Resolving Co-eluted Oligostilbenes using Recycling High Performance Liquid Chromatography (R-HPLC)

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Natural oligostilbenes occur as closely related isomers and many have similar plane structures. Similar compounds have a tendency to co-elute in a chromatographic process, hence difficult to separate. A recycling high performance liquid chromatography (HPLC) was used to separate co-eluted oligostilbenes in a fraction from *Dipterocarpus semivestitus* leaves extract. Prior to the isolation process, a chromatographic method was developed on an ultra-high performance liquid chromatography (UHPLC). An oligostilbene tetramer, vaticaphenol A was successfully isolated and purified in ten cycles of chromatographic runs. The compound was identified by NMR and mass spectrometry data. The ability of the system to recirculate the unresolved peaks into the column increases the separation efficiency of the chromatographic process.

INTRODUCTION

Oligostilbenes are naturally occurring phenolic compounds that polymerized from resveratrol or other stilbene units. Resveratrol in plants is a phytoalexin, which is synthesized by a plant for protection during stress condition such as infections, injuries and diseases (Zain *et al.*, 2010). Oligostilbenes were widely studied for their variety of chemical structures and bioactivities. Their polymeric structures vary from a dimer to an octamer (Ito *et al.*, 2001). As natural compounds are often tested against many bioactivities (Srinivasan *et al.*, 2014; Abdulkadir *et al.*, 2015), some of reported bioactivities of oligostilbenes are antimicrobial activity (Peng *et al.*, 2008; Yim *et al.*, 2010; Nitta *et al.*, 2002), cytotoxicity (Rohaiza *et al.*, 2011), antioxidant and lipoxygenase inhibitory effects (Kim *et al.*, 2009) as well as chemopreventive and anti-inflammatory activity (Lin and Yao, 2006).

The complex structure of oligostilbene makes them difficult to be separated by conventional chromatographic methods. In an HPLC separation method, they often co-elute makes the process sometimes end up with mixtures. This required repetitive HPLC run, which is time consuming and requires high volume of organic solvents. More often than not, a longer HPLC column was needed in order to improve the separation. Recycling HPLC increases the separation efficiency by recirculate the unresolved compounds into the column. It benefits the researchers by lowering solvent consumption, fast, economical and practical. The commonly used technique on natural product isolation is the closed loop recycling (Teoh, Sorensen and Hooker, 2003) in which the sample mixture that eluted from a column is delivered into the same column repetitively until a desired

separation is achieved (Fig. 1). In this article, we report the application of his technique in the purification of oligostilbenes from a fraction of *Dipterocarpus semivestitus* extract.
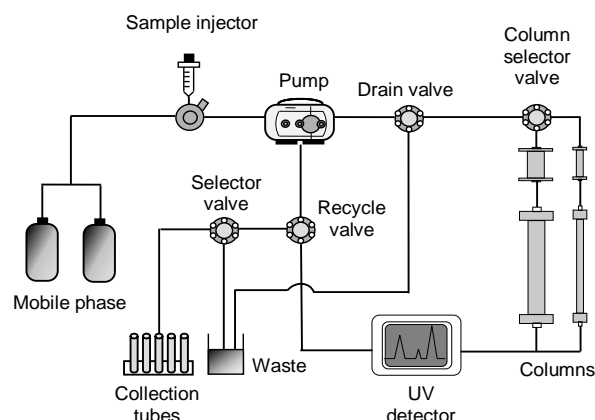
Fig. 1: Schematic diagram of a closed loop recycling HPLC system.

MATERIALS AND METHODS

General experimental procedures:

Organic solvents were of HPLC grade from RCI Labscan. Ultrapure water (18 MΩ.cm^{-1}) was obtained from PURELAB$^{®}$ Option water purification system (ELGA).

Chromatographic development was carried out on a Dionex^{TM} Ultimate$^{®}$ 3000 Thermo Scientific^{TM} UHPLC system, fitted with a vacuum degasser, a quaternary pump, an automated liquid sampler and a DAD detector. The chromatographic profiles and the integrated data were recorded using Chromeleon^{TM} Chromatography software.

Compound purification was achieved by a JAI recycling HPLC, LC-9103, equipped with a liquid feed pump type L-7150, a UV detector type UV-3702, a connecting unit involving a recycle valve and a selector valve.

The NMR spectra were measured on Bruker Ascend FT_NMR 600 in acetone-d_6 without TMS. MS data were obtained from an LC-MS system consists of an Agilent RRLC 1200 series UHPLC and an Agilent 6300 Series Ion Trap LC-MS, hyphenated by an ESI interface.

