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# Comprehensive two-dimensional normal-phase liquid chromatography × reversed-phase liquid chromatography for analysis of toad skin

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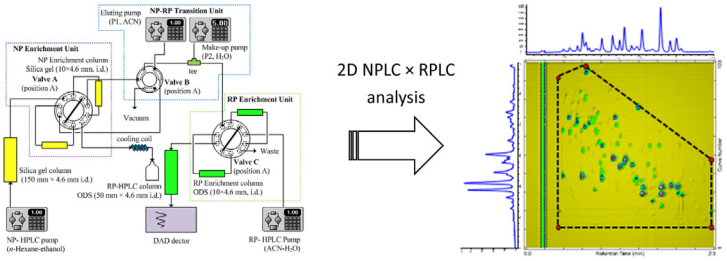
## HIGHLIGHTS

- A novel thermal evaporation assisted adsorption (TEAA) interface was designed for 2D NPLC × RPLC system.
- Rapid on-line solvent exchange between NP and RP dimensions was achieved within a short modulation time of 190 s.
- The first time to realize on-line comprehensive analysis of a moderate polar natural product by coupling of NPLC and UHPLC.
- A high orthogonality of 75.2% was achieved within 300 min' analysis.

## GRAPHICAL ABSTRACT

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## ABSTRACT

An analytical two−dimensional normal-phase liquid chromatography × reversed-phase liquid chromatography (2D NPLC × RPLC) system was constructed with a newly developed thermal evaporation assisted adsorption (TEAA) interface. This novel TEAA interface with heating temperature above solvent boiling point allowed fast removal of organic NPLC solvent and successfully solved the solvent incompatibility problem between NPLC and RPLC. The system achieved rapid on-line solvent exchange between the two dimensions within a short modulation time of 190 s and was applied in the analysis of an extract from the skin of *Bufo bufo gargarizans*. This is the first time to realize the on-line comprehensive analysis of a moderate polar natural product by coupling NPLC with reversed phase ultra-high performance liquid chromatography (UHPLC). To be highlighted, with the TEAA interface, the 2D NPLC × RPLC system provided excellent resolution and orthogonality (75.2%), when compared with that of 2D RPLC × RPLC.



## 1. Introduction

In recent years, two-dimensional liquid chromatography (2D-LC) has outperformed conventional one-dimensional (1D-LC) techniques in term of complex sample separation with higher peak

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capacity. In 2D-LC, many separation mechanisms, such as normal phase liquid chromatography (NPLC), hydrophilic interaction (HILIC), reversed phase liquid chromatography (RPLC), size exclusion (SEC), ion exchange (IEX), and affinity chromatography (AC), usually apply in pairs [1]. Of all the combination, NPLC × RPLC is probably the most orthogonal [2], though, difficult to achieve because of the problem of mobile phase immiscibility in each dimension.

Recently, Yun Zhang [3] developed an off-line 2D NPLC × RPLC method for the isolation and identification of bufadienolides in toad skin, where orthogonality of 49.5% was achieved. However, the off-line mode suffered from the disadvantages of difficult automation, poor reproducibility and time-consuming. In contrasted to that, the on-line comprehensive NPLC × RPLC method was preferred. As a typical example, Murphy et al. [4] applied aqueous solvents in both NPLC and RPLC systems to isolate alcohol ethoxylates, which successfully avoided the solvent immiscibility. However, the NPLC selectivity and the orthogonality of the 2D-LC system were affected. Wei et al. [5] developed a NPLC × RPLC system based on the modification of mobile phases, without considering its versatility. Dugo et al. [6] constructed a 2D-LC system with the use of a microbore silica column operated in first dimensional NP mode and a monolithic type C18 column operated in second dimensional RP mode. The small NPLC column inner diameter and the low NPLC flow rate permitted the direct injection of a small volume of NPLC solvents onto the second dimension. However, the small sized column reduced sample capacity and detection sensitivity. Tian et al. [7,8] and Ding et al. [9] designed a new vacuum assisted dynamic evaporation loop-type valve interface for 2D-LC system. In this design, desired fractions from NPLC were eluted into the loop-interface and underwent solvent evaporation, before RP column separation. To be mentioned, due to the limitation of second dimension's long separation time, the system could only be operated in a stop-flow mode.

In previous reports, we have showed the construction of airflow assisted adsorption (AAA) interface [10] and vacuum evaporation assisted adsorption (VEAA) interface [11] for on-line preparative 2D NPLC × RPLC systems. In details, the NPLC solvent was evaporated when it flowed into the NP enrichment column, under the blowing of airflow (for AAA) or a vacuum condition (for VEAA). To be highlighted, both AAA and VEAA interfaces could achieved the NPLC solvent removal, adsorption of the solutes on the NP enrichment column, and further solute transition from the first dimension to the second one, which completely solved the problem of mobile phase incompatibility.

