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Zirconia based monoliths used in hydrophilic-interaction chromatography for original selectivity of xanthines

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ABSTRACT

Monolithic capillary columns based on zirconia were prepared directly from zirconium alkoxide. They were also prepared coating a classical silica based monolithic column with zirconium butoxide. Using the gradual evolution of the theophylline/caffeine separation factor, it was found that successive zirconia coatings produced the progressive fading of surface silanols replaced by Zr–OH groups. The behavior of a silica monolith coated four times with zirconium butoxide was very similar to that of a pure zirconia monolith. The dramatic change in xanthine separation factor observed with zirconia stationary phases and the theophylline and caffeine probe solutes was used to develop a complete separation of xanthines on zirconia stationary phase in less than 6 min. The three dimethylxanthine isomers, theophylline, theobromine and paraxanthine, are very difficult to separate in RPLC with classical C_{18} stationary phases. The three isomers were easily separated in HILIC mode on a zirconia based stationary phase.

1. Introduction

Reversed-phase liquid chromatography (RPLC) on octadecyl silica columns is the most widely used separation system to date. The major limitations of RPLC are the lack of adequate retention of polar molecules and the chemical solubility of silica at elevated pHs. Normal phase liquid chromatography (NPLC) with silica or alumina stationary phases can partially solve the first limitation: the retention of polar molecules. However, the solubility of polar molecules in non-aqueous apolar normal mobile phases is very limited, drastically reducing the NPLC capability.

To overcome the solubility dilemma, hydrophilic-interaction chromatography (HILIC) was introduced by Alpert [1]. HILIC works with a polar stationary phase and a less polar mobile phase, usually a polar organic solvent such as acetonitrile (MeCN) or methanol to which small amounts of water are added. HILIC has been steadily gaining interest in the last few years as it has emerged as a viable alternative to RPLC when dealing with polar and hydrophilic analytes [2]. However, silica is most often used as the classical HILIC stationary phase. Various polar stationary phases such as silica particles bonded with amide, amine, sulfobetaine 2-mercaptoethanol, 1-thioglycerol, polyhydroxyethyl or phosphocholine have been investigated as HILIC stationary phases [3–6]. To overcome the silica limitation, other metal oxides were tested as potential new stationary phases. Titanium oxide was used in several studies [7,8]. Zirconium oxide was packed in columns for liquid chromatography in the early 90s [9].

Monolithic columns were developed during the last decade to reduce the mobile phase flow resistance without loss of efficiency. In micro-column technology for electrochromatography and/or nano-LC applications, the monolithic structure avoids the problems associated with frits needed in packed bed capillary columns. The continuous monolithic bed is formed in situ in the capillary and cannot move not needing any frits. If silica is the most popular material used in chromatography, alternative inorganic materials such as ZrO_2, TiO_2, Al_2O_3 and HfO_2 have been proposed [10,11] as chromatographic support. Most of the studies were devoted to zirconia particles after the successful production of zirconia porous particles in the early 90s [12,13]. Using such metal oxide as a monolithic support, we could expect benefits from the properties of the monolithic structure as well as from the chemical resistance of the material.

In a previous paper [14], we reported two different strategies to produce zirconia based monolithic columns. The first way was to coat zirconia onto a silica monolith; the second was the complete sol–gel preparation of zirconia monolithic columns. In this work, we investigated the capabilities of the HILIC mode used with zirconia capillary monolithic columns prepared in our laboratory. Polar xanthine alkaloids were used as probe compounds to compare the

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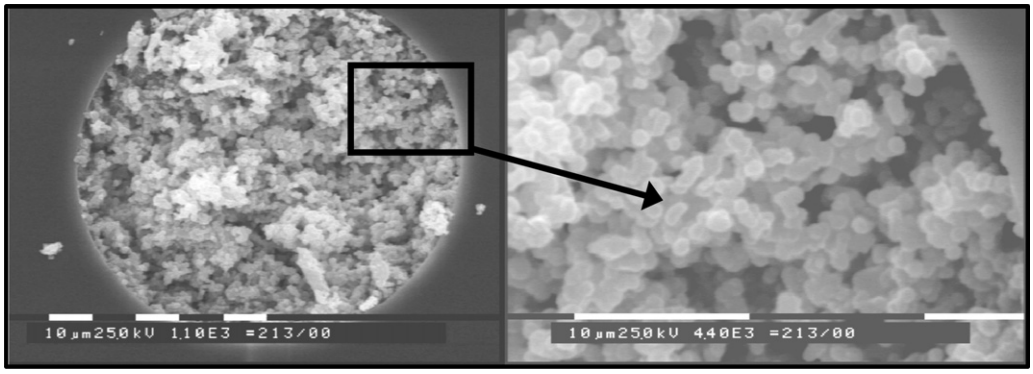