Sample materials:

The fraction from *D. semivestitus* acetone extract was provided by Atta-ur-Rahman Institute for Natural Products Discovery (AuRINs), Universiti Teknologi MARA, Malaysia. The plant sample was collected in a freshwater swamp forest in UiTM Kampus Sri Iskandar, Perak, Malaysia. 2 kgs of dried stem of *D. semivestitus* were extracted with hexane to remove non polar constituents. Further extraction with acetone (3 x 24 h) at room temperature afforded phenolic rich extract and the solvent was then evaporated using rotary evaporator to afford 42 g of crude extract.

Sample preparation and chromatographic method:

Crude acetone extract (10 g) of *D. semivestitus* was fractionated using MPLC. The separation was performed on 5L Hi-flash column (250g silica, 40 µm) at flowrate of 20 ml/min, visualized at 215 nm and eluted by step-wise gradient of n-hexane/ethyl acetate (3:2, 3:7, 1:4, 0:10; 30 mins each) followed by ethyl acetate/methanol (95:5, 4:1, 1.1; 15 minutes each). The sample was dissolved in acetonitrile to the concentration of 1000 ppm according to the weight and filtered through a 0.45 μm membrane filter into an HPLC vial. The separation was achieved through a Phenomenex$^{®}$ Luna 5 μm C18 column (150 X 4.6 mm) equipped with a guard column of similar chemistry. The chromatographic condition was a gradient of ACN:H$_2$O (5:95 to 95:5 for 10 minutes) at 1.0 ml/min. and were detected at 215, 254 and 283 nm.

Sample purification on recycling HPLC:

The isolation and purification were performed on a JAIGEL-ODS-AP preparative column (20 x 250 mm, 10μm) eluted at 4 ml/min. The analyses were detected at 215 nm. Partial loop injections were performed on a 3 ml injection loop. Purified compound was obtained after 10 cycles of chromatographic run.

Vaticaphenol A:

Light brown amorphous powder; ^{1}H NMR (600 MHz, acetone-*d6*, δ ppm): 7.24 (2H, d, J=8.4 Hz, H-2a,6a), 7.20 (2H, d, J=8.4 Hz, H-2d,6d), 7.17 (2H, d, J=8.4 Hz, H-2b,6b), 6.79 (4H, d, J=8.4 Hz, H-3a,3d,5a,5d), 6.71 (2H, d, J=8.4 Hz, H-3b,5b), 6.52 (2H, d, J=8.4 Hz, H-3c,5c), 6.48 (1H, d, J=2.4 Hz, H-14c), 6.41 (2H, d, J=8.4 Hz, H-2c,6c), 6.31 (1H, t, J=2.4 Hz, H-12d), 6.29 (1H, d, J=2.4 Hz, H-12a), 6.20 (1H, d, J=1.8 Hz, H-12c), 6.13 (1H, d, J=1.8 Hz, H-14a), 6.10 (2H, brs, H10d,14d) 6.07 (1H, s, H12b), 5.78 (1H, d, J=11.4 Hz, H-7a), 5.38 (1H, d, J=5.4 Hz, H-7d), 5.21 (1H, d, J=3.6 Hz, H-7b), 4.69 (1H, d, J=5.4 Hz, H-8d), 4.56 (1H, d, J=10.8 Hz, H-8c), 4.44 (1H, d, J=12.0 Hz, H-8a), 4.10 (1H, t, J=11.4 Hz, H-7c), 3.13 (1H, brd, J=12.0 Hz, H-8b); IT-ESI-MS *m/z* [M+H]$^+$ 907.5, MS^2 (813.4, 707.4), MS^3 (613.2, 479.2, 371.2), MS^4 (409.1).

RESULT AND DISCUSSIONS

In the development of a chromatographic method, the sample underwent a full-range gradient method, which is a standard gradient elution analysis run to determine the overall performance of the chromatographic profile. The analysis run can be a preliminary guide tool to determine the best possible elution mode for a specific sample, column and eluent system. In the analysis, the solvent composition was of acetonitrile (ACN) and water with a gradient elution of ACN:H$_2$O (9:95 to 95:5) for 16 minutes chromatographic run, followed by 5 minutes column flushing and another 5 minutes post-run for column conditioning.

