



# Development and optimization of a method for the separation of platycosides in *Platycodi Radix* by comprehensive two-dimensional liquid chromatography with mass spectrometric detection

Eun-Kyung Jeong<sup>a,b</sup>, Hyun-Jeong Cha<sup>a</sup>, Young Wan Ha<sup>c</sup>, Yeong Shik Kim<sup>d</sup>, In Jin Ha<sup>d</sup>, Yun-Cheol Na<sup>a,\*</sup>

<sup>a</sup> Seoul Center, Korea Basic Science Institute, 126-16 Anam-dong, Sungbuk-gu, Seoul 136-713, South Korea

<sup>b</sup> College of Pharmacy, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, South Korea

<sup>c</sup> Life/Health Division, Korea Institute of Science and Technology, 39-1 Hawolgok-dong, Sungbuk-gu, Seoul 136-791, South Korea

<sup>d</sup> Natural Products Research Institute, College of Pharmacy, Seoul National University, 599 Gwanakno, Gwanak-gu, Seoul 151-542, South Korea

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## ABSTRACT

Comprehensive two-dimensional chromatography (LC × LC) using combinations of two columns ( $C_{18} \times CN$  and  $C_{18} \times NH_2$ ) was employed with electrospray (ESI) mass spectrometry to analyze platycosides from root extract. Based on the capability of the  $C_{18}$ , CN and  $NH_2$  columns to separate the platycosides, the orthogonality in two-dimensional space according to each combination of columns was predicted from the correlation coefficients between the retention times of the 17 compounds separated by the independent CN and  $C_{18}$  columns, and  $NH_2$  and  $C_{18}$  columns. The expected distribution of the peaks was also compared with the two-dimensional plots obtained by practical separation in an LC × LC system. The increased peak capacities using  $C_{18} \times NH_2$  allowed three minor components and five isomers of the platycosides to be newly separated, which were not identified with 1D-LC using the individual  $C_{18}$  column, whereas the combination of  $C_{18} \times CN$  did not result in any improvement of the separation performance.

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## 1. Introduction

Platycosides, a class of glycosides, are constituents of the roots of *Platycodon grandiflorum* A.DC. (Campanulaceae), which have been used as a traditional medicine for the treatment of bronchitis, tonsillitis, sore throat and other respiratory ailments [1–9]. The characterization and determination of the active platycosides have previously been carried out by high performance liquid chromatography (HPLC) using a UV detector [5,10–14], a differential refractometer [15] or an evaporative light scattering detector (ELSD) [16,17]. Especially, mass spectrometry coupled with HPLC provided information on their molecular weights as well as their chemical structure by MS<sup>n</sup> techniques [17,18].

Crude samples extracted from the natural products are made up of complex constituents whose concentrations vary widely. Therefore, the 1D chromatography of the bioactive compounds derived from the complex matrices does not provide sufficient separating efficiency, due to the various polarities and relatively low presence of the target compounds. In order to achieve the separation of the co-eluting peaks, additional separation techniques are needed to

increase peak capacity, defined by Giddings [19,20] as the number of peaks separated with unit resolution between the retention volumes of the first and last eluting peaks in a fixed separation time. One approach is two-dimensional separation (LC–LC) by heart cutting, where a particular fraction of interest in the first column is separated through a second column with different separation mechanisms connected in series [21–23]. On the other hand, comprehensive techniques can perform secondary separations of all of the compounds eluted from the first dimension column. Each fraction transferred from the first dimension is continuously sampled with finite volume aliquots using a switching valve, and the fraction from the first column is re-separated by the other column to achieve enhanced peak capacity [24]. This comprehensive 2D-LC (LC × LC) technique provides increased peak capacities, as well as fast and reproducible separation, without any sample loss or contamination, with the employment of automation.

In the early studies of LC × LC, proteins or peptides were mainly separated by a combination of size-exclusion chromatography (SEC) × reversed-phase liquid chromatography (RPLC) or ion-exchange chromatography (IEC) × RPLC [25–28] to reduce the time required for the tedious sample treatment in the typical 2D gel electropherogram. The 2D-LC techniques aim to increase the peak capacity, i.e. the number of peaks separated on the chromatogram [19]. Orthogonal peaks across the 2D separation space

\* Corresponding author. Tel.: +82 2 920 0794; fax: +82 2 920 0779.

E-mail address: [nyc@kbsi.re.kr](mailto:nyc@kbsi.re.kr) (Y.-C. Na).

arise from independent separation mechanisms and the compounds having at least two different properties by which they can be separated. It can maximize peak capacity. To accomplish this, various LC × LC techniques with different separation mechanisms, such as RPLC × SEC [29–31], normal phase liquid chromatography (NPLC) × RPLC [32,33] and IEC × SEC [24] have been studied. Although all possible pairs of columns can be fundamentally utilized in an LC × LC system, the consideration of the mobile phases used in each dimension is critical for successful system development, because a strong solvent in the first dimension (1st-D) column acts as a weak solvent in the second dimension (2nd-D) column and *vice versa* [34–37]. The incompatibility of the mobile phases will result in the poor peak resolution of the compounds and peak broadening. Consequently, peak distortion in the second dimension will follow [38], which diminishes peak capacity.

For the separation and identification of platycosides from crude root extract, RPLC, especially with a C<sub>18</sub> stationary phase, has been used previously [39]. Furthermore, mass spectrometry with electrospray ionization (ESI) was a powerful tool which could provide information about the platycosides' structures, with or without chromatographic separation [18,40]. Platycosides have similar polarities and the major platycosides are well separated using a gradient mobile phase of water/acetonitrile. However, minor compounds, which co-elute with the major platycosides or suffer from ion suppression, reduce the signal intensity of the target compounds. Therefore, they are difficult to identify and confirm, although mass spectra provide information on their molecular weights, except in the case of isobaric compounds. For the reliable characterization of the platycosides, LC × LC separation can provide sufficient peak capacity to separate the latent platycosides in the major component. The aim of this study is to establish a comprehensive 2D-LC method for the separation of platycosides extracted from *Platycodi Radix* and to determine the separated platycosides with mass spectrometric detection using this system.

