



Chromatographic behavior of xanthenes in aqueous normal phase chromatography using titania stationary phase

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ARTICLE INFO

Article history:

Received 19 September 2010

Received in revised form 1 December 2010

Accepted 7 December 2010

Available online 13 December 2010

Keywords:

Titania

Zirconia

Silica

HILIC

ANP

Xanthine derivatives

Caffeine

Selectivity

ABSTRACT

The chromatographic behavior of native titania was investigated in aqueous normal phase chromatography using a set of N-methylated xanthenes as polar test solutes. In agreement with a hydrophilic interaction on a polar bed, the retention of xanthine models increased mainly along their molecular polarity. Adsorption of these molecules on the hydrated surface of titania prevailed as a retention mechanism for low water contents in the mobile phase. Several N-methylated xanthenes could be easily discriminated along the number and position of their methyl groups while the nitrogen atom at position 3 was found deeply involved in the retention on titania. To get further insights on the interactions occurring on the surface of titania, the retention of xanthine derivatives based on ligand-exchange was evaluated as a function of the buffer concentration and type. The separation efficiency of native titania for the set of N-methylated xanthenes was comparable to that observed on zirconia but lower than that obtained on native silica due to mixed-mode interactions. However, titania exhibited a superior ability to recognize several isomeric positions of xanthine derivatives in comparison to zirconia and silica.

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1. Introduction

Titania material, thanks to its specific surface properties, appears as an original support for chromatographic separations or sample treatment. Titania offers an enhanced column long-term stability due to a great thermal and chemical resistance toward highly aggressive mobile phases such as extreme low or high pH (1–14) and can withstand high temperature conditions (up to 150 °C) [1–5].

Compared to common silica-based materials, titania is characterized by several surface properties. Its surface exhibits either anion- or cation-exchange at low or high pH respectively, in correlation to its isoelectric point which is in the range from 4 to 7 [4,6–8]. Titania can also selectively adsorb Lewis hard bases such as carboxylate and phosphate through ligand-exchange interactions [9,10]. So, owing to its rich surface chemistry, titania can generate complex selectivities often leading to discrimination between isomers of substitution [6,11,12]. Such great potential of bare titania has already found several applications in liquid chromatography. For example, titania has been used as a preconcentration media in pre-columns for trapping organophosphate compounds of proteolytic digests such as amino acids and peptides in phosphoproteomic analysis [13–17]. Other studies have led to its use as

a chromatographic bed in normal phase (NP) [2,4,11], ion exchange (IE) [18] and more recently in hydrophilic interaction liquid chromatography (HILIC) [19,20].

In the recent years, HILIC has gained increasing attention for the analysis of hydrophilic compounds such as nucleotides, amino acids and carbohydrates [21–24]. Separation in HILIC is based on partition of solutes between a water-enriched layer at the surface of the hydrophilic bed and the bulk of the aqueous/organic mobile phase generally containing from 5% to 40% of water, and in opposition to RP chromatography, water in HILIC works as the strongest eluting solvent in the mobile phase. HILIC offers two main advantages over NP chromatography: acetonitrile/water mixtures enhance the solubility of polar compounds in comparison to hexane-based eluents, and the mobile phase composition fits well with electrospray mass spectrometry (ESI-MS) leading to high sensitivity of detection [25]. The terminology aqueous normal phase (ANP) chromatography [22,26] is used when partition is not the main retention mechanism of hydrophilic compounds but where interactions such as adsorption, ion and/or ligand-exchange may also be involved.

