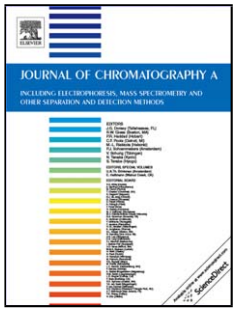

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PII: S0021-9673(09)01515-5
DOI: doi:10.1016/j.chroma.2009.10.019
Reference: CHROMA 350476

To appear in: *Journal of Chromatography A*

Received date: 19-6-2009
Revised date: 5-10-2009
Accepted date: 8-10-2009

Please cite this article as: M. Xue, H. Huang, Y. Ke, C. Chu, Y. jin, X. Liang, "Click dipeptide": A novel stationary phase applied in two dimensional liquid chromatography, *Journal of Chromatography A* (2008), doi:10.1016/j.chroma.2009.10.019

"Click dipeptide": A novel stationary phase applied in two dimensional liquid chromatography

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ABSTRACT

2D-HPLC is an important technique for the separation of complex samples. Developing new types of stationary phases is of great interest to construct 2D-LC systems with high orthogonality. In this study, a novel stationary phase-Click dipeptide (L-phenylglycine dipeptide) was prepared by immobilization of α-azido L-phenylglycine dipeptide on alkyne-silica via click chemistry. In the preparation of this new material, an efficient, inexpensive and shelf-stable diazo transfer reagent (imidazole-1-sulfonyl azide hydrochloride) was utilized to transfer the amino group of L-phenylglycine to corresponding azido group under mild conditions. The Click dipeptide thus prepared was confirmed by FT-IR, solid state CP/MAS ^{13}C NMR and elemental analysis. The Click dipeptide packing showed high orthogonality with C18, which reached 63.5%. An off-line 2D-RP/RPLC system was developed to analyze a traditional Chinese medicine (TCM)-*Rheum Palmatum* L.. The results showed high orthogonality between Click dipeptide and C18 as well as great separating power in the practical separation of complex samples.

Keywords: Click dipeptide stationary phase; α-azido L-phenylglycine dipeptide; Orthogonality; 2D-RP/RPLC; complex sample

1. Introduction

Reversed phase liquid chromatography (RPLC) is the most popular analytical tool in separation science, and has dominated analysis field for more than 30 years [1]. However, with the increasing demand for the separation and analysis of complex samples in the fields of proteomics, metabolomics, and natural products [2], the

traditional RPLC could not satisfy some of these requirements. Attempting to overcome these limitations, two dimensional liquid chromatography (2D-LC) was gradually developed [3]. Up to now, great progress in 2D-LC has been achieved in methodology, technique and application [4], and many different 2D-LC modes have been constructed, such as SEC-RP [5], IEX-RP [6], NP-RP [7], HILIC-RP [8], and so on. Even so, there are still many problems in practical application of 2D-LC, such as orthogonality, solvent compatibility and low peak capacity [9, 10].

2D-LC systems based on RP/RP should be the very useful combinations due to its high separation efficiency, great peak capacity and the completely miscible mobile phases used in each dimension [4, 9]. But 2D-RP/RPLC system is not completely perfect because of the limited orthogonality between the two dimensions [11]. This problem can be tackled partially by varying the mobile phases in the two dimensions，such as changing the gradient conditions in both dimensions [12], taking different mobile phase composition (i.e., methanol, acetonitrile, tetrahydrofuran) into account [13], or utilizing mobile phases with different pH value in both dimensions [14]. However, the results are not satisfactory enough, because the improvement of orthogonality is always limited. Another alternative is to combine different kinds of stationary phases with totally different physicochemical surfaces. In other words, there is still a demand to develop independent retention mechanisms in each dimension, such as Click OEG-C18 [4, 15]. Nevertheless, the types of stationary phases which can be combined with C18 for the purpose of high orthogonality in RP-RP mode are still rare. In this case, it is extremely important to develop new types

of stationary phases with different selectivity from that of C18 column.

As far as we know, few peptide bonded-silica stationary phases have been reported [16, 17]. Short peptide composed of hydrophobic amino acid residue is obviously different from long-chain alkane on the structures. Thus, combinating this kind of peptide-bonded silica with C18 stationary phase may bring about high orthogonality for 2D-RP/RPLC separation. The immobilization of peptide onto silica is usually accomplished by N,N'-Dicyclohexylcarbodiimide (DCC) coupling method [18-20]. However, this bonding method is not efficient enough for heterogeneous reaction, thus, the resultant surface coverage is usually low. Moreover, long reaction time and anhydrous solvent are required. Recently，Sharpless's "click chemistry" [21] has been widely used in preparation of covalently bonded silica separation materials [15, 22-26]. This strategy showed powerful ability for immobilization of different stationary phases. In 2007, an efficient, safe and conveniently prepared diazotransfer reagent was reported to transfer amino group to corresponding azido group with high selectivity [27].

In this paper, we describe an easy route for preparation of a novel Click dipeptide stationary phase by introducing azido group into dipeptide artfully. The results of orthogonality evaluation and off-line 2D-LC separation of complex samples show that Click dipeptide and C18 stationary phases are highly orthogonal.

2. Experiment

2.1. Chemicals and Reagents

Spherical silica (5 μm partical size; 10 nm pore size; 300 $m^2 g^{-1}$ surface area) was purchased from Fuji Silysia Chemical Ltd. (Japan). Copper iodide was purchased from Acros (USA). HPLC-grade acetonitrile and formic acid were purchased from Tedia (USA) and Acros (USA), respectively. Water was purified on a Milli-Q system (USA). 3-Isocyanatopropyl-triethoxysilane and propargylamine were domestic reagents and purified by distillation before use. L-phenylglycine was purchased from Shanghai Kayon Biological Technology Co., Ltd. (Shanghai, China). N,N'-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Shanghai Medpep Co., Ltd. (Shanghai, China). The test solutes (listed in Table 1) used for orthogonality evaluation were commercially available and used without further purification.

2.2. Sample preparation

The test probes (uracil, phenylamine, phenol, toluene, phenylethane), used to evaluate the performance of Click dipeptide column, were dissolved in acetonitrile /water (20/80, v/v) to afford 1 mg mL^{-1} concentration.

The solutes (listed in Table 1) used for orthogonality evaluation were dissolved in acetonitrile to form about 1 mg mL^{-1} concentration.

Rheum palmatum L. was used to further investigate the orthogonality of the 2D-RP/RPLC system. The sample was prepared as follows: 100 g *Rheum palmatum* L.

was ground into powder and decocted in 1 L water at 100 ^{o}C for 2 h. The supernatant liquid was filtered and the residue was re-decocted in another 1 L water at 100 ^{o}C for 1.5 h. The combined solutions were condensed to fine powder (about 1.7 g), which was re-dissolved in 30 mL of methanol/water (75:25, v/v), then the mixture was kept in a refrigerator overnight. The extract was filtered through a 0.2 μm regenerated cellulose membrane prior to injection.

2.3. Preparation of Click dipeptide stationary phase and column packing

The diazotransfer reagent imidazole-1-sulfonyl azide hydrochloride **1** was prepared according to the reference [27].

Condensation of 3-isocyanatopropyl-triethoxysilane with propargylamine in anhydrous DMF afforded terminal alkynyl triethoxysilane **2**, which was then directly polymerized with silica beads to afford the terminal alkyne-silica beads **3** [26].

The amino group in L-phenylglycine was smoothly transferred to corresponding azido L-phenylglycine by using an efficient, inexpensive and shelf-stable diazo transfer reagent **1** [27]. Subsequent condensation of the azido L-phenylglycine **4** with L-phenylglycine methyl ester **5** afforded the dipeptide azide **6**. Then "clicking" between the dipeptide azide and the terminal alkyne-silica afforded Click dipeptide silica **7**. The general synthesis scheme is shown in Fig. 1.

Imidazole-1-sulfonyl azide hydrochloride **1** [27]: a suspension of NaN_3 (3.2 g, 50 mmmol) in anhydrous MeCN (50 mL) in 100 mL bottom round flask was cooled in an ice bath, then sulfuryl chloride (4.0 mL, 50 mmol) was added dropwisely to this flask.

Then the mixture was stirred overnight at room temperature. At this stage, imidazole (6.8 g, 100 mmol) was added in one portion to the ice-cooled mixture. After stirring for 3 h at room temperature, the mixture was diluted with EtOAc, the organic layer was in sequence washed with H_2O and saturated aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4 and filtered. A solution of HCl in EtOH which was prepared by the drop-wise addition of AcCl (5.4 mL, 76 mmol) to ice-cooled dry ethanol (19 mL), was added slowly to the filtrate while stirring in an ice bath. Then the suspension was filtered and the filter cake was washed with EtOAc to get imidazole-1-sulfonyl azide hydrochloride **1** (7.66 g, 73%) as white crystal. ^{1}H-NMR (D_2O) δ: 9.32 (dd, 1H, J=1.3, 1.6 Hz, H-2), 8.02 (dd, 1H, J=1.6, 2.2 Hz, H-5), 7.58 (dd, 1H, J=1.3, 2.2 Hz, H-4).

