo Park and Jong-Shik Shin*

Department of Biotechnology, Yonsei University, Shinchon-Dong 134, Seodaemun-Gu, Seoul 120 749, South Korea

Table of contents

1. Chemicals

Racemic form of α-methylbenzylamine (**1a**), 4-fluoro-α-methylbenzylamine (**1b**), α-ethylbenzylamine (**1c**), 1-methyl-3-phenylpropylamine (**1d**), 1-aminoindan (**1e**), *sec*-butylamine (**1f**), 2-aminopentane (**1h**), 1-methoxy-2-propylamine (**1j**) and alaninol (**1k**) were purchased from Sigma-Aldrich Co. In the case of cyclopropylethylamine (**1g**) and 2-aminooctane (**1i**), we purchased chemical stocks of enantiopure forms from Alfa-Aesar Co. and made up racemic mixtures by combining stoichiometric amounts of the enantiopure forms. (*S*)-**1a**, (*R*)-**1a**, (*S*)-**1c**, (*R*)-**1c**, (*S*)-**1e** and (*S*)-**1f** were purchased from Sigma-Aldrich Co. Pyruvate was obtained from Kanto Chemical Co. (Tokyo, Japan). L-Alanine, D-alanine, L-threonine, L-homoalanine, D-homoalanine, 2-oxobutyrate, pyridoxal 5′-phosphate (PLP), acetophenone, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) were obtained from Sigma-Aldrich Co. Materials used for preparation of culture media including yeast extract, tryptone and agar were purchased from Difco.

2. Cloning of (*R*)-selective ω-transaminase from *Aspergillus terreus*

The (*R*)-selective ω-TA gene from *Aspergillus terreus* (NCBI gene ID: 115385557) was synthesized and cloned into a pGEM-T vector by gene synthesis service of Bioneer, Inc. (Daejon, Korea). The (*R*)-selective ω-TA gene was amplified using PCR primers: forward (5′-AGAAGGAGATATACCATGGCCTCCATG GACAAAGTCT-3′) and reverse (5′-GGTGGTGGTGGTGCTCGAGGTTCCTCTCGTTATAATC-3′) which carry a NcoI and XhoI restriction site, respectively, as underlined. Subcloning of the gene into a pET28a(+) expression vector (Novagen, WI) was carried out using an Infusion HD cloning kit (Clontech, CA).

3. Preparation of purified enzymes

Overexpression and purification of TD and ω-TA were performed as described elsewhere[1, 2] with a few modifications. *E. coli* BL21 (DE3) cells transformed with pET21-TD[1] or pET28-ω-TA[2] vector were cultivated in 300 mL LB medium at 37 ºC in the presence of appropriate antibiotics. Protein expression was induced by IPTG (final concentration=1 mM) at around 0.5 OD_{600} value and then the cells were allowed to grow further for 6 h. Cells were harvested by centrifugation (10,000 × g, 20 min, 4 ºC) and resuspended in 15 mL resuspension buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 20 μM PLP, pH 7.0). Ultrasonic cell disruption followed by centrifugation (17,000 × g, 30 min, 4 ºC) provided cell-free extracts. Protein purification was carried out on ÄKTAprime plus (GE Healthcare Life Sciences). The His_6-tagged proteins were purified by a HisTrap HP column using an elution buffer (20 mM sodium phosphate, 0.5 M sodium chloride, 0.5 mM PLP, pH 7.4) with a linear gradient of imidazole (0 - 0.5 M). Then, buffer exchange of the purified enzyme solution was carried out by

a HiTrap Desalting column using an elution buffer (50 mM sodium phosphate, 0.15 M sodium chloride and 0.5 mM PLP).

4. Enzyme assay

Typical enzyme assays were carried out at 37 °C and pH 7.5 (50 mM phosphate buffer). The enzyme reaction (100 µl reaction volume) was stopped by adding 600 µL acetonitrile after 10 min reaction. One unit of TD activity is defined as the enzyme amount that catalyzes the formation of 1 µmole of 2-oxobutyric acid (**3**) in 1 min at 50 mM L-threonine (**2**) in the presence of 0.1 mM PLP. One unit of (*S*)-selective ω-TA activity is defined as the enzyme amount that catalyzes the formation of 1 µmole of acetophenone (**5**) in 1 min at 10 mM pyruvate and 10 mM (*S*)-**1a** in the presence of 0.1 mM PLP. For TD and ω-TA activity assays, **3** and **5**, respectively, were analyzed by HPLC. In case of the assay for (*R*)-selective ω-TA, (*R*)-**1a** was used instead of (*S*)-**1a**.

5. Product analysis

For determination of product concentration and enantiomeric excess, chiral amines and amino acids were analyzed after pre-column derivatization using a chiral GITC[3] or Marfey's reagent.[4,5] In a typical GITC derivatization procedure, reaction samples appropriately diluted with acetonitrile (total amine concentration less than 0.5 mM) were mixed with the same volume of derivatizing solution (1.5 mM GITC and 1.5 mM triethylamine in acetonitrile), which was then allowed to stand for 35 min at room temperature. Aliquots of the mixtures (20 µL) were analyzed with a Waters HPLC system (Milford, MA) using a Symmetry C18 column. HPLC analysis of the GITC derivatives of chiral amines and amino acids are listed in Table S1. All the chiral compounds except **1g** and **1k** were well resolved after the GITC derivatization on the Symmetry column at a flow rate of 1 ml/min under elution conditions shown in Table S1 with 254 nm UV detection. The GITC derivatives of the (*R*)-isomers of amines **1a-f** and **1h-i** elutes before the corresponding (*S*)-isomers, which was confirmed by comparing with the GITC derivative of (*S*)-**1a** and also by the consumption of (*S*)-isomers in the ω-TA reaction (Table S1). In the case of **1j**, the elution order was reversed.

In a typical pre-column derivatization procedure by the Marfey's reagent, 10 µL reaction samples of **1g** and **1k** appropriately diluted with acetonitrile (the molar ratio of the Marfey's reagent to amine is 1.4:1) were mixed with 8 µL of 1 M sodium bicarbonate solution and 40 µL of 1 % Marfey's reagent in acetonitrile. The reaction mixture was thoroughly mixed, heated at 40 °C for an hour and then cooled at room temperature. Eight µL of 1 M hydrochloric acid was added to the mixture to quench the derivatization reaction. The mixture was diluted with 434 µL of 40 % acetonitrile/water, vortexed and transfered to HPLC vials. Aliquots of the sample (10 µL) were analyzed by a Waters HPLC system (Milford, MA) using a Symmetry C18 column at a flow rate of 1 ml/min with and detection at 320 nm at

40 ºC. The derivative of (*R*)-**1g** elutes before the corresponding (*S*)-isomer, whereas an inversion in the elution order was observed with **1k** (Table S1).

