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Hydrophilic interaction liquid chromatography of hydroxy aromatic carboxylic acid positional isomers

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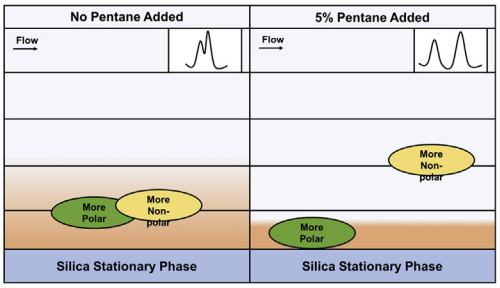
HIGHLIGHTS

- Hydroxybenzoic and hydroxycinnamic acid isomers separated by HILIC.
- Pentane as a mobile phase modifier facilitates peak resolution.
- Method has potential mass spectrometry compatibility.
- Four silica columns from different companies tested.

GRAPHICAL ABSTRACT



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ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) has become increasingly popular as an alternative to reversed phase LC due to its ease of separating complex polar compound mixtures and the compatibility of the mobile phase with mass spectrometry (MS). Using a plain silica column (150 mm × 4.6 mm), we have shown a mixture containing three hydroxybenzoic acid isomers plus syringic and vanillic acid and three hydroxycinnamic acid isomers plus ferulic and sinapic acid can be separated using a mobile phase comprised of 90% acetonitrile (MeCN) and 10% 20 mM ammonium acetate at pH 6 in under 45 min. This method is appropriate when using UV detection at 275 nm. However, for improved MS compatibility, a buffer concentration of 10 mM is recommended but this greatly decreases the analyte retention factors. A second more nonpolar organic solvent component in the mobile phase (particularly pentane which has not been previously considered for HILIC) is found to offset this loss in retention. The optimum mobile phase is found to be 90% MeCN, 5% 10 mM ammonium acetate pH 6, and 5% pentane with resolution of eight of the ten compounds with a separation time of 30 min. Using UV detection, we have shown that detection limits range from 36 to 133 pmole and quantitation limits are spread from 94 to 376 pmole for six of the analytes. Upon testing this method on two other silica columns from different manufacturers, it is found that while resolution is similar, further optimization of the mobile phase is recommended.

1. Introduction

Since its emergence in the 1990s, hydrophilic interaction liquid chromatography (HILIC) has proven to be a powerful mode of liquid chromatography (LC). For HILIC, the stationary phase is polar while

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the mobile phase is comprised of water or an aqueous buffer phase and a water-miscible organic solvent like acetonitrile. While this mobile phase is similar in composition to that of reversed-phase liquid chromatography (RPLC), the strong solvent in HILIC is water while the weak solvent is acetonitrile. The retention mechanism in HILIC is primarily partitioning of the analyte between the aqueous layer and the organic layer [1]. Ideally, HILIC is an excellent approach for the separation of polar or hydrophilic compounds, like neurotransmitters [2], parasitic metabolites [3], and various classes of lipids [4]. Mass spectrometry (MS) has become increasingly popular as the detector of choice for HILIC because of its mobile phase compatibility [5,6].

Many studies have been done that investigate the various HILIC stationary phases available including zwitterionic sulfobetaine groups, diols, diisopropyl-cyanopropylsilane, amide, and amino types however bare silica is also effective. These columns are all hydrophilic, though they vary in the strength of electrostatic interaction and hydrogen bonding capability [7,8]. It has been shown that polar stationary phases extract water from the mobile phase more strongly than less polar stationary phases. When using a binary mobile phase, the extraction of water is stronger when the water concentration is lower. Through comparison of 14 stationary phases, it was found that mainly the stationary phase polarity determines the strength of the water extraction from the mobile phase [9]. The effect of salt, organic solvent, and mobile phase pH are commonly tested parameters for HILIC. A higher salt content tends to lead to higher retention since this increases the water layer thickness, while the pH of the mobile phase should be at a value where most analytes are in their ionized form and therefore more retained due to the increased hydrophilic character. The choice of organic solvent can greatly affect the retention and elution order. Acetonitrile has been found to be one of the weaker eluting solvents, which tends to work best for HILIC as the primary mobile phase component, though methanol, ethanol, and isopropyl alcohol have also been used as co-solvents [7,8].