In this report, we design a novel thermal evaporation assisted adsorption (TEAA) interface, which allows complete NPLC solvent removal without vacuum condition at a relative low flow rate., TEAA technique overcomes the defect of VEAA interface and is more suitable for establishing on-line analytical 2D NPLC × RPLC system. Furthermore, the performance of the on-line NPLC × RPLC system with new TEAA interface has also been evaluated by using toad skin as a complex model.

## 2. Experimental

### 2.1. Chemicals and materials

Acetonitrile, *n*-hexane and anhydrous alcohol (HPLC grade) were obtained from TEDIA (Fairfield, USA). A Milli-Q system (Billerica, MA, USA) was utilized for water supply. Analytical grade solvents applied for the toad skin pretreatment were purchased from Xilong Chemical Company, Shantou, China. The dried toad skin of *Bufo bufo gargarizans* were supplied by Luyan Pharmaceuticals (Xiamen, China) and identified by Professor Quan-Cheng Chen (Xiamen University, Xiamen, China).

### 2.2. Instrumentation

Two Agilent 1100 series HPLC systems were utilized for 2D-LC analysis. Acetonitrile was used as an eluent of the NP enrichment column and water was applied as a makeup fluid, with assistance of an Agilent 1100 series dual pump. Switching valves, tubing, and fittings were obtained from Valco Instruments. NP enrichment column heating was achieved by using a water bath (Jinghong Shanghai Industry Co., Ltd., Shanghai, China). Agilent Chemstation Rev. B.04.03 was applied for data collection and handling. ACD/SepcManager v. 12.0 (Advanced Chemistry Development Inc., Toronto, Canada) was utilized to visualize 2D HPLC spectra.

### 2.3. System setup

As shown in Fig. 1a, the novel 2D-LC system was constructed by the combination of the NPLC system and the RPLC system with the newly designed TEAA interface, comprising NP enrichment unit, RP enrichment unit, and NP-RP transition unit. The first dimension was operated by using a silica gel column (150 mm × 4.6 mm i.d., 5 μm, Shimadzu, Kyoto Japan) in NP mode, while the second dimensional RPLC system was carried out by using a Kinetex C18 column (50 mm × 4.6 mm i.d., 5 μm, Phenomenex). The NP enrichment unit, which was used to remove the NP solvent and adsorb the solute, consisted of two equivalent silica gel enrichment columns (10 mm × 4.6 mm i.d., 5 μm, Nakalai Tesque Co. Ltd., Kyoto Japan) and an electronically controlled 2-position 10-port switching valve (Valve A) (Valco Cheminert, EDU10UW, VICI, Schenkon, Switzerland). A water bath was applied to heat the silica gel enrichment columns, whose outlets were connected with organic solvent recovering device. The RP enrichment unit, a preconcentration equipment before RP separation, contained two equivalent C18-PAQ enrichment columns (10 mm × 4.6 mm i.d., 5 μm, Nakalai Tesque Co. Ltd., Kyoto Japan) and another 2-port 10-position valve (Valve C). Furthermore, a new NP-RP transition unit, consisting of tee, a 2-port 6-position valve (Valve B), an eluting pump (P1) and a makeup pump (P2), was utilized to connect NP and RP enrichment units. As shown in Fig. 1b (and detailed in supporting information Fig. S1), the switching procedure of valve was as follows: switch of Valve A and C were scheduled every 190 s; Valve B was kept at position A for 10 s and position B for 3 min, respectively. Under elevated temperature, the flow of NPLC effluent into the NP enrichment unit experienced rapid solvent evaporation, which rendered fast solute adsorption in the NP enrichment column. With the switching of Valve A, the NPLC effluent continued to flow into the other silica gel enrichment column. At the same time, acetonitrile was pumped from P1 to wash the solute in the previous column to the mixer in the NP-RP transition unit, where it was diluted with water pumped from P2 and further transferred to the RP enrichment unit. The diluted solvent was enriched in one of the RP enrichment columns with the switching of Valve C and analyzed in the second dimensional RPLC continuously. Once the complete solute elution in the silica gel enrichment column was achieved, Valve B was switched to another position, in order to directly control the flow of P1 and P2 to the RP enrichment unit and to establish connection between the NP enrichment column and vacuum pump for column regeneration (10 s every cycle).

### 2.4. Sample preparation

A Soxhlet extractor was utilized to extract the dried skin powder of *Bufo bufo gargarizans* (10.0 g) in methanol (100 mL) for 2 h. After removal of methanol in vacuum at 40 °C, the residue was extracted