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# A simple way to configure on-line two-dimensional liquid chromatography for complex sample analysis: Acquisition of four-dimensional data

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ABSTRACT

An on-line comprehensive two-dimensional liquid chromatography (HPLC × UPLC-TOF MS) was set up just using the injection valve of the ultra performance liquid chromatography (UPLC) as the interface through which the effluent of high performance liquid chromatography (HPLC) was injected automatically to UPLC coupled to time-of-flight mass spectrometry (TOF MS). As a demonstrative application, a complex sample of Traditional Chinese Medicine, Qingkailing was analyzed. As a result, a four-dimensional (4D) data containing 2D retention times, peak intensity and m/z ratios was plotted, where 398 peaks were counted and low concentration components were distinguished from the high concentration ones with a total peak capacity of 1090. Comparing with traditional 3D data acquired by HPLC × HPLC, the 4D data generated by HPLC × UPLC-TOF MS can increase the number of recognized components by three times, reduce the analysis time by 75%. Such a configuration of HPLC × UPLC-TOF MS can realize easily upon commercial chromatographs while exhibited enhanced separation efficiency, high sensitivity, huge peak capacity and great potential in complex sample analysis.



## 1. Introduction

High-resolution analytical techniques are essential for dealing with complex samples [1], because single technique can hardly be used to fully handle samples like those encountered in proteomic [2] and metabolomic research [3]. Comprehensive two-dimensional liquid chromatography (LC × LC) is attracting increasing attention with its dramatically high resolving power. In principle, LC × LC can greatly increase the peak capacity of highly optimized one-dimensional HPLC [4]. In recent researches, LC × LC systems have been used to separate molecules in biological systems [5–7] polymers [8], natural products [9–15] and other complex mixtures [16].

The peak capacity of LC × LC can be calculated by the following formula: $n_c = n_1 \times n_2$ [17,18], in which $n_1$ is the peak capacity of the first dimension, and $n_2$ is the peak capacity of the second dimension. Li [4] has introduced many corrected equations; however, increasing peak capacity of the first and the second dimension ($n_1$, $n_2$) will raise the total peak capacity ($n_c$) in all corrections. In addition, the LC × LC system combines two columns with different separation mechanisms, for example, normal phase (NP), reverse phase (RP), size exclusion chromatography (SEC), iron exchange chromatography (IEX) and hydrophilic interaction chromatography (HILIC), to achieve optimal separation. The speed of the second dimension is a key feature of a successful separation. Shen [19] developed an off-line approach which achieved a considerably high peak capacity ( > 10,000) in the separation of peptide digests from the human plasma proteome but required a rather long analysis time (2 day). The results confirm the great potential of application of off-line comprehensive two-dimensional HPLC in the analysis of highly complex samples. The off-line comprehensive two-dimensional HPLC also underlines the considerable cost of time that these analyses might entail. Monolithic columns and parallel columns have been used as the second dimension to improve the throughput of the multiple dimensional analyses [20–26]. Short columns, high column temperature and fast gradient elution have been applied in the second dimensional separation which maintains one of the best performances ever achieved in on-line comprehensive two-dimensional HPLC separation with a peak capacity of 1350 and an analysis time of 20 minutes only [27,28].

At pressures of up to 15 000 psi and flow rates as high as 2 mL/min, the application of UPLC with small particles ( < 2 μm) could produce up to 8-fold improvement in sensitivity, a 1.4-fold increase in resolution, and a 9-fold increase in sample throughput [29–31]. Ultra performance liquid chromatography combined with mass spectrometry (UPLC-MS and UPLC-MS/MS) offers

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unprecedented on-column resolving power, sensitivity, speed of analysis, and mass selectivity [32]. UPLC coupled to time-of-flight mass spectrometers (UPLC-TOF MS) has been applied in the analysis of complex samples, such as metabolites[33–35], pollutants [19], polymers [36] and pharmaceutical [32]. Ultra performance liquid chromatography as the second dimension is another possibility for multidimensional liquid chromatography analysis (HPLC × UPLC). An elevated column temperature, short columns and a high flow rate can improve the resolution of HPLC × UPLC. Peak capacity of HPLC × UPLC will be much higher than that of conventional HPLC × HPLC in the same chromatographic conditions. Recently, UPLC as the second dimension in an off-line comprehensive two-dimensional liquid chromatography has been investigated for the screening of pharmaceutical samples [37]. However there remain challenges in the exploitation of on-line HPLC × UPLC system, for example, the limitation of the ultra high pressure interface of HPLC and UPLC.

