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Consequences of transition from liquid chromatography to supercritical fluid chromatography on the overall performance of a chiral zwitterionic ion-exchanger

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ABSTRACT

Major differences in the chromatographic performance of a zwitterion ion-exchange type (ZWIX) chiral stationary phase (CSP) in supercritical fluid chromatography (SFC) and high-performance liquid chromatography (HPLC) have been observed. To explain these differences, transition from HPLC to SFC conditions has been performed. The amount of a protic organic modifier in supercritical carbon dioxide ($scCO_2$) was stepwise increased and the effect of this change studied using acidic, basic and ampholytic analytes. At the same time, the effect of various basic additives to the mobile phase and transient acidic buffer species, formed by the reaction of $scCO_2$ with the organic modifier and additives, was assessed. Evidence is provided that a transient acid together with the intrinsic counter-ions present in the ZWIX selector structure drive the elution of analytes even when no buffer is employed. We show that the tested analytes can be enantioseparated under both SFC and HPLC conditions; the best conditions for the resolution of ampholytes are in the so-called enhanced-fluidity mobile phase region. As a consequence, subcritical fluid and enhanced-fluidity mobile phase regions seem to be chromatographic modes with a high potential for operating ZWIX CSPs.

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1. Introduction

In the last years, supercritical fluid chromatography (SFC) has gained importance, especially, for the separation of chiral compounds [1–3]. In comparison to HPLC, SFC offers several advantages including lower analysis/purification costs due to enhanced speed of analysis, lower organic solvent consumption, and consequently reduced waste management costs. Most importantly, SFC offers a great variability in tuning chromatographic conditions.

Although supercritical carbon dioxide ($scCO_2$) is claimed to be an apolar solvent, it is miscible with a wide assortment of organic modifiers which makes it possible to adjust the overall solvation strength and polarity of the resulting mobile phase composition in SFC. This is achieved by the addition of polar protic and nonprotic solvents, typically alcohols (methanol, ethanol, propan-2-ol), to $scCO_2$. The enhanced polarity and elution strength of the resulting fluid enables routine elution, separation and enantioseparation of polar analytes from diverse statiunary phases under SFC conditions [1,4–15]. It is important to note that the addition of organic solvents to $scCO_2$ changes the properties of the otherwise highly fluidic solvent [16] such as viscosity, density and diffusion properties leading to a fluid of subcritical nature [17,18]. As a consequence, the term supercritical fluid chromatography may be misleading when a certain amount of a polar organic modifier is added to $scCO_2$. However, the term SFC has been well established for $scCO_2$-based mobile phases with up to 50% of a polar mostly protic organic modifier.

For mobile phases consisting of higher amount of an organic modifier than $scCO_2$, the term enhanced-fluidity mobile phase was proposed [19]. Such a mobile phase is typically composed of a mixture of organic solvents and a low density fluid, typically $scCO_2$ (methanol/$scCO_2$, hexane/ethanol/$scCO_2$, and others).The resulting mobile phases retains to a great part the solvation strength and polarity of the organic solvents (modifiers), while offering viscosities and diffusivities close to supercritical fluids [19–21]. Thus, both temperature and pressure remain major parameters for fine tuning separation conditions and broadening the retention and selectivity window. The high amount of organic solvents in the $scCO_2$ containing mobile phase leads to the "enhanced-fluidity chromatography"

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(EFC) which allows efficient separations of even highly polar substances, such as nucleotides and nucleosides [22,23].

Enantioseparation of acids and bases using chiral ion-exchange type CSPs in SFC has already been documented. It has been shown that chiral separations with a quinine-based weak anion-exchange type CSP is feasible even without any added buffer into the subcritical mobile phase composed of $scCO_2$ and methanol [9]. Furthermore, strong cation-exchange type CSPs have also been employed for the enantioseparation of racemic amines. In this case, the presence of a basic additive in the organic modifier to $scCO_2$ was essential to facilitate reasonable elution times of the basic analytes following on exchange retention mechanisms. It has also been described that the basic additive as a primary or secondary amine not only adopts the role of a counter-ion to the analyte, but can also react with $scCO_2$ adding new acidic substances to the pool of transiently formed buffer species [14,15]. Recently, a study focusing on the enantioseparation of *N*-protected proteinogenic amino acids on zwitterion ion-exchange type CSPs (Chiralpak ZWIX (+) and Chiralpak ZWIX (−)) has been published [7], clearly documenting the applicability of a zwitterionic CSP for the enantioseparation of acidic analytes under SFC conditions. Moreover, it has been demonstrated that the ion-exchange mechanism works well under subcritical fluid conditions. However, the possible changes in the ion-exchange mechanism and the underlying stoichiometric displacement model on increasing the ratio of a polar organic modifier (alcohol) and $scCO_2$ entering enhanced-fluidity chromatographic conditions have not yet been studied in detail.

In the present study, this has been investigated using Chiralpak ZWIX (+) as a stationary phase. Continuous change in chromatographic conditions from subcritical and enhanced fluid chromatography to classical liquid chromatography brings new evidence in the debate on the influence of transient buffer species and actually identifies the boundaries between the applied chromatographic modes.