Fig. 1. *Left*: Scanning electron microscopy (SEM) image of the zirconia monolith inside a 75 μm fused silica capillary. *Right*: Enlargement showing the micrometer sized particle arrangement.

HILIC behavior of zirconia capillary monoliths to that of classical silica capillary monoliths.

2. Experimental

2.1. Reagents and instrumentation

Fused silica capillaries (75 μm I.D., 375 μm O.D.) were obtained from Cluzeau (Bordeaux, France). Ethanol, trishydroxymethylaminomethane (Tris), tetramethoxyxilane (TMOS), polyoxyethylene glycol (PEG) (MW = 10,000 g mol^{-1}), urea, acetic acid, zirconium butoxide, ethanol, propanol, butanol, naphthalene, thiourea, sodium hydroxide, caffeine, theophylline, theobromine, paraxanthine and dyphylline (7-dihydroxypropyl theophylline) were purchased from Sigma (Saint-Quentin Fallavier, France). HPLC-grade acetonitrile, (SDS, Vitry sur Seine, France) was used throughout. Mobile phases were prepared from acetonitrile and water. When buffer was used, the buffer concentration reported was the buffer concentration in water.

Electro- and nano-chromatography characterizations were performed on an Agilent capillary electrophoresis system HP^{3D}CE equipped with a diode array detector (Agilent, Massy, France). Separations were carried out at 25 °C and test analytes were monitored at 254 nm. The morphology of the zirconia monolith was examined by a scanning electron microscope (SEM 515) from Philips (Eindhoven, the Netherlands).

2.2. Columns preparation

Silica monolithic capillaries were prepared following the protocol described by Puy et al. [15]. Typically, a fused silica capillary was activated filling it with a 1 M NaOH solution followed by heating at 40 °C for 3 h. PEG (1.054 g) and urea (1.081 g) were dissolved in 12 mL 0.01 M acetic acid in an ice bath under stirring for 20 min. Next 4.81 g of TMOS were added to the cold solution. After 40 min at 0 °C under stirring, the activated capillary was filled with the mixture. It was heated at 120 °C for 3 h in order to create the mesopores and then cured at 40 °C for 16 h. The monolith filled capillary was finally washed with pure water to open the pores dissolving urea and partially removing PEG.

To perform the zirconia coating, a monolithic silica capillary must be washed with absolute ethanol to remove any trace of water. Zirconium alkoxide was dissolved in dry ethanol (1/2, v/v), and the solution was flushed through the monolith capillary at 10 μl min^{-1} for 3 h. Next the capillary was rinsed with ethanol to remove any excess of zirconium alkoxide, and finally water was flushed through the monolith capillary to hydrolyze all remaining alkoxide groups on the zirconium coated surface. These different stages were repeated to increase the zirconium coating coverage.

The total zirconia monolith synthesis was performed as follows: first, the hydrolysis solution was prepared with 0.01 M acetic acid, PEG and n-butanol and placed in an ultrasonic bath until complete dissolution at room temperature. The suitable amount of liquid zirconium alkoxide was weighted, dissolved in dry ethanol and stored in a sealed glass vessel in order to avoid any contamination by air moisture. Next the hydrolysis solution was poured into the zirconium alkoxide solution and the mixture was immediately introduced into the activated (activation was realized in the same way than for silica monolith) capillary. The composition of the final reaction solution was characterized by four parameters: C, the alkoxide concentration (C = 1 M); h, the hydrolysis ratio which is the number of water molecules over the number of alkoxide molecule (h = 1); R, the complexation ratio, which is equal to the number of complexant molecules over the number of alkoxide molecule (R = 3); and the PEG concentration ([PEG] = 5.10^{-2} mol L^{-1}). Afterwards, the capillary was kept at 30 °C for 24 h, and then heated at 150 °C for 6 h.

