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Separation of targeted ganoderic acids from *Ganoderma lucidum* by reversed phase liquid chromatography with ultraviolet and mass spectrometry detections

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Abstract

Ganoderic acids are valuable bioactive secondary metabolites produced by a traditional medicinal mushroom *Ganoderma lucidum* ("Ling-zhi" in Chinese and "Reishi" in Japanese). In this work, a fast and efficient method for the recovery and purification of ganoderic acid T (GA-T) and ganoderic acid Me (GA-Me) from triterpene-enriched extracts of *G. lucidum* mycelia was developed by using reversed phase HPLC (RP-HPLC) on a C_{18} column with an acidified methanol–water mobile phase in combination with ultraviolet (UV) detection and electrospray ionization mass spectrometry (ESI-MS). The presence of each targeted GA (GA-T and GA-Me) in its corresponding peak was easily identified and confirmed by UV and MS. The chemical structures of the purified GA-T and GA-Me were further confirmed by ^{1}H NMR. The retention behaviors of the two GAs over a temperature range of 15–55 °C were also investigated. From the retention time data, van't Hoff plots were obtained. The estimated enthalpy (ΔH) and entropy (ΔS) data suggest that the retention time difference between GA-T and GA-Me might be driven by an enthalpy difference. Furthermore, a semi-preparative HPLC purification was achieved on a semi-preparative C_{18} column using the conditions optimized for the analytical column. The method presented in this work can be a valuable tool for the rapid semi-preparative purification of targeted GAs, and it may also be applicable to some other natural products.
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Keywords: Bioseparation; Purification; Natural products; RP-HPLC; Electrospray ionization mass spectrometry (ESI-MS)

1. Introduction

Since ancient times, *Ganoderma lucidum* (Fr.) Krast (Polyporaceae) ("Ling-zhi" in Chinese and "Reishi" in Japanese) has been used as a medicinal herb to treat many diseases in Asia, such as hepatitis, hypertension, hypercholesterolemia and gastric cancer [1]. Lanostane-type triterpenes, such as ganoderic acids (GAs) and lucidenic acids, were identified as main bioactive ingredients in *G. lucidum* [1,2]. Ganoderic acid T (GA-T) and ganoderic acid Me (GA-Me) (Fig. 1) were found to have significant anti-tumor activities [3,4]. GA mixtures can be produced in quantity through mushroom fermentation [1,5]. However, to obtain a large amount of pure GAs for biological activity tests, efficient separation and purification methods still need to be developed.

Chromatographic purification is widely used for various bioproducts such as plasmid DNA, enzymes and natural products [6–8]. In the analysis and isolation of triterpenes from *G. lucidum*, the crude triterpene extracts are usually subjected to qualitative analysis and semi-preparative separation using silica gel TLC plates or silica gel column chromatography [9,10]. RP-HPLC methods were also used for the complete separation of triterpenes isolated from the *Ganoderma* mycelia [11,12]. However, modern hyphenated techniques, such as GC–MS, HPLC-MS, HPLC-MS-MS and HPLC-NMR, have not been applied, even though these techniques may provide useful structural information online on these triterpene metabolites and allow the rapid structural determination of known plant constituents

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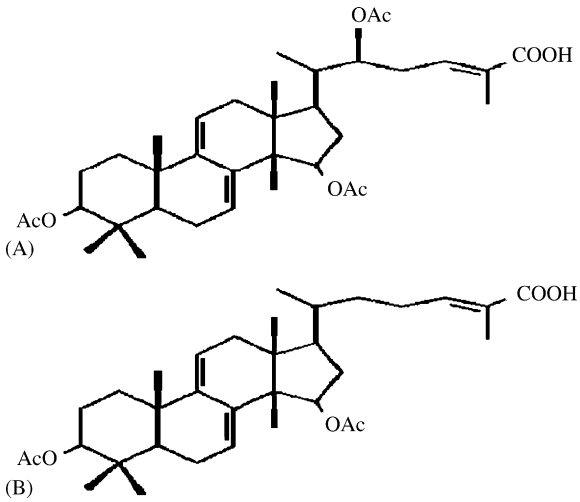

Fig. 1. Chemical structures of ganoderic acids GA-T (A) and GA-Me (B). The structures were identified with ^{1}H NMR, ^{13}C NMR, MS, IR and UV spectra in our lab. Data are consistent with literature [3,4].

with only a minute amount of materials [13,14]. It is worthwhile to apply modern hyphenated chromatographic techniques to the characterization and determination of a variety of components in *Ganoderma* extracts. The objective of this work was to develop an effective HPLC-UV-ESI-MS hyphenated method to determine the presence and distribution of targeted triterpenes in the methanol extract of *Ganoderma* mycelia and then to purify them efficiently.

