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Preparation and chromatographic evaluation of a newly designed steviol glycoside modified-silica stationary phase in hydrophilic interaction liquid chromatography and reversed phase liquid chromatography

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ABSTRACT

A diterpene glycoside compound, rebaudioside A (commonly abbreviated as RA), was immobilized onto porous silica surface through "thiol-ene" click chemistry strategy. The successful immobilization of the RA on the silica support was confirmed by FT-IR and elemental analysis. Chromatographic characteristics of the new stationary phase, named Click TE-RA, were evaluated by a set of diverse analytes such as carbohydrates, nucleosides, and organic acids in hydrophilic interaction liquid chromatography (HILIC) mode. The effects of water content, buffer pH and concentration were investigated and a typical HILIC retention feature of Click TE-RA was observed at high organic modifier content. The Click TE-RA stationary phase was further studied by a series of glycoside compounds. Tunable retention mechanisms from hydrophilic to hydrophobic interactions were observed. Separation of very polar compounds including oligosaccharides, nucleic acid bases and nucleosides using Click TE-RA in HILIC mode was successfully accomplished. In addition, separation of saponins both in HILIC and reversed-phase liquid chromatography (RPLC) modes was performed, demonstrating the presence of orthogonality between two different modes on Click TE-RA column. The multiple interactions induced by polar sugar group and hydrophobic aglycone group allowed this Click TE-RA to serve as a multi-mode stationary phase in two-dimensional liquid chromatography.

1. Introduction

As an alternative to reversed-phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC) [1] has become a powerful technique for separation of polar and hydrophilic compounds. Nowadays, HILIC has been accepted as a common separation mode and applied in a broad range of applications such as carbohydrates [2,3], amino acids [4,5], peptides [6], glycopeptides [7], and highly polar natural products [8,9].

For HILIC stationary phases, besides bare silica and aminopropyl modified silica [10], a number of silica-based bonded polar phases including diol [11,12], amide [13,14], saccharine [15,16] and zwitterionic ligands [17,18], have been developed to meet separation needs. Among these stationary phases, saccharide bonded silica is a very attractive stationary phase withunique chromatographic characteristics. Due to their excellent hydrophilic property, saccharides have been immobilized on silica surface and used as HILIC stationary phases in recent literatures [15,16,19,20]. However, the synthesis of saccharide modified-silica stationary phase is a difficult process, especially for saccharides with higher degree of polymerization. Besides saccharide, glycoside is a kind of glycoconjugates consisting of saccharide and aglycone components widely distributed in nature. The conjugated saccharides result in distinctive

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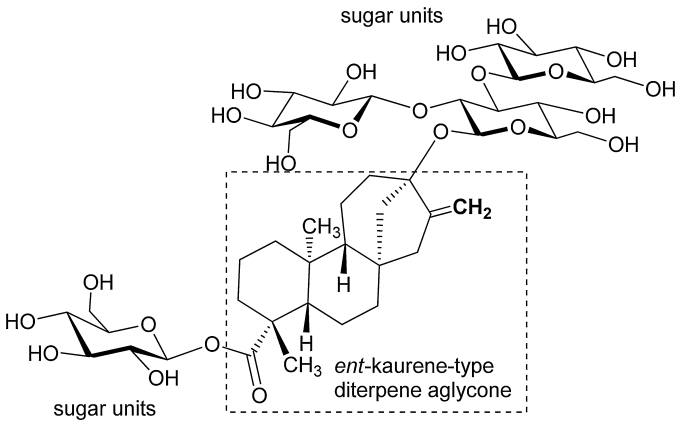

Fig. 1. Structure information of rebaudioside A (RA).

hydrophilic characteristics of glycosides. A variety of reactive groups present in the structure of glycosides provide possible reactive sites for synthesis, which is beneficial for immobilization of stationary phase. In addition, glycosides with special spatial configuration and spatial folding features exhibit both hydrophilic and hydrophobic characteristics. Therefore, glycoside modified-silica may be used as a mixed-mode hydrophilic interaction/reversed-phase stationary phase to increase application coverage of HILIC stationary phase in separation.

Steviol glycosides, belonging to *ent*-kaurene-type diterpene glycoside compounds, are the major sweet components present in *Stevia rebaudiana* Bertoni [21]. Rebaudioside A (RA) is one of the most abundant steviol glycosides in *stevia*, which is found increasingly versatile applications as a low caloric sugar substitute. It should be noted that the structure of RA is consisted of hydrophobic aglycone and hydrophilic sugar units (Fig. 1), which just meet the requirement of the HILIC/RPLC mixed-mode stationary phase properties. The sugar units containing four glucoses are suitable for HILIC mode, while the *ent*-kaurene-type diterpene aglycone has hydrophobic features, which have potential for RPLC mode. In addition, a terminal alkene group present in the structure of aglycone provides a reactive site for "thiol-ene" click chemistry. The "thiol-ene" click chemistry has proven to be an efficient tool, which has been utilized to synthesize a series of HILIC stationary phases by Guo et al. [18,22,23]. In this strategy, thiol groups present on the silica surface acted as an intermediate silica species to provide a reactive site for the immobilization of a chromatographic ligand on the surface.