Concentration of each enantiomer of chiral compounds was determined by a standard calibration curve method. Enantiomeric excess (*ee*) was determined by the following equation where C_R and C_S represent concentrations of (*R*) and (*S*)-enantiomer, respectively.

$$\%\ ee^R = \frac{(C_R - C_S)}{(C_R + C_S)} \times 100$$

Table S1. HPLC analysis of the derivatives of chiral amines and amino acids

analyte	elution condition[a]	retention time (min)	
		(*R*)-form	(*S*)-form
GITC derivatization:			
1a	isocratic elution of 55 % A / 45 % B	14.5	16.2
1b	isocratic elution of 55 % A / 45 % B	18.7	21.5
1c	isocratic elution of 55 % A / 45 % B	24.5	26.4
1d	isocratic elution of 55 % A / 45 % B	39.4	42.0
1e	gradient elution from 60 A / 40 B to 50 A / 50 B	14.1	16.6
1f	isocratic elution of 50 % A / 50 % B	13.3	14.4
1h	isocratic elution of 50 % A / 50 % B	27.5	30.6
1i	isocratic elution of 65 % A / 35 % B	23.6	25.4
1j	gradient elution from 40 A / 60 B to 65 A / 35 B	24.9	27.0
4	isocratic elution of 55 % A / 45 % B	5.3	4.2
alanine	isocratic elution of 50 % A / 50 % B	5.6	4.6
Derivatization by the Marfeys' reagent:			
1g	isocratic elution of 50 % A / 50 % B	26.9	28.5
1k	isocratic elution of 55 % A / 45 % B	4.9	3.3

[a] A: MeOH (0.1 % TFA), B: TDW (0.1 % TFA)

6. Amino donor specificity

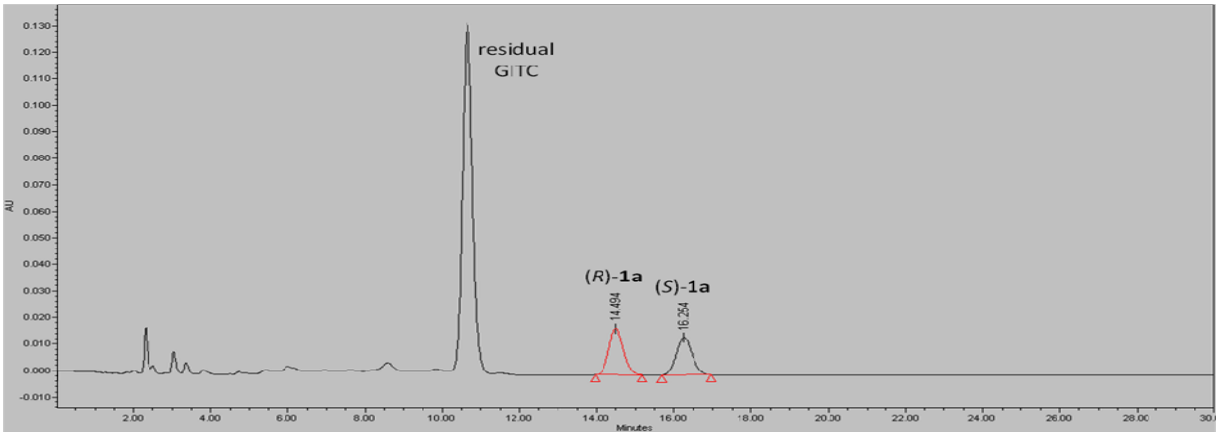
To examine amino donor specificity of (*S*)-selective ω-TA from *Ochrobactrum anthropi*, initial reaction rates of 11 racemic amines were compared. Reaction conditions were 10 mM **1a**–**1k**, 10 mM pyruvate, 0.1 mM PLP in 50 mM phosphate buffer (pH 7.5). Initial rates were measured by analyzing produced L-alanine after GITC derivatization. Reaction conversions were kept less than 5 % for the initial rate measurements.

7. Coupled enzyme reactions

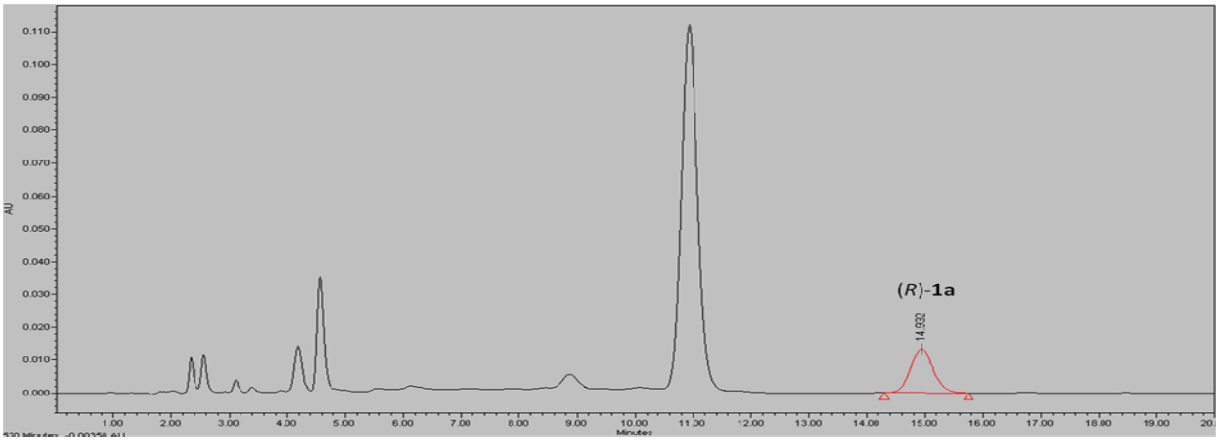
Coupled enzyme reactions were performed at 100 mM **1a**–**1k**, 60 mM **2**, 0.1 mM PLP in 50 mM phosphate buffer (pH 7.5). To make up the reaction mixture, 144.2 μL of 26 U/mL (*S*)-selective ω-TA solution and 14.1 μL of 635 U/mL TD solution were added to the substrate solution, so the final concentrations of ω-TA and TD became 3.75 and 9 U/mL, respectively. The reaction volume was 1 mL and the reaction mixture was incubated under magnetic stirring at 37 °C. For time-course monitoring of the coupled enzyme reactions, aliquots of reaction mixture (20 μL) were taken at predetermined reaction times and subjected to HPLC analysis. The reaction mixture aliquots were mixed with 120 μL of acetonitrile to stop the reaction, and the resulting solution was diluted 10-fold with acetonitrile and then subjected to pre-column derivatization and HPLC analysis of **4**, (*R*) and (*S*)-**1a**–**1k**. In the case of the coupled reaction using (*R*)-selective ω-TA, 1.5 U/ml (*R*)-selective ω-TA from *A. terreus* and 4.5 U/ml TD were used.