For chromatographic separation, temperature is an important factor affecting selectivity, resolution and column efficiency [15]. Investigations on the effects of temperature on separation characteristics of terpene, cellulose derivatives and phenol were reported [15–17]. However, there have been no reports regarding the effect of temperature on the chromatographic separation of triterpenoids. In this study, the retention behavior of GA-T and GA-Me over the range of temperature of 15–55 °C was also examined in order to reveal the effect of temperature on selectivity. The differences of enthalpy and entropy changes of GAs were discussed.

2. Materials and methods

2.1. Chemicals and materials

Methanol was of HPLC grade, and acetic acid was of analytical reagent grade. Deionized water was passed through a 0.5 μm membrane filter before being sparged with helium gas. The working mobile phase solutions were prepared daily as needed. They were heated to the column bath temperature before use and maintained during HPLC runs. The mycelia of *G. lucidum* were harvested from liquid cultures fermented in bioreactors using a liquid static cultivation method [1].

2.2. Sample preparation

The harvested mycelia were dried at 60 °C for 3 days in an air-circulation oven. They were ground to pass an 80-mesh screen. Five hundred grams of the dried mycelia powder was extracted successively using a solution consisting of 70% (v/v) methanol and 30% water in an extractor for 6 days. The methanol extract was evaporated to near dryness under vacuum and dissolved in 500 ml water. The water solution was then extracted with chloroform. The chloroform extract (70 g) was subsequently dried in vacuum to obtain a sticky paste. A stock solution was prepared by dissolving 100 mg of the paste containing mostly triterpene acids in 1 ml methanol and stored at −20 °C.

2.3. Instrumentation

A Shimadzu (Shimadzu Ltd., Kyoto, Japan) HPLC system equipped with a Rheodyne (Cotati, CA, USA) Model 7725 injection valve fitted with a 20 μl sample loop was used in this work. This HPLC system consisted of two Model LC-10ADvp pumps, an SPD-10AVP diode-array UV–vis detector and an SCL-10AVP system controller. A Fisher Scientific (Pittsburgh, PA, USA) Model 9105 circulating water bath was used to regulate the column temperature.

A Shim-pack 250 mm × 4.6 mm i.d. analytical HPLC column from Shimadzu was used for sample analysis. Its stationary phase consisted of 4.6 μm C_{18} particles. In the HPLC analysis, each peak was scanned in the range of 190–370 nm with the diode-array UV detector. Chromatographic data were collected and analyzed using the Shimadzu Class-VP software. A semi-preparative column (250 mm × 10.0 mm i.d.) packed with 5 μm Hypersil ODS2 C_{18} (Elite Co., Dalian, China) was used for semi-preparative separations. The operating conditions of the semi-preparative chromatography were identical to those used for the analytic column, except that its flow rate was 4 ml/min instead of 1 ml/min.

2.4. Chromatography and ESI-MS operating conditions

A 20 μl sample in methanol prepared from the chloroform extract was injected into the analytical HPLC column for analysis. Each analytical chromatographic run was repeated twice. The mobile phase for the isocratic elution was prepared by adding water to a mixture containing 99.5% (v/v) methanol and 0.5% acetic acid. The methanol–acetic acid mixture was mixed with water at a ratio of 9:1 (v/v). The mobile phase flow rate for analytical HPLC runs was 1 ml/min. Because the triterpene acids exhibit a maximum UV absorbance at 245 nm, this wavelength was used for UV detection. Identification of peaks was based on retention times.