## 2. Experimental

### 2.1. Solvents and chemicals

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was purified with a Milli-Q system (Bedford, MA, USA). Formic acid (FA), as an additive of the mobile phase, was purchased from Aldrich (Milwaukee, WI, USA). The solvents were filtered through Millipore 0.45 μm membrane filters and degassed for 10 min by sonication before use.

### 2.2. Sample preparation

The extraction of platycosides from *Platycodi Radix* followed a previously described method [17]. In brief, the water extract of finely powdered *Platycodi Radix* was purified using a Sep-Pak C<sub>18</sub> cartridge (Waters, Milford, Ma, USA) with 70% MeOH. The eluent was filtered through a 0.45 μm syringe filter and 5 μL of the filtrate was injected into an LC/MS system.

### 2.3. Instrumentation for LC × LC

The 1st-D HPLC system (NANOSPACE SI-2, Shiseido, Japan) consisted of two pumps, a 2-way degasser, a column oven and an autosampler. The RPLC column was a Luna C<sub>18</sub>(2) (150 mm × 1 mm I.D., 5 μm particle size, Phenomenex, Torrance, CA) using a mobile phase composed of differing proportions of 5% ACN (A) and 95% ACN (B) in water containing 0.1% of FA. The gradient program used was: 0–5 min (15% B in A) and 5–200 min (15–20% B) at a flow rate of

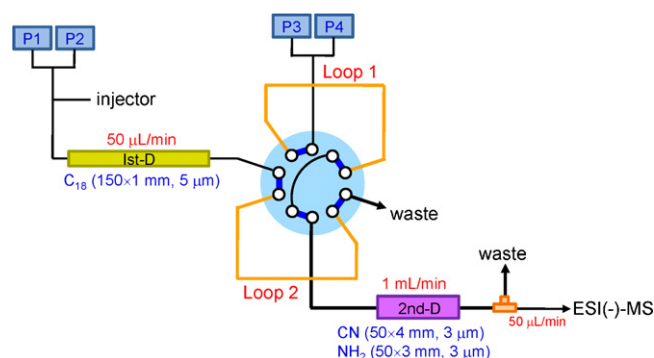
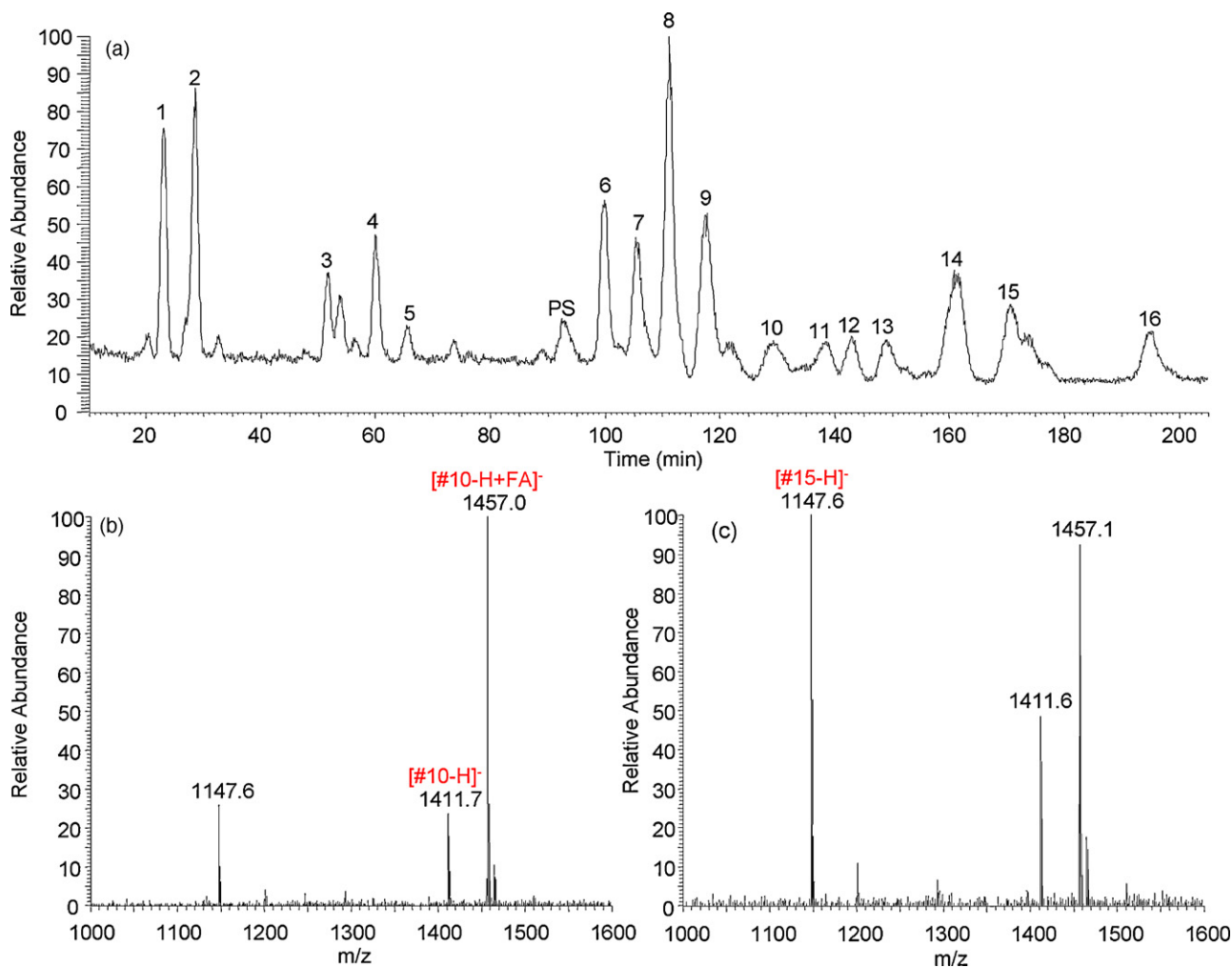


Fig. 1. Schematic diagram of LC × LC system used for the separation of platycoside extract.