8. HPLC chromatograms of chiral analysis of racemic amines and enzymatic reactions

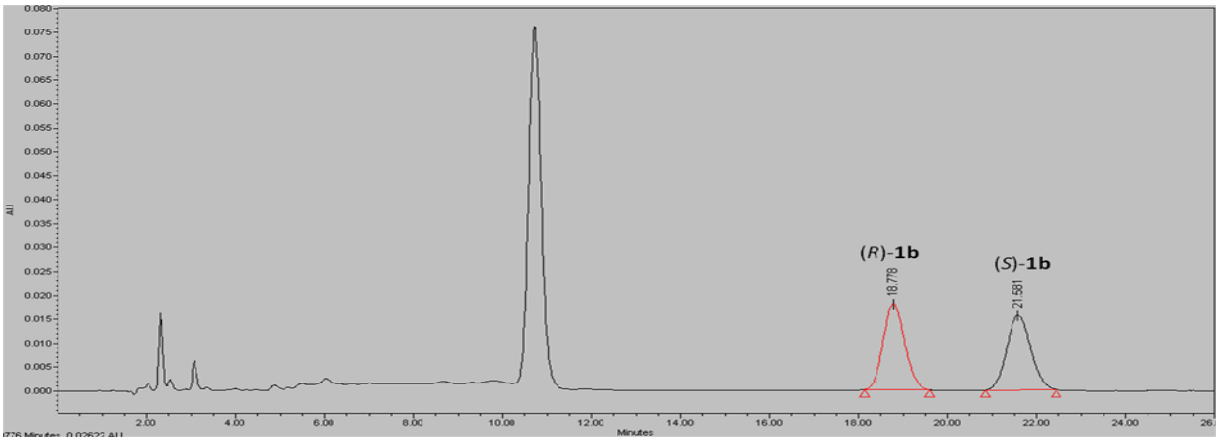
HPLC separation of GITC derivatives of **1a**



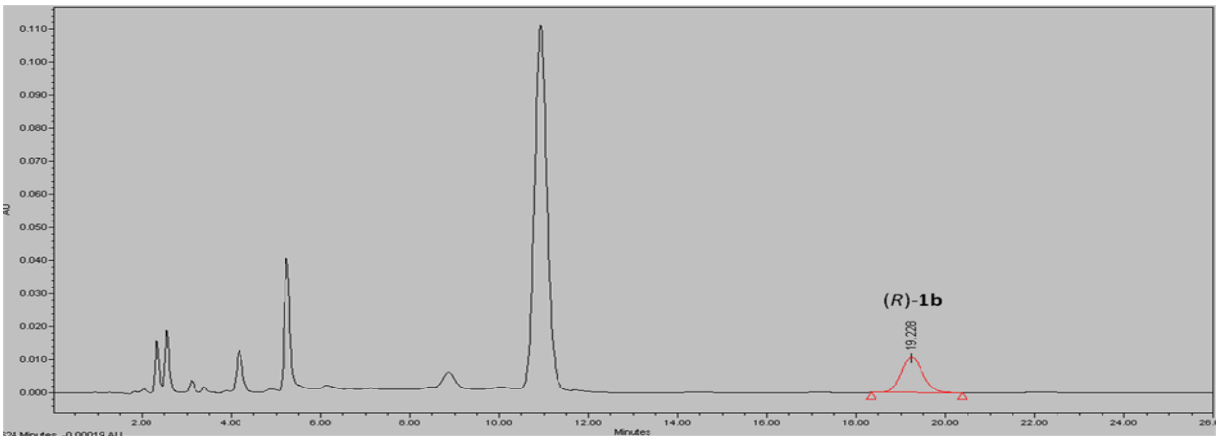
ω-TA catalyzed resolution of **1a**



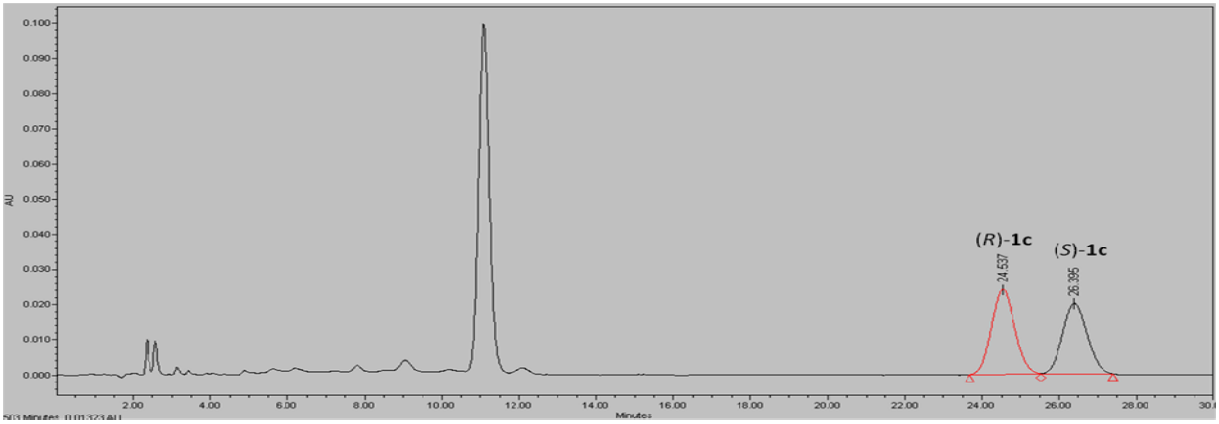
HPLC separation of GITC derivatives **1b**