Electrospray ionization mass spectrometry (ESI-MS) measurements were performed using a Quattro LC triple-quadrupole mass spectrometer (Micromass, Manchester, UK). The sample was directly infused into the MS system using a syringe pump. The effluent at a flow rate of 10 μl/min from the analytical HPLC column was diverted to the mass spectrometer. It was analyzed by the MS in the positive ion mode with an electrospray capillary potential of 3.5 kV and a cone potential of 21 V. The optimization of MS operating conditions was based on the stability of the spray and intensity of the $(M + Na)^{+}$ signal.

2.5. Equations of resolution, capacity factor and van't Hoff isotherm

The resolution, capacity factor and van't Hoff isotherm from the chromatogram were calculated in this work. The following formulas were used for their calculations:

Resolution:

$$R = \frac{1.18(t_2 - t_1)}{w_1 + w_2} \quad (1)$$

where t_1 and t_2 are the retention times of two indicated peaks and w_1 and w_2 are their baseline peak widths.
Capacity factor (k'):

$$k' = \frac{t_R - t_0}{t_0} \quad (2)$$

where t_R is the retention time of indicated peak and t_0 is the column dead volume time.
The van't Hoff equation [18]:

$$\log k' = \frac{-\Delta H}{2.3RT} + \frac{\Delta S}{2.3R} + \log \Phi \quad (3)$$

where ΔH and ΔS are the system enthalpy and entropy, T the absolute temperature, R the universal gas constant and Φ is the phase ratio of the system, respectively. For neutral solutes, the van't Hoff plot of $\log k'$ versus $1/T$ is usually linear with a slope of $-\Delta H/2.3R$ and an intercept of $\Delta S/2.3R + \log \Phi$, provided that ΔH and ΔS are invariant with temperature [19,20].

2.6. ^{1}H NMR spectroscopy

The purified samples were dissolved in deuterated DMSO that was from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). The ^{1}H NMR spectroscopy was performed on the samples using a Varian UNITY plus 500 spectrometer with a triple resonance probe. An 8 s relaxation delay was used, along with a 90° tip angle and a 2 s acquisition time (6500 Hz sweep width with 26 K data points). The data were processed using a Sun workstation model Sparc station 10 and zero filling to 16 K data points. No presaturation delay or exponential multiplication was used. Resonances are reported here relative to transcranial magnetic stimulation (TMS).

2.7. Statistical analysis

All experiments were performed at least three times ($n = 3$) unless otherwise indicated. Data are expressed as mean value ± S.D., and significance was assessed by the t test. Differences with $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Detection of presence and distribution of targeted GAs

Fig. 1 shows that the structures of GA-T and GA-Me were derived from lanostane, whose molecular weight is 612 and 554, respectively [3,4]. Comparing their structures, GA-T has an extra acetyl group at C_{22}. The GAs have only weak solubilities in aqueous media due to their high hydrophobicity. Methanol mixed with water was selected as the mobile phase because of the good elution behaviors and higher solubilities of the GAs. Fig. 2A shows the chromatogram of an analytical RP-HPLC run. The operating conditions were mentioned above. In Fig. 2A, the compounds indicated by peaks A and B were subsequently identified as GA-T and GA-Me, respectively. The figure shows that the analytical RP-HPLC method used in this work achieved a baseline separation of the GAs using the chloroform extract of *G. lucidum*. The GA-T and GA-Me peaks were found to be single-compound peaks using diode-array UV peak-purity scans.