From the initial analysis of the chromatographic profile, the analyses were continued by changing the solvent composition of the initial and the end of a chromatographic run. In a preliminary analysis, the initial solvent composition was maintained at ACN:H$_2$O (5:95), while at the end of the chromatographic run, the solvents were adjusted as ACN:H$_2$O (90:10, 80:20, 70: 30, 60:40 and 50:50). The chromatographic profiles for all analyses were analyzed individually by examining the peak resolutions. Based on this result, further analyses were carried out by maintaining the selected solvent composition at the end of the chromatographic run, while adjusting the initial composition at ACN:H$_2$O (10:90, 20:80, 30: 70, 40:60 and 50:50).
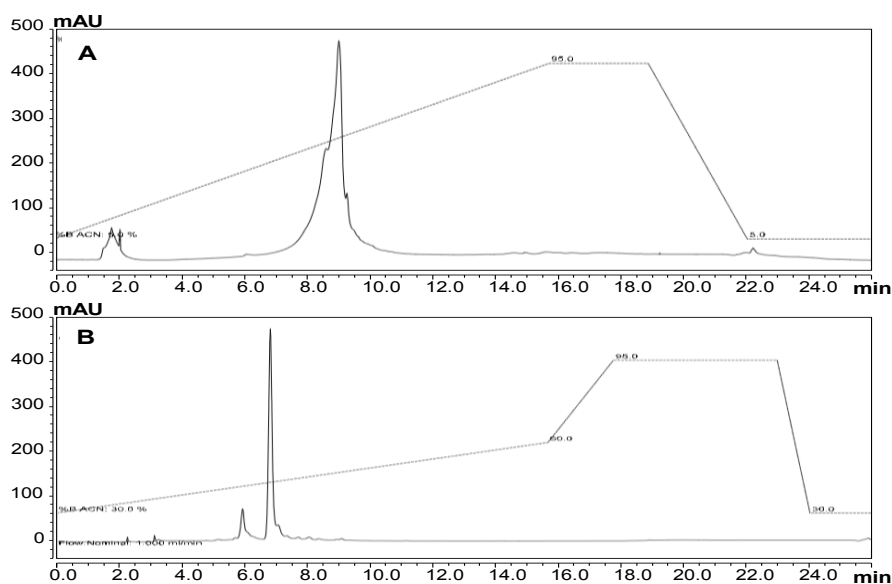
Fig. 2: The chromatograms of *D. semivestitus* extract; (A) a full-range gradient elution of ACN:H$_2$O (5:95 to 95:5) and (B) an adjusted gradient elution of ACN:H$_2$O (30:70 to 60:40) in 16 minutes.

All chromatographic profiles were analyzed and after some adjustment and modification, a gradient of ACN:H$_2$O (30:70 to 60:40 in 20 minutes) was considered performing a reasonably good separation for the sample. Fig. 2 shows how the chromatographic profiles improved from a full-range gradient method to the selected chromatographic conditions.

The first peak was eluted at 5.7 minutes while the second peak at 6.5 minutes. Using higher initial percentage of acetonitirile, the peaks become narrower, taller and sharper, as the higher acetonitrile concentration which is an organic compound pushes the tailing part of the peak forward. These characteristics of peak shape are important in trace analysis. The peaks also separated better with a lower gradient slope. The solvent system was chosen for the purification process using recycling HPLC.

The main purpose of using RHPLC is to improve resolution by increasing number of plates which shows the efficiency. This can be achieved when the sample passing repetitively through the existing column

(Tarafder, Aumann and Morbidelli, 2008). Recycling HPLC has advantages such that it increase throughput, reduce time of separation, reduce the consumption of the solvent and complete recovery of the compounds. The aim is achieved by incorporating a recycle valve in the HPLC, enabling the system to recirculate the unresolved peaks into the column.

The recycle valve switches between recycling and collection mode, enable the analytes to pass through the same column repetitively, thus improve the separation during every recycle run. The drain valve channels the eluent to the waste in the middle of a recycle run, making it possible to remove impurities while a chromatographic separation is running. The selector valve switches between collection and draining, for the compound of interest to be collected. The column selector valve switches the two columns, making a separation on different phases is possible in a recycling mode.
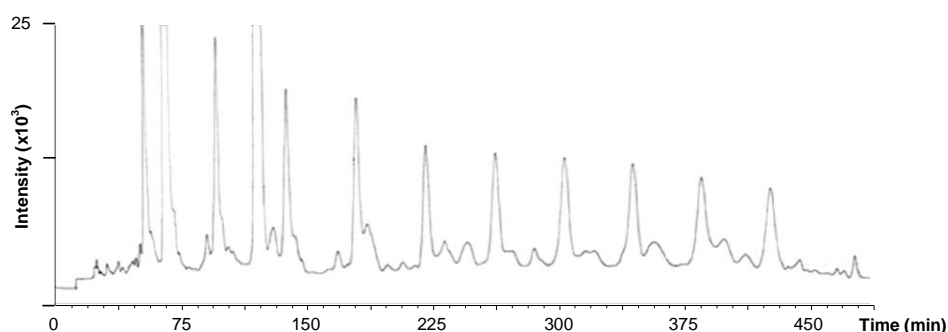