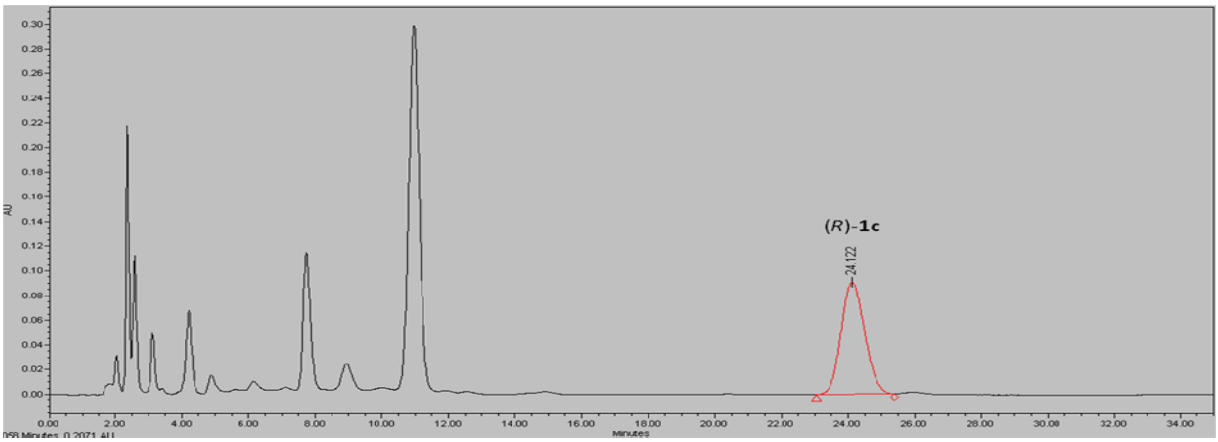
ω-TA catalyzed resolution of **1b**



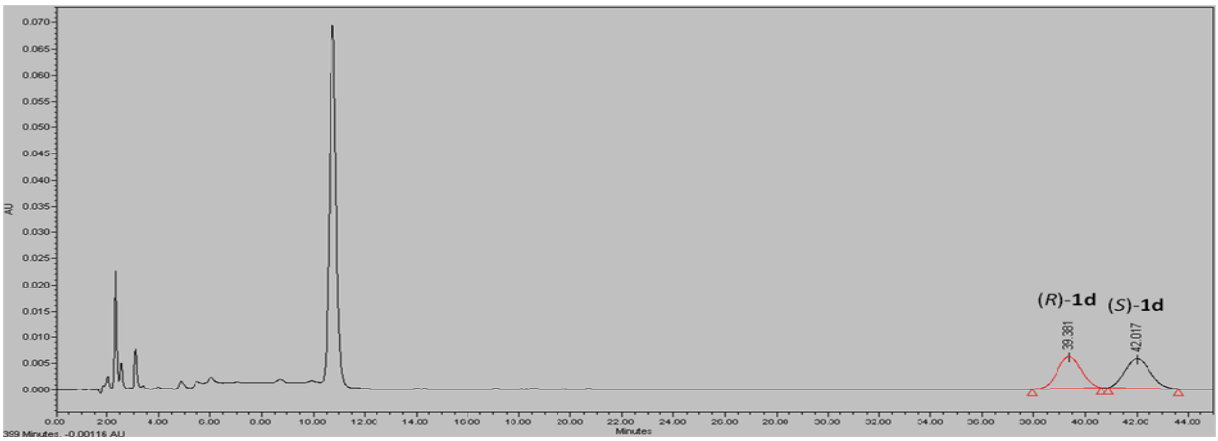
HPLC separation of GITC derivatives of **1c**



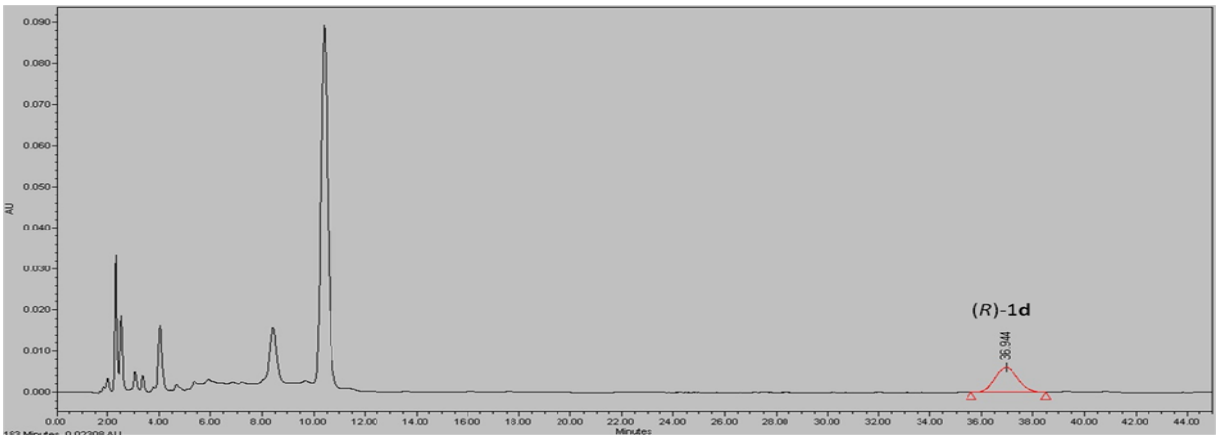
ω-TA catalyzed resolution **of 1c**



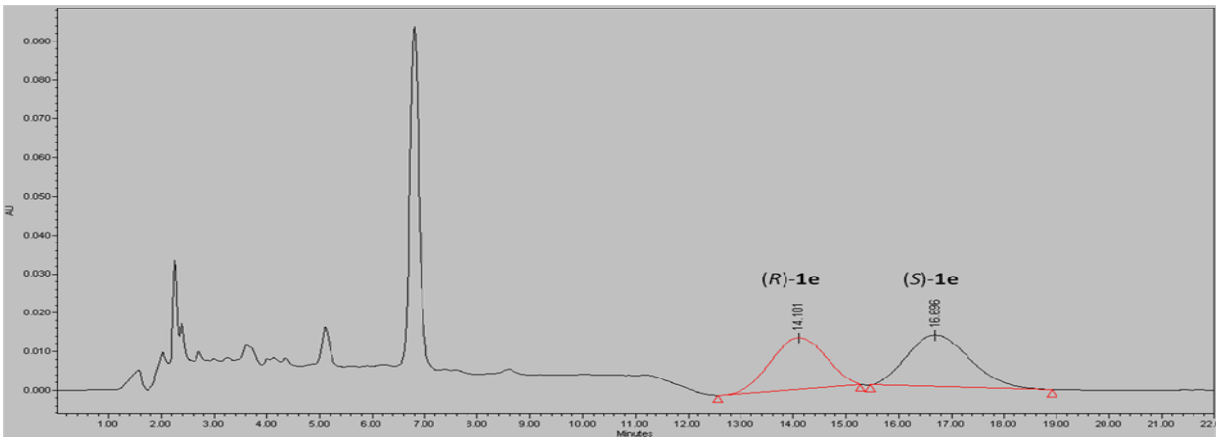
HPLC separation of GITC derivatives of **1d**