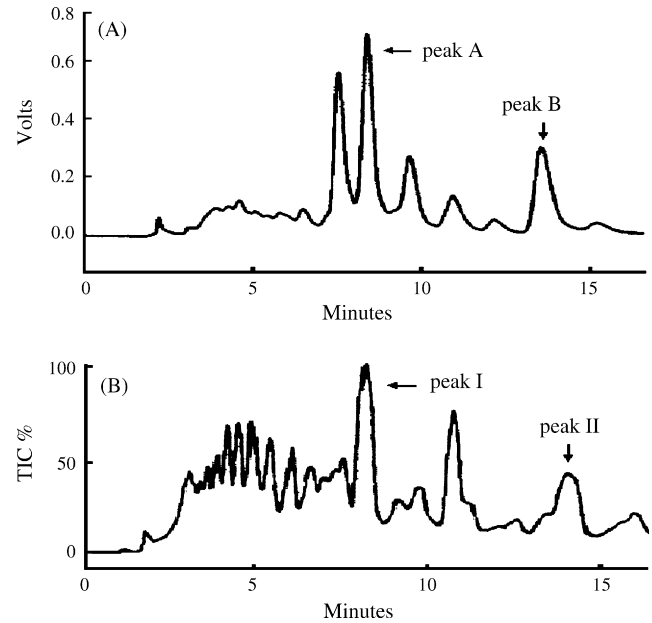

Fig. 2. Analytical HPLC chromatogram with UV detection at 245 nm (A) and reconstructed TIC chromatogram (B). Peaks I and II in (B) correspond to peaks A and B in (A). The 20 μl sample was a methanol solution of 50 μg of the chloroform extract of triterpene-enriched mixture obtained from the crude methanol extract.

To validate the identities of the GA peaks in Fig. 2A, ESI-MS detection was applied. An ESI voltage set at 3.5 kV for the detection of the positive ion spray yielded the most abundant $(M + Na)^+$ signal. In the analysis of the chloroform extract of *G. lucidum*, all peaks recorded by the UV detector at 245 nm gave mass responses in the total ion current (TIC) trace (Fig. 2B). It is interesting to note that some individual peaks in the UV spectrum gave multiple peaks in the TIC spectrum. This indicates that these chromatography peaks were not single-compound peaks, meaning that each peak contained several compounds with the same retention time on the analytical C_{18} column. Moreover, some large UV peaks gave only small ion currents in the TIC chromatogram, indicating that the corresponding compounds had strong UV absorbance but small mass amounts. The ESI-MS spectrum in this case provided important additional information on the RP-HPLC peaks.

Fig. 3 shows typical results acquired using HPLC-UV-ESI-MS. The positive ion ESI mass spectrum of peak I (at 8.5 min in the TIC spectrum) gave an $(M + Na)^+$ signal at m/z 635.4 cor-

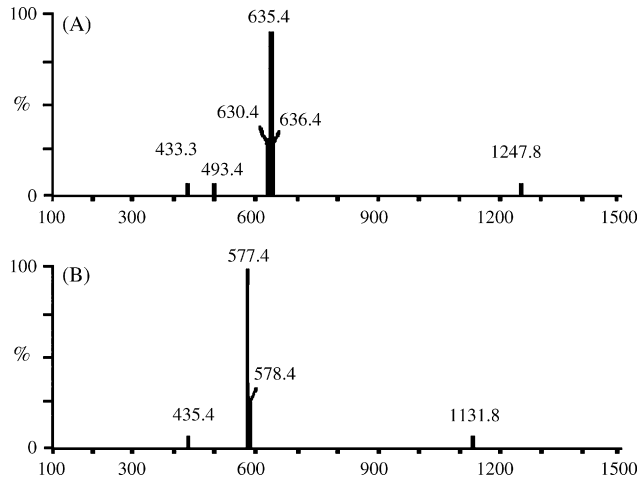

Fig. 3. LC–ESI-MS spectra showing GA-T and GA-Me. The positive ion ESI-MS of: (A) GA-T and (B) GA-Me. The figure shows an enlarged region between scans 300 and 1500.

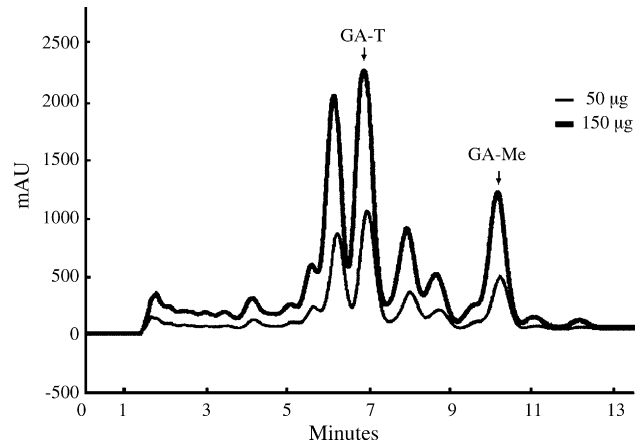

Fig. 4. Semi-preparative HPLC chromatograms showing GA-T and GA-Me peaks. The HPLC conditions are mentioned in the experimental section. The light curve corresponds to the 50 μg sample injection and the heavy curve 150 μg sample injection.