Zhou et al. [19] first inspected the chromatographic behavior of native titania in ANP chromatography using a set of phosphorylated nucleotides. These authors have found ligand-exchange and partition as the two mechanisms of retention of the test solutes depending on the mobile phase conditions. In their study, the acetonitrile (ACN) content, eluent pH and buffer concentration were tuned to control the chromatographic retention of phosphorylated nucleotides. Their investigation allowed the separation of fifteen

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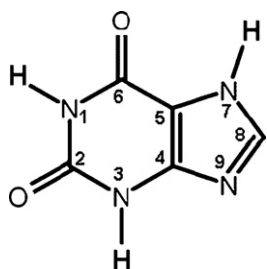


Fig. 1. Molecular structure of xanthine.

phosphorylated nucleotides and their intermediates on bare titania using a gradient of mobile phase composition. The same authors have later used carboxylates as probe molecules [20] and found a mixed retention mechanism on titania: both electrostatic repulsion and HILIC were involved, the latter being favored at high %ACN in the mobile phase. The acetonitrile content, the buffer concentration, the pH of the eluent and the column temperature influenced the chromatographic retention of carboxylic molecules, and a fine tuning of these parameters was essential to achieve the separation of carboxylate solutes on a bare titania column.

To broaden the investigation of the chromatographic behavior of native titania in aqueous normal phase chromatography, our study focused on a series of N-methylated xanthines (oxopurine derivatives) as probe molecules (Fig. 1). N-methylated xanthines are among the alkaloid compounds that are mainly found in tea, cocoa and coffee. They exhibit biological activity [27] as stimulants and they have also been used for pharmacological purposes [28] in view of their high physiological importance in various enzyme systems and metabolic pathways [29]. HILIC analysis of xanthine derivatives was previously reported on native silica monoliths in capillary electro-chromatography (CEC) [30] and on native titania monoliths in nano-liquid chromatography (nano-LC) using a mobile phase of (95:05, v/v) ACN/Tris [31]. A more detailed study of the thermodynamic aspect of the separation was later carried out in nano-LC on silica, and compared to that on zirconia-modified silica monoliths [32]. The chromatographic retention of xanthine probes was reported to decrease with increasing water content up to 20% in the ACN-based mobile phase for either native silica or zirconia-modified silica monoliths. A larger retention and higher theophylline–caffeine selectivity was however observed on the zirconia modified silica monoliths.

In this work, we investigated the capabilities of the aqueous normal phase with a bare titania column. The impact of several chromatographic parameters on the retention of xanthine derivatives (xanthine, mono-, di- and tri-methyl xanthines) and on the efficiency of the separation was considered.

2. Experimental

2.1. Materials

Naphthalene, uracil, 1-methylxanthine (1MX), 3-methylxanthine (3MX), 7-methylxanthine (7MX), 1,3-dimethylxanthine (1,3DMX or theophylline), 3,7-dimethylxanthine (3,7DMX or theobromine), 1,7-dimethylxanthine (1,7DMX or paraxanthine), 1,3,7-trimethylxanthine (1,3,7TMX or caffeine), ammonium acetate, and ammonium formate were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). HPLC-grade ACN (SDS, Vitry sur Seine, France) was used as organic modifier in the mobile phase. An Elga Pure Lab UHQ system provided by Veolia Water STI (Le Plessis Robinson, France) was employed for water purification. When buffers were applied, the reported concentration was that of the total amount of buffer in the hydro-organic mobile phase.

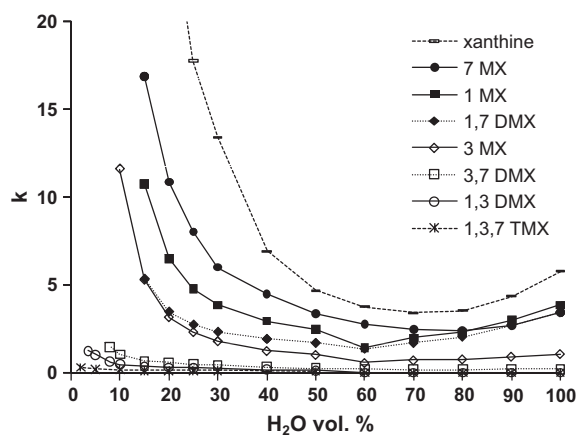


Fig. 2. Effect of water content on the retention of xanthine derivatives on native titania in ANP chromatography. Experimental conditions: chromatographic column titania Sachtapore®-NP (100 mm × 2.1 mm I.D., 5 μm); mobile phase: ((100 – x):x, v/v) ACN/ammonium acetate (1×10^{-3} mol L⁻¹); flow rate, 0.4 mL min⁻¹; UV detection at 254 nm; column temperature, 25 °C; column hold-up volume marker, naphthalene.