α-azido L-phenylglycine **4** [27]: To the mixture of L-phenylglycine (3.02 g, 20 mmol), K_2CO_3 (6.92 g, 25 mmol) and $CuSO_4.5H_2O$ (0.05 g, 0.5 mmol) in MeOH (100 mL), Imidazole-1-sulfonyl azide hydrochloride **1** (5.04 g, 24 mmol) were added, and the reaction system was stirred at room temperature overnight. Removal of the solvents left a residue, which was diluted with H_2O and acidified with conc. HCl until pH to 2, the mixture was extracted with ethyl acetate (100 mL×3). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated. Flash chromatography gave the α-azido L-phenylglycine **4** (2.14 g, 60%) as light yellow solid. ^{1}H-NMR ($CDCl_3$) δ: 9.84 (br, 1H, CO_2H), 7.43 (m, 5H, Ar-H), 5.05 (s, 1H, N_3-CH).

L-phenylglycine methyl ester **5** was synthesized according to a method described in

the literature [28]. AcCl (12.0 mL, 169 mmol) was added drop-wise to ice-cooled MeOH (75 mL), then L-phenylglycine (8.61 g, 57 mmol) was added. The mixture was heated under reflux for 3 h. The solvent was evaporated to get L-phenylglycine methyl ester hydrochloride as white solid. Then the hydrochloride was dissolved in saturated aqueous Na_2CO_3 and subsequently extracted with EtOAc (200 mL×3). The organic phase was collected and dried over anhydrous Na_2SO_4. Removal of all volatiles gave L-phenylglycine methyl ester **5** (8.16 g, 86.8%) as yellow oil.

General procedure for preparation of α-azido L-phenylglycine dipeptide **6**: DMAP (89 mg, 0.73 mmol) was added to the solution of L-phenylglycine methyl ester (1.33 g, 8.0 mmol) in anhydrous CH_2Cl_2 (40 mL) in an ice bath and the mixture was stirred for 20 min. Then α-azido L-phenylglycine (1.30 g, 7.33 mmol) and HOBt (0.99 g, 7.33 mmol) were added to the ice-cooled mixture and stirred for 4 h at 0 ^oC then another 12 h at room temperature. Flash chromatography gave α-azido L-phenylglycine dipeptide (1.66 g, 69.9%) as white solid. ^1H-NMR ($CDCl_3$) δ: 7.42-7.30 (m, 10H, Ar-H), 5.56 (d, 1H, J=7.2 Hz, NH-CH), 5.10 (s, 1H, N_3-CH), 3.75 (s, 3H, O-CH_3). FT-IR (KBr): 3045 cm^{-1} (Ar-H), 2118 cm^{-1} (-N_3), 1744 cm^{-1} (CO_2Me) and 1668 cm^{-1} (CONH).

Immobilization of α-azido L-phenylglycine dipeptide **6** on alkyne-modified silica: CuI (29 mg, 0.15 mmol) was dissolved in CH_3CN/CH_3OH (v/v, 1:1, 100 mL) by ultrasonic, then the dipeptide **6** (0.99 g, 3.05 mmol) and alkyne-silica **3** (2.5 g) were added successively. The mixture was stirred slowly at room temperature for 72 h then filtered. The filter cake was washed with MeOH (200 mL), CH_3CN (200 mL), 10% EDTA (300 mL), water (300 mL), CH_3OH (200 mL), acetone (200 mL) in turn. The

177 solid material was collected and dried in vacuo before packing.

178 The Click dipeptide stationary phase was slurry-packed into stainless-steel column
179 (100 mm×2.1 mm i.d.) with ethanol/acetone (1:1, v/v) as slurry solvent and
180 propulsion solvent.

181

182 *2.4. Apparatus and chromatographic conditions*

183 FT-IR measurements were performed on a Nicolet 5SXC (USA) with KBr pellets.
184 ^{1}H-NMR identifications were carried out on a Bruker 400 (Germany). ^{13}C CP/MAS
185 NMR characterization was performed on a Bruker AVANCE 500 (Germany).
186 Elemental analysis was measured on an elementar vario EL III (Germany).

187 All the chromatographic separations were performed on an Agilent 1200 HPLC
188 system, which comprised a binary pump, a degasser, an autosampler, an automatic
189 thermostatic column compartment and a diode array detector (DAD). Click dipeptide
190 column (5 μm, 100 mm×2.1 mm i.d., laboratory-made); XTerra MS C18 column (5
191 μm, 150 mm×2.1 mm i.d., Waters). The columns temperatures were maintained at 30
192 ^{o}C and the flow rate was set at 0.2 mL min^{-1} during all of the analyses.

193 The column efficiency of Click dipeptide was evaluated with 85% water and 15%
194 acetonitrile as the mobile phases. The injection volume was 1 μL. During the
195 orthogonality evaluation between Click dipeptide and C18, mobile phases were
196 composed of 70% water and 30% acetonitrile. The injection volume was 1 μL, and the
197 chromatogram was extracted at 254 nm. In 2D-RP/RPLC analysis of *Rheum*
198 *palmatum* L., Click dipeptide column was employed as the first dimension and XTerra

MS C18 column as the second dimension. The mobile phases for both dimensions consisted of water with 0.1% (v/v) formic acid (A) and acetonitrile (B). In the first dimension, the injection volume was 5 μL and the corresponding linear gradient eluent started from 5% B to 30% B within 15 min, then to 90% B in the next 15 min, and kept at this concentration for 6 min. 35 fractions were collected from 1 min to 36 min at an interval of 1 min, and marked as Fraction 1 to Fraction 35 in succession. Afterwards, 50 μL of each fraction was re-injected onto the second dimension without concentration, followed by linear gradient eluent from 5% B to 30% B in 25 min, to 90% B in another 25 min, and kept for 10 min.

2.5. Data analysis

The Origin 7.0 and Microsoft 2003 were used for data plots and calculations.

The geometric approach for orthogonality evaluation was according to the reference [29]. First, retention times of 25 solutes on each one-dimensional LC setup were normalized according to Eq. (1):

$$RT_{i(norm)} = \frac{RT_i - RT_{min}}{RT_{max} - RT_{min}} \quad (1)$$

where RT_{max} and RT_{min} were the retention times of the of the most and least retained solute in the data set, respectively. In this way, the retention times RT_i of solutes were converted into normalized $RT_{i(norm)}$. Then, a two-dimensional (2D) separation space was divided into 5 × 5 bins based on the number of solutes, and the normalized retention data were plotted into this 2D separation space. The orthogonality *O* was calculated according to Eq. (2):

$$O=\frac{\sum \text{bins}-\sqrt{P_{max}}}{0.63P_{max}} \quad (2)$$

in which ∑bins was the number of bins containing data points in the 2D plot. P_{max} was the sum of all bins, which represented the total peak capacity, i.e, 25 in this evaluation system.

3. Results and discussion

3.1. Preparation and characterization of Click dipeptide stationary phase

In this work, the dipeptide was synthesized successfully by coupling two kinds of L-phenylglycine derivates. The key intermediate α-azido L-phenylglycine was prepared utilizing imidazole-1-sulfony azide hydrochloride as diazo donor, which was an efficient, safe and conveniently prepared diazotransfer reagent. By this way, azido group can be introduced easily under mild condition. Subsequent condensation α-azido L-phenylglycine with L-phenylglycine methyl ester gave the product of α-azido L-phenylglycine dipeptide. The structure of α-azido L-phenylglycine dipeptide was confirmed by FT-IR and ^{1}H-NMR (listed in section 2).

3-Isocyanatopropyl-triethoxysilane reacted with propargylamine in anhydrous DMF to afford terminal alkynyl triethoxysilane, which was then directly polymerized with silica beads to achieve the terminal alkyne-silica beads. The ‘‘clicking’’ between the terminal alkyne-silica and α-azido dipeptide in the presence of Cu (I) catalyst yielded the functionalized silica, which was packed as column.

The alkyne-silica and Click dipeptide stationary phase was characterized by FT-IR and elemental analysis. $\nu_{\equiv CH}$ on alkyne-silica at 2928 cm^{-1} was weakened after “click”

243 step. The results of elemental analysis and the surface concentration are shown in
244 Table 2. The carbon content of Click dipeptide is 12.77% (RSD=0.5%, n=5), and the
245 nitrogen content is 3.37% (RSD=0.4%, n=5). The increase of carbon content
246 demonstrated that dipeptide was bonded to alkyne-silica successfully. According to
247 the equation proposed by Kibbey and Meyerhoff [30], the surface concentration
248 (calculated from carbon content) of alkynyl group on alkyne-silica was 2.73 μmol m^{-2},
249 and the surface concentration of dipeptide (calculated from the increment of carbon
250 content) on the Click dipeptide stationary phase was 0.98 μmol m^{-2} (RSD=1.1%, n=5).
251 Solid state ^{13}C CP/MAS NMR result (Fig. 2) further supported the immobilization
252 of dipeptide on alkyne-silica. Compared with the ^{13}C CP/MAS NMR datum of
253 alkyne-silica [26], the signals on Click dipeptide could be assigned as follows: the
254 intense signals around at 159 ppm and 127 ppm were ascribed to the carbon atoms on
255 the carbonyl groups and aromatic carbons respectively. The signals between 66 ppm
256 and 59 ppm were assigned to carbon atoms linked with oxygen atoms. The signals
257 between 50 ppm and 40 ppm were attributed to the methylene (**c**, **d**) and the chiral
258 carbons (**e**, **f**) bounded to nitrogen atoms. The signals at 9.2 ppm and 22.8 ppm were
259 assigned to the carbon atom **a** and **b** respectively.
260
261 *3.2. Orthogonality evaluation of Click dipeptide stationary phase with C18*
262 Before the orthogonality evaluation, the column efficiency of Click dipeptide was
263 investigated with uracil, phenylamine, phenol, toluene and phenylethane as test probes.
264 As shown in Fig. 3, the peak symmetry is 1.07, retention factor is 5.46 (RSD=1.3%,

n=5) and column efficiency is 33,800 plates m^{-1} (RSD=8.8%, n=5) calculated from phenylethane according to the USP method. At the same time, the detection limit of standard compounds (at 254 nm) on Click dipeptide was investigated and compared with that on C18 column. It is showed that the detection limit on Click dipeptide was approximately 2 times as high as that on C18. In a conclusion, the column efficiency, peak shape and detection limit can satisfy the separation, and it is possible for Click dipeptide to develop a 2D-LC system with C18 for further orthogonality evaluation.