Natural products are very complex mixtures containing hundreds or even thousands of constituents of different structural types and concentrations. Only a few of them have been elucidated as being responsible for their pharmacological activity and/or toxicity. Derived from Traditional Chinese Medicine, *Qingkailing* injection is prepared from eight herbs, including *Radix Isatidis*, *Flos Lonicerae*, *Fructus Gardenise*, *Cornu Bubal*, *Concha Margaritifera*, *aicalinum*, *Acidum Cholicum*, and *Acidum Hyodesoxy-cholicum*. It has excellent curative effects on circulation system disease, phlogistic disease, virosis and some inexplicable fever. The main chemical components of *Qingkingling* injection are bile acids, amino acids, flavonoids, organic acids, nucleotides, iridoid glycoside, pigments, volatile compounds, inorganic compounds, etc. [38,39]. Due to such complex components, *Qingkailing* injection can be suitably analyzed and assayed by the comprehensive two-dimensional liquid chromatography system.

In this study, UPLC coupled to time-of-flight mass spectrometry was investigated as the second dimension of the comprehensive HPLC × UPLC system. The UPLC injection valve was used as the interface of HPLC and UPLC as it solved problems caused by pressure difference in the two dimensions and avoided the use of additional high pressure valve. HPLC × UPLC-TOF MS system was used to analyze *Qingkailing* injection, a complex herbs extraction. A 4D data containing HPLC and UPLC retention time, peak intensity and m/z was plotted, which indicated higher resolution than the conventional 3D data. Low concentration components were identified in the 4D data. The total peak capacity of HPLC × UPLC-TOF MS system was calculated and compared with conventional off-line HPLC × HPLC.

## 2. Experimental

### 2.1. Chemicals and reagents

Acetonitrile and methanol of HPLC grade were purchased from J. T. Baker (NJ, USA). Formic acid, ammonium formate, leucine-enkephalin, uridnine, guanosine, adenosine, phenylalanine, tryptophane and caffeic acid were obtained from Sigma-Aldrich (MO, USA). Genipin-1-gentiobioside, geniposide, baicalin, shanzhiside, neochlorogenic acid, gardenoside, scandoside methyl ester, wogonoside, hyocholic acid and cholic acid were standards (NICPBP, China). Ultrapure water (18.2 MΩ) used in all experiments was prepared with a Milli-Q water purification system (Millipore, France). *Qingkailing* injection was prepared by a Chinese pharmaceutical factory and filtrated by the microvoid filter film (0.22 μm).

### 2.2. Construction of HPLC × UPLC system

The first dimension HPLC consists of Jasco HPLC system equipped with a binary pump (Jasco PU-980, Japan), an injection valve (Rheodyne 7725i, USA), and a UV detector (Jasco UV-975, Japan). A PEAKSIMPLE chromatography data system (SRI Instruments Inc., USA) was used to record the detector signal as well as to calculate the retention time and peak area. The data was recorded at the rate of 1 Hz. The flow rate range of PU-980 pump is from 0.001 mL to 10 mL per minute. The volume of sample loop is 20 μL. The packed-column (200 mm × 2.1 mm i.d.) was used in the first dimension. The packing material is Toyopearl HW-40 S (TOSOH, Japan), a semi-solid globoid which is polymerized by vinyl alcohol and esters of methacrylic acid for size exclusion chromatography. This packing material has many advantages such as high physical and chemical stability, high column pressure, high flow rate, etc. Besides, with many hydroxides and ether bonds on its surface, it is highly hydrophilic and able to separate components of water-solubility well. SEC column packed with Toyopearl HW-40 S can separate components whose molecular weights are between 100 and 10,000. Due to the hydrophobic group, ion exchange and hydrogen bond, deviation of size exclusion chromatographic behavior of some components will be found. The mobile phase of the first dimension is 10 mmol/L ammonium formate and the flow rate range of the first dimension should be 0.005–0.1 mL/min.

Chromatographic separations of the second dimension were performed on a 100 × 2.1 mm ACQUITY 1.7 μm column (Waters Corp, USA) in a Waters ACQUITY UPLC system equipped with a binary solvent delivery system, and an autosampler. The mobile phase was water with 0.1% (by volume) formic acid (A) and acetonitrile (B). The column was maintained at 80 °C and eluted at a flow rate of 0.7 mL/min. The column effluent was directed to mass spectrometer. The gradient conditions of mobile phase were shown in Table 1. The total analysis time was 5 min. The data of mass spectrometry was recorded from 0 to 4.5 min and column equilibration and injection were finished from 4.5 min to 5.0 min.