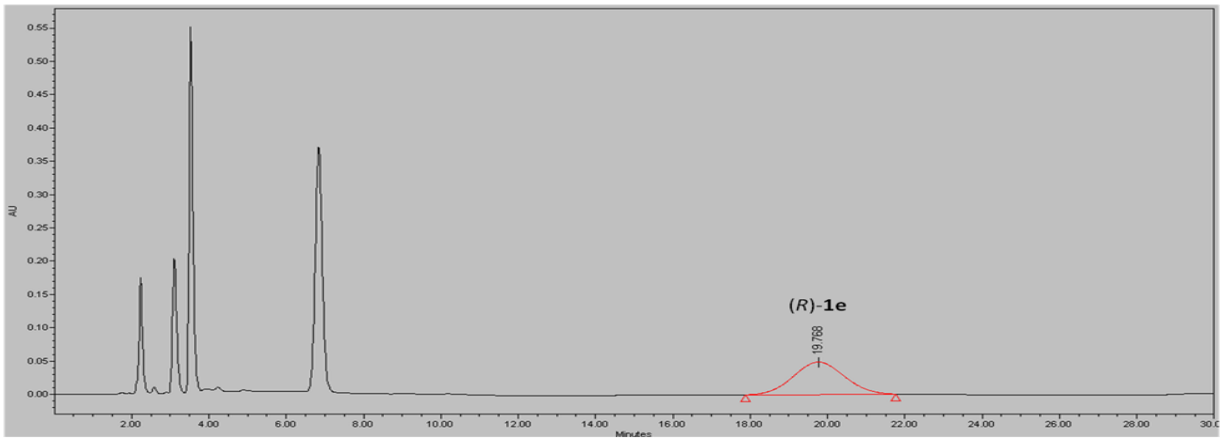
ω-TA catalyzed resolution of **1d**



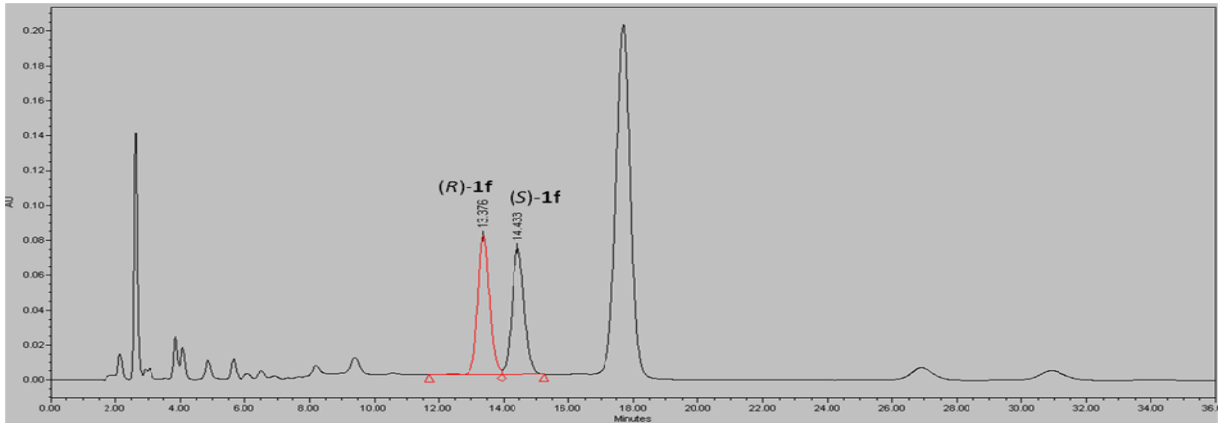
HPLC separation of GITC derivatives of **1e**



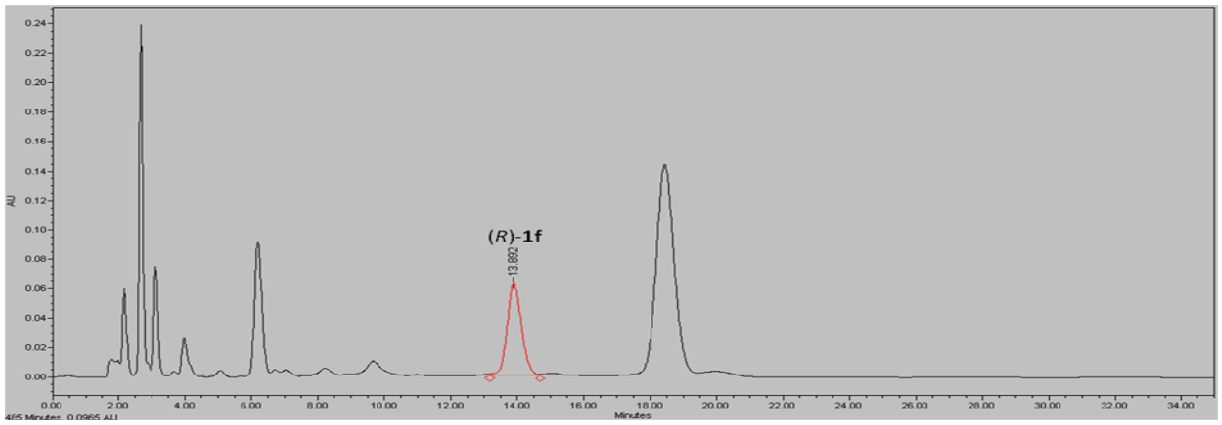
ω-TA catalyzed resolution of **1e**



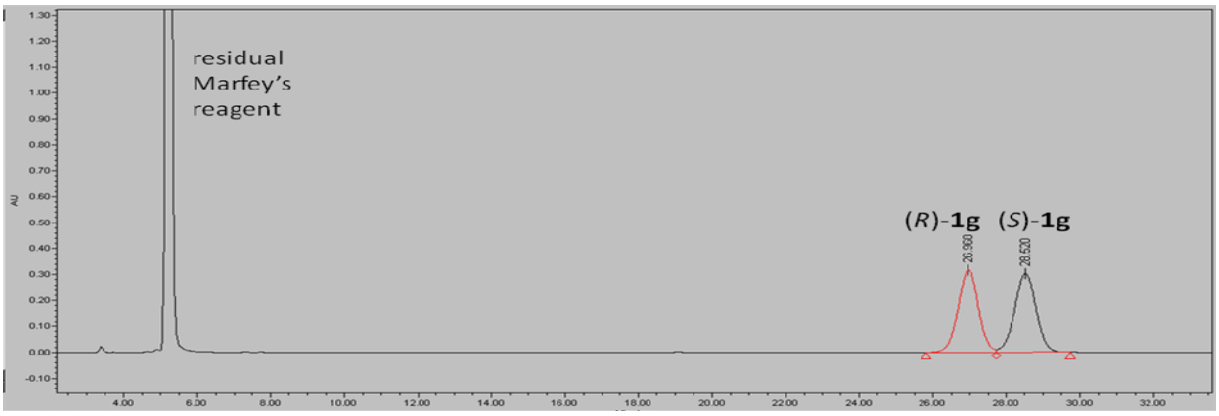
HPLC separation of GITC derivatives of **1f**