Additionally, a third solvent can be added to the mobile phase to further affect the retention and elution order of analytes. A hydrophilicity gradient exists between the organic and aqueous layers in HILIC due to the difference in hydrophilic character between the two layers. More hydrophilic and polar analytes will be retained longer with a steeper gradient. The largest effect on retention of hydrophilic compounds should be when a nonpolar solvent, such as hexane, is used [10]. Polar compounds like methacrylic acid, cytosine, nortriptyline, and nicotinic acid showed an increase in retention and resolution as the mobile phase modifier was changed from methanol to ethanol to isopropanol, suggesting that as the polarity of the modifier is decreased, the retention of polar analytes will increase [11,12]. However, to the best of our knowledge, neither a hydrocarbon nor an alcohol has been used as a co-solvent to enhance the HILIC retention of aromatic compounds with polar substituents.

Aromatic hydroxy carboxylic acids, commonly found in wine, beer, and fruit juices, are a well-studied class of compounds. They have been separated using various modes of chromatography including RPLC, ion-pair RPLC, ion exchange chromatography, normal phase liquid chromatography, and thin-layer chromatography [13–21]. Although RPLC methods have advanced to shorter analysis times, they continue to use complex gradients, and these methods tend to leave out one isomer in a positional aromatic isomeric set. Micellar ultra high performance LC (UHPLC) has been used to separate aromatic carboxylic acids, including two sets of isomers, with baseline resolution in less than 35 min, however the surfactant mobile phase is not compatible with MS [22]. Recent work using UPLC-MS/MS has shown the separation of seventeen compounds with two isomeric sets in about 10 min, although the method requires a complicated five step RPLC acetic acid-acetonitrile mobile phase gradient [19].

Hydroxy aromatic carboxylic acids are naturally occurring compounds that possess anti-oxidative qualities and exist as secondary metabolites in many plants. Because of their antioxidant properties, they are important to the human diet. Apart from appearing in food and beverages, they also play a role in pharmaceuticals and cosmetics. There are two primary groups that make up these hydroxy aromatic carboxylic acids, or phenolic acids: hydroxybenzoic and hydroxycinnamic acids [23–25]. The HILIC separation of isomeric hydroxy- and amino-benzoic acids has been characterized using a zwitterionic sulfobetaine stationary phase [26]. Although no chromatograms were shown and discussion of peak resolution was absent, separation of the isomeric sets of aromatic acids seemed apparent at 90% acetonitrile-10% 15 mM ammonium acetate. Such a study using a plain silica column was not evident in the literature.

In this work, we present an isocratic HILIC method to separate these two previously indicated classes of compounds, including positional isomers (structures represented in Fig. 1), that is compatible with MS detection. To extend the versatility of silica beyond normal phase LC to separate aromatic positional isomers, a plain silica column is used with a ternary mobile phase comprised of acetonitrile, ammonium acetate buffer, and pentane. Although pentane is volatile ensuring MS compatibility and has the best water solubility of hydrocarbon solvents, it has not been previously considered as a co-solvent for HILIC. Additionally, few isocratic methods are available to separate positional isomers with even fewer methods available that are MS compatible.

2. Methods

2.1. Apparatus

Chromatographic separations were conducted on a Dionex UltiMate 3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA) equipped with a ternary pump, online degasser, autosampler, temperature-controlled column oven, and diode array UV detector. Chromeleon 6.8 software (Thermo Scientific, Sunnyvale, CA, USA) was used for instrument control and data acquisition. Mobile phase optimization as well as all separations were done using a plain silica stationary phase involving a Phenomenex Nucleosil silica column (Torrance, CA, USA) (150 × 4.6 mm, 3 μm). Both an Agilent Poroshell 120 HILIC column (Santa Clara, CA, USA) (150 × 4.6 mm, 2.7 μm) and a Grace (Columbia, MD, USA) silica column (150 × 4.6 mm, 3 μm) were compared to the Nucleosil column to determine chromatographic reproducibility between columns with the same dimensions and particle size. A Thermo Scientific Accucore HILIC column (Sunnyvale, CA, USA) (100 × 2.1 mm, 2.6 μm) was used to compare the final method on a dimensionally smaller silica column.

A 2 μL injection size was used. The column was kept at ambient temperature. UV detection was monitored at 254 and 275 nm, however 275 showed greater response for most analytes.

2.2. Chemicals and procedures

All solutions were made using 18.2 MΩ water that was distilled and de-ionized before being passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA) and a UV photolyzer. Acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) comprised the majority of the mobile phase. The concentration of ammonium acetate (Fisher Scientific, Fair Lawn, NJ, USA) as was its pH using acetic acid (Sigma-Aldrich, St. Louis, MO, USA) was varied in the mobile phase. All analyte chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were made in 90% acetonitrile,