As shown in Fig. 1, the UPLC two-position six-port injection valve (dash and solid lines) controlled by the UPLC software was used as the interface of SEC and UPLC. During one injection circle, 100 μL sample loop was used to collect the effluent of SEC column

**Table 1**
Parameters of comprehensive HPLC × UPLC chromatographic conditions.

| Chromatographic conditions | First dimension HPLC | Second dimension UPLC |
|---|---|---|
| Columns | Toyopearl HW-40S 200 × 2.1 mm | ACQUITY UPLC RP $C_{18}$ SB 100 × 2.1 mm 1.7 μm |
| Packing material | Toyopearl HW-40S | ODS C18 |
| Chromatographic column | SEC | RPLC |
| Mobile phase | Ammonium formate 0.01 mol/L, pH 6.9 | A: 0.1% formic acid–water B: acetonitrile |
| Flow rate | 0.02 mL/min | 0.7 mL/min |
| Eluting mode | Isocratic elution | 0-1-3-3.5-4.5-5 min 5-5-30-50-5-5%B |
| Column temperature | 25 °C | 80 °C |
| Wave length | 254 nm | 254 nm |
| Sample size | 20 μL | 100 μL elution of SEC |
| Total separation time | 250 min | 5 min |

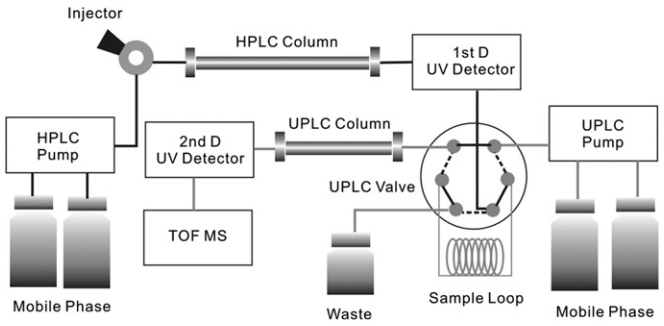


**Fig. 1.** Schematic of comprehensive two-dimensional HPLC × UPLC-TOF MS system.

(solid line). The effluent collected in the sample loop was injected into UPLC (dash line). The UPLC separation started. After injection at $t=0.3$ min, the valve changed and the sample loop was used to collect the first dimensional effluent again. The injection circles repeated until the whole separation was completed. TOF MS was connected with the UPLC column. The collected data were analyzed by the MATLAB 6.5 and MarkerLynx software. The PEEK tubing (0.10 mm i.d.) was used to connect the instruments together.

### 2.3. Time-of-flight mass spectrometry analysis

Mass spectrometry was performed by exploiting a LCT Premier XE (W mode of operation) mass spectrometer (Waters MS Technologies, UK) in negative ion mode. The TOF data were collected from m/z 50 to m/z 1000. The capillary voltage was set to 2200 V and the cone voltage 35 V in both positive and negative ion modes. Nitrogen was used as the drying gas. The desolvation gas flow rate was set to 1000 L/h, and the cone gas flow rate was maintained at 40 L/h. The desolvation temperature was 350 °C, and the source temperature was set to 120 °C. The scan time and inter-scan delay were set to 0.2 s and 0.02 s respectively. All analyses were acquired by using an independent reference lock-mass ion via the LockSpray interface to ensure accuracy and reproducibility. Leucine-enkaphalin was used as the reference compound ($[M-H]^-=554.2615$). The data were collected in the centroid mode, and the LockSpray frequency was set to 10 s and averaged over 10 scans for correction.

## 3. Results and disscusion

### 3.1. Interface of HPLC × UPLC system

Parallel columns trapping technique is widely used in conventional on-line comprehensive two-dimensional HPLC separations. The effluent of the first dimension column is enriched in the trap columns or on the head of the second dimensional columns. Two-position ten-port automatic valves are frequently used as the interface of HPLC × HPLC. However, this technique does not fit for HPLC × UPLC system. The maximum pressure of HPLC pump and valves is 5,000 psi and that of UPLC column is over 10,000 psi. Neither the HPLC pumps of the first dimension nor trap columns can afford the high pressure of the HPLC × UPLC system. Sample loops-valve interface technique is also used in comprehensive HPLC × HPLC. The effluent of the first dimension column is collected in the sample loops and then injected into the second dimensional column. The automatic valve is the key device of the interface. In the analysis of HPLC × UPLC system, the common automatic valves cannot afford the ultra high pressure in the UPLC analysis. The expensive high-pressure two-position interfacing valves like VICI Valco valve (VICI Valco, USA) might be used as the interface of HPLC and UPLC.

In this study, injection valve of UPLC was used as the interface of HPLC × UPLC system. It is a two-position six-port valve and can afford 14,000 psi. The switching time of UPLC injection valve was controlled by the UPLC software. No additional valves and pumps were used in this system. It is a simple and practical method that can be widely used in the HPLC × UPLC analysis in the future.

### 3.2. Optimization of HPLC × UPLC system

There are several parameters to be optimized in HPLC × UPLC system. The mobile phase for SEC columns is usually phosphate and Tris–HCl buffer, however, the TOF MS is incompatible with nonvolatile solvent and salt. Here we used ammonium formate as mobile phase additive. As shown in Fig. 2, 20 μL *Qingkailing* injection was separated by the SEC column. The mobile phase of the first dimension is 10 mmol/L ammonium formate. The volume of sample loop for collecting the SEC column effluent is determined by the product of flow rate on the SEC column and analysis time of UPLC column. As the volume of UPLC sample loop is 100 μL and the analysis time of UPLC is 5 min, the flow rate range of the first dimension is 1–20 μL/min. After optimization, the flow rate was chosen as 20 μL/min. The wavelength was 254 nm. More than 8 peaks can be seen in Fig. 2.