All chemicals were of analytical grade quality and were used as received except for prepared buffer solutions which required additional filtration and sonication prior to use. Solutions of test solutes were prepared by dissolving each compound individually with naphthalene (the column hold-up volume marker) in (50:50, v/v) ACN/water and later diluted with the mobile phase, prior to injection (injection volume, 5 μL). Liquid chromatography on titania was performed on Sachtapore®-NP column (100 mm × 2.1 mm I.D., 5 μm, 100 Å, 55 m² g⁻¹ Zirchrom Separations, Anoka, MN, USA). Other chromatographic columns were also tested: Sachtapore®-NP column (100 mm × 2.1 mm I.D., 5 μm, 300 Å, 15 m² g⁻¹), ZirChrom®-PHASE (100 mm × 2.1 mm I.D., 5 μm, 300 Å, 30 m² g⁻¹) from Zirchrom Separations and HYPERSIL® SILICA (50 mm × 4.6 mm I.D., 3 μm, 120 Å, 170 m² g⁻¹) from Thermo Electron Corporation (Runcorn, Cheshire, UK).

2.2. Instrumentation

Liquid chromatography was performed with a Thermo Scientific Accela™ HPLC-UHPLC system (Waltham, MA, USA), with an autosampler and an integrated diode array UV detector. The results were then processed using the chromatography data system software, Thermo Scientific ChromQuest. Log *P* values of the test compounds were calculated with HyperChem™ 8.0 (Hypercube, USA) after a geometry optimization based on the Polak Ribiere algorithm.

3. Results and discussion

3.1. Effect of water content

In order to study the influence of water content on the retention of xanthine probes on titania column, ACN/H₂O mobile phases have been used. By varying the water content of the mobile phase from 5% to 100%, two opposite regimes of retentions were observed as illustrated in Fig. 2. Increasing water contents from 2% to 60% led to a decrease of the retention factors of xanthine derivatives: in this range, water acted as the strongest eluting solvent as commonly established for HILIC on a variety of polar hydrophilic beds [21,22,33]. However, further addition of water (i.e. contents above 60%) increased the retention factors of xanthine derivatives as water worked as the least eluting solvent. Such trend, which still remains unclear, was already observed on silica based HILIC sup-

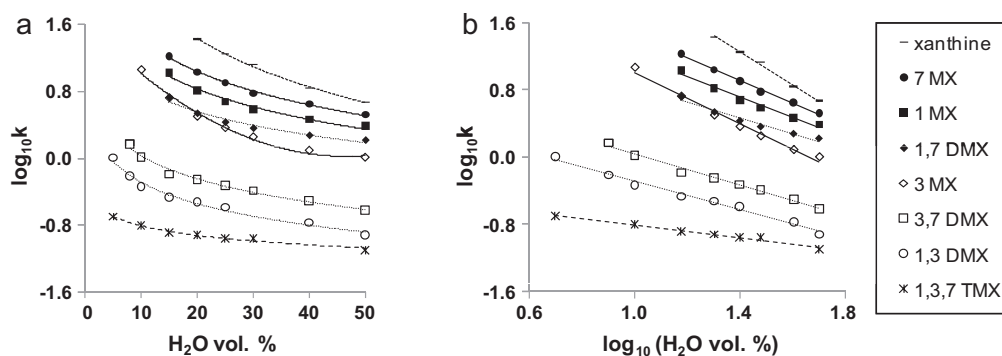


Fig. 3. Elution patterns of xanthine derivatives on titania for water contents less than 50%. Plots of the logarithm of retention factors (k) against the volume percentage of water in the hydro-organic mobile phase: (a) normal scale for partition; (b) logarithmic scale for adsorption. Experimental conditions are listed in Fig. 2.

ports and was related to a partition of some polar test solutes involving probably hydrogen bonding with the siloxane bonds on the surface [34,35].

In the following sections, the detailed studies were limited to low water contents (i.e. <50%) where aqueous normal phase occurs.