The monolith permeability, B_0, was determined by recording the retention time, t_M, of an unretained analyte (naphthalene) as a function of the driving pressure, ΔP, according to relation:

$$u = \frac{B_0}{\eta L}\Delta P \quad (1)$$

in which η is the mobile phase viscosity and L is the capillary length.

3. Results and discussion

3.1. Zirconia and zirconia coated silica monoliths

Scanning electron microscopy images of the zirconia monolith prepared in fused silica capillary (internal diameter 75 μm) are displayed in Fig. 1. The monolith is made of porous zirconia globules with diameters close to 1 μm attached together with micrometer through pore size. The permeability, B_0, for such monolith, measured using a nano-LC system at constant pressure, was typically in the range 2–6 × 10^{-13} m^2. With such very high permeability compared to the one observed with a packed bed column, the capillary electrophoresis system HP^{3D}CE can be used for chromatographic column characterizations simply using its pressure driven mode. The HP^{3D}CE system was able to apply an external pressure up to 10 bars on the inlet vial producing a maximum mobile phase velocity of 0.2 cm s^{-1} (hold-up time of 50 s with a 10 cm capillary).

Fig. 2 is a SEM picture of a typical zirconia coated silica monolith. The zirconium butoxide coating did not modify the macroscopic structure of the silica backbone. Because the first reaction between silanol groups and zirconium butoxide is performed in an anhydrous solution, the created "zirconia" layer does not contain anymore hydroxyl group preventing any possibility of expansion

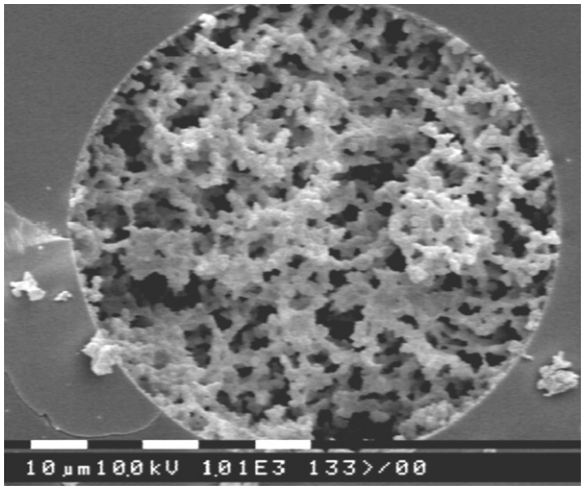

Fig. 2. Typical SEM image of a zirconia coated silica monolith (four coating inside a fused silica capillary (internal diameter 75 μm)).

from the surface:

$$\text{Si–OH} + \text{BuO–Zr(OBu)}_3 => \text{Si–O–Zr(OBu)}_3 + \text{BuOH} \quad (2)$$

The hydrolysis step generates new hydroxyl groups as $Si–O–Zr(OH)_3$ groups. These hydroxyl groups can react with more zirconium butoxide in the next coating application. However, the four successive zirconium coatings did not modify the morphological backbone of the silica monolith as shown in Fig. 2 (column permeability did not change after the multiple coating stages).

3.2. Zirconia coverage and xanthine retention

3.2.1. The xanthine probes

Naphthalene, caffeine and theophylline were used as probe solutes to compare the HILIC behavior of silica and zirconia based monolithic capillary columns working with MeCN/Tris 1 mM pH 7.5 98/2 (v/v) mobile phase (Fig. 3). The HILIC elution order is from