50 μL/min. For the 2nd-D HPLC, a dual pump and a 2-way degasser were additionally installed on the 1st-D HPLC system. The columns used for the 2nd-D HPLC were a Hypersil GOLD CN (50 mm × 4 mm I.D., 3 μm particle size, Waters, MA, USA), Luna NH<sub>2</sub> (50 mm × 3 mm I.D., 3 μm particle size, Phenomenex, Torrance, CA) and Luna HILIC (50 mm × 3 mm I.D., 3 μm particle size, Phenomenex, Torrance, CA). The mobile phase was of the same composition as that of the 1st-D HPLC and flow rates were adjusted to 1 mL/min under isocratic conditions. The back pressures of the pumps used in the CN and NH<sub>2</sub> columns were 13.9 and 15.6 MPa, respectively. To optimize separation, each dimension of the HPLC was operated independently. The columns were maintained at 30 °C throughout. For the LC × LC system, the composition of the mobile phase, the gradient condition, the type of column, and the flow rate for the 1st-D separation were the same as those described above for C<sub>18</sub> separation. The injected volume was 5 μL. The 1st-D column outlet was connected to a 10-port 2-way switching valve (VICI Valco, Houston, TX, USA) with a jumper and two loops of 75 μL, corresponding to a switching time of 1.5 min. The valve was controlled to be automatically switched at each modulation time by interface hardware (Model 1B-S, Kroungold Analytical Inc., PA, USA). The mobile phases of the CN and NH<sub>2</sub> columns used for the 2nd-D were composed of 20% and 70% ACN in H<sub>2</sub>O containing 0.1% FA, respectively, and the flow rate was 1 mL/min. During LC × LC separation, the flow was split by a T connector and 0.125 mm I.D. PEEK (polyetheretherketone) tubing to achieve a flow rate of 50 μL/min towards the ESI-MS system (LCQ Deca XP, Thermo Finnigan, San Jose, CA, USA). Fig. 1 shows the configuration of the LC × LC system. The ion trap analyzer's parameters were optimized according to the manufacturer's instructions. The electrospray voltage was 4 kV in negative mode under N<sub>2</sub> sheath gas flow at 50 arbitrary units. The capillary temperature was 275 °C. The measured mass range was *m/z* 1000–1600.

### 2.4. Data processing and visualization

The total ion chromatograms (TICs) obtained by mass spectrometry were converted to ASCII data format with Xcalibur software (Thermo Finnigan, San Jose, CA, USA) and then converted into a matrix with the rows corresponding to the switching time of the valve using Excel software. For its visualization, the matrix was plotted with Matlab 7.4 (The Mathworks, Natick, USA). The compounds corresponding to the peaks observed on the map generated by the surf function, which is a particular routine within Matlab software, were identified by their molecular weight information in the mass spectrum of the untransformed comprehensive LC chromatogram.



**Fig. 2.** Total ion chromatogram (TIC) of platycosides extract on the  $C_{18}$  column for 1st-D (a). The mass spectra of peak 10 (b) and 15 (c) obtained by electrospray ionization (ESI) in negative ion mode.

### 3. Results and discussion

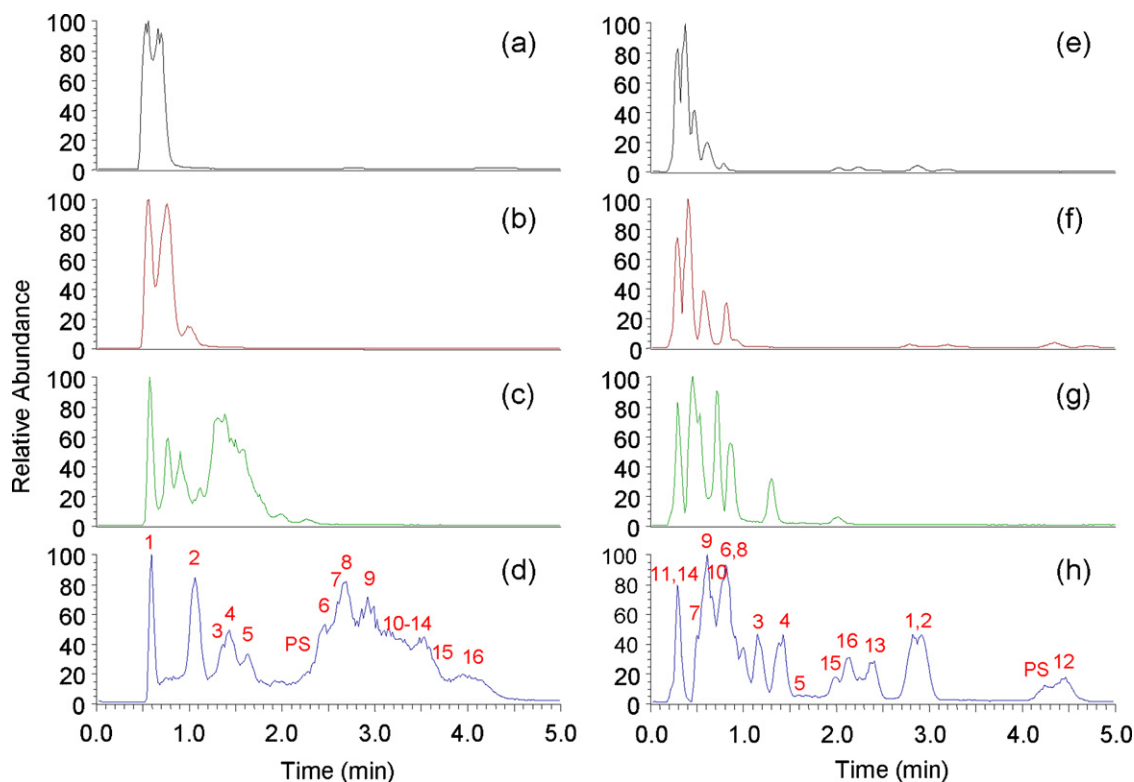
#### 3.1. Separation of platycoside extract on $C_{18}$ for 1st-D-LC