responding to a molecular weight of 612 (Fig. 3A). Peak II (at 13.6 min in the TIC spectrum) gave an $(M+Na)^+$ signal at *m/z* 577.4 corresponding to a molecular weight of 554 (Fig. 3B). The UV and mass spectra suggested that peaks I and II in the TIC spectrum were most likely GA-T and GA-Me corresponding to peaks A and B in the UV chromatogram based on their strong absorbance at 245 nm and molecular weights. Moreover, the reduplicated molecular weights $[(2M+Na)^+]$ were 1247.8 and 1131.8 in aforementioned peaks, which also confirmed the molecular weight results. Meanwhile, signals at *m/z* 433.3 and 493.4 in Fig. 3A and at *m/z* 435.4 in Fig. 3B might be random fragments of target molecule, or other contamination since their abundance was very low. However, more proof is needed as shown in the discussion below.

3.2. Semi-preparative purification of targeted GAs

The semi-preparative HPLC separation of the GAs was accomplished after the establishment of the HPLC-UV-ESI-MS hyphenated method. Sample injection volume as a key operating parameter in preparative- and large-scale HPLC was examined [21]. Fig. 4 shows HPLC chromatograms for the same sample mixture with two different sample sizes (50 and 150 μl) using the semi-preparative C_{18} column with the aforementioned mobile phase at 4 ml/min flow rate. The two chromatograms in Fig. 4 are very similar, and this indicates that some operating conditions for the analytical runs could be successfully adopted in the semi-preparative separation in this case. The chromatograms in Fig. 4 also resemble the analytical chromatograms in Fig. 2A. Moreover, GA-T and GA-Me samples purified from the semi-preparative column runs were re-injected into the analytic column, and retention times were found to be consistent with those shown in Fig. 2A. Fractions containing GA-T and GA-Me from several semi-preparative runs were pooled together. After the evaporation of methanol, 0.568 g of GA-T and 0.12 g of GA-Me were purified from the 70 g semi-dried paste of chloroform extract that was obtained originally from the 500 g dried mycelia of bioreactor-cultivated *G. lucidum*. Their structures were elucidated by ^{1}H NMR. These data are consistent with those in literature [3,4]. This further validated the identities of GA-T and GA-Me in Fig. 2A. Compared with the published works [11,12], the separation method demonstrated here is more effective in determining the presence and distribution of those targeted triterpenes in the methanol extract of *Ganoderma*.

3.3. Effect of temperature on the separation of targeted ganoderic acids on C_{18} HPLC column

Until now, information on the effect of temperature on GAs purification in RP-HPLC separations is not yet available. An insight into the retention behaviors of GAs on silica-based C_{18} column based on a thermodynamic analysis will enhance our understanding of the separation of triterpenes. In this work, the retention behaviors of GA-T and GA-Me on analytical C_{18} column over a temperature range of 15–55 °C were investigated (Fig. 5). The results indicated that temperature affected the separation efficiency. The capacity factor was decreased with the increase of temperature. In contrast, the resolution was increased slightly when the temperature increased from 15 to 40 °C, while a further increase to 50 °C did not further improve the resolution.

The effect of temperature on solute retention factor (k') is also a function of the energy changes in the interaction between the solute and the stationary phase according to the van't Hoff equation [18]. Eq. (3) shows that a more negative ΔH value yields a larger k' that indicates a stronger retention. Fig. 6 shows the van't Hoff plots obtained using experimental data. The methanol and water mixture in the mobile phase could form protic environment [22] and made GA molecules neutral by hydrogen bonds, so the two lines shown in Fig. 6 are almost linear. Many researchers observed linear van't Hoff plots with hydroorganic mobile phases for temperature ranges within 10–60 °C [20]. The van't Hoff plots for GAs in this work are similar to those in the literature. From the slope and intercept values in Fig. 6, ΔH and ΔS could be assessed for both GA-T and GA-Me. Since the phase ratio of the column (Φ) is believed to be constant over a reasonable temperature range [23], the values of ΔS could