2. Experimental

2.1. Materials

HPLC grade solvents (methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN)) were purchased either from VWR or from Sigma-Aldrich. Additives for mobile phase: formic acid (FA); ammonia (7 N in methanol); ammonium formate (NH_4FA); diethylamine (DEA) and triethylamine (TEA) were from Sigma-Aldrich or Fluka. Carbon dioxide was purchased from Air Liquide and was of standard grade (food chemistry). The analytes were either commercially available or kindly donated by cooperating partners in frame of other projects. *N*-Ac-Trp, Trp, and clenbuterol were purchased from Sigma Aldrich, whereby *N*-Bz-Leu, *N*-Bz-Phe and *N*-Bz-Ala were from Fluka. Mefloquine was obtained from Kreamer & Martin Pharma Handels-GmbH (Germany). For comparison reasons (see Electronic Supporting Information – ESI) the QN-AX column (Chiralpak QN-AX, Chiral Technologies Europe) (150 × 4 mm, 5 μm) which is a weak anion-exchange type CSP and a homemade strong cation-exchange type SCX CSP (150 × 4 mm, 5 μm) were employed (for details see ESI). The synthesis of the SCX CSP was previously described [14]. The ZWIX CSP is commercially available as Chiralpak® ZWIX (+) (150 × 3 mm, 3 μm) from Chiral Technologies Europe.

2.2. Instrumentation and chromatography

2.2.1. HPLC

HPLC measurements were carried out on an 1100 Series HPLC from Agilent Technologies equipped with a binary pump, degasser, photo diode array detector (PDA), solvent tray and an auto sampler. The flow rate was 0.5 mL/min and the injection volume was 5 or 10 μL. Sample concentration was 1–2 mg/mL and the void volume (t0) was determined by three consecutive injections of a methanolic solution of acetone at the beginning of each sequence of samples.

2.2.2. SFC and EFC

These measurements were carried out on an Acquity Ultra Performance Convergence Chromatography (UPC^2) System equipped with a binary solvent manager, sample manager, convergence manager, column manager and PDA detector (Waters). The flow rate was set to 0.5 mL/min, in order to ensure that the system pressure does not exceed the instrument pressure limit over the whole range of mobile phase compositions or to 2 mL/min for temperature studies. The injection volume was 5–10 μL. If not stated otherwise, the backpressure was set to 150 bar and the column temperature to 40 °C. Sample concentration was 1–2 mg/mL and the void volume (t0) was determined from the first negative peak observed after the injection. The detector wavelength was 254 nm in both HPLC and SFC modes.

3. Results and discussion

3.1. General aspects of using zwitterion ion-exchange type stationary phases

The chromatographic performance of zwitterion ion-exchange type CSPs can be freely modulated by the choice of the mobile phase composition. The primary interaction between the zwitterionic CSP (Fig. 1) and a charged analyte is based on electrostatic forces between the (ionized) groups (cationic, anionic) of the analytes (selectands) and the ionized groups of the selector (sulfonate and quinuclidinium). Thus, retention and elution of ionizable analytes is driven by balanced electrostatic interactions using appropriate amounts of counter-ions into mobile phase [24]. Retention and elution of analytes can thus be adjusted, while enantioselectivity is usually barely affected. An additional intrinsic intramolecular counter-ion of the zwitterionic selector motif (see Fig. 1) can become effective as will be discussed later. Retention and enantioselectivity can be optimized by the choice of organic solvent, usually methanol and/or acetonitrile in various ratios, in order to favour either hydrophobic and aromatic π–π interaction or hydrogen bond formation, respectively. Solvation effects of the ionized group will play a major role for the adjustments of the electrostatic interaction forces and in consequence of the retention behaviour. In the following, the influence of the mobile phase composition using $scCO_2$ and organic solvents, including organic acids and bases, on the chromatographic performance for acidic, basic and zwitterionic analytes on the Chiralpak ZWIX (+) CSP will be presented.

3.2. Enantioseparation of racemic acids and bases in SFC/EFC with ZWIX (+) CSP

A set of *N*-protected amino acids and basic analytes was used for the initial evaluation of the chromatographic performance of ZWIX (+) in SFC mode. As the organic modifier, MeOH, EtOH, a mixture of ACN/MeOH (1/1) and ACN/MeOH/H_2O (49/49/2) with NH_4FA, NH_4OAc or FA with DEA were used. To ensure good peak shape of the polar analytes, gradient elution was employed. The initial concentration of $scCO_2$ was set to 90%. It was gradually decreased to 70% in 8 min, kept constant for 4 min and returned to initial $scCO_2$ concentration and kept for 3 min to equilibrate the column.

Generally, retention decreased with increasing polarity and content of protic solvents in sequence ACN/MeOH > ACN/MeOH/H_2O > EtOH > MeOH. Please note, in using only ACN without a protic solvent retention will increase very strongly which deals with

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