As we know, orthogonality is one of the main concerns in designing a 2D separation system. Recently, the geometric method for evaluation of orthogonality developed by Gilar et al. [29] has been used to describe the orthogonality of 2D-LC separation system [4]. In this work, this geometric method was employed to evaluate the orthogonality between Click dipeptide and C18 with 25 aromatic compounds (listed in Table 1). The evaluation results showed that the orthogonality between Click dipeptide and C18 was as high as 63.5%. As shown in Fig. 4, the normalized retention times of test solutes on Click dipeptide were plotted against those on C18. The value of correlation coefficient r was 0.534 for this two dimensional separation plot, which illustrated the dispersion of test solutes on the two columns. Some of the test probes getting poor resolution in one-dimensional separation can be well resolved with the orthogonal system. This instance can be well described by the data points of 5 different test probes on the dashed line **a** and **b**. It was visible that solutes 9 (4-nitroacetophenone), 25 (4-ethylaniline) and 1 (benzene) on the transverse line **a** could hardly be separated on Click dipeptide, but easily be differentiated by C18. With

287 regard to solutes 15 (5-methoxyindole), 25 (4-ethylaniline) and 20 (2-nitrophenol) on
288 the vertical line **b**, the case was just reverse. Obviously, if a sample contained above
289 five constituents, one-dimensional separation would fail to provide sufficient
290 resolution, but the orthogonal combination of Click dipeptide with C18 could resolve
291 this problem.

292 The greatly different selectivity of these two columns was attributed to the dramatic
293 difference of the stationary phase surfaces. As we know, hydrophobic interaction
294 provided by the long chain alkane is the main effect on C18. While on Click dipeptide,
295 the hydrophobic interaction gained from phenyl group is weaker than that on C18,
296 which can be confirmed by the less retention of test solutes. Meanwhile, phenyl group
297 provide π-π interaction, which may have special effects on aromatic compounds.
298 Moreover, peptide bond, ester group and triazole ring may bring about hydrogen
299 bonding and dipole-dipole interactions. Furthermore, the triazole ring can be
300 protonated at low pH and makes electrostatic interaction during the separation of
301 acidic or basic compounds [31]. All of these may lead to high orthogonality from the
302 combination with C18.

303 The evaluation of orthogonality was still important for applying the new Click
304 dipeptide column in combination with C18 to separate complex samples. The
305 orthogonality evaluation may offer a reference for separation of complex compounds
306 such as TCM, which contained many compounds that was difficult to be separated
307 only by one dimension.

308

309 *3.3. 2D-RP/RPLC analysis of the complex TCM sample*

A complex TCM sample-*Rheum palmatum* L. was analyzed beforehand on one-dimensional Click dipeptide column and C18 column respectively. The separations were performed under approximately the same gradient conditions in both dimensions, because the gradient time increased in proportion to the length of the column. In this case, the system orthogonality can be ascribed to the different separation mechanisms and the properties of the samples involved. Different selectivity to the samples between the two columns can be observed easily from the chromatograms (Fig. 5), thus high orthogonality can be anticipated.

The off-line 2D-RP/RPLC analysis was carried out by repeating fractionation-reinjection process. The eluents were collected in vials manually from the first dimension (Click dipeptide column) at 1 min interval, and 35 fractions in total were collected. Then these fractions were re-injected onto the second dimension (C18 column). Herein, an off-line 2D-LC setup was preferred, because it allowed the use of a second column with much higher efficiency, offered a greater flexibility, and claimed low demands on the equipments than on-line setup [10]. The reason why C18 column was selected as the second dimension and Click dipeptide as the first one could be elucidated as following: the better resolution capability and higher efficiency of C18 column in the second dimension is beneficial for the further analysis of complex samples [4, 32]. Moreover, a relatively lower retention column in the first dimension is helpful for focusing fractions on the top of the second dimension column ("on-column focusing") [6, 32, 33]. The most important benefit of this "on-column focusing" technique is that the fractions eluted from the first dimension can be

directly analyzed in the second dimension with high injection volume without peak broadening. With this approach, there was no need to concentrate samples prior to the second dimensional analysis, so sample loss and contamination could be avoided during the process of analysis.

The practical 2D-LC analysis results further validated that the system based on Click dipeptide and C18 was highly orthogonal. When the simple fractions from the Click dipeptide column were analyzed on the C18 column, many peaks were detected. For example, when fraction 5 (eluent between 5 min and 6 min in Fig. 5A) was re-analyzed on Click dipeptide column (Fig. 6A), only two peaks (peak a_1 and b_1) could be observed, while on C18 column (Fig. 6B), not only the peak a_1 and b_1 were better separated, but also many new peaks with low intensity appeared. Fraction 11 (eluent between 11 min and 12 min in Fig. 5A) was a pure ingredient (Fig. 7A) on Click dipeptide column, however, more low abundance components in fraction 11 could be separated in the second dimension analysis (Fig. 7B). These facts showed that low-abundance components buried under the main peak in the first dimensional column could be well resolved in the second dimensional column. Similar results could be observed from other fractions. Based on the information mentioned above, 2D-LC analysis could provide a more reliable view for the minor compounds in complex samples. Also it is a very useful access to find the low-abundance components with highly pharmacological activity or toxicity [34]. The combination of Click dipeptide column with C18 column may play an important role for better recognition of traditional Chinese medicine, in which a wide range of relative low

354 abundance compounds exist.

355 In order to further illustrate the orthogonality and separation ability of this
356 combination comprehensively, a three-dimensional projection chromatogram (Fig. 8)
357 for the fractions 1 to 35 was given. This 3D chromatogram was constructed from each
358 HPLC/UV chromatogram stacked side by side and viewed from a side perspective.
359 Calculated according to the literature method [32], the theoretical peak capacity of
360 Click dipeptide and C18 column was about 30 and 64 respectively, thus the peak
361 capacity of this off-line 2D-LC system was about 1920 in theory. Compared with
362 other combinations such as CN/C18 [32], the same level of peak capacity can be
363 obtained as long as manipulated under the same column length and mobile phase
364 conditions. With the great improvement in peak capacity and good orthogonality,
365 more than 400 peaks can be separated with this new 2D-LC system, while on the
366 one-dimensional separation (Fig. 5B), only about 60 peaks can be observed. So the
367 separation ability of this new system was powerful, which was beneficial for the
368 analysis of complex samples.

369 Our group has reported “Click OEG/C18 RP/RP” [4] and “C18/Click β-CD
370 RP/HILIC” [34] 2D-LC systems. Both of them exhibited excellent orthogonality in
371 the separation of complex TCM samples. Comparing the structures of Click OEG
372 with Click dipeptide, we suppose that the more diversity of functional groups on Click
373 dipeptide may lead to more effect such as π-π interaction than that on Click OEG.
374 While Click β-CD was used in HILIC mode mainly due to the high density of
375 exposed hydroxy groups [35]. Compared with other systems [36, 37], this new

combination keeps the advantage of 2D-RP/RPLC，such as high separation efficiency, great peak capacity and mobile phases' compatibility with MS detection. Furthermore, Click dipeptide possessed greatly different stationary phase surface, thus the chromatographic property is unique. In summary, Click dipeptide is a good partner to C18 for the RP/RP 2D-LC separation of complex samples.

4. Conclusions

A novel stationary phase termed Click dipeptide was synthesized via click chemistry. The key intermediate α-azido L-phenylglycine dipeptide was prepared easily utilizing an efficient, inexpensive and shelf-stable diazo transfer reagent. The successful immobilization was confirmed by FT-IR, solid state ^{13}C CP/MAS NMR and elemental analysis. The orthogonality evaluation result not only showed the high orthogonality between Click dipeptide and C18, but also validated the dramatically different selectivity between them. This new packing material was then applied in 2D-RP/RPLC combined with C18 column for the separation of complex TCM sample. An off-line 2D-LC approach was adopted, and the same mobile phases were used in both dimensions, thus avoided the problems of immiscibility and incompatibility. "On-column focusing" method enabled the suppressing band broadening. Besides, sample loss and contamination would not occur. With the combination of Click dipeptide and C18, much more information could be obtained from the complex samples. Click dipeptide served as an excellent complementary stationary phase to traditional C18 stationary phase due to its unique structure. The coupled-column

displayed great separation power as well as excellent orthogonality. It is reasonable to believe that Click dipeptide will be a promising RPLC packing material, and its combination with C18 as 2D-RP/RPLC system will find widespread application in the separation of complex samples.