3.1.1. Retention model of xanthine derivatives

Despite the amphoteric character of titania surface (i.e. cation- and anion-exchange properties), ion exchange cannot be involved with this set of xanthine owing to their neutral state [36] (see Section 3.2). To gain insight on the interaction mechanism of xanthine derivatives on titania, retention data obtained for water contents lower than 50% were analyzed according to the two well-established models associated to partition and adsorption mechanisms [22,23,37–40]. These relations have been written in order to take into account the order of elution strength of water and acetonitrile in aqueous normal phase chromatography:

$$\text{partitioning: } \log k = \log k_{\text{ACN}} - S\phi_w \quad (1)$$

$$\text{adsorption: } \log k = \log k_w - \left(\frac{A_s}{n_w}\right) \log N_w \quad (2)$$

where k , k_{ACN} and k_w represent, respectively, the retention factors in the hydro-organic mobile phase, in pure acetonitrile (the weakest eluent) and in pure water (the strongest eluent). ϕ_w is the volume fraction of the strongest eluent (water in this case), S is the observed slope. A_s and n_w are the cross-sectional areas occupied by the solute and water molecules on the surface, and N_w is the molar fraction of water.

The retention data were plotted according to Eqs. (1) and (2) in Fig. 3, and the linearity of elution patterns best fitted the adsorption mechanism. The correlation coefficients assuming each of the retention mechanisms on native titania (i.e. partition and adsorption) are summarized in Table 1. The calculated values of these coefficients were closest to 1 for the adsorption model.

3.1.2. Retention order of xanthine derivatives

The retention of N-methylated xanthine derivatives was investigated as a function of the number and position of their methyl groups. As a general trend, hydrophilic retention on titania increased when decreasing the number of methyl groups of the molecule: k tri-methylated xanthine < k di-methylated < k mono-methylated < k unsubstituted xanthine (refer to Fig. 2, for water contents less than 50%). As expected, the hydrophilic retention on native titania increases with molecular polarity according to the octanol–water partition coefficients reported in Table 1. This calculation of the K_{ow} coefficients was however not able to distinguish the position of methyl groups on the molecular backbone which appeared to be fundamental in the molecule interaction with the surface. Indeed, an unexpected order of retention, that is not

consistent with the global polarity of the molecule, was observed comparing 1,7DMX which is a di-methylated xanthine and 3MX which is mono-methylated one.

For all molecules in the data set having the same number of methyl groups, the highest chromatographic retentions were always observed for N-methylated xanthenes having a hydrogen atom at N3 (i.e. 7MX, 1MX and 1,7DMX). So we can assume that the hydrogen atom at N3 is deeply implicated in the interaction process of xanthine derivatives via adsorption on the surface of titania.

3.2. Effect of buffer concentration and nature

Fig. 4 shows the influence of the concentration of ammonium acetate on the retention behavior of some N-methylated xanthenes on titania using ACN/H₂O (85:15, v/v) as mobile phase. The decreasing retention of these solutes on native titania with increasing salt concentrations supports the adsorption theory and underlines a competition phenomenon between the buffer and the test solutes for specific sites on titania surface. To analyze this result let us review first in brief, the chemistry of xanthine N-methylated derivatives.

Xanthine N-methylated derivatives are polar and neutral solutes, as their aptitude to display electrostatic charges is limited by their significantly weak Brønsted acid–base behavior. Ogston [36] reported that the basicity of nitrogen atoms of xanthine derivatives is too weak to undergo protonation under acidic conditions. The same author related the acidic properties of N-methylated xanthenes (except for caffeine which is not acid at all) to enolisation of their 6-carbonyl group involving its adjacent part of the imidazolium ring. The enol remains either in its normal acid form or evolves to a zwitterionic state. Due to this uncharged overall

Table 1

Log P values of methylated xanthine derivatives calculated using HyperchemTM 8.0 and the Polak Ribiere algorithm; linear correlation coefficients of retention models of xanthine derivatives considering the partition and the adsorption mechanism in chromatography.