The total analysis time of HPLC × UPLC is determined by the analysis time of UPLC and the total number of SEC column fractions [40]. The peak eluted from the SEC column should be divided into at least three fractions in order to maintain the first dimension separation in UPLC column. Analysis time of UPLC separation is the most critical parameter in HPLC × UPLC analysis. More fractions of peaks can be obtained by reducing the analysis time of UPLC. Therefore, the low flow rate of the first dimension is chosen to get broader peaks for the division and the high flow rate of UPLC is chosen to reduce the total analysis time of HPLC × UPLC system. The separation efficiency of UPLC is higher than that of HPLC under the same chromatographic conditions. Therefore, UPLC is more suitable for the second dimension separation. The flow rate of UPLC is 0.7 mL/min. The column temperature is controlled at 80 °C to reduce the column pressure. Fig. 3 shows the BPI chromatogram for the UPLC separation of 1 μL *Qingkailing* injection. 50 peaks can be seen in the chromatogram. The base peak chromatogram (BPI) is similar to the TIC chromatogram, however it monitors only the most intense peak in each spectrum.

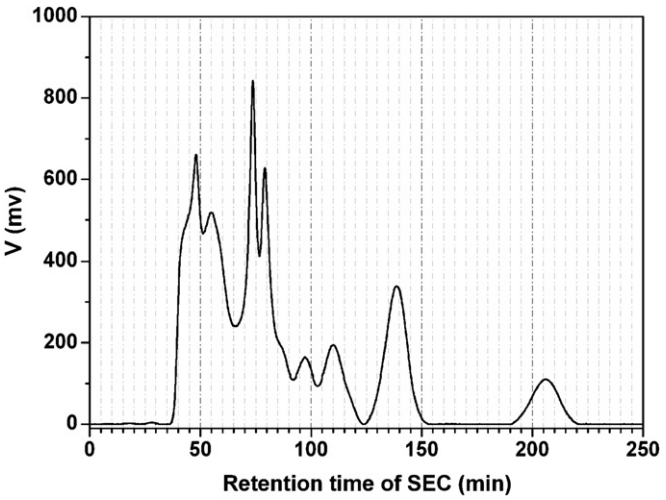


**Fig. 2.** First dimensional UV chromatogram of *Qingkailing* injection. SEC column: Toyopearl HW-40S, 200 × 2.1 mm i.d.; mobile phase: 10 mmol/L ammonium formate; flow rate: 0.020 mL/min; injection volume: 20 μL; detector: UV 254 nm.

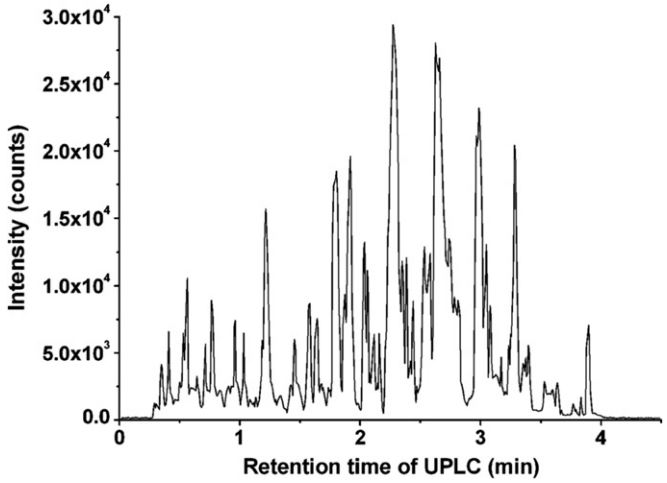


**Fig. 3.** Second dimensional BPI chromatogram of *Qingkailing* injection. UPLC column: ACQUITY UPLC RP $C_{18}$ SB 100 × 2.1 mm 1.7 μm; mobile phase A: 0.1% formic acid–water, mobile phase B: acetonitrile; gradient conditions: 5%-5%-30%-50%-5%-5%B (0-1-3-3.5-4.5-5 min); flow rate: 0.7 mL/min; column temperature: 80 °C; injection volume: 1 μL; detector: LCT Premier XE time-of-flight mass spectrometer.