In a previous study [17], the major platycosides in *Platycodon Radix* were well separated by a  $C_{18}$  column with gradient elution of identical stationary phase as employed in this study. The similar polarities of the platycosides necessitated a change of mobile phase composition within the narrow range of 15–25% ACN in aqueous 0.1% FA. The structures of compounds corresponding to the separated peaks were identified by their ESI mass spectra obtained in negative ion mode. However, separation of the minor components from the major platycosides of same polarity was not possible, although their mass spectra allowed the mixed constituents to be discriminated by their mass differences. Besides, trace compounds suffering from ion suppression by the major components may not reveal themselves as a peak on the TIC. From the chromatographic point of view, the increase of the peak capacity obtained using the comprehensive 2D-LC technique may play an important role in discovering the unidentified platycosides on the 1D chromatogram.

When extending 1D-LC to comprehensive 2D-LC, the sampling rate of the first dimension is crucial to maintaining the resolution of peaks obtained from the 1D chromatogram [30,41–46]. Murphy et al. [30] proposed, as a criterion for the sampling rate, that the effluent from the 1st-D must be sampled at least three or four times over the first dimension peak to avoid severe loss of first dimension

resolution by incomplete sampling. To determine the appropriate sampling rate across the first dimension peaks, the peak widths obtained by the  $C_{18}$  column on our previous chromatogram were calculated. The  $4\sigma$  peak width ( $w$ ) of the 13 major compounds was between 0.9 and 1.7 min [17] and the peak capacity ( $n = t_r/w$ ) of the separation was 18–34 with a total run time ( $t_r$ ) of 31 min. The peak widths were too narrow to apply the sampling rate criteria described above. Prolonging the total run time with the same gradient having the same composition of the mobile phase produced a wider peak width of between 2.7 and 7.0 min, while maintaining the same peak separation (Fig. 2a). This provided adequate peak width to apply comprehensive 2D-LC. Under these conditions, 16 platycosides and a prosapogenin (PS) were separated with an increased peak capacity of 24–63 when run time was 172 min, whereas only 13 major compounds were identified in the previous study [17]. Additionally, varying other experimental conditions, such as increasing the flow rate to 100  $\mu\text{L}/\text{min}$ , changing the pH of the eluent and using other columns of  $\text{NH}_2$  and amide phases, did not improve the efficiency of peak separation and resulted in incompatibility with the sampling rate criteria.

The mass spectrum of each platycoside contains molecular information corresponding to deprotonated ions  $[M-H]^-$ , formic acid adducted ions  $[M-H+FA]^-$  or both. However, some mass spectra of the peaks presented the characteristic ions of mixed platycosides due to co-eluting. The mass spectrum of peak 10 in Fig. 2a, for example, shows the presence of two platycosides, which



**Fig. 3.** Total ion chromatograms (TICs) of platycoside extract on CN (a–d) and  $\text{NH}_2$  (e–h) columns for 2nd-D; (a) 40%, (b) 30%, (c) 20%, (d) 15%, (e) 50%, (f) 60%, (g) 70%, and (h) 80% ACN in aqueous 0.1% FA at flow rate of 1 mL/min.

are assumed to be the major 3''-O-acetyl-polygalacin  $\text{D}_3$  and the minor dexyl-3''-O-acetyl-polygalacin  $\text{D}_3$  (Fig. 2b). Also, the mass spectrum of peak 15 in Fig. 2a appeared to be the same as that of peak 10, except for the relative intensity ratios of compounds 10 to 15i and compounds 10i to 15 (Fig. 2c), and may correspond to the isomers of the compound in peak 10. A stationary phase having dissimilar hydrophobic selectivity from the  $\text{C}_{18}$  column may be suitable for orthogonal chromatographic separation of these compounds. The MS/MS spectra and the proposed chemical structures of peaks 10 and 15 observed in the 2D chromatogram are presented in the [Supplementary data](#).

### 3.2. Separation of platycoside extract on CN and $\text{NH}_2$ columns for 2nd-D-LC

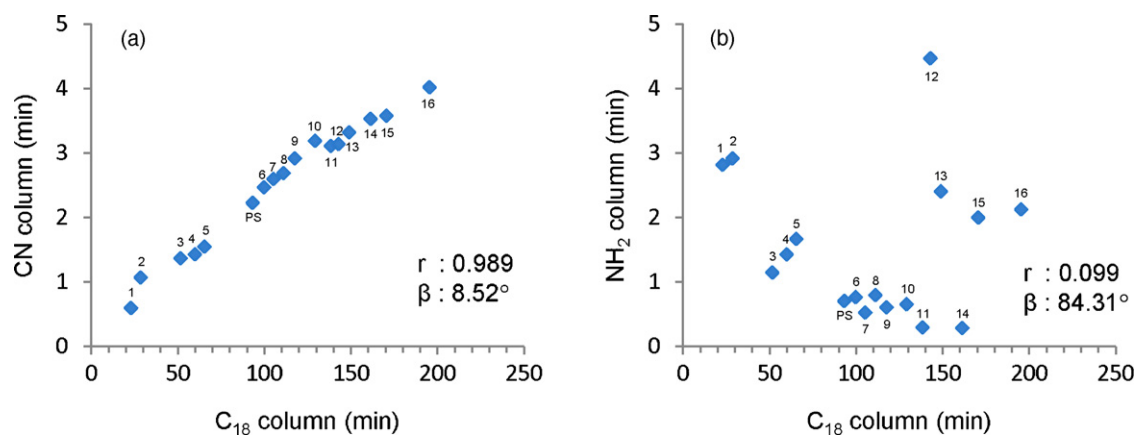
Combinations of NPLC and RPLC have provided the orthogonal selectivity required for comprehensive 2D-LC [33,47–49]. However, in order to maintain the peak resolution obtained by the  $\text{C}_{18}$  column, the use of NPLC as the 2nd-D is limited, because the weak solvent in the 1st-D acts as a strong solvent in the 2nd-D, which results in the broadening of the solute peaks. To minimize this drawback and enhance the polar selectivity, various columns, such as cyano (CN), amino ( $\text{NH}_2$ ) and HILIC providing normal phase selectivity with the solvents used in RPLC, were employed for the 2nd-D separation system.