Acknowledgements

This work was sponsored by the start-up fund from East China University of Science and Technology and National Natural Science Fund for Distinguished Young Scholars (20825518).

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FIGURE CAPTION

Fig. 1 Scheme for preparation of Click dipeptide

Fig. 2 Solid State ^{13}C CP/MAS NMR spectrum of Click dipeptide stationary phase.

Fig. 3 Chromatogram for column efficiency evaluation of Click dipeptide (5μm, 100 mm × 2.1 mm i.d.). Mobile phase: ACN/H_2O=15/85, flow rate: 0.2 mL min^{-1}, column temperature: 30 ^oC, UV detection: 220 nm.

Fig. 4 Normalized retention time plot for 2D-LC system of Click dipeptide and C18. Solutes information are listed in Table 1. Experimental conditions are given in section 2.

Fig. 5 Chromatograms of one-dimensional separation of *Rheum palmatum* L. on Click dipeptide column (A) and C18 column (B). UV detection: 280nm. More detailed experimental conditions are given in Section 2.

Fig. 6 Chromatograms of Fraction 5 analyzed on Click dipeptide column (A) and C18 column (B). UV detection: 280 nm. More detailed experimental conditions are given in Section 2.

Fig. 7 Chromatograms of Fraction 11 analyzed on Click dipeptide column (A) and C18 column (B). UV detection: 280 nm. More detailed experimental conditions are listed in Section 2.

Fig. 8 Three-dimensional chromatogram of fractions 1 to 35 analyzed on C18 column. X-axis: Fractions 1 to 35; Y-axis: retention time/min; Z-axis: absorbance/mAu. UV detection: 280 nm. More detailed experimental conditions are given in Section 2.

Fig. 1

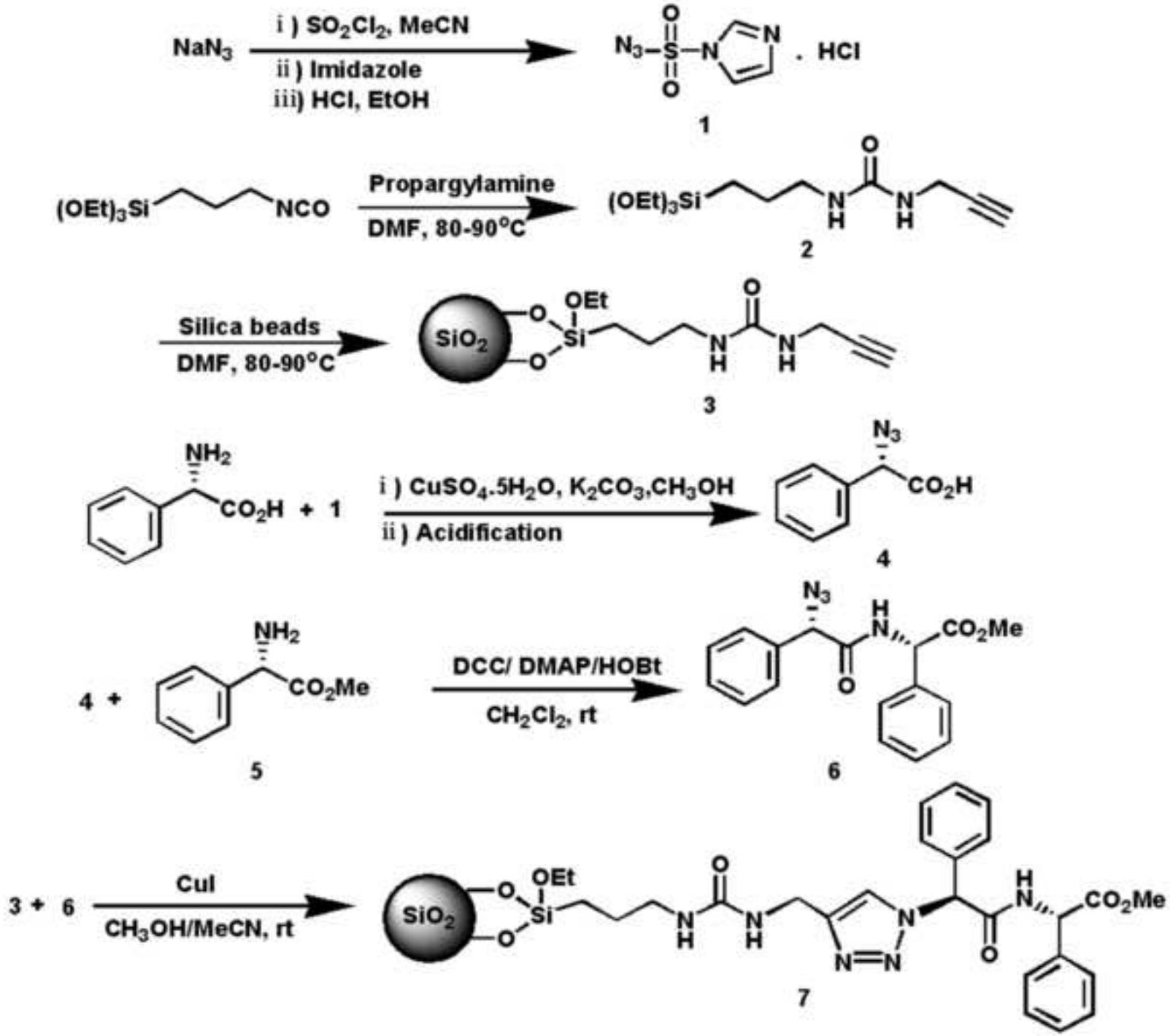

NaN3
i) SO2Cl2, MeCN
ii) Imidazole
iii) HCl, EtOH
. HCl
1
(OEt)3Si
NCO
Propargylamine
DMF, 80-90°C
2
Silica beads
DMF, 80-90°C
SiO2
OEt
3
NH2
CO2H + 1
i) CuSO4.5H2O, K2CO3,CH3OH
ii) Acidification
4
4 +
CO2Me
5
DCC/ DMAP/HOBt
CH2Cl2, rt
6
3 + 6
CuI
CH3OH/MeCN, rt
7