Xanthine derivative	Hydrogenated nitrogen sites	Log P	R^2	
			Partition	Adsorption
Xanthine	N1,N3,N7	−1.80	0.986	0.996
7MX	N1,N3	−1.55	0.944	0.995
1MX	N3,N7	−1.55	0.896	0.976
3MX	N1,N7	−1.55	0.876	0.985
1,7DMX	N3	−1.31	0.870	0.962
3,7DMX	N1	−1.31	0.905	0.986
1,3DMX	N7	−1.31	0.894	0.984
1,3,7TMX	N/A ^a	−1.06	0.903	0.982

^a Not applicable.

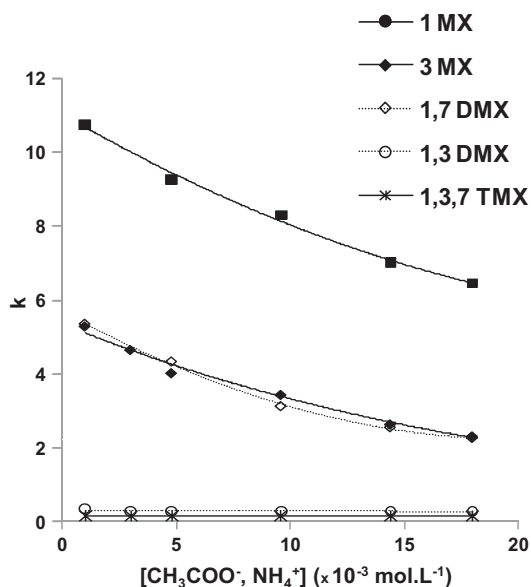


Fig. 4. Effect of ammonium acetate buffer total concentration on the retention of several N-methylated xanthines in ANP mode on titania. Mobile phase mixture: (85:15, v/v) ACN/water. Other experimental conditions are identical to Fig. 2.

state, N-methylated xanthines behave as neutral solutes excluding therefore the possibility of an ion-exchange mechanism with the titania surface as an explanation to the competition behavior observed in Fig. 4. All these previous assumptions have been confirmed experimentally by measuring the electrophoretic mobility of xanthenes. For all mobile phase conditions, the electrophoretic mobility of xanthine was always equal to zero and so this neutral state excludes ion exchange mechanism. On the other hand, xanthine derivatives are characterized by Lewis base properties, as they display electron-pair donor centers on their nitrogen atoms which are capable of interacting via ligand-exchange with Lewis acid sites on titania [41–43].

The ligand-exchange behavior of titania originates from the presence of water molecules that are coordinatively bonded to Ti^{4+} atoms on the surface of the titanium dioxide particles in order to fulfill the coordination sphere of these atoms [12]. Machesky et al. [44] have established that water molecules interact with the under-coordinated surfaces of metal oxides, via either associative or dissociative adsorption mechanisms. Under the former mecha-

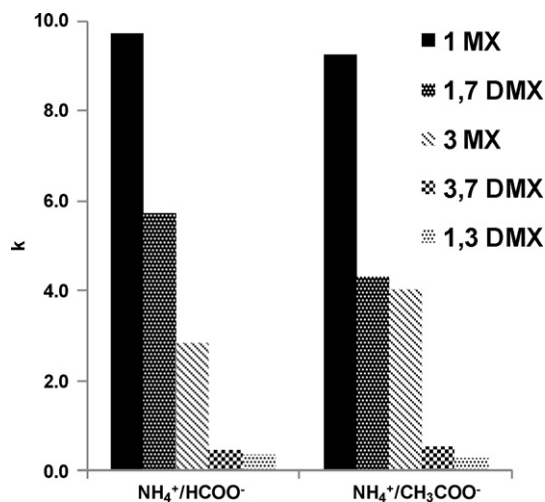
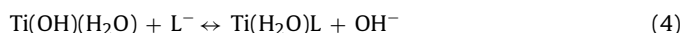
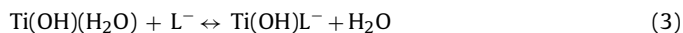