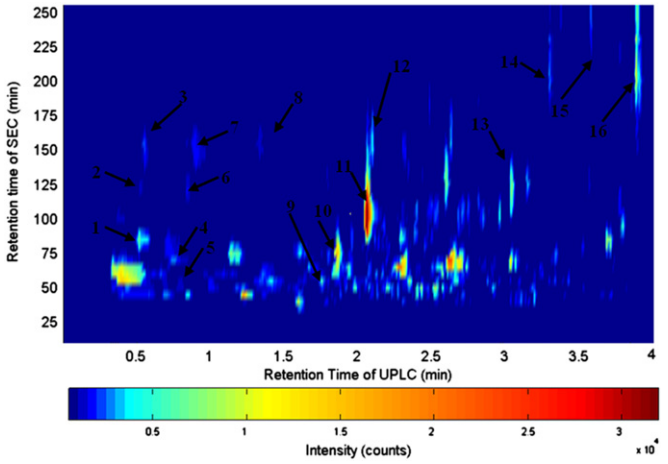


**Fig. 4.** Three-dimensional BPI chromatogram of HPLC × UPLC-TOF MS. Chromatographic conditions were shown in Table 1. Components were marked as 1–16 in Table 2.

This means that the BPI chromatogram represents the intensity of the most intense peak at every point in the analysis. Base peak chromatograms often have a cleaner look and thus are more informative than TIC chromatograms because the background is reduced by focusing on a single analyte at every point

In HPLC × UPLC analysis, peaks of SEC column were divided into more than three fractions and were diluted by the mobile phase of the first dimension. In Figs. 3, 1 μL *Qingkailing* injection was injected into UPLC column. In HPLC × UPLC separation, increasing the injecting volume can avoid reducing the detection limit. In this study, 20 μL *Qingkailing* was selected to inject into SEC column.

### 3.3. Qualitative analysis

With retention time of the SEC and the UPLC columns as *y*-axis and *x*-axis respectively, and the peak height of UPLC as *z*-axis, the MATLAB 6.5 software converted the collected data of the two dimensions into a three-dimensional (3D) data. Fig. 4 is the 3D BPI chromatogram. The retention time of a peak on UPLC column and SEC column can be obtained from the *x*-axis and the *y*-axis respectively. Each spot represents a peak of HPLC × UPLC separation and different colors indicate different height of chromatographic peaks. The results show that the HPLC × UPLC separations are reasonably orthogonal to each other, which makes it possible to fractionate each peak nearly independently.

16 components of *Qingkailing* injection were identified by the qualitative analysis of standards. 100 μg of each standard substance was dissolved in 100 μL methanol and 1 μL of mixed solution was injected into UPLC column respectively in the chromatographic conditions shown in Table 1 to position the peaks on the $x-$axis in the 3D data and obtain the exact m/z of each component. As what is shown in Table 2 and Fig. 4, 1–16 are uridine, guanosine, adenosine, phenylalanine, shanzhiside, neochlorogenic acid, tryptophane, caffeic acid, gardenoside, genipin-1-gentiobioside, geniposide, scandoside methyl ester, baicalin, wogonoside, hyocholic acid and cholic acid respectively. SEC column packed with Toyopearl HW-40 S separates components in accordance with molecular size; therefore components with big molecular size elute first. With many hydroxyls, specific adsorption occurs between geniposide, baicalin, hyocholic acid, cholic acid and the SEC packing material, therefore retention time of them was relatively late and the tailing peaks appeared.

A 4D data of HPLC × UPLC-TOF MS was investigated for the improvement of 3D data resolution and the low concentration components identification. The raw data of UPLC-TOF MS were analyzed by the MarkerLynx Applications Manager version 4.1 (Waters, UK). MarkerLynx incorporates an ApexTrack-peak detection package, which allows detection and retention time alignment of the peaks eluting in each chromatogram. The data of 50 fractions were combined into a single matrix by aligning peaks with the exact mass/retention time pair (EMRT) together from each data file in the data set, along with their associated intensities. The ion intensities for each peak detected are then normalized, within each fraction, to the sum of the peak intensities in that fraction. The data processing result was then exported for a 4D data by MATLAB 6.5 (retention time of SEC and UPLC, m/z, peak intensity). High resolution TOF MS not only offers the additional m/z, but also improves the resolution of HPLC × UPLC chromatograms. Low concentration components were effectively identified by HPLC × UPLC. Fig. 5 shows the 4D data of HPLC × UPLC-TOF MS. The 4D data provides not only narrower peak width than BPI 3D data but also exact m/z of each peak. Peak overlapping was reduced and more low concentration components were obtained. In Fig. 4, 116 peaks were counted by the software, and in Fig. 5 398 peaks were found, while in all the reports in the literatures only tens of peaks could be identified [11,41–43]. Therefore, such 4D data improve the resolution and reduce peak identification errors caused by retention time differences of peaks in UPLC analysis.

According to the results of TOF MS and our previous research, 15 extra low concentration components were identified in the 4D data which are leucine, tyrosine, rutin, loganin acid, vogeloside, secologanoside-7-methy ester, deacetylasperulosidic acid methy ester, geniposidic acid, crocetin, salicylic acid, linarin, laricresinol glucosides, malefic acid, PBD-5,11-dione, 4-(1,2,3-trihydroxypropyl)-2 and 6-dimethoxyphenyl-O-β-D-glucopyranoside.