A HILIC column, used to retain polar compounds, is highly orthogonal to RPLC, but its use for platycoside separation is limited to non-aqueous mobile phases. As a result, the platycosides were poorly discriminated with low peak resolutions and the constituents were eluted at the void volume, due to the incompatibility of the mobile phase, whether the HILIC column was used alone or in the  $\text{C}_{18} \times \text{HILIC}$  system (not shown).

The second dimension separation should be as fast as the sampling time of the 1st-D will allow. It was set to 1.5 min to

sample each peak three or four times in this study. The total run time of the platycosides in the separation was altered by varying the proportion of mobile phase used. Fig. 3a–d shows the TICs of the platycoside extract obtained with the CN column. Although the hydrophobicity of the stationary phase is low when compared with that of  $\text{C}_{18}$ , the eluting order of the platycosides was maintained. The isocratic elution at 20% ACN in aqueous 0.1% FA provided the optimal conditions for the 2nd-D, where all of the peaks were eluted within 1.5 min (Fig. 3c), which is equal to the switching time for  $\text{LC} \times \text{LC}$ . Of the various possible methods of evaluating orthogonality [50–57], the geometric approach to the factor analysis was applied in this study. The correlation coefficient ( $r$ ) and spreading angle ( $\beta$ ) are used to evaluate orthogonality in both dimensions. These were measured by evaluating the retention time of each dimension [50]. The retention times of the 16 platycosides and the one prosapogenin obtained from the TICs on the  $\text{C}_{18}$  column in Fig. 2a and on the CN column in Fig. 3d were used to evaluate the orthogonality. Fig. 4a shows the correlation between  $\text{C}_{18}$  and CN for these 17 compounds, indicating that  $r$  and  $\beta$  are 0.989 and  $8.52^\circ$ , respectively. This implies that the platycosides are highly correlated with the two columns and that, it is difficult to improve the peak capacity in a practical  $\text{LC} \times \text{LC}$  system.

An alternative  $\text{NH}_2$  column providing hydrogen bonding interaction under normal phase conditions provided good separation with a higher proportion of organic solvent and better peak resolution than the CN column (Fig. 3e–h). Different interactions from those in the  $\text{C}_{18}$  column were responsible for changing the order of elution of the platycosides. The retention times obtained from the TICs of the  $\text{C}_{18}$  column in Fig. 2a and  $\text{NH}_2$  column in Fig. 3h allowed calculation of the correlation factors,  $r$  and  $\beta$ , as 0.099 and  $84.31^\circ$  (Fig. 4b), respectively. Accordingly, the platycosides would be expected to be well distributed in 2D space by the combination of  $\text{C}_{18}$  and  $\text{NH}_2$  columns. In the second dimensional separation, the peak capacity was 8–15 with a total run



**Fig. 4.** Correlation between the retention times of 17 compounds separated by independent CN column with 15% ACN in aqueous 0.1% FA at flow rate of 1 mL/min and  $C_{18}$  column (a), and  $NH_2$  column with 80% ACN in aqueous 0.1% FA at flow rate of 1 mL/min and  $C_{18}$  column (b). Experimental conditions of  $C_{18}$  column are described in Section 2.3.

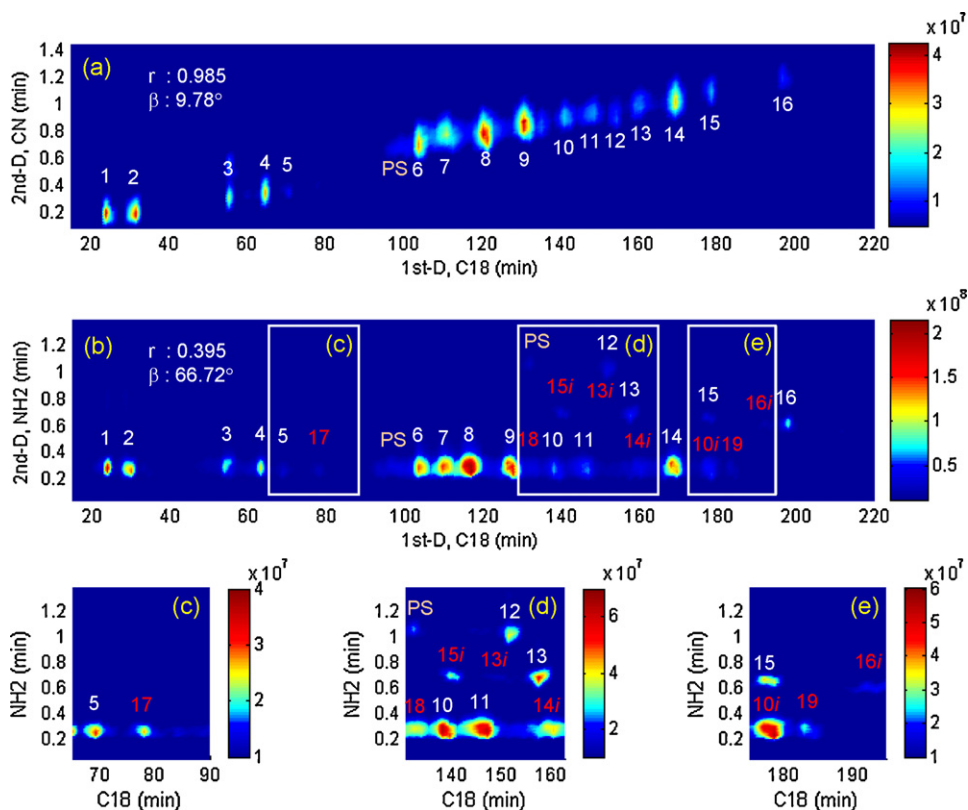
time of 1.5 min. Therefore, the peak capacity in the 2D separation plane could theoretically be increased to 192–945. This approach allowed prediction of the separating ability of the platycosides using the various combinations of columns before undertaking comprehensive 2D-LC. The mobile phase of 70% ACN in aqueous 0.1% FA in the  $NH_2$  column was optimal in the 2nd-D (Fig. 3g). It was suitable for separating platycosides during a switching time of 1.5 min.