Fig. 2

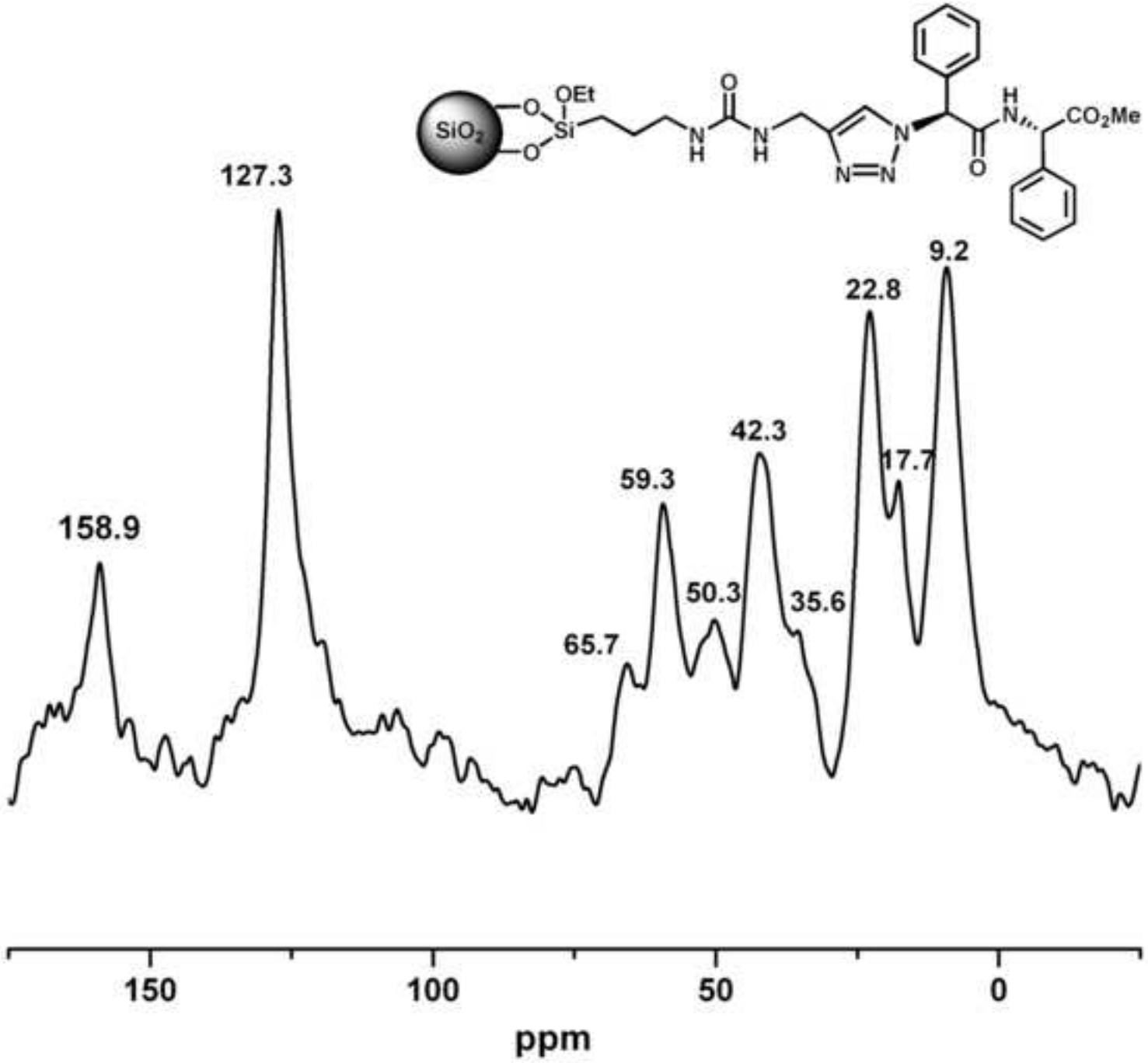
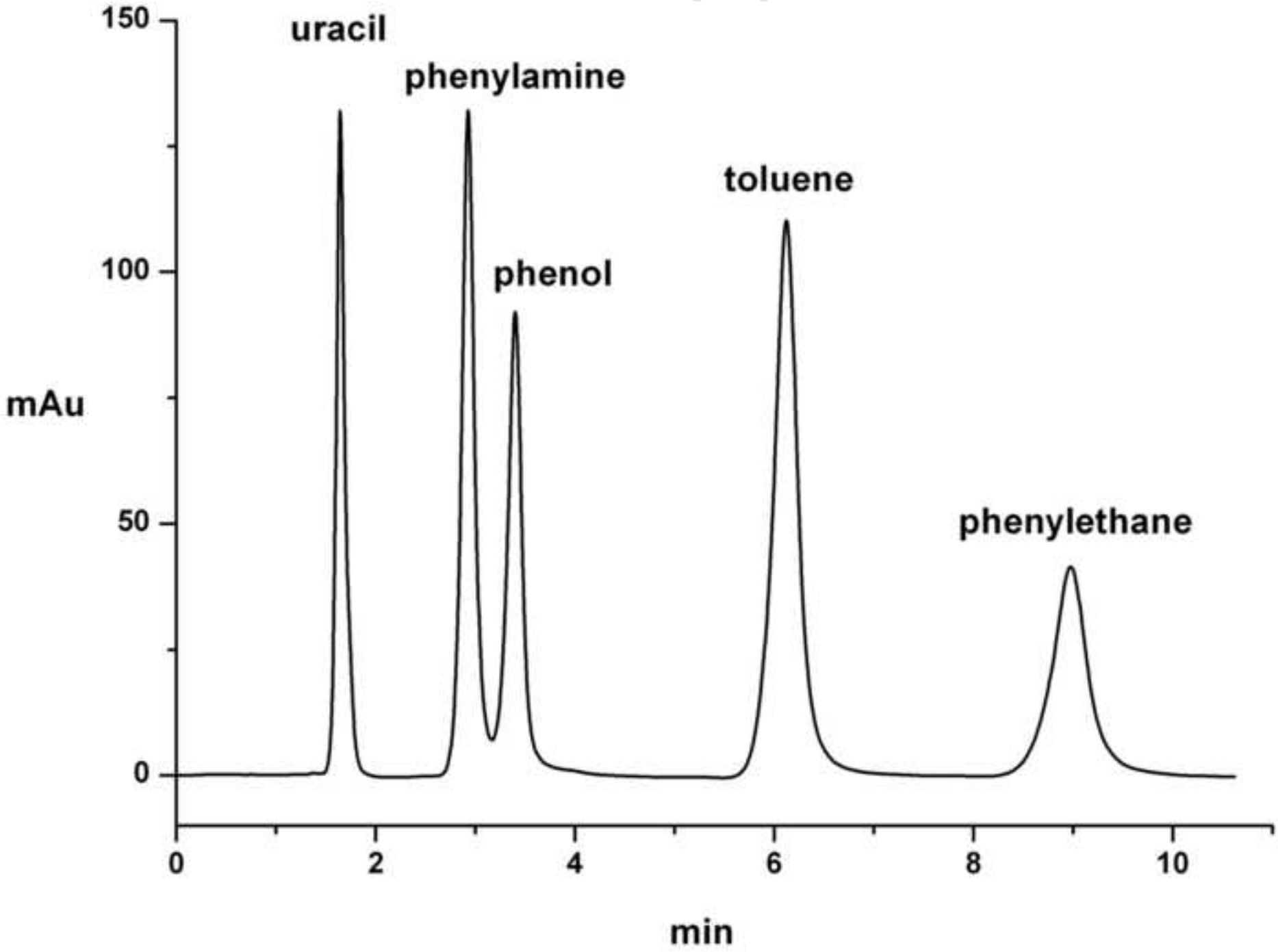

SiO2
OEt
Si
O
N
H
N
N
N
O
H
N
CO2Me
127.3
158.9
65.7
59.3
50.3
42.3
35.6
22.8
17.7
9.2
150
100
50
0
ppm

Fig. 3


150
100
50
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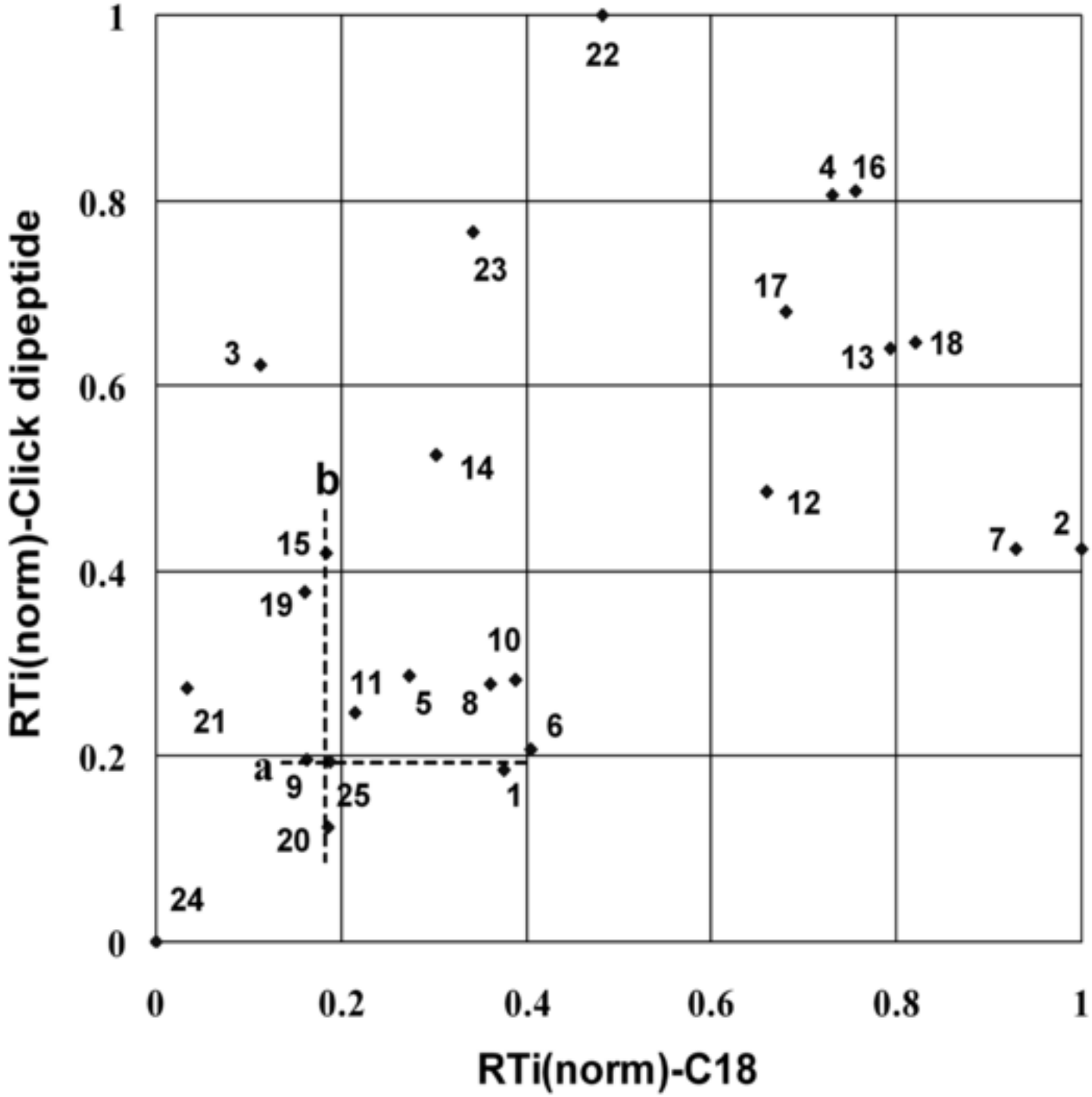
uracil
phenylamine
phenol
toluene
phenylethane
0
2
4
6
8
10
min

Fig. 4


RTi(norm)-Click dipeptide
RTi(norm)-C18
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0.2
0.4
0.6
0.8
1
22
4
16
23
17
3
13
18
14
12
b
15
7
2
19
10
11
5
8
21
6
a
9
25
1
20
24