Fig. 5. Effects of ammonium formate and ammonium acetate buffers on the retention of xanthine derivatives on titania. Conditions: flow rate, 0.4 mL min^{-1} ; mobile phase: (85:15, v/v) ACN/buffer ($5 \times 10^{-3} \text{ mol L}^{-1}$); UV detection at 254 nm; column temperature, 25°C ; column hold-up volume marker, naphthalene.

nism, water molecules remain intact after they adsorb to surface metal cations (Lewis acid sites), while under the latter, they dissociate, resulting in a surface populated by hydroxyl groups (i.e., $Ti-OH$). The interaction of ligand molecule (xanthine or mobile phase component) in the mobile phase can be described using the following equilibria [12]:



Because acetate ions have Lewis basic properties, it has a competitive role to the retention of xanthenes on titania [45] displacing the solute to the mobile phase and reducing their retention factor.

In our study on the retention of N-methylated xanthenes on native titania, formate- and acetate-based buffers were compared for their influence on retention (Fig. 5) using an equal concentrations of $5 \times 10^{-3} \text{ mol L}^{-1}$ in (85:15, v/v) ACN/ H_2O . The same general trend was observed confirming the competitive effect of both ligands. A difference in selectivity can be noticed comparing 1,7DMX and 3MX and so the control of the selectivity on titania could be performed by changing the Lewis base in the buffer.

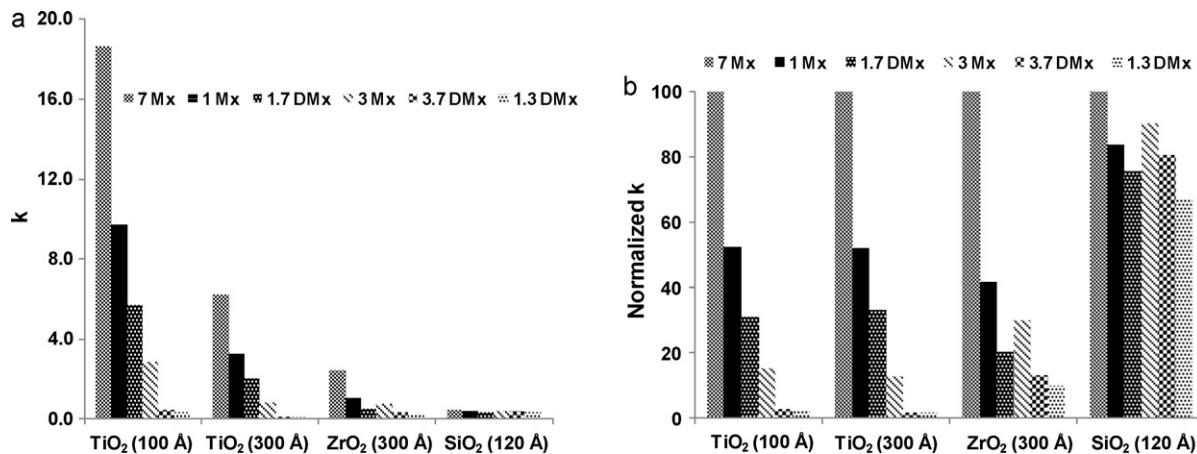


Fig. 6. Original retention factors (a) and normalized retention (b) of N-methylated xanthines on titania, zirconia and silica columns using ACN/ammonium formate ($5 \times 10^{-3} \text{ mol L}^{-1}$) (85:15, v/v); UV detection at 254 nm; column temperature, 25°C ; column hold-up volume marker, naphthalene. Eluent flow rates: 0.25 mL min^{-1} for titania Sachtapore®-NP columns (100 mm \times 2.1 mm I.D., $5 \mu\text{m}$, 100 Å and 300 Å) and zirconia ZirChrom®-PHASE column (100 mm \times 2.1 mm I.D., $5 \mu\text{m}$, 300 Å); 0.3 mL min^{-1} for HYPERSIL® SILICA (50 mm \times 4.6 mm I.D., $3 \mu\text{m}$, 120 Å).