### 3.4. Total peak capacity of HPLC × UPLC system

To obtain maximum separation efficiency in a comprehensive two-dimensional separation, the two separation systems should have different separation mechanisms; and in the optimum case, the two separation systems should be orthogonal. Separation mechanisms of size exclusion chromatography and reversed phase chromatography were not significantly correlated. The peak

**Table 2**
Qualitative analysis of *Qingkailing* injection. The components were marked in Fig. 4.

| No. | Components | Selected ion | Formula | Calculated mass(m/z) | Retention time of UPLC/min | Measured mass(m/z) |
|---|---|---|---|---|---|---|
| 1 | Uridine | $[M-H]^-$ | $C_9H_{12}N_2O_6$ | 243.0622 | 0.52 | 243.0612 |
| 2 | Guanosine | $[M-H]^-$ | $C_{10}H_{13}N_5O_5$ | 282.0843 | 0.53 | 282.0838 |
| 3 | Adenosine | $[M+HCOO]^-$ | $C_{10}H_{13}N_5O_4$ | 312.0944 | 0.56 | 312.0931 |
| 4 | Phenylalanine | $[M-H]^-$ | $C_9H_{11}NO_2$ | 164.0712 | 0.72 | 164.0668 |
| 5 | Shanzhiside | $[M-H]^-$ | $C_{16}H_{24}O_{11}$ | 391.1240 | 0.77 | 391.1238 |
| 6 | Neochlorogenic acid | $[M-H]^-$ | $C_{16}H_{18}O_9$ | 353.0873 | 0.86 | 353.0867 |
| 7 | Tryptophane | $[M-H]^-$ | $C_{11}H_{12}N_2O_2$ | 203.0821 | 0.91 | 203.0794 |
| 8 | Caffeic acid | $[M-H]^-$ | $C_9H_8O_4$ | 179.0349 | 1.35 | 179.0328 |
| 9 | Gardenoside | $[M-H]^-$ | $C_{17}H_{24}O_{11}$ | 403.1240 | 1.73 | 403.1236 |
| 10 | Genipin-1-gentiobioside | $[M+HCOO]^-$ | $C_{23}H_{34}O_{15}$ | 595.1879 | 1.89 | 595.1877 |
| 11 | Geniposide | $[M+HCOO]^-$ | $C_{17}H_{24}O_{10}$ | 433.1346 | 2.07 | 433.1429 |
| 12 | Scandoside methyl ester | $[M-H]^-$ | $C_{17}H_{24}O_{11}$ | 403.1240 | 2.13 | 403.1246 |
| 13 | Baicalin | $[M-H]^-$ | $C_{21}H_{18}O_{11}$ | 445.0776 | 2.99 | 445.0767 |
| 14 | Wogonoside | $[M-H]^-$ | $C_{22}H_{20}O_{11}$ | 459.0927 | 3.30 | 459.0930 |
| 15 | Hyocholic acid | $[M+HCOO]^-$ | $C_{24}H_{40}O_5$ | 453.2852 | 3.58 | 453.2851 |
| 16 | Cholic acid | $[M+HCOO]^-$ | $C_{24}H_{40}O_5$ | 453.2852 | 3.88 | 453.2851 |

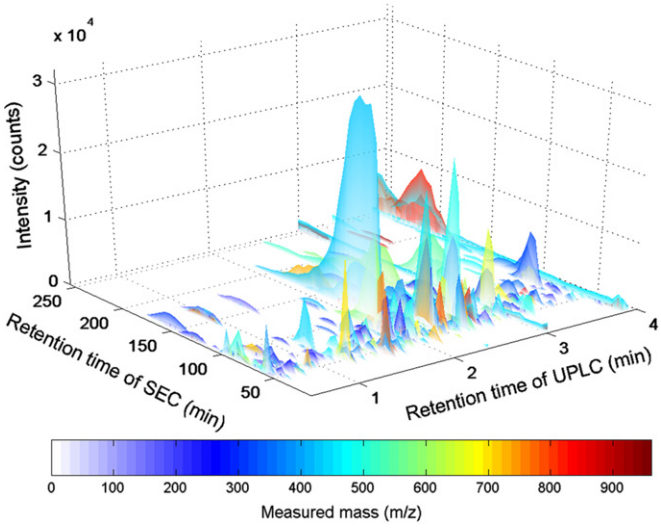


**Fig. 5.** Four-dimensional data of HPLC × UPLC-TOF MS. Color bar represents the measured mass of components by TOF MS.

capacity for columns was calculated by the following equation:

$$n_c = (t_{R,n} - t_{R,1})/W,$$

where $t_{R,n}$ and $t_{R,1}$ are the retention time of the last and the first eluting peaks, and $W$ is the average peak width [44].