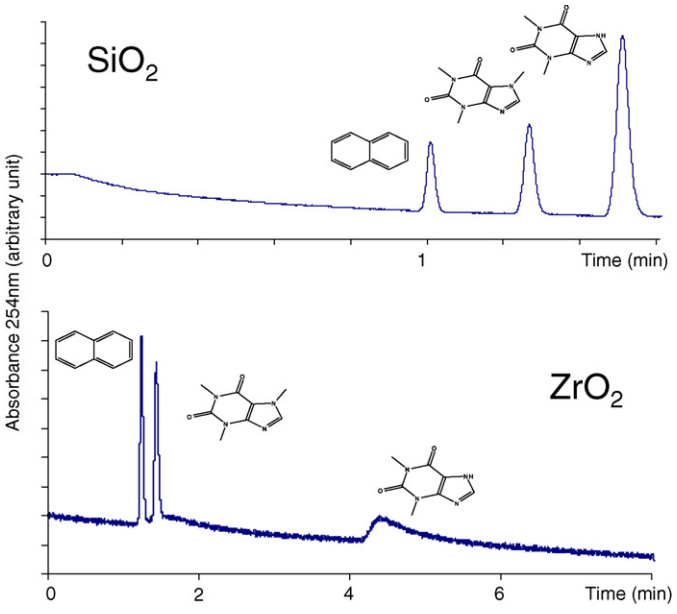

Fig. 3. LC separation of naphthalene, caffeine and theophylline on silica (top) and zirconia (bottom) monolithic capillary columns (8.5 cm length, 75 μm I.D. Mobile phase MeCN/Tris 1 mmol L^{-1}, pH 7.5, 98/2 (v/v); UV detection 254 nm).

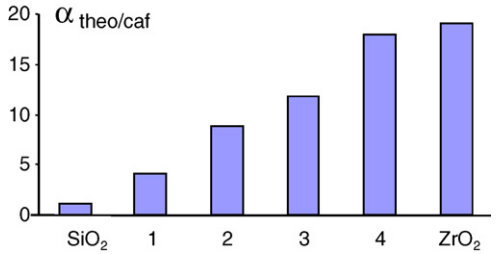

Fig. 4. Changes in theophylline/caffeine separation factor from silica to zirconia monolith as a function of the number of coating stages (8.5 cm length, 75 μm I.D. Mobile phase MeCN/Tris 1 mmol L^{-1}, pH 7.5, 98/2 (v/v); UV detection 254 nm).

least to most polar analyte—the reverse of that observed in RPLC. Naphthalene was used as an unretained hold-up volume marker. As expected for HILIC retention mechanism, caffeine with three methyl groups is less polar and will elute earlier than theophylline that has only two methyl groups on the xanthine ring. Dyphylline (7-dihydroxypropyl theophylline) is more polar than theophylline hence it is the most retained solute. It could not be used with zirconia and zirconia coated monolith being retained too long. With silica and zirconia stationary phases, a very high MeCN content was required in order to retain caffeine molecules. If the caffeine retention factor is very low for both silica and zirconia columns, the theophylline retention factor was very sensitive to the nature of the stationary phase. The main retention mechanism in HILIC mode is solute partitioning between a water-rich layer associated to the polar stationary phase surface and the less polar (high %MeCN) mobile phase. However, this mechanism is not suitable to fully explain the very strong theophylline retention observed on the zirconia coated stationary phase. A specific theophylline adsorption on zirconia surface is suspected. This assumption is reinforced by the significant peak broadening observed on zirconia (Fig. 3).

3.2.2. Zirconia coating

In order to study the change in surface properties produced by the successive zirconia coatings onto a silica monolith, the probe compounds were injected after each coating. Fig. 4 shows the changes observed in the theophylline/caffeine separation factor as a function of the number of coatings. With the same mobile phase, this separation factor was close to 2 on the silica monolith and increased continuously up to the value of 17 observed with a pure zirconia monolith. It can be considered that the silica surface was fully transformed to a zirconia like surface after four coatings producing an $\alpha_{theo/caf}$ of 16 (Fig. 4). However, the column efficiency measured using the theophylline peak width decreased continuously with the number of coatings and could be a limiting factor of use for such kind of monolith. But, the efficiency of caffeine did not decreased on the same way than theophylline as it can be seen in Fig. 3. Specific interactions of theophylline with the metal oxide surface seem to be the main reason for efficiency reduction: a ligand exchange mechanism with low kinetic was suspected as already observed on zirconia particles [12]. So zirconia coated monolith could be used using other solutes or using mobile phase additives in order to reduce ligand exchange mechanism.

