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# Hydrophilic interaction chromatography for selective separation of isomeric saponins

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ABSTRACT

A method based on a Click XIon zwitterionic stationary phase was developed to separate isomeric saponins efficiently in hydrophilic interaction chromatography (HILIC) mode using methanol as a weak eluent. The retention times of a set of isomeric saponins, ginsenoside Rc (S1), ginsenoside $Rb_2$ (S2), and ginsenoside $Rb_3$ (S3) on five kinds of HILIC columns were compared using acetonitrile/water or methanol/water as mobile phase. All results indicated that the Click XIon column showed the highest retention times of all these isomeric saponins. Then the retention behaviors of these isomeric saponins on the Click XIon column were investigated. The results demonstrated that the retention behaviors of saponins on Click XIon in methanol/water mobile phase were different from that in acetonitrile/water. Methanol/water binary eluent offered enhanced selectivity to these isomeric saponins ($\alpha_{S2/S3(MeOH)} = 1.16 > \alpha_{S3/S2(ACN)} = 1.04$), and the elution order of ginsenoside $Rb_2$ (S2) and ginsenoside $Rb_3$ (S3) was reversed compared with acetonitrile/water. Moreover, methanol/water solvent has better solubility for saponins than acetonitrile/water, resulting in improved preparative peak shape, which is greatly beneficial for saponin purification. Application to the preparative separation of saponins from leaves of *P. notoginseng* was also investigated, and eleven saponins, including three sets of isomeric saponins with one new saponin were isolated and identified. All these results indicated that this method was efficient for analytical and preparative separation of saponins, especially for isomeric saponins, containing xylopyranosyl, arabinofuranosyl or arabinopyranosyl units.

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## 1. Introduction

Saponins are widely distributed in plant species, and are considered to be the main bioactive principles of various medicinal herbs [1]. They consist of aglycones coupled to sugar chain units. Different linkages of saccharides and functionalization in aglycones provide the structural diversity of the natural saponins with different bio-activities including antitumor, antiviral, antifungal, and anti-inflammatory, etc. [2–5]; however, at the same time this also increases the difficulty of structural elucidation of these compounds, especially for identification of isomeric saponins.

Currently, reversed-phase liquid chromatography (RPLC) is among the most popular chromatographic modes for separation of saponins owing to its high column efficiency and good repeatability [6,7]. However, the separation selectivity between isomeric saponins which differ from each other only in an aldohexosyl or aldopentosyl unit is not always sufficient in RPLC, making their purification greatly difficult. Ginsenosides Rc (S1), $Rb_2$ (S2), and $Rb_3$ (S3) (Fig. 1), which are isomers of the terminal aldopentosyl unit, were difficult to be completely separated within 25 min on reversed-phase columns, especially for ginsenosides $Rb_2$ and $Rb_3$ [8–10]. Moreover, high-performance liquid chromatography (HPLC) of borate complexes was also investigated for the separation of these isomeric saponins (S1, S2, and S3) on a basic ion-exchange column, but they were also not completely separated [11]. The separation selectivity of the above methods is therefore limited, requiring a separation mode with improved polar selectivity to resolve this kind of isomeric saponins.

Hydrophilic interaction chromatography (HILIC) has been shown to be a powerful alternative for separation of polar compounds in the past two decades [12–15]. The presence of polar glycosyl moieties in saponin molecules enables their retention in HILIC mode in most cases [16–20]. In HILIC, the adjuvant QS-21, a RPLC purified triterpene glycoside deriving from *Quillaja saponaria*, was analyzed on a poly(hydroxyethyl aspartamide) stationary phase and was resolved into two peaks. When those two peaks were purified, they were characterized as isomers of the terminal aldopentosyl unit [21]. HILIC using diol-bonded [19], or polyvinyl alcohol-bonded [17,22,23], or polyamine-bonded [24] stationary phases was developed for the separation and quantitative determination of ginseng saponins in ginseng formulations.