### 3.3. Comprehensive LC $\times$ LC separation of platycoside extract

To optimize comprehensive LC  $\times$  LC separation, the flow rate in the second dimension, the sample loop volume and valve switch-

ing time must be balanced. With the correct choice of mobile phase in the second dimension, all of the platycosides were eluted within 1.5 min at a flow rate of 1 mL/min. Therefore, a loop volume of 75  $\mu$ L was applied to the  $C_{18} \times$  CN and  $C_{18} \times$   $NH_2$  systems.

As explained in Section 3.2, the CN phase showed a linear correlation with the  $C_{18}$  phase, due to its similar interactions with platycosides during the separation. In a practical comprehensive separation system, the relationship between the two column phases corresponded to  $r$  and  $\beta$  values of 0.985 and 9.78 $^\circ$ , respectively (Fig. 5a), indicating that two phases were of limited orthogonality, as predicted by the data in Fig. 4a. Therefore, this combination of columns was near useless in enhancing peak capac-



**Fig. 5.** Two dimensional plots of platycoside extract on  $C_{18} \times$  CN (a) and  $C_{18} \times$   $NH_2$  (b) on the basis of the TIC. The composition of mobile phase of CN and  $NH_2$  column composed with 20% and 70% ACN in aqueous 0.1% FA at flow rate of 1 mL/min, respectively. Experimental conditions of the  $C_{18}$  column are described in Section 2.3. Extended 2D plots of three different time windows in (b); first dimension ( $C_{18}$ ) from 65–90 min (c), 130–163 min (d) and 175–195 min (e).

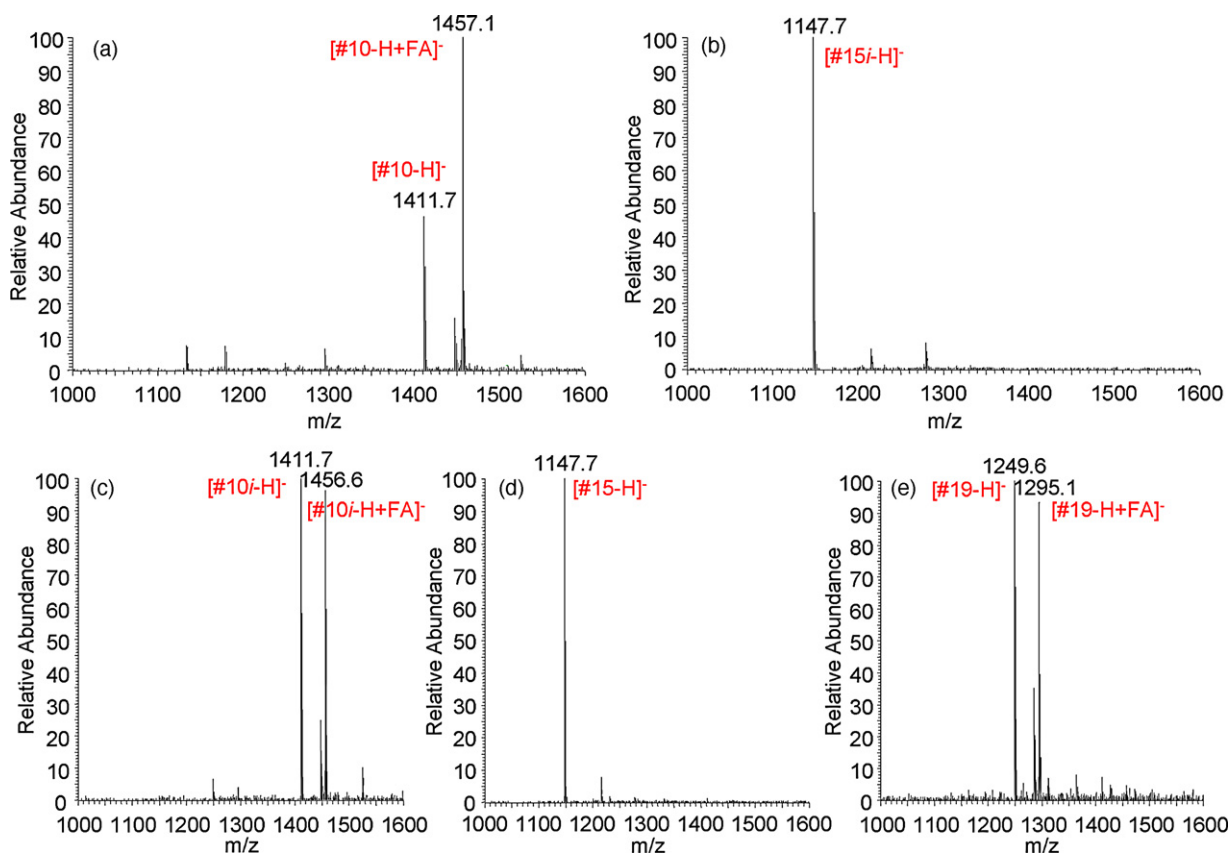


Fig. 6. Mass spectra of peaks 10 (a), 15i (b), 10i (c), 15 (d), and 19 (e) in Fig. 5b in negative ion mode.

ity, selectivity of the platycosides and resolving power, compared with even individual  $C_{18}$  separation.