This peak capacity depends strongly on all of the experimental conditions of the gradient elution program including temperature, flow rate, initial and final elution compositions, as well as the column parameters and the sample's properties. Thus, it is useful for evaluating the peak capacity of the experimental system.

In Fig. 2, the total separation time was 190 min, the average width of peak was 16.0 min, therefore the peak capacity of SEC was 12. In Fig. 3, the total separation time was 4.0 min, the average width of peak was 0.044 min, and the peak capacity of RPLC was 91. Because the retention mechanism in the two columns were not significantly correlated, the total peak capacity ($n_c$) could be calculated as $n_c = n_{c1} \times n_{c2} = 1090$, where $n_{c1}$ and $n_{c2}$ are the peak capacities in SEC and UPLC columns respectively.

### 3.5. Comparison of HPLC, HPLC × HPLC and HPLC × UPLC

In our previous study, qualitative analysis of *Qingkailing* injection was accomplished with HPLC-TOF MS and off-line HPLC × HPLC [11,42]. Table 3 shows the comparison of HPLC, HPLC × HPLC and HPLC × UPLC. Obviously, the peak capacity of multidimensional liquid chromatography is significantly higher than that of one-dimensional liquid chromatography. UPLC as the second dimension will help increase peak capacity per unit time and reduce the analysis time. The peak capacities of off-line HPLC × HPLC and HPLC × UPLC are nearly the same, but the analysis time of HPLC × UPLC is a quarter of HPLC × HPLC. Peak capacity per unit of time is more suitable for the evaluation of HPLC × HPLC and HPLC × UPLC. The HPLC × UPLC peak capacity per unit of time is 4-fold higher than that of HPLC × HPLC. Due to the high peak capacity, multidimensional liquid chromatography separated more components than HPLC. Due to the high sensitivity of UPLC, HPLC × UPLC system separated 116 components, nearly twice of that in HPLC × HPLC. Thus, HPLC × UPLC is method featured in great separation efficiency, high sensitivity and huge peak capacity. High resolution TOF MS as the detector of HPLC × UPLC offers additional m/z in a 4D data, which is more convenient for the identification of the peaks. Also TOF MS improves the resolution of HPLC × UPLC. The peak number counted in a 4D data is 398, which is over 3-fold of that in a 3D data. Thus, HPLC × UPLC-TOF MS system is a high resolution method.

Due to the advantages of HPLC × UPLC TOF MS system, low concentration components were effectively separated and identified. Fig. 6a–c shows BPI chromatogram of UPLC-TOF MS, 3D and 4D HPLC × UPLC-TOF MS of *Qingkailing* injection respectively. In Fig. 6a, only 6 peaks were separated completely. In Fig. 6b, 104 peaks were counted. Fig. 6c is the 4D data of the dark area in Fig. 6b. After qualitative analysis, the highest green peak was identified as genipin-1-gentiobioside. In our previous research, qualitative and quantitative analysis of genipin-1-gentiobioside were carried out by both HPLC and UPLC [42]. Due to the low resolution and different concentrations, low intensity peaks were hidden in the high intensity peak, which will cause interference in the qualitative and quantitative analysis. In Fig. 6c, 18 low intensity peaks hidden in the genipin-1-gentiobioside were separated completely and the colors of the peaks indicated the m/z with reference to the colorbar. The results demonstrate that more unidentified components will be separated respectively which will help to explain their pharmacological activity and/or toxicity.

UPLC as the second dimension of comprehensive 2DLC has shown great improvement. As the development of UPLC, the HPLC × HPLC will be updated to a comprehensive UPLC × UPLC system which will significantly increase peak capacity, resolution, sensitivity and reduce the total analysis time in the future.

Application of UPLC in comprehensive two-dimensional liquid chromatography will become the trend in the future. HPLC × UPLC-TOF MS system shows great potential in analysis of complex samples.

**Table 3**
Comparison of HPLC, HPLC × HPLC and HPLC × UPLC separation.

| Method | Analysis Time ($_{min}$) | Peak Capacity ($n_c$) | Peak capacity per unit time ($n_c$/min) | Peak number | Chromatograms |
|---|---|---|---|---|---|
| HPLC-TOF MS[40] | 60 | 120 | 2 | 40 | 2D-plot |
| HPLC × HPLC-MS [12] | 1110 | 1134 | 1 | 54 | 3D-plot |
| HPLC × UPLC-TOF MS | 250 | 1090 | 4 | 116 | 3D-plot |
| | | | | 398 | 4D-plot |