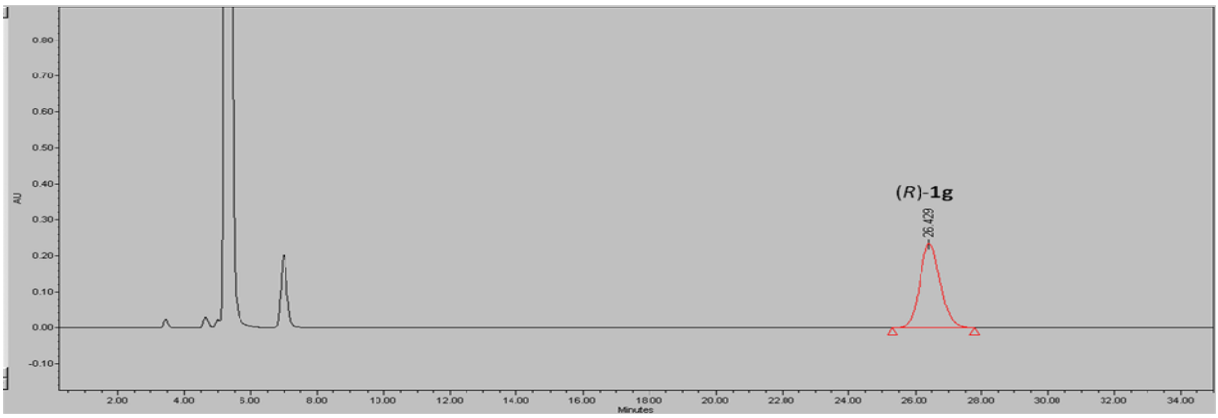
ω-TA catalyzed resolution of **1f**



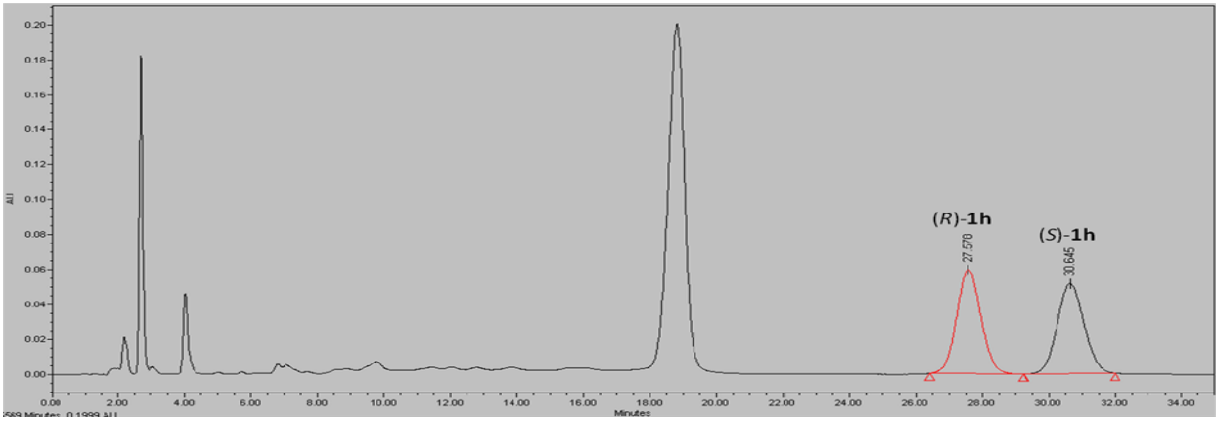
HPLC separation of Marfey's reagent derivatives of **1g**



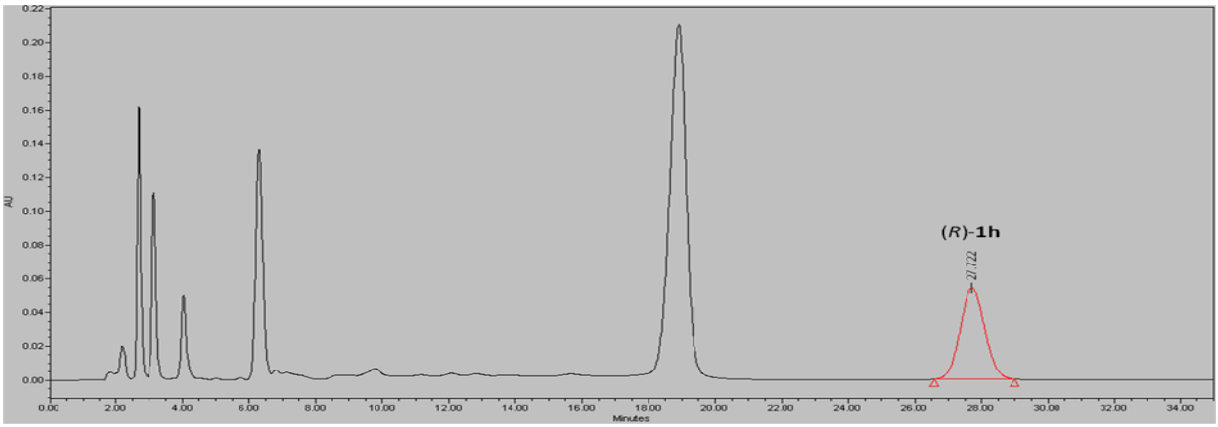
ω-TA catalyzed resolution of **1g**



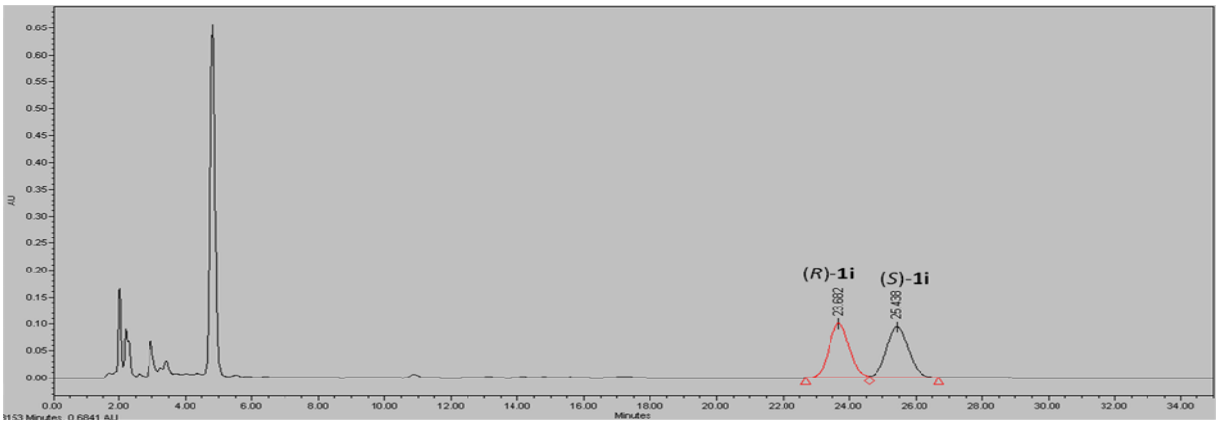
HPLC separation of GITC derivatives of **1h**



