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Destructive stationary phase gradients for reversed-phase/hydrophilic interaction liquid chromatography

Caitlin N. Cain, Anna V. Forzano, Sarah C. Rutan*, Maryanne M. Collinson*

Department of Chemistry, Virginia Commonwealth University, 1001 W. Main St.,Richmond, VA, 23284-2006, USA

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ABSTRACT

The use of stationary phase gradients for liquid chromatography (LC) is a promising new strategy to allow for specific control over the selectivity of a separation by having a gradual change in the ligand density along the length of the column. Unfortunately, there have been very few, if any, methods to prepare continuous stationary phase gradients on traditional packed LC columns. In this work, destructive methodologies are used to create stationary phase gradients on commercial C_{18} columns by infusing trifluoroacetic acid (TFA) onto the column through controlled rate of infusion (CRI). The introduction of TFA via CRI while the column is heated at 80 °C promotes acid hydrolysis of the alkylsilane ligand in a gradient fashion. Characterization with scanning electron microscopy and Barrett-Joyner-Halenda pore size distributions of the stationary phase after fabrication of the destructive gradient establishes that the chromatographic support was not damaged during the procedure. The shape of the gradient was examined using thermogravimetric analysis (TGA) and attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy. TGA and ATR-FTIR showed an increase in the percent carbon loss along the length of the column, indicating that there was an increase in the C_{18} ligand from the front to the end of the column. Two selectivity tests demonstrated a decrease in the hydrophobicity and increase in the silanol activity of the stationary phase gradient from the uniform C_{18} counterpart. Additionally, the fabrication of the destructive stationary phase gradient resulted in two different surface functionalities allowing hydrophobic *and* hydrophilic interactions with analyte species depending on the mobile phase composition. Plots of the log of retention factor versus percent acetonitrile illustrated that these stationary phase gradients have two separation mechanisms: reversed-phase (RP) and hydrophilic interaction. Coupling the stationary phase gradient with a mobile phase gradient shows differences in the peak widths and the resolution of phenolic compounds, indicating that the orientation of the stationary phase gradient has the potential to enhance the resolution of a separation. With this methodology, stationary phase gradients can be fabricated on previously used RP columns, allowing for these columns to be repurposed.

1. Introduction

Method development for liquid chromatography (LC) has been dependent on the use of gradients in the mobile phase, flow rates, or temperature programming [1,2]. Traditionally, an LC separation is optimized through the use of a mobile phase gradient while using a uniformly functionalized stationary phase. An alternate paradigm to this approach is to vary the polarity of the stationary phase along its length via the creation of a stationary phase gradient. A stationary phase gradient could be beneficial in achieving an increase in resolution and/or a decrease in analysis time. Gradients present in the stationary phase in combination with isocratic or gradient mobile phases could provide another optimization approach in method development for LC separations.

Stationary phase gradients can be classified into two categories, discontinuous and continuous, based on their configuration. One approach for the preparation of discontinuous stationary phase gradients involves serially connecting two or more columns with different ligands together. This category of stationary phase gradients has been popularized by Bischoff Chromatography's (Leonberg, Germany) Phase Optimized Liquid Chromatography (POPLC) kits [3]. Serially coupling two or more stationary phases with different chemically bonded ligands has been an attractive idea, not only for the new possibilities in selectivity, but also for an increase in plate count [4]. Therefore, discontinuous stationary phase gradients, mainly using POPLC, have been implemented to provide new selectivities in a wide array of separations,

* Corresponding authors.
E-mail addresses: srutan@vcu.edu (S.C. Rutan), mmcollinson@vcu.edu (M.M. Collinson).

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from metabolites to flavonoids [3–12]. However, the connections involved in coupling columns creates issues with excess void volume and fronting peaks [4,5,11]. Another drawback is that serially connected columns do not allow for cooperative interactions to take place between various stationary phase ligands [13,14].

Continuous stationary phase gradients, as the name implies, consists of a gradual change in chemical functionality(ies) along the length of the substrate or column. This type of gradient not only allows for cooperative interactions, but also eliminates excess void volumes and coupling issues that occur with serially connected columns. Continuous stationary phase gradients were first introduced by Pucci et al., who photografted ionizable chains onto a hydrophobic polymer monolithic column in a gradient fashion for capillary electrochromatography [15]. These stationary phase gradients showed an improvement in the resolution of probe solutes compared to uniform counterparts; however, the limited material available for characterization of the stationary phase gradient profile lead to inconsistent results [16,17]. Continuous stationary phase gradients have also been prepared on thin layer chromatography (TLC) plates using silane chemistry and controlled rate of infusion (CRI) [18]. Compared to unmodified or uniformly modified TLC plates, the gradient plates provided different selectivities in the separation of various mixtures including vitamins and metal ions on TLC plates [19–21]. Recently, CRI was employed to fabricate an amine continuous stationary phase gradient on in-house prepared silica monoliths for LC, which showed different selectivity on the amine stationary phase gradient when compared to both uniform silica and amine counterparts using a mixture of weak acids and bases [13]. The profiles of the stationary phase gradients developed by CRI were successfully characterized through the use of X-ray photoelectron spectroscopy [13,19–21].