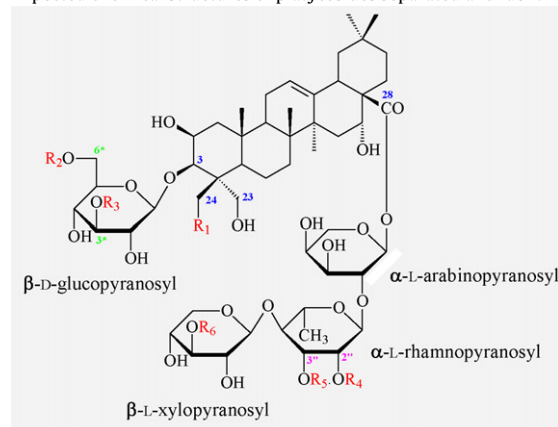
Comprehensive separation by another combination of  $C_{18}$  and  $NH_2$  columns is shown in Fig. 5b. The population of platycosides spread over the 2D space maintained a similar correlation ( $r=0.395$ ) and spreading angle ( $\beta=66.72^\circ$ ) to those calculated from the retention times of each column in Fig. 4b. This indicates that the system was well set up to provide successfully comprehensive separation. However, incompatibility of the solvents led to the 2D plot showing a small amount of peak spreading. This effect was prominent at the fraction of the aqueous aliquot transferred from the first dimension, especially for peaks 1 and 2. It was due to the mobile phase in  $C_{18}$  causing it to act as a relatively strong solvent for  $NH_2$ . On the other hand, the peaks eluted in the abundant organic solvent portion of the mobile phase on the 1st-D separation were less sensitive to this effect.

The varying concentrations of platycosides in *Platycodi Radix* make their visualization on a 2D full map difficult. To display those less abundant platycosides, three sections of the map were replotted with adjustment of the axis scale of the peak intensity (Fig. 5c–e). Fig. 5c shows the peaks in the space of 65–90 min on  $C_{18}$  and 0–1.5 min on  $NH_2$ . Two apparent platycosides, viz. deapi-polygalacin E and polygalacin  $D_2$  corresponding to peaks 5 and 17, respectively, whose molecular weight information was obtained by mass spectrometry, are shown in Fig. 5c, in spite of the trace compounds in the 2D full map. As a result of the increased peak capacity, some major peaks containing minor platycosides were separated in the 2D space. As an example, peak 10 in Fig. 2a referred to in Section 3.1 was a mixture of 3''-O-acetyl-polygalacin  $D_3$  and dexyl-3''-O-acetyl-polygalacin  $D_3$ , which were separated as two apparent peaks, 10 and 15i (the isomer of peak 15), on the 2D plot in Fig. 5d. The mass spectra of these peaks are

shown in Fig. 6a and b, respectively. Additionally, the benefit of the increased peak capacity was revealed at peaks 15, 10i and 19 in Fig. 5e. Two platycosides, dexyl-2''-O-acetyl-polygalacin  $D_3$  and 2''-O-acetyl-polygalacin  $D_3$ , corresponding to peaks 15 and 10i on the 2D map, respectively, were observed in the mass spectrum of peak 15 in Fig. 2a, whereas the 2''-O-acetyl-polygalacin D of peak 19 was newly observed in this 2D system (Figs. 6c–e). Additionally, one prosapogenin (PS) [58] was separated. It may have been generated during the extraction procedure. Although deapi-3''-O-acetyl-polygalacin  $D_3$  corresponding to peak 13i in Fig. 5d was not observed in the mass spectra of peaks 11 or 12 in Fig. 2a, because it was a trace compound and may have a matrix effect, its molecular information was obtained from the mass spectrum of this peak. Other isomers, of peaks 14 and 16, corresponding to peaks 14i and 16i in Fig. 5d and e, respectively, were also observed in the space adjacent to them. The MS/MS spectra and the proposed chemical structures of the compounds corresponding to peaks 17, 18 and 19 observed in the 2D chromatogram are presented in the Supplementary data.

In the  $C_{18} \times NH_2$  system, eight platycosides and one prosapogenin were separated which were not identified in the individual  $C_{18}$  system. Five of these platycosides were isomers of the major peaks, which are platycosides acetylated at the  $R_4$  or  $R_5$  site of the rhamnopyranosyl sugar moiety. In general, in the case of the acetylated isomers with the same molecular weight, those platycosides acetylated at  $R_5$  eluted more rapidly than those acetylated at  $R_4$  in  $C_{18}$  separation, while they eluted with same retention times in  $NH_2$  separation. Based on their chromatographic properties, the chemical structures of these separated isomers can be inferred. The chemical structures of the platycosides observed in this 2D system are shown in Table 1. Unfortunately, the chemical structural analysis by the  $MS^n$  technique did not allow the discrimination of the