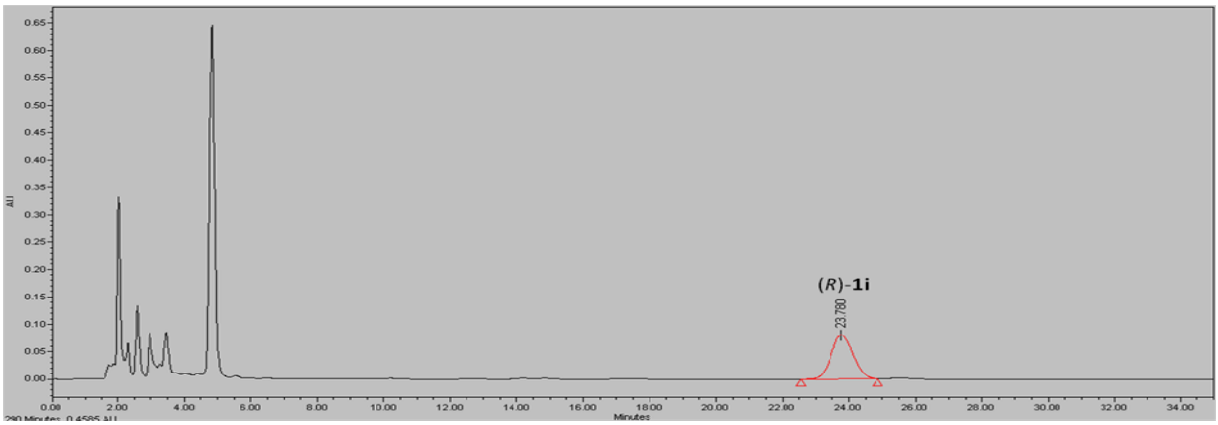
ω-TA catalyzed resolution of **1h**



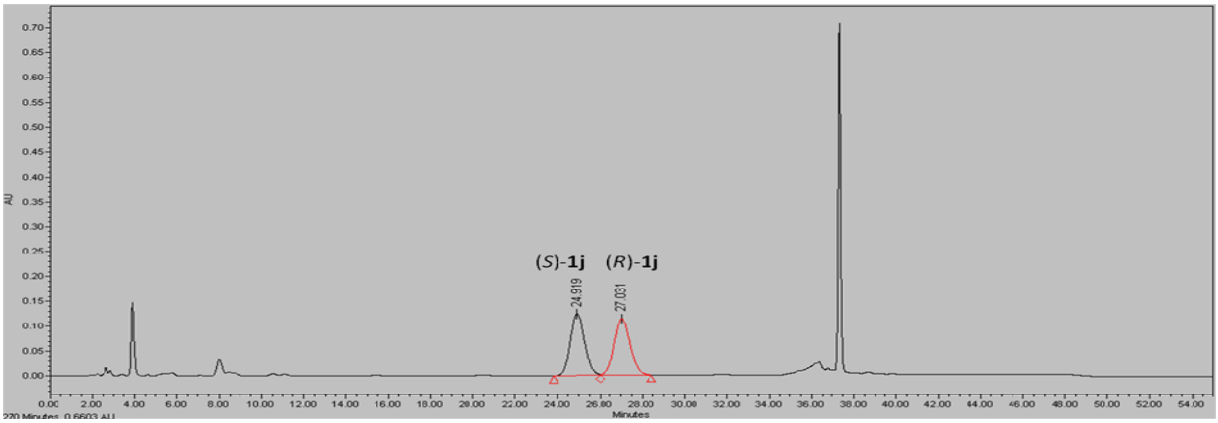
HPLC separation of GITC derivatives of **1i**



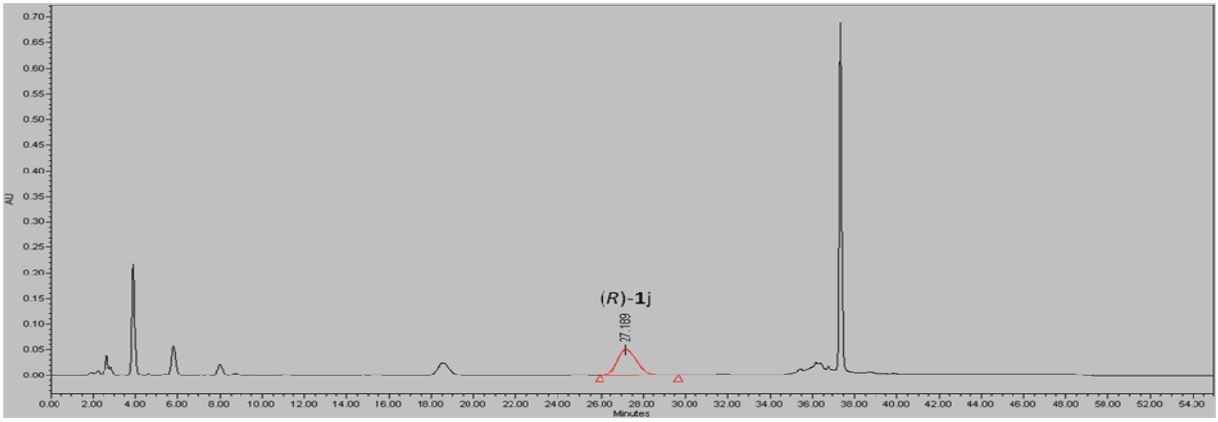
ω-TA catalyzed resolution of **1i**



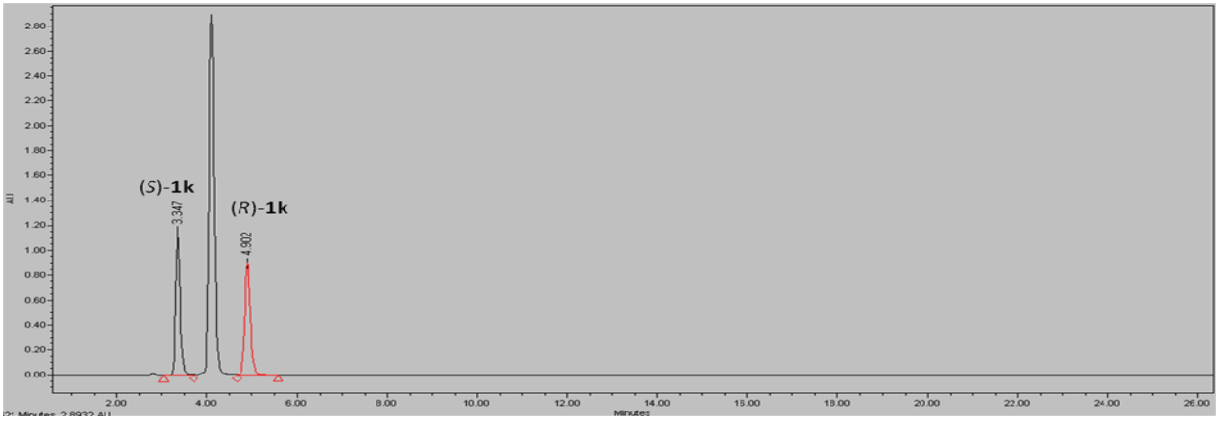
HPLC separation of GITC derivatives of **1j**