3.3. HILIC mode and xanthine retention

3.3.1. Mobile phase water content

The elution order of naphthalene and the xanthine compounds is typical of what is observed in HILIC mode: the retention factors increase with the solute polarity. Also, an increase of mobile phase polarity, obtained by increasing the water content (or decreasing the MeCN percentage) produces a decrease of all retention factors. Fig. 5 shows the plots of the log k of the xanthine compounds

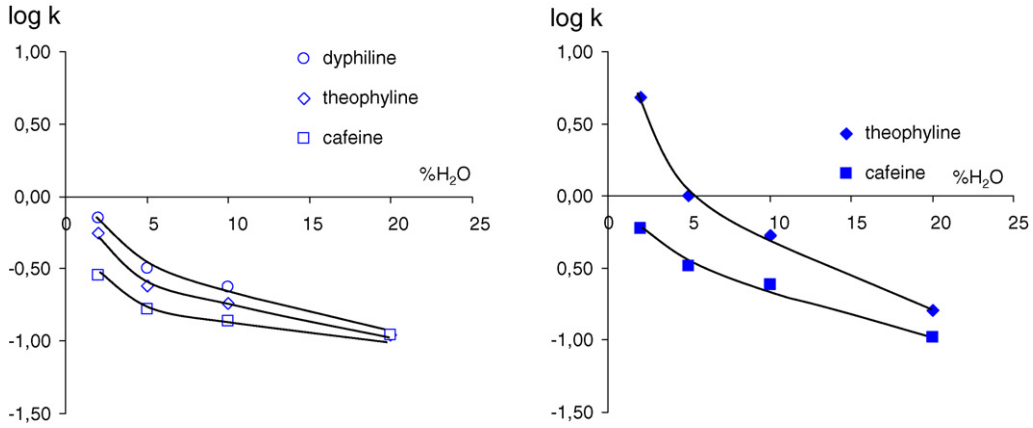

Fig. 5. Influence of the mobile phase water content on the retention factor of caffeine, theophylline and dyphylline (7-dihydroxypropyl theophylline) for left: silica monolith and right: two times coated zirconia monolith (mobile phase MeCN/H_2O).

versus the mobile phase water content. Silica monolith was compared to a two times coated monolith; such coating lead to a good compromise between theophylline/caffeine separation factor and efficiency of theophylline. All retention factors were very low on silica monolith stationary phase even for dyphylline which is the most polar and hydrophilic xanthine in the probe mixture. The retention factors lower than unity produced only negative values for the log *k* observed on silica (Fig. 5 left plot). The retention of all probe compounds increased dramatically on zirconia coated monolith. For example, the retention factors of caffeine was 0.28 on silica and 0.60 on zirconia with a 98/2 MeCN/water mobile phase, the caffeine retention is more than doubled. For theophylline with the same mobile phase, the retention factors were respectively 0.52 and 4.8. Going from a silica stationary phase to a zirconia coated stationary phase with a 98/2 MeCN/water mobile phase produced an increase of almost one order of magnitude of the theophylline retention factor. Dyphylline was retained so long on the zirconia monolith that it was removed from the mixture (broad peak). The curvatures of the log *k* versus water content seen in Fig. 5 demonstrate that the solutes are not retained through a pure partition mechanism.

3.3.2. *pH and ionic strength*

Different buffers were used to control the mobile phase pH and maintain it at pH 7.4. It was observed that, with zirconia coated monoliths, the retention depended on the buffer concentration as well as the buffer nature. The retentions of caffeine and theophylline were monitored using a MeCN/H_2O 98/2 (v/v) solution as mobile phase with various buffer concentrations. With the Tris buffer added from 1 to 100 mM to the mobile phase, the retention factors of both xanthines decreased by less than 10% with the highest 0.1 M Tris buffer mobile phase. The picture was very different with ammonium acetate: the retention factor of theophylline was 5.6 with a 10 mM ammonium acetate MeCN/water 98/2 (v/v) mobile phase. It decreased down to 1.9 with a 100 mM ammonium acetate concentration in the same mobile phase. The caffeine retention factor changed by less than 5% in the same conditions. In HILIC mode, an increase of the buffer concentration can lead to higher or lower solute retention depending on the ion chaotropicity. Higher retentions are usually related to the thickness of the water layer which is enlarged when chaotropic ions are present in the mobile phase assuming that the ion species are located in the water-rich layer close to the surface [3,4]. Lower solute retention can be associated to a competitive effect of the buffer ions on the adsorption sites of the stationary phase. The chloride and acetate anions of the Tris and ammonium acetate buffers have similar weak chaotropicity. However, the ammonium cation is a much stronger chaotropic cation than the big tris(hydroxymethyl)methane ammonium cation.