Fig. 5A

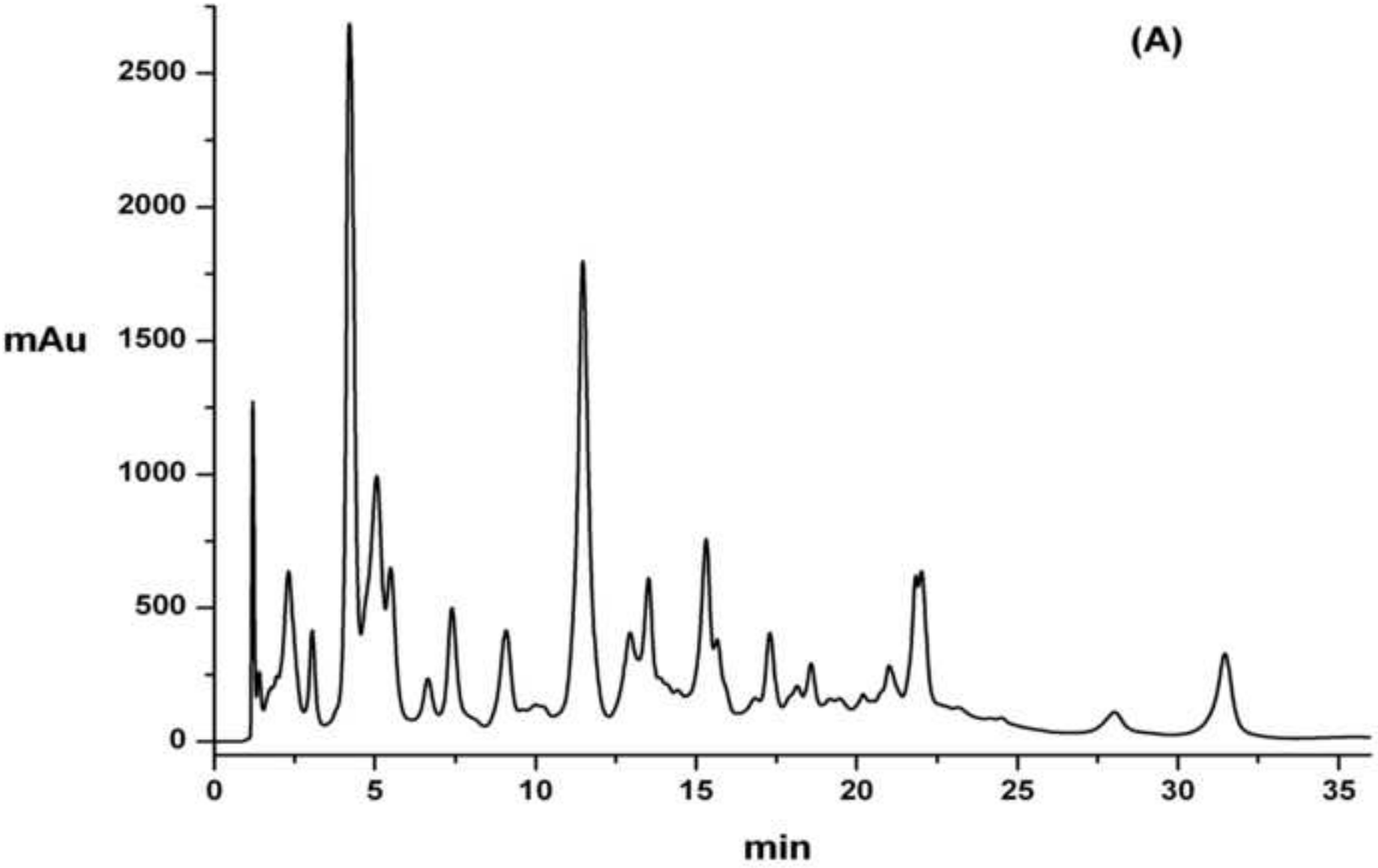

Fig. 5B

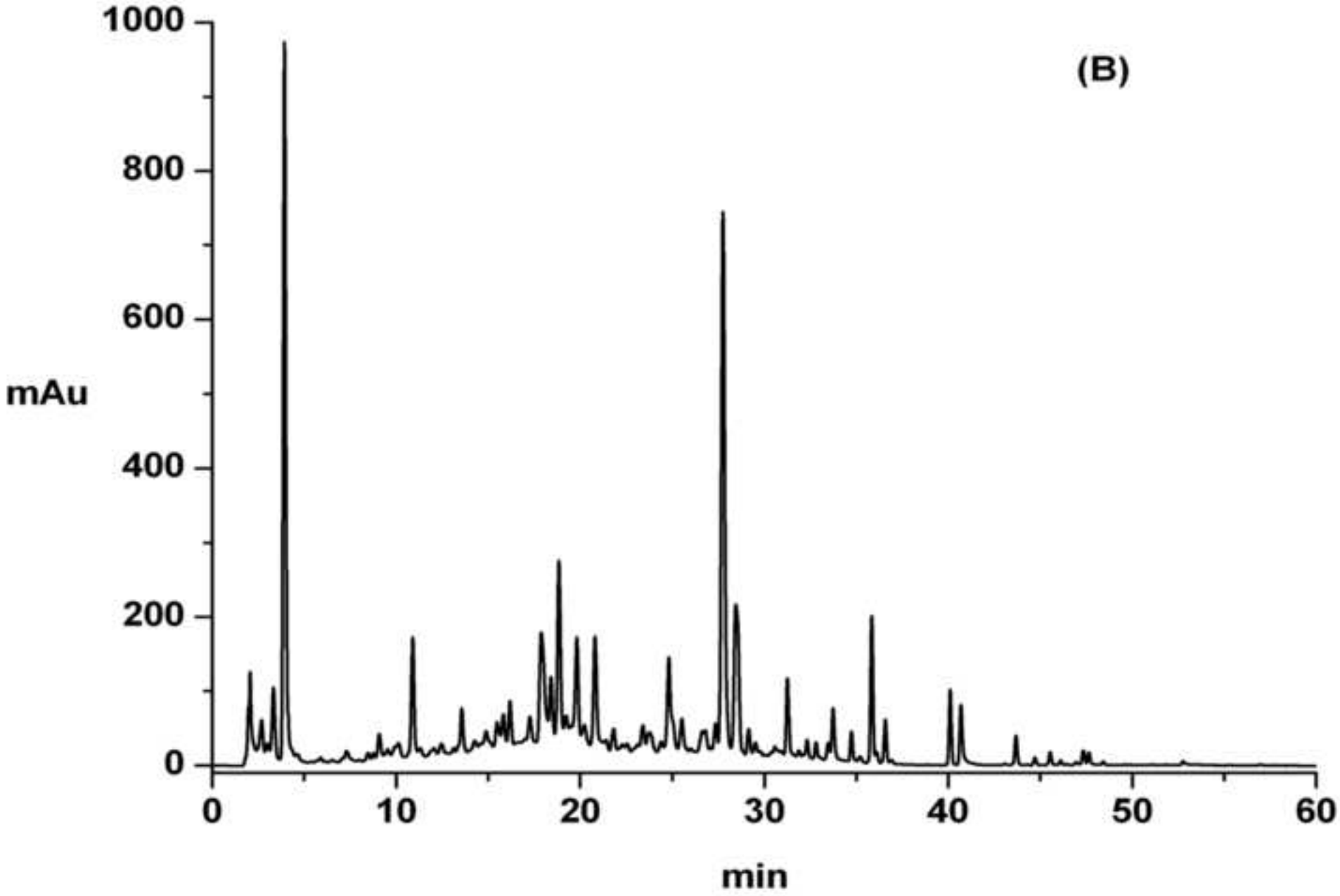

Fig. 6A

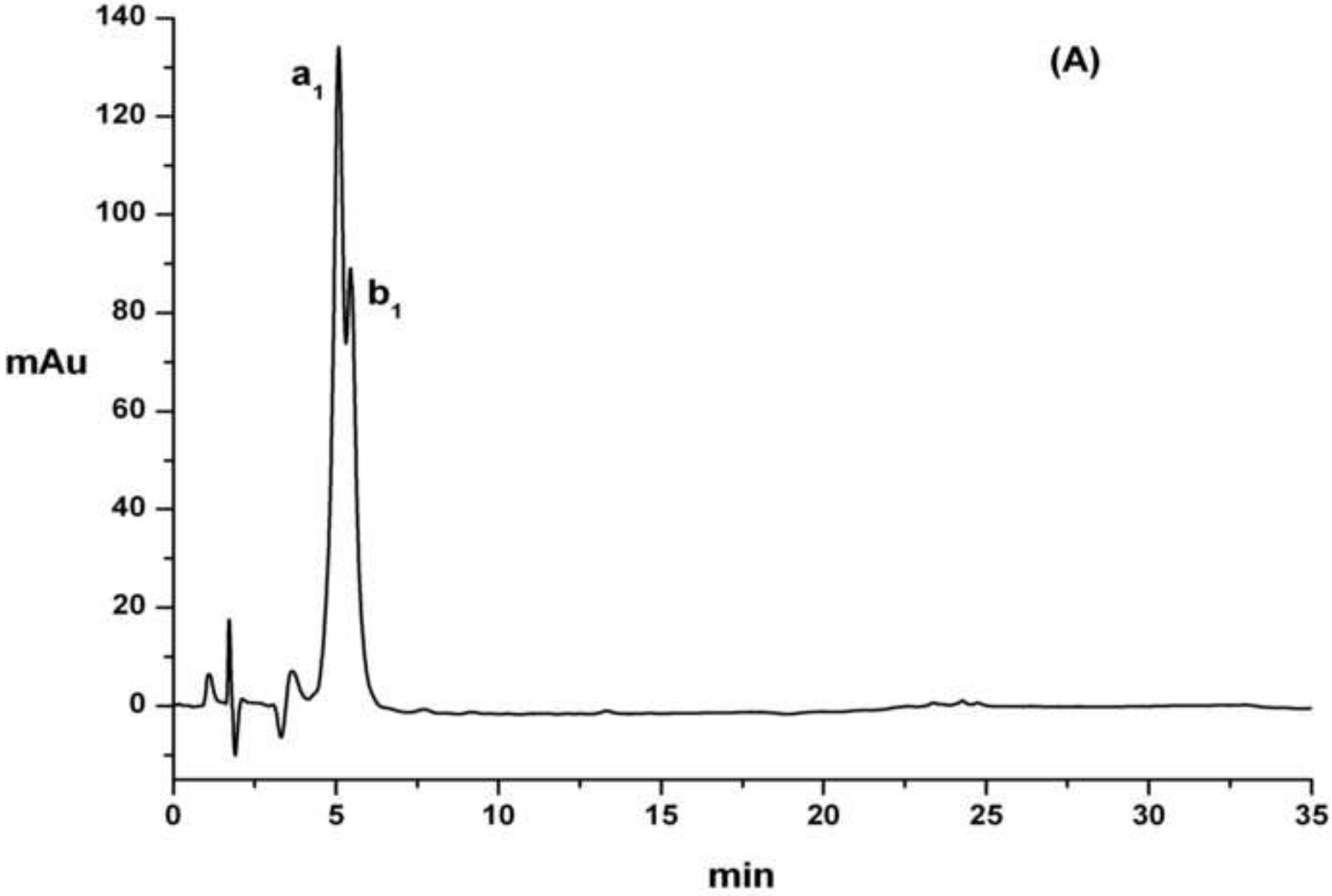

Fig. 6B

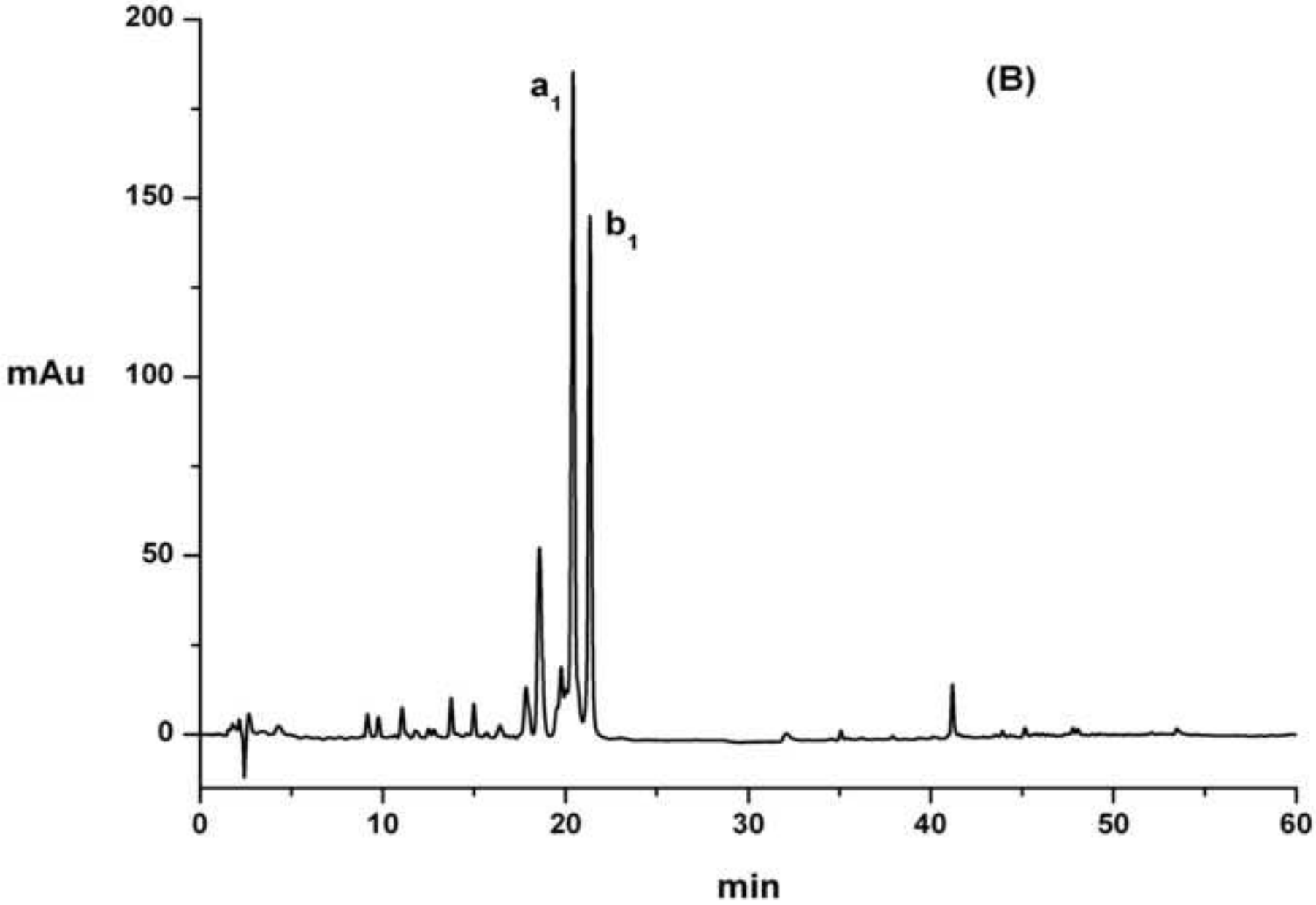

Fig. 7A

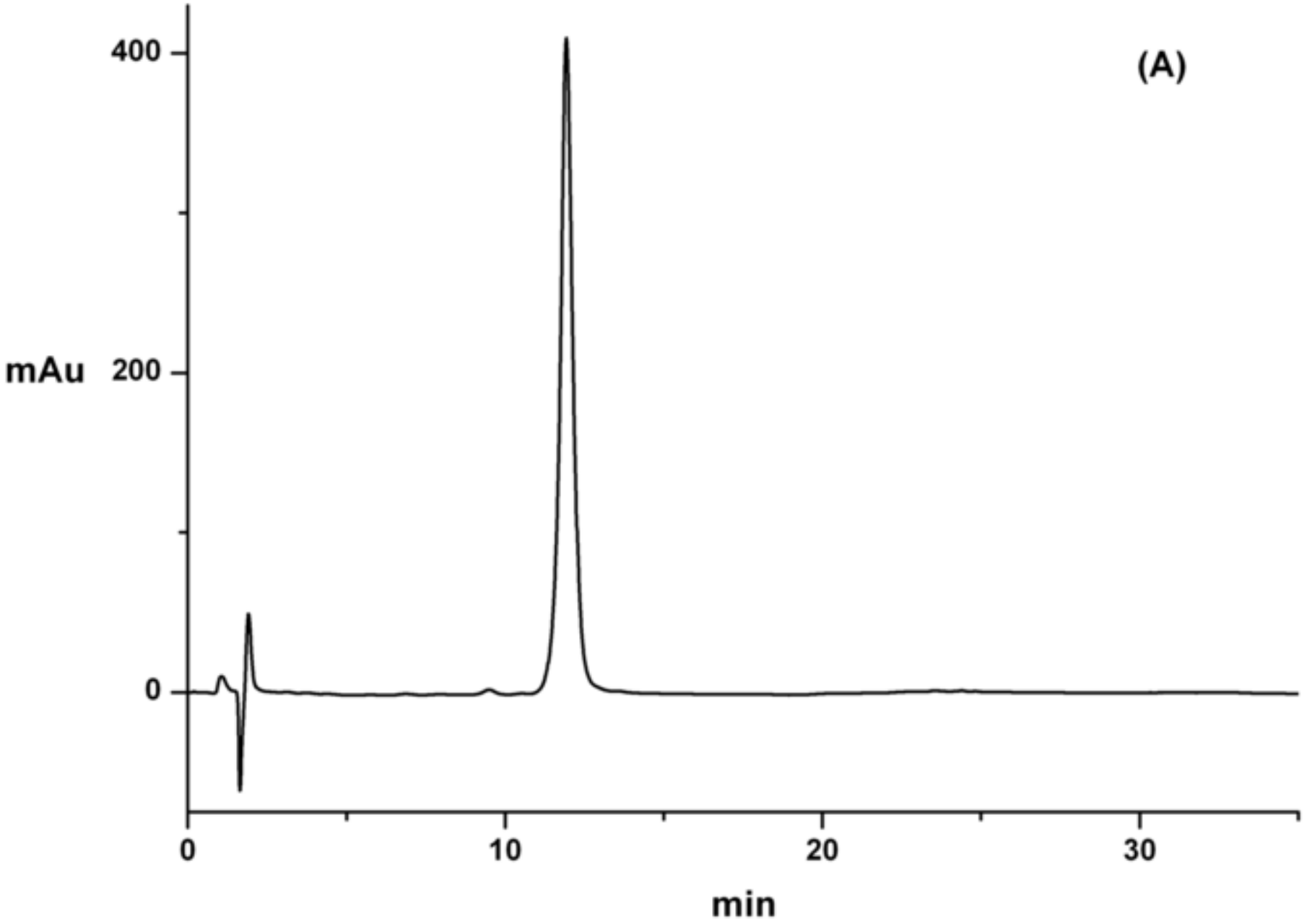

Fig. 7B

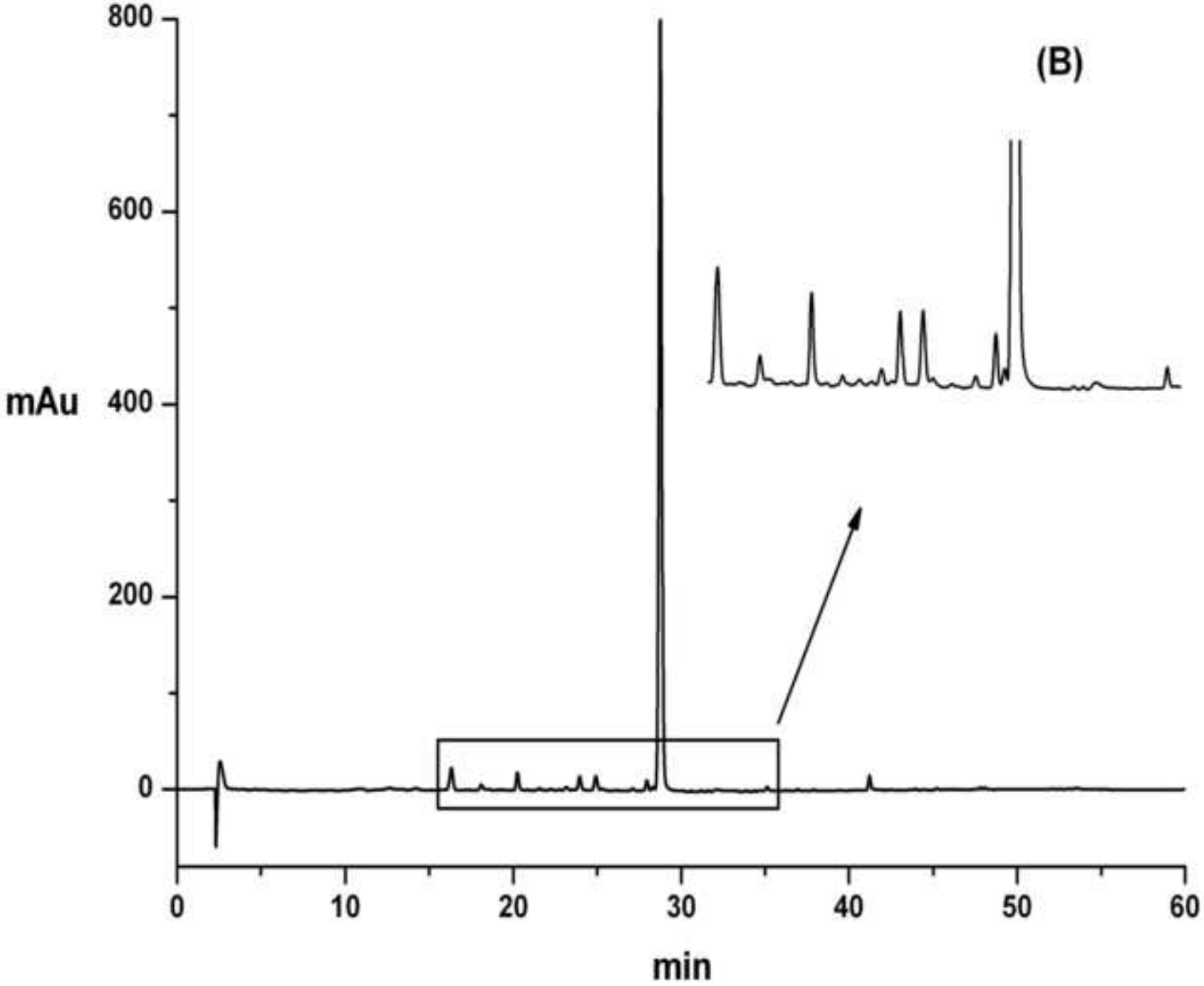

Fig. 8

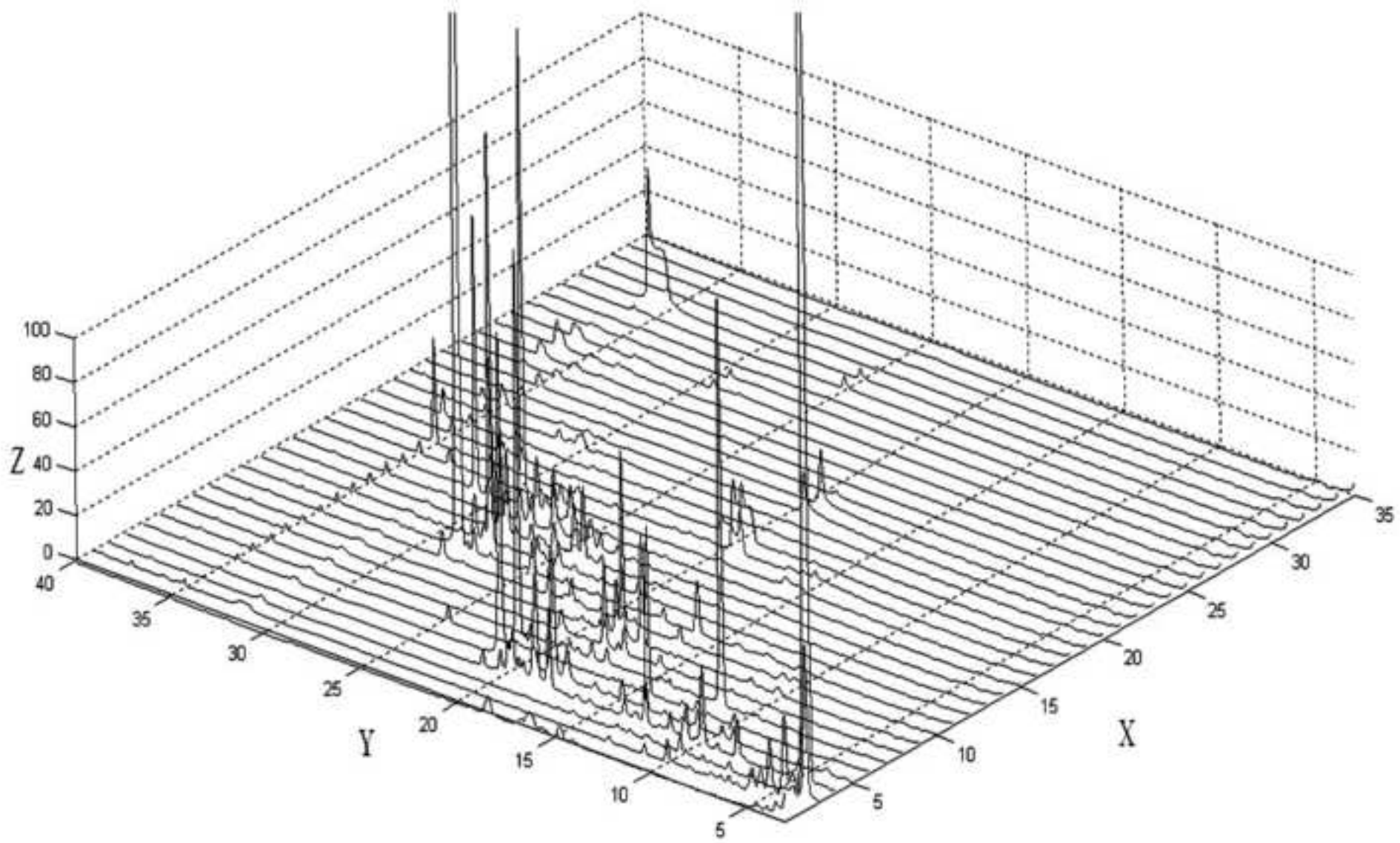

Table 1

List of test solutes used for orthogonality evaluation

No.	Aromatic compounds
1	Benzene
2	Toluene
3	Chlorobenzene
4	Bromobenzene
5	Nitrobenzene
6	Anisole
7	Ethyl benzoate
8	2-Chloroacetophenone
9	4-Nitroacetophenone
10	2-Chlorobenzaldehyde
11	Cinnamaldehyde
12	3-Methylcinnamaldehyde
13	2-Chlorocinnamaldehyde
14	Indole
15	5-Methoxyindole
16	6-Methylindole
17	2-Chloronitrobenzene
18	4-Chloronitrobenzene
19	4-Chlorophenol
20	2-Nitrophenol
21	3-Nitrophenol
22	1-Naphthol
23	2-Naphthol
24	2-Methylaniline
25	4-Ethylaniline

Table 2

Elemental analysis of Alkyne-silica and Click dipeptide stationary phase

Stationary Phase	C%	N%	Surface coverage (μmol m^{-2})
Alkyne-silica	7.85	2.02	2.73
Click dipeptide	12.77	3.37	0.98