To date, we are not aware of any reports on the fabrication of continuous gradients on traditional packed LC columns. In this work, we describe for the first time the fabrication of a stationary phase gradient from a uniformly functionalized support on a commercially packed column using destructive methodologies to explore the fundamentals of stationary phase gradients. Inducing hydrolytic cleavage by exposing reversed-phase (RP) columns to low pHs at an elevated temperature develops excess silanols on the stationary phase [22–29]. In this work, gradients are fabricated on C_{18} columns through acid hydrolysis of the C_{18} ligand, creating a gradient of excess silanols along the stationary phase from one end to the other. To understand the properties of this new stationary phase, the aim of this paper is to show the physical and chromatographic characterization of this new stationary phase gradient. Physical characterization was carried out to show the effects of the acid hydrolysis methodology on the silica support and to illustrate the profile of the C_{18} gradient. Chromatographic characterization with an isocratic mobile phase was performed to illustrate the hydrophobic and hydrophilic properties of the stationary phase gradient. Additionally, the stationary phase gradient was coupled with a mobile phase gradient to illustrate the effects of the orientation of the stationary phase gradient on the separation. The methodology for gradient formation is not only simpler, but also cost effective and provides a unique means to repurpose old, used columns into new gradient columns that all chromatographers can employ to understand the effects of stationary phase gradients on separations.

2. Methods

2.1. Reagents

Acetonitrile (ACN, HPLC grade), methanol (HPLC grade), and isopropanol (99.9%, ACS grade) were purchased from Fisher Scientific. Hexane (HPLC grade) and chloroform (HPLC grade) were acquired from Sigma-Aldrich. Trifluoroacetic acid (TFA, 99%) was obtained from Acros Organic. Ammonium formate (99%) and formic acid (98%) were purchased from Honeywell Fluka. Uracil (98%), naphthalene (99%), and anthracene (99.9%) were acquired from Sigma-Aldrich. Benzoic acid (99.5%) and toluene (99.9%, ACS grade) were obtained from Fisher Scientific. Benzene (99.9%) and diethyl-*m*-toluamide (DEET, 98%) were purchased from EM Science and Acros Organic, respectively. Gallic acid (98%) and caffeic acid (99%) were purchased from Acros Organic and coumaric acid was acquired from Sigma-Aldrich. Protocatechuic acid and tyrosol were obtained from Alfa Aesar.

2.2. Destructive stationary phase gradient fabrication

Three commercial Alltech Econosphere C_{18} columns were used in the development of the destructive stationary phase gradient. Columns A and B had dimensions of 150×4.6 mm with a particle size of 5 μm and pore size of 80 Å. Column C had dimensions of 50×4.6 mm with a particle size of 3 μm and pore size of 80 Å. The columns were flushed at 1 mL/min with 10 column volumes (V_M) of a 50:50 (v/v) methanol/water mixture, 10 V_M of methanol, 20 V_M of ACN, 10 V_M of isopropanol, 10 V_M of hexane, 10 V_M of isopropanol, and 20 V_M of ACN with a LC pump (1100 series, Agilent Technologies).

The columns were attached to a LC pump (1100 series, Agilent Technologies) for 27 V_M of concentrated TFA (99%) to be slowly infused into the column with CRI. To date, we have not seen any significant corrosion of pump parts and frits, which would arise if a stronger acid was used. The flow rate for the CRI infusion was determined by dividing the dead volume of the column by the four hour infusion process. Acid hydrolysis of the ligand was promoted by heating the column in an 80 ^{O}C column oven (1100 series, Agilent Technologies) during the CRI process. (*Caution: Concentrated TFA can decompose into hydrofluoric acid when heated.*) The front of the column was exposed to the acid for a longer period of time, which causes more ligand bonds to be cleaved versus the end of the column. Therefore, a gradient of C_{18} functionalities was formed along the length of the column. The water for the reaction comes from the small amount found in TFA as well as that adsorbed to the stationary phase. To remove the acid reagent and cleaved ligands, the columns were back-flushed with the washing procedure described above using the 1100 series LC pump.

2.3. Destructive stationary phase gradient characterization

The column end-fitting from the front of the column (most exposed to TFA) was removed and the column end-fitting at the end of the column was attached to a LC pump (1100 series, Agilent Technologies). The stationary phase bed was extruded from the column by pushing ACN through the column at 0.5 mL/min. The stationary phase bed was cut into 5 mm discs while the bed was still wet and then dried in a 40 °C oven overnight.

Physical characterization of the destructive stationary phase gradient included an examination of the morphology of the chromatographic particles. Images of uniformly modified C_{18} particles (Alltech Econosphere C_{18}, 5 μm, 80 Å), bare silica particles (Alltech Econosphere Si, 5 μm, 80 Å), and particles from the stationary phase gradients were collected with scanning electron microscopy (SEM) (Hitachi FE-SEM SU-70). A suspension of the particles in ACN was placed onto the SEM sample holder with adhesive carbon tape. The sample holders were then placed into a 40 °C oven overnight before being sputtered coated with platinum for one minute. The pore size of the bare silica, uniformly modified C_{18}, and stationary phase gradient particles were obtained from N_2 adsorption-desorption isotherms (Quantachrome NOVA 2200e Surface Area and Pore Ana-

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