We can conclude that for water contents lower than 50%, the retention of the set of N-methylated xanthines used in our study, as nitrogen-containing compounds, follows an adsorption pattern mainly governed by ligand-exchange on titania surface.

3.3. ANP chromatography on titania vs native silica and zirconia

3.3.1. Separation efficiency

The separation efficiencies observed on the chromatograms of N-methylated xanthines ($k > 0.5$) using 85% ACN in the mobile phase and formate- or acetate-based buffers ($5 \times 10^{-3} \text{ mol L}^{-1}$) appeared as low as 2000 plates m^{-1} at a flow rate of 0.2 mL min^{-1} (this flow correspond to the classical optimum flow observed in RP mode with a 2.1 mm internal column diameter with a $5 \mu\text{m}$ particle diameter). Changing the salt concentrations in the mobile phase had no major effects on the overall separation efficiencies of the test solutes. Flow rates lower than 0.2 mL min^{-1} mildly increased the chromatographic efficiency up to 10,000 plates m^{-1} at 0.02 mL min^{-1} , highlighting therefore the slow desorption kinetics of test solutes between the titania surface and the mobile phase (i.e. a large C-term of the Van Deemter equation). The slow process, which highly contributed to band broadening on the chromatograms of N-methylated xanthines, supports the theory of their ligand-exchange on titania. Moreover, using the same mobile phase conditions on native zirconia (i.e. 85% ACN and ammonium-formate or acetate buffers at $5 \times 10^{-3} \text{ mol L}^{-1}$), band broadening and low efficiencies were also observed for this set of xanthine derivatives. This observation attests that both native titania and zirconia hold comparable surface properties and hence similar chromatographic behavior (i.e. mixed-mode retention mechanism). In contrast, xanthine derivatives exhibited a totally different behavior on native silica considering the same mobile phase conditions. For example, using a column geometry of 4.6 mm I.D. packed with silica particles of $3 \mu\text{m}$ with a total porosity of 0.74, low peak dispersions and chromatographic efficiencies as high as about 80,000 plates m^{-1} were recorded at 0.3 mL min^{-1} . The behavior observed on native silica compared to native titania and zirconia is only related to the absence of Lewis acid surface sites on silica.

3.3.2. Retention and selectivity of N-methylated xanthines

The retention factors of N-methylated xanthines in aqueous normal phase chromatography on two titania columns which differ by their mean pore size distribution (100 \AA and 300 \AA) were compared to those of native silica and zirconia using (85:15, v/v) ACN/ammonium formate ($5 \times 10^{-3} \text{ mol L}^{-1}$) as mobile phase (Fig. 6). For titania based columns, the retention factors decreased with increasing mean pore size due to the modification of the specific surface area of the support and the selectivity was similar on both columns. Compared to silica, higher chromatographic retentions were found on native titania owing to additional ligand-exchange interactions which are absent on the surface of silica.

The retention of xanthines has been normalized for each column to the retention of 7MX (the most retained compound on both titania and zirconia columns) in order to make a selectivity comparison (Fig. 6b). Significantly different retention mechanisms still point out for both titania and zirconia and the great ability of native titania to recognize isomeric positions between the set of mono- and di-N-methylated xanthines originates from the mixed-mode interaction mechanism (i.e. adsorption and ligand-exchange) that was identified in aqueous normal phase chromatography.

Based on the comparative study between unmodified silica, titania and zirconia, native titania appear to be more suited to sample preparation due of its high specificity rather than to its analytical separation efficiency (the high solute selectivity can however justify the application of titania for specific analytical separations).

4. Conclusions

The retention of N-methylated xanthines exhibited a mixed-mode adsorption/ligand-exchange on titania in aqueous normal phase chromatography. It was shown that increasing the buffer concentration decreased the retention factors of a series of N-methylated xanthines. The buffer type had also a great influence on the chromatographic retention and on the selectivity on bare titania. The separation efficiency in ANP chromatography on native titania was similar to that observed on zirconia. Moreover, titania offered a superior recognition ability of isomeric positions between the set of mono- and di-N-methylated xanthines than native zirconia and silica.

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