In recent years, the mixed-mode stationary phase designed with dual HILIC/RPLC retention mechanism has also been reported [24–27]. Ihara et al. designed a new peptide-modified silica stationary phase that combined multiple interactions induced by polar carbonyl group and hydrophobic phenyl group, which was used for the separation of hydrophobic compounds, small polar molecules, and drug molecules [28]. In general, the HILIC/RPLC mixed-mode stationary phases have both hydrophilic and hydrophobic moieties, providing multiple retention mechanism and unique chromatographic selectivity.

In order to increase the diversity of HILIC stationary phases to enlarge their application ranges, we here design a RA modified-silica stationary phase for use in both HILIC and RPLC modes. The immobilization of RA on silica was achieved through the "thiol-ene" click chemistry reaction between terminal alkene group in RA and thiol groups on the silica surface. The new stationary phase (named Click TE-RA) will be evaluated with a set of polar compounds including carbohydrates, organic acids, and nuleosides in HILIC mode. The HILIC/RPLC mixed-mode chromatographic behaviors of Click TE-RA will also be investigated using steviol glycosides and triterpene saponins. At last, applications in the separation of complex samples on Click TE-RA will be demonstrated.

2. Experimental

2.1. Chemicals and materials

Spherical silica (5 μm, 10 nm, 300 $m^2 g^{-1}$ surface area) was purchased from Fuji Silysia Chemical (Kasugai, Japan). 3-mercaptopropyltrimethoxysilane was obtained from ABCR (Karlsruhe, Germany), α,α′-azodiisobutyronitrile (AIBN) was purchased from Shanghai Chemical Reagents (Shanghai, China). Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were purchased from Fisher (Fair Lawn, NJ, USA). Formic acid and ammonium formate of HPLC grade were obtained from Merck (Darmstadt, Germany). Water was from a Milli-Q water purification system (Billerica, MA, USA). All other reagents were analytical grade reagents and used without purification. An analytical Inertsil Diol column (5 μm, 150 mm × 4.6 mm i.d. Tokyo, Japan) and an analytical NH_2 column (5 μm, 150 mm × 4.6 mm i.d. Waters) were used for comparative experiments.

Standards of xylitol, maltitol, glucose, glucosamine hydrochloride, ribose, sucrose, turanose, raffinose, and melezitose were purchased from Sigma–Aldrich (St. Louis, MO). Standards of uracil, cytosine, thymine, cytidine, uridine, adenosine, guanosine, inosine, 5-methyluridine, and orotic acid were from Acros (Fair Lawn, NJ, USA). Oligosaccharides (chitooligosaccharides and sodium alginateoligosaccharides) were kind gifts from natural products and glyco-biotechnology group (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, P. R. China). Steviol glycosides (stevioside, rebaudioside A, and rebaudioside C) were purchased from Sigma–Aldrich. Triterpene saponins (ginsenoside Mc, gypenoside XIII, ginsenoside Rg3, notoginsenoside Fe, notoginsenoside Fd, ginsenoside Rd, ginsenoside Rc, and notoginsenoside Fa) were purified in our laboratory. Their structures were confirmed by UV, ESI-MS, ^1H and ^{13}C NMR, and by comparison with literature values [29]. Structural information of these compounds was shown in Fig. 2. All standards were dissolved in the corresponding HILIC mobile phase at an appropriate concentration and filtered through a nylon membrane (0.22 μm).

2.2. Sample of oligosaccharide in natural product

The water extract of *Lycopus lucidus* Turcz. was purchased from Shaanxi Province, China. The sample was dissolved in ACN/H_2O (1:1, v/v) solution at an appropriate concentration for chromatographic evaluation of Click TE-RA stationary phase.

2.3. Preparation of Click TE-RA stationary phase and column packing

The synthesis of RA-bonded stationary phase was shown in Fig. 3a. The mercapto-silica was firstly synthesized. 3-mercaptopropyltrimethoxysilane (6 mL) and silica (10 g) were added into toluene (50 mL) under nitrogen atmosphere. Pyridine (2 mL) was added to the mixture with stirring and the reaction was reflux for 24 h. Then the mixture was cooled to 40 °C and filtered. The product was washed with toluene, methanol, water and methanol successively and then dried at 80 °C overnight to afford mercapto-silica. The carbon content was 2.79% for mercapto-silica by elemental analysis.

Secondly, rebaudioside A (RA) was bonded to the mercapto-silica *via* thiol-ene click chemistry. The mercapto-silica (10 g) was added into the solution of RA (5 g) in water–methanol (7:5, v/v, 120 mL) under nitrogen atmosphere. AIBN (160 mg) was added