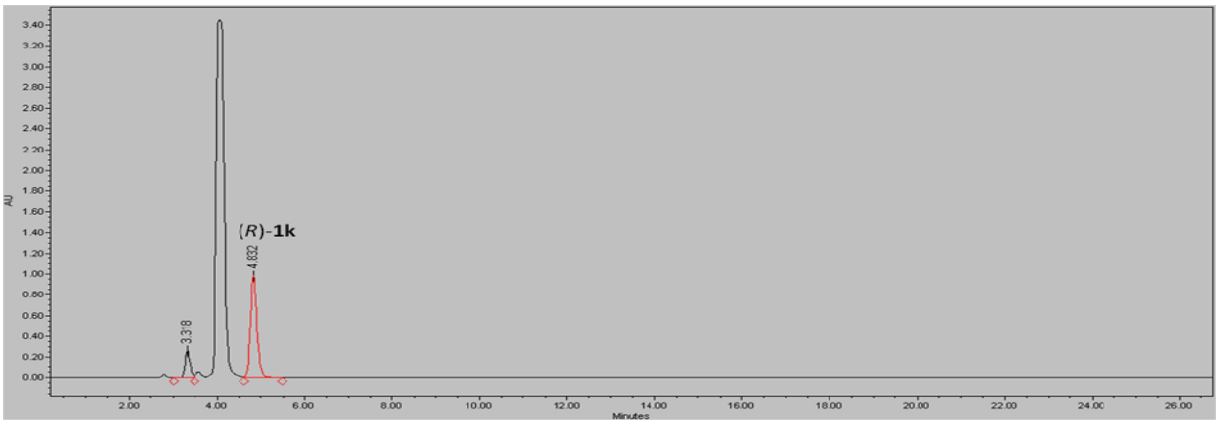
ω-TA catalyzed resolution of **1j**



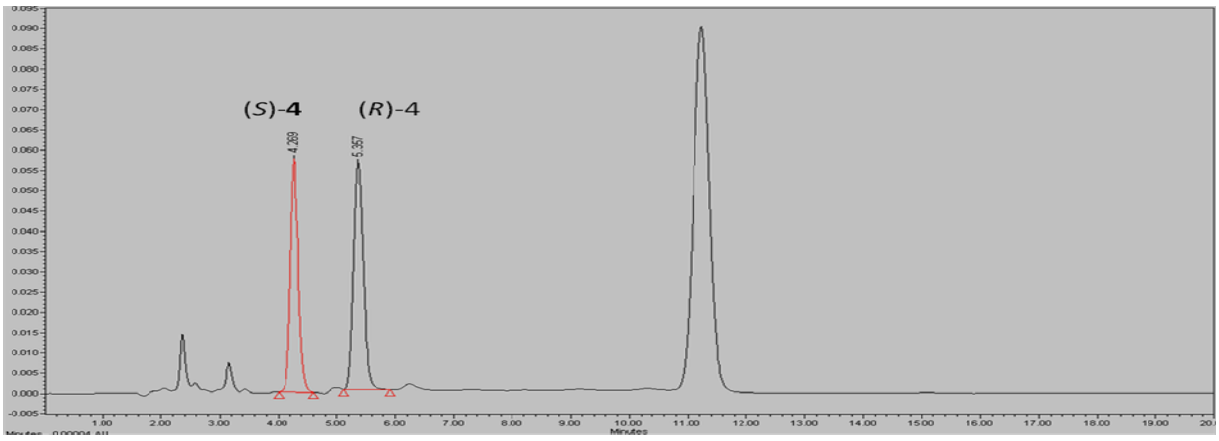
HPLC separation of Marfey's reagent derivatives of **1k**



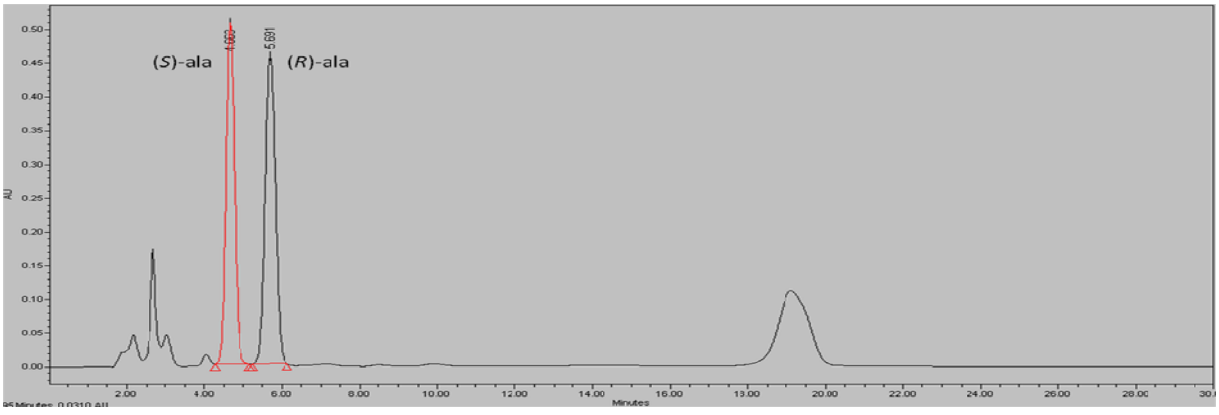
ω-TA catalyzed resolution of **1k**



HPLC separation of GITC derivatives of racemic **4**



HPLC separation of GITC derivatives of racemic alanine



9. References

1. E. Park, M. Kim and J. S. Shin, *Adv. Synth. Catal.*, 2010, **352**, 3391-3398.
2. E. S. Park, M. Kim and J. S. Shin, *Appl. Microbiol. Biotechnol.*, 2012, **93**, 2425-2435.
3. S. Ito, A. Ota, K. Yamamoto and Y. Kawashima, *J. Chromatogr.*, 1992, **626**, 187-196.
4. C. B'Hymer, M. M. Bayon and J. A. Caruso, *J. Sep. Sci.*, 2003, **26**, 7-19.
5. R. Bhushan, H. Bruckner, *Amino Acids*, 2004, **27**, 231-247.