**Table 1**  
Expected chemical structures of platycosides separated and identified on C<sub>18</sub> × NH<sub>2</sub>.



No.	Platycosides	Molecular formula	Molar mass (u)	[M-H] <sup>-</sup> (m/z)	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>
1	Deapi-platycoside E	C <sub>64</sub> H <sub>104</sub> O <sub>34</sub>	1416.6	1415.7	OH	Gen <sup>a</sup>	H	H	H	H
2	Platycoside E	C <sub>69</sub> H <sub>112</sub> O <sub>38</sub>	1548.7	1547.7	OH	Gen	H	H	H	Api <sup>b</sup>
3	Deapi-platycodin D <sub>3</sub>	C <sub>58</sub> H <sub>94</sub> O <sub>29</sub>	1254.6	1253.7	OH	Glc <sup>c</sup>	H	H	H	H
4	Platycodin D <sub>3</sub>	C <sub>63</sub> H <sub>102</sub> O <sub>33</sub>	1386.6	1385.7	OH	Glc	H	H	H	Api
5	Deapi-polygalacin E	C <sub>64</sub> H <sub>104</sub> O <sub>33</sub>	1400.7	1399.7	H	Gen	H	H	H	H
6	Deapi-platycodin D	C <sub>52</sub> H <sub>84</sub> O <sub>24</sub>	1092.5	1091.6	OH	H	H	H	H	H
7	Deapi-3''-O-acetyl-platycodin D	C <sub>54</sub> H <sub>86</sub> O <sub>25</sub>	1134.6	1133.5	OH	H	H	H	Ac <sup>d</sup>	H
8	Platycodin D	C <sub>57</sub> H <sub>92</sub> O <sub>28</sub>	1224.6	1223.7	OH	H	H	H	H	Api
9	Platycodin A	C <sub>59</sub> H <sub>94</sub> O <sub>29</sub>	1266.6	1265.6	OH	H	H	H	Ac	Api
10	3''-O-Acetyl-polygalacin D <sub>3</sub>	C <sub>65</sub> H <sub>104</sub> O <sub>33</sub>	1412.7	1411.7	H	Glc	H	H	Ac	Api
10 <sup>i</sup> e	2''-O-Acetyl-polygalacin D <sub>3</sub>	C <sub>65</sub> H <sub>104</sub> O <sub>33</sub>	1412.7	1411.7	H	Glc	H	Ac	H	Api
11	Deapi-2''-O-acetyl-platycodin D	C <sub>54</sub> H <sub>86</sub> O <sub>25</sub>	1134.6	1133.6	OH	H	H	Ac	H	H
12	Deapi-polygalacin D <sub>3</sub>	C <sub>58</sub> H <sub>94</sub> O <sub>28</sub>	1238.6	1237.6	H	Glc	H	H	H	H
13	Deapi-2''-O-acetyl-polygalacin D <sub>3</sub>	C <sub>60</sub> H <sub>96</sub> O <sub>29</sub>	1280.6	1279.6	H	Glc	H	Ac	H	H
13 <sup>i</sup> e	Deapi-3''-O-acetyl-polygalacin D <sub>3</sub>	C <sub>60</sub> H <sub>96</sub> O <sub>29</sub>	1280.6	1279.6	H	Glc	H	H	Ac	H
14	2''-O-Acetyl-platycodin D	C <sub>59</sub> H <sub>94</sub> O <sub>29</sub>	1266.6	1265.7	OH	H	H	Ac	H	Api
14 <sup>i</sup> e	3''-O-Acetyl-platycodin D	C <sub>59</sub> H <sub>94</sub> O <sub>29</sub>	1266.6	1265.7	OH	H	H	H	Ac	Api
15	Dexyl-2''-O-acetyl-polygalacin D <sub>3</sub>	C <sub>55</sub> H <sub>88</sub> O <sub>25</sub>	1148.6	1147.6	H	Glc	H	Ac	H	-Xyl
15 <sup>i</sup> e	Dexyl-3''-O-acetyl-polygalacin D <sub>3</sub>	C <sub>55</sub> H <sub>88</sub> O <sub>25</sub>	1148.6	1147.6	H	Glc	H	H	Ac	-Xyl
16	Deapi-2''-O-acetyl-polygalacin D <sub>2</sub>	C <sub>60</sub> H <sub>92</sub> O <sub>29</sub>	1280.6	1279.6	H	H	Glc	Ac	H	H
16 <sup>i</sup> e	Deapi-3''-O-acetyl-polygalacin D <sub>2</sub>	C <sub>60</sub> H <sub>92</sub> O <sub>29</sub>	1280.6	1279.6	H	H	Glc	H	Ac	H
17	Polygalacin D <sub>2</sub>	C <sub>63</sub> H <sub>102</sub> O <sub>32</sub>	1370.6	1369.6	H	H	Glc	H	H	Api
18	Polygalacin D	C <sub>57</sub> H <sub>92</sub> O <sub>27</sub>	1208.6	1207.5	H	H	H	H	H	Api
19	2''-O-Acetyl-polygalacin D	C <sub>59</sub> H <sub>94</sub> O <sub>28</sub>	1250.6	1249.6	H	H	H	Ac	H	Api

<sup>a</sup> Gen: β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl.

<sup>b</sup> Api: β-D-apiofuranosyl.

<sup>c</sup> Glc: β-D-glucopyranosyl.

<sup>d</sup> Ac: acetyl.

<sup>e</sup> The structure of acetylated isomer is assumed based on a mass spectrum obtained by ESI-MS.

acetylated isomers [18] and their content in the extract was too low in the 2D map for another technique to be used to obtain the structural information.

#### 4. Conclusion

The correlation plots obtained from retention times of platycosides observed in each dimension's chromatogram provided information permitting the separating efficiency in comprehensive 2D to be predicted by geometric factor analysis [50]. The high linear correlation of the C<sub>18</sub> × CN combination did not provide any increase in peak capacity, whereas the high orthogonality of the C<sub>18</sub> × NH<sub>2</sub> combination provided a powerful separation method for analyzing platycosides in a complex extract. With this increased peak capacity, the minor peaks and isomers, which were mixed with some of the major peaks or were not observed by a single the C<sub>18</sub> column, were chromatographically separated by this 2D system. This strategy for evaluating the separation of each dimension before applying comprehensive 2D separation was useful to the separation of platycosides.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.04.053.

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