The surface of zirconia is very complex and as such it is difficult to attribute just HILIC mechanism for the separation. The zirconia surface is well known to have ligand exchange capabilities [16,17], so the acetate anions could be involved through a specific adsorption that could also explain the observed modifications of the retention of theophylline.

3.3.3. *Xanthine separation*

Based on the knowledge of the influence of the operating conditions (water content, nature and concentration of mobile phase additive) on the retention of xanthines, the separation of five xanthines has been performed on zirconia monolithic capillary as shown in Fig. 6. The efficiency of zirconia monolithic columns decreased with the retention and a gradient elution mode was required in order to minimize band broadening of the latest eluted solutes (not shown). It is interesting to note that the dimethylxanthine isomers eluted in HILIC mode on zirconia stationary phases in the order: the 1,3 derivative eluted first followed by the 3,7 and the 1,7 eluted last (Fig. 6). In the RPLC mode with a conventional C_{18} stationary phases and a water rich mobile phase, the dimethylxanthine elution order was: the 3,7 derivative eluted first followed by the 1,7 derivative and the 1,3 derivative eluted last [18–20]. The last two isomers (1,3-theophylline and 1,7-paraxanthine) are difficult to separate in RPLC. Using HILIC mode with a zirconia stationary phase, the xanthine separation factor and also the resolution was dramatically improved compared to RPLC and all three dimethyl

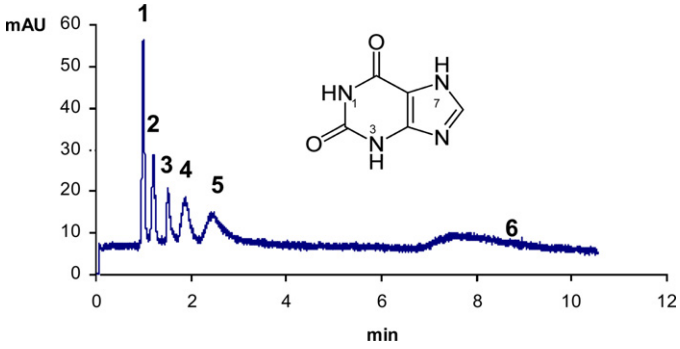

Fig. 6. LC separation of xanthines on zirconia monolithic capillary columns (8.5 cm length, 75 μm I.D. Mobile phase MeCN/Tris 1 mM pH 7.5 90/10; UV detection 254 nm, $u = 0.14$ cm s^{-1}). Compounds: (1) naphthalene; (2) caffeine; (3) 7-hydroxyethyl theophylline (etofylline); (4) 1,3-dimethylxanthine (theophylline); (5) 3,7-dimethylxanthine (theobromine); (6) 1,7-dimethylxanthine (paraxanthine).

isomers were easily separated. This is an example of the unique selectivity that can be obtained with a zirconia stationary phase toward basic compounds.

4. Conclusion

The association of the HILIC mode of elution with zirconia based stationary phases can produce original and unique selectivity in the analysis of polar compounds as demonstrated in the case of xanthines. Zirconia stationary phases can be obtained easily by coating bare silica using zirconium butoxide. The coating can be applied to silica monoliths producing zirconia monoliths. In the zirconia coating procedure, the theophylline–caffeine separation factor was demonstrated to depend on the concentration of either silanol Si–OH and Zr–OH groups onto the surface of the monolithic stationary phase. Successive coatings decreased the silanol concentration leaving only Zr–OH surface group. This change produced a dramatic shift from 2 to 17 in theophylline–caffeine separation factor. Since the separation factor change is progressive, the packing selectivity could be controlled adjusting the zirconia coverage of the silica surface. If all three dimethylxanthine isomers are difficult to separate in RPLC, in HILIC mode with a zirconia stationary phase, the separation of the 1,3-, 1,7- and 3,7-dimethylxanthines was easily obtained.

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