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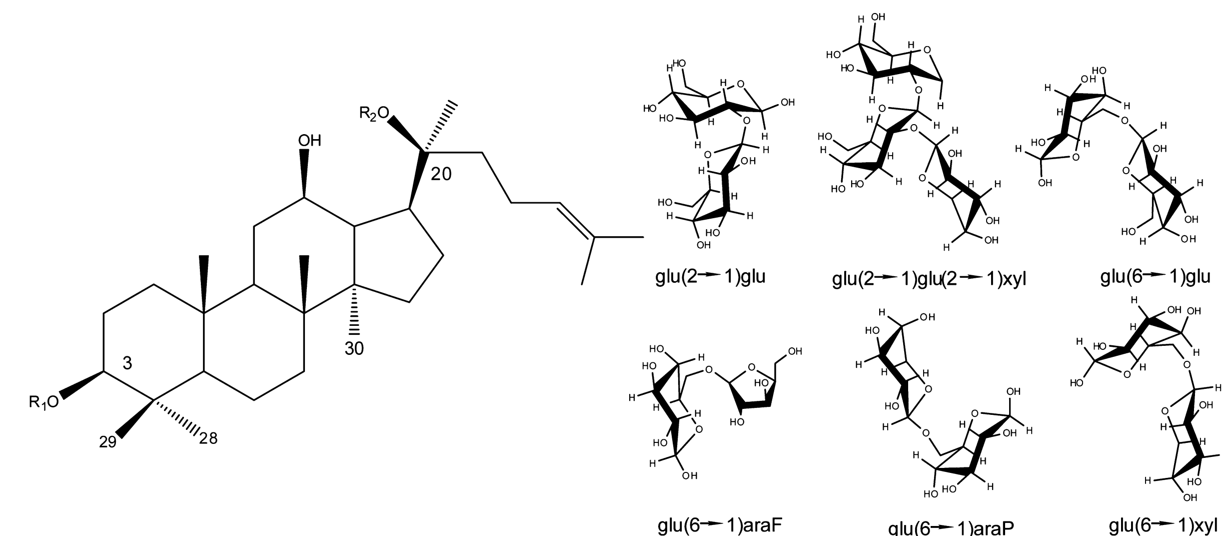


| No. | Compounds | MW | Formula | $R_1$ | $R_2$ |
|---|---|---|---|---|---|
| S1 | ginsenoside Rc | 1078 | $C_{53}H_{90}O_{22}$ | glu(2→1)glu | glu(6→1)araF |
| S2 | ginsenoside $Rb_2$ | 1078 | $C_{53}H_{90}O_{22}$ | glu(2→1)glu | glu(6→1)araP |
| S3 | ginsenoside $Rb_3$ | 1078 | $C_{53}H_{90}O_{22}$ | glu(2→1)glu | glu(6→1)xyl |
| P1 (F10-1) | ginsenoside $Rb_1$ | 1108 | $C_{54}H_{92}O_{23}$ | glu(2→1)glu | glu(6→1)glu |
| P2 (F10-2) | notoginsenoside FP2 | 1210 | $C_{58}H_{98}O_{26}$ | glu(2→1)glu(2→1)xyl | glu(6→1)araF |
| P3 (F10-3) | notoginsenoside Fc | 1210 | $C_{58}H_{98}O_{26}$ | glu(2→1)glu(2→1)xyl | glu(6→1)xyl |
| P4 (F10-4) | new compound | 1210 | $C_{58}H_{98}O_{26}$ | glu(2→1)glu(2→1)xyl | glu(6→1)araP |
| P5 (F10-5) | notoginsenoside Fa | 1240 | $C_{59}H_{100}O_{27}$ | glu(2→1)glu(2→1)xyl | glu(6→1)glu |
| P6 (F17-1) | notoginsenoside Fe | 916 | $C_{47}H_{80}O_{17}$ | glu | glu(6→1)araF |
| P7 (F17-2) | notoginsenoside Fd | 916 | $C_{47}H_{80}O_{17}$ | glu | glu(6→1)xyl |
| P8 (F17-3) | quinquenoside-L1 | 916 | $C_{47}H_{80}O_{17}$ | glu | glu(6→1)araP |
| P9 (F26-1) | ginsenoside Mc | 754 | $C_{41}H_{70}O_{12}$ | H | glu(6→1)araF |
| P10 (F26-2) | gypenoside XIII | 754 | $C_{41}H_{70}O_{12}$ | H | glu(6→1)xyl |
| P11 (F26-3) | ginsenoside Y | 754 | $C_{41}H_{70}O_{12}$ | H | glu(6→1)araP |

**Fig. 1.** Structures of dammarane-type triterpene saponins from *P. notoginseng*. (MW) molecular weight, (glu) $\beta$-D-glucopyranosyl, (xyl) $\beta$-D-xylopyranosyl, (araF) $\alpha$-L-arabinofuranosyl, (araP) $\alpha$-L-arabinopyranosyl.

Shorter analysis time (15–30 min compared with more than 60 min in RPLC) and better resolution were achieved. HILIC has comparative separation power and good orthogonality to RPLC, making it a good choice for constructing a two-dimensional hyphenated system to improve the separation of saponins [16,18,20]. However, there are also some problems for the separation of saponins in HILIC mode. Firstly, the number of stationary phases in HILIC for separation of saponins is growing [25], and it is a difficult task to select an appropriate one [26]. Secondly, acetonitrile is commonly used as a weak eluent and water or aqueous buffer as a strong eluent in HILIC [25], but mobile phases with high acetonitrile concentration decrease the solubility of saponins, resulting in peak fronting and low loadability. It makes almost impossible to separate saponins in preparative scale. Thirdly, the percentage of acetonitrile in the mobile phase is a main parameter affecting saponin separation selectivity in HILIC, so trying different elution gradients of acetonitrile is almost the only means to obtain better separation resolution. Therefore, if these problems mentioned

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