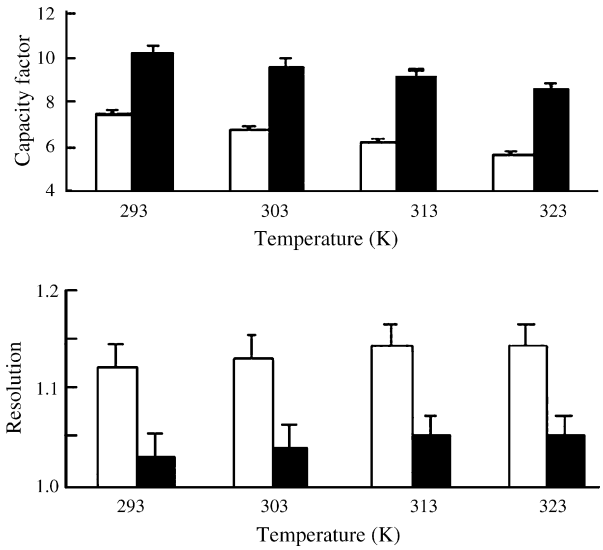

Fig. 5. Influence of temperature on the resolution and capacity factor of ganoderic acids in analytic HPLC. (□) GA-T; (■) GA-Me.

be calculated as 32.0 and 28.4 kJ/mol for GA-T and GA-Me, respectively, based on Eq. (3). These two ΔS values are not significantly different according to the t test ($p < 0.05$, $n = 4$). The calculated ΔH values are −7.3 and −4.6 kJ/mol for GA-T and GA-Me, respectively. They are significantly different based on the t test ($p < 0.001$, $n = 4$). These ΔH values are similar to the reported value for benzacridine (−6.6 kJ/mol), a tetracyclic aromatic hydrocarbon, in methanol mobile phase on a C_{18} column [22]. The ΔH and ΔS data indicate that the retention behavior of GA-T and GA-Me might be driven by an enthalpy difference, so the participation of the enthalpy may be a contributing factor in the selectivities of GAs on the C_{18} stationary phase surface. This may be explained by the hydrophobic interactions of the GA molecules with the non-polar stationary phase and the aqueous mobile phase, since the hydrophobic effect governs the separation process in RP-HPLC. As RP-HPLC employs a strongly hydrophobic stationary phase surface, the retention and selectivity behaviors of elutes were determined by the hydrophobic interactions between the non-polar elutes and the hydrophobic C_{18} ligands on the stationary phase pore surfaces [23]. Moreover, the acidified methanol and water mixture as eluent was able to form hydrogen bonds with the solutes [24], thereby reducing the interactions between GAs and C_{18} particle surfaces by forming hydrogen bonds with GAs. The GA-T molecule has an extra acetyl group and could form more hydrogen bonds, hence its retention time compared to GA-Me was substantially smaller.

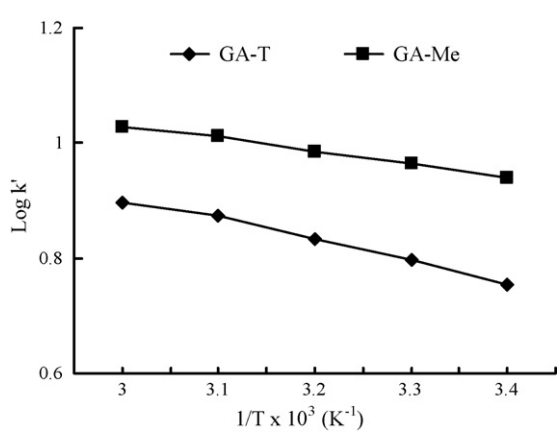

Fig. 6. The van't Hoff plots of GA-T and GA-Me separated on the semi-preparative C_{18} column.

4. Conclusions

An HPLC-UV-ESI-MS approach was proven successful in the rapid screening of complex triterpene mixtures in pretreated chloroform extracts of *Ganoderma*. The use of two detectors, UV and ESI-MS, allowed us to obtain the UV absorbance and the molecular weights of two GA-T and GA-Me, which aided in their identifications. Baseline separations of the two GAs from a dried chloroform extract obtained from a methanol extraction were achieved on analytical and semi-preparative C_{18} columns. Furthermore, a thermodynamic investigation indicated that the retention behaviors of GAs on C_{18} surface with temperature change might be driven by an enthalpy difference. The separation method presented in this work can be a valuable tool for the identification and rapid semi-preparative purification of targeted GAs and it may also be helpful in the separation of other natural products.

Acknowledgments

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