Fig. 3: The chromatogram of the recycling HPLC for the isolation of compound **1**

The attempt of using the previously developed method on a UHPLC was not very practical for a recycling HPLC. For a gradient elution, a post-run of 5 times the column void volume is necessary in every cycle in order to keep the same condition for every chromatographic cycling. The process therefore defeats the purpose of recycling, which should be fast and reproducible. A method with an isocratic elution was developed with the solvent composition closely resemble the one of the gradient.

The purification was continued using an isocratic elution of 35% ACN and 65% H_2O. It was observed that minor peaks were recorded, prior to the elution of the desired peaks. Further recycling may lead to the remixing of the solutes. To avoid this, the minor peaks were flushed out and the rest of the sample was recycled into the column. This is called peak-shaving (Teoh, Sorensen and Hooker, 2003). Two major peaks were recorded after 23 minutes of the chromatographic run. It takes 48 minutes for a complete chromatographic cycle.

At the second cycle, the two peaks appear to be well resolved from each other. The second peak however appeared too close to the end of the chromatographic cycle. To avoid sample remixing, the compound was flushed out from the system and was collected. Upon checking on an analytical HPLC, the compound was found to be at high purity. The chromatographic cycles were continued until a separation at baseline resolution was achieved (Fig. 3). During the process, the impurities were eluted out whenever necessary. At the end of the 10th cycle, the compound correspond to the only peak remaining was collected. Its amount was however too little for spectroscopic measurement.

The structure of the purified compound **1** was elucidated by interpretation of NMR and mass spectra. Analysis of the chemical shifts and coupling constants from the ^{1}H-NMR (Fig. 4) spectrum revealed a known compound.
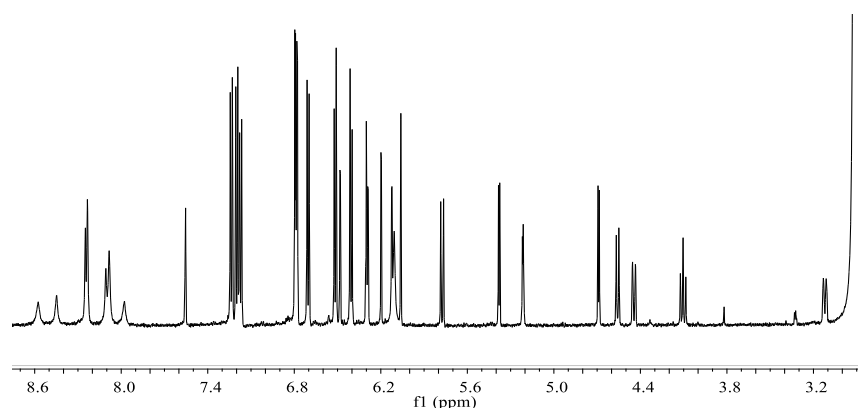
Fig. 4: The 600 MHz ^{1}H-NMR spectrum of compound **1**.

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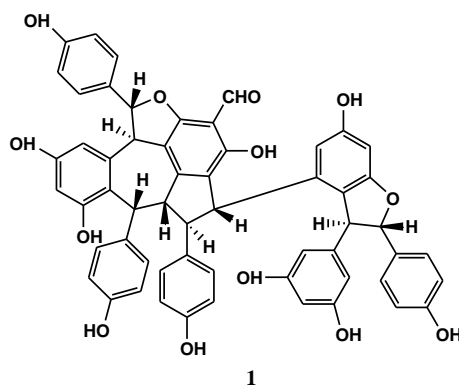
Comparison of its mass spectrometric data with those in our in-house library confirms the identity of the compound. Therefore, further spectroscopic measurement was discontinued. The compound is vaticaphenol A, was first isolated from *Vatica diospyroides* (Seo *et al*., 1999), *Vatica oblongofolia* (Zgoda-Pols *et al.*, 2002) and *Neobalanocarpus heimii* (Bayach *et al.*, 2015) It was also isolated from a non-dipterocarpaceous plant, *Vitis vinifera* (Yan, Terashima and Niwa 2002).

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