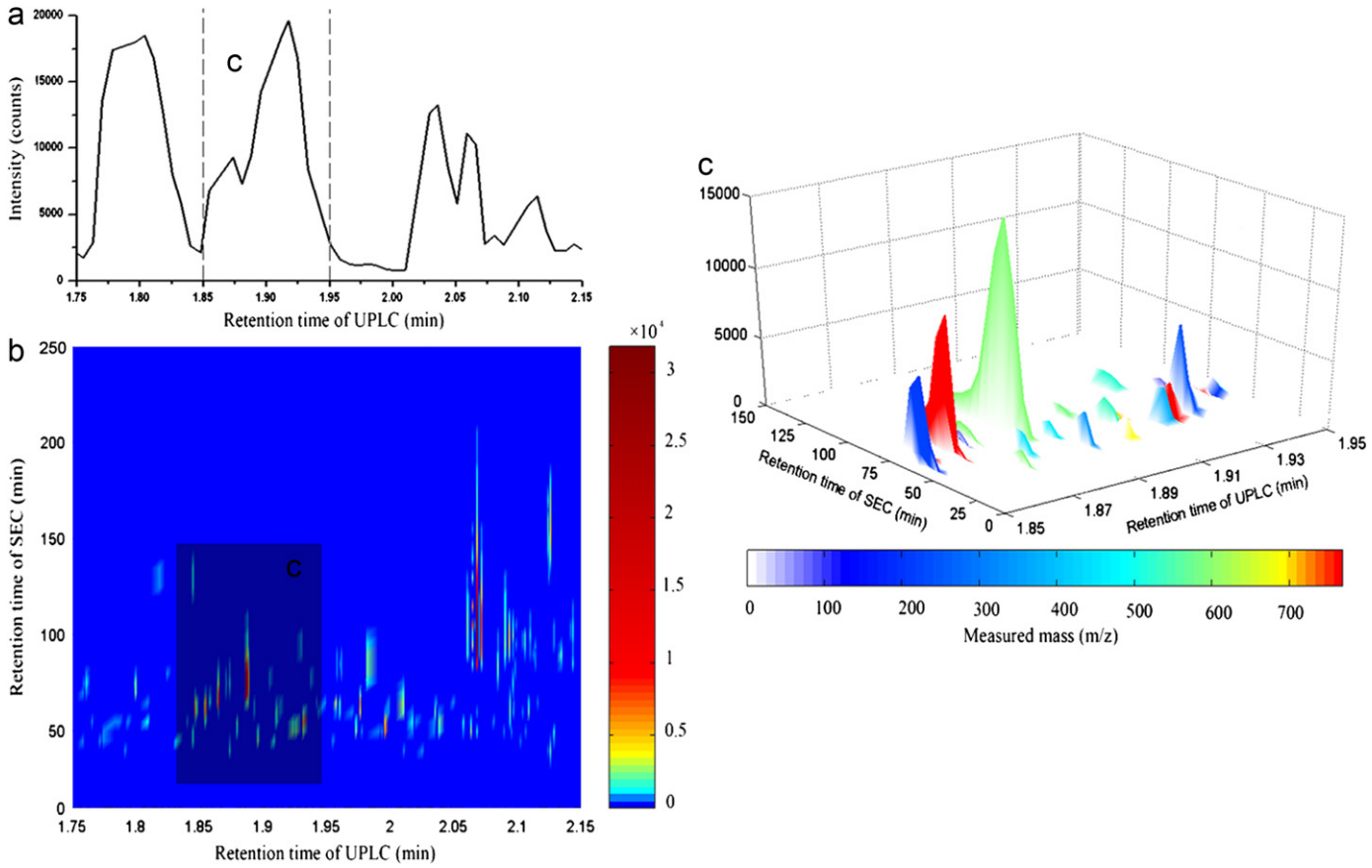


**Fig. 6.** Comparison of UPLC-TOF MS and HPLC × UPLC-TOF MS separation in 2.4–2.6 min. (a) BPI chromatogram of UPLC-TOF MS. (b) 3D data of HPLC × UPLC-TOF MS. (c) 4D data of HPLC × UPLC-TOF MS; enlarged chromatogram of dark area in b.

For processing large amount data, new qualitative and quantitative analysis methods for HPLC × UPLC-TOF MS will be well developed in the future research.

## 4. Conclusions

A simple and practical connecting technique was developed as the interface of HPLC × UPLC system which exhibited higher peak capacity, separation efficiency and sensitivity than conventional HPLC × HPLC and was more powerful for identification of lower-concentration components in complex samples. HPLC × UPLC-TOF MS can generate 4D data with the information of accurate m/z ratios useful for identification. As a demonstration, as many as 398 components of a TCM product, Qingkailing, were recognized in a run, which is the largest numbers of components detected in a natural product. Such a configuration of HPLC × UPLC-TOF MS with enhanced separation efficiency, high sensitivity and huge peak capacity can be achieved easily in any laboratory and similarly, UPLC × UPLC can also be developed with the development of UPLC device and orthogonal UPLC separation columns which may be more powerful for the analysis of complex samples.

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