Preparation and characterization of a poly(methyloctadecylsiloxane) thermally immobilized onto zirconized silica stationary phase for high-performance liquid chromatography

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ABSTRACT

This study describes the preparation and characterization of a HPLC stationary phase (SP) obtained by deposition of poly(methyloctadecylsiloxane) (PMODS) onto the surface of porous zirconized silica particles, followed by thermal immobilization. The SP were characterized using elemental analysis and infrared and ^{29}Si NMR spectroscopies, while their chromatographic behaviors were investigated by separation of selected neutral, acidic and basic compounds of different test mixtures (Engelhardt, SRM 870 and Tanaka tests). The preparation of the metalized support and the thermal immobilization of PMODS onto zirconized silica, optimized by experimental design, produced a SP based on PMODS that showed high values of efficiency (up to 85 000 plates/m) and appropriate asymmetry factors. The presence of zirconium on the surface significantly decreases silica solubility in severe conditions (alkaline mobile phase at pH 10 and high temperature), increasing column lifetime.

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1. Introduction

In many analytical laboratories worldwide, high-performance liquid chromatography (HPLC) has become an indispensable technique for the analysis of samples, the determination of physical constants and the isolation of purified components from complex mixtures. Today HPLC is the most popular technique for the qualitative and quantitative analysis of a wide range of compounds and the popularity of this technique is undoubtedly due to its versatility [1–3]. Reversed-phase (RP) separations are by far the most common mode in HPLC, due to advantages that include the use of gradient elution, compatibility with aqueous samples, versatility of retention mechanisms, allowing changes in the separation to be brought about by changes in pH, organic modifier or additives, and the use of less noxious and less expensive mobile phases, as well as the extensive literature about the technique [4,5].

Increasing stability and reducing the high activity of residual groups from stationary phases (SP) have been the main focuses of research on the preparation of reversed-phase high-performance liquid chromatography (RP-HPLC) phases over the last four decades [6]. These developments are focused principally on the novel selectivities of SP that are required for the separation of complex mixtures, and on improving the peak shapes of basic compounds, which remains a challenge in developing SP for HPLC [7].

Silica is the most widely used material for column packing supports in RP-HPLC. However, silica supports and silica-based stationary phases present critical problems: moderate to severe peak tailing in the chromatography of basic compounds and limited pH stability in acidic (pH < 2) or basic (pH > 9) media often recommended for the analysis of pesticides and pharmaceuticals [8,9]. In acidic media (pH < 2) hydrolysis of the Si—C bonds responsible for attachment of the silane agent to the silica surface occurs, resulting in stationary phase degradation. In basic media (pH > 9) silica dissolution is accelerated, causing the destruction of the silica backbone of the stationary phase [1,10]. Thus, RP-HPLC column technologies focus on innovations to improve selectivity, efficiency, chemical stability and better asymmetry for the analysis of basic compounds.

An interesting alternative is the procedure of reacting the silica support with zirconium [11–14] or titanium [15–17] oxides, combining the chemical stability of zirconia or titania with the excellent chromatographic properties of the silica chromatographic support. Although the chemical properties of these metalized supports are different, they have proven to be excellent for immobilization of a polysiloxane layer over their surfaces. Metalized supports have also proven to produce good chemically bonded C18 phases [18]. SP based on zirconized silica have demonstrated better stability and chromatographic performance than similar stationary phases based on unmodified silica in stability tests using both neutral and

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Table 1
Conditions for thermal immobilization (for the variables time and temperature) evaluated by the central composite design with the respective chromatographic parameters for each procedure.

Experiment	Variables			Chromatographic parameters				%C	τ (nm)
	Temperature (°C)	Time (h)	N/m[a]	As[a]	As[b]	k[a]	Rs[c]		
1	80	8	46,300	1.6	2.9	3.3	4.7	17.2	0.77
2	120	8	48,900	1.6	2.6	4.3	4.0	19.2	0.89
3	80	16	85,400	1.2	4.1	3.5	5.1	15.7	0.95
4	120	16	89,900	1.1	2.1	5.0	5.7	19.8	1.01
5	100	12	52,400	1.8	3.0	4.2	4.1	17.8	0.82
6	100	12	52,250	1.7	3.2	4.2	4.1	17.8	0.82
7	100	12	52,650	1.8	3.4	4.1	4.1	17.7	0.81
8	72	12	9500	0.7	4.1	3.5	2.8	18.6	0.66
9	128	12	60,900	1.3	2.6	4.6	4.7	18.4	0.87
10	100	6.4	78,350	1.0	2.8	3.8	5.1	18.8	0.91
11	100	17.6	18,300	1.8	2.3	4.3	5.1	18.2	0.86

[a] Calculated for the naphthalene peak.
[b] Calculated for the N,N-dimethylaniline peak.
[c] Calculated for the toluene–naphtalene pair.

alkaline mobile phases, mainly when more drastic conditions are applied [12,14].

An alternative to the development of procedures based on the chemical attachment of silylant agents to the silica surface is represented by the preparation of stationary phases based on the immobilization of pre-synthesized organic polymers onto silica or metalized-silica surfaces [6]. Immobilized polymer stationary phases have advantages that make them attractive for use in RP-HPLC, such as better protection of residual silanols (silica) or Lewis acidic sites (zirconols and titanols) compared to covalent bonded SP, with more effective protection of the support matrix against the chemical attack of alkaline mobile phases, as well as an easy preparation procedure [6,18].

The aim of this paper is to describe the preparation and characterization of new SP for HPLC based on the polymer poly(methyloctadecylsiloxane), PMODS, thermally immobilized onto zirconized silica, with focus on the optimization of the thermal immobilization, using a central composite design and response surface methodology [19,20], evaluating the physical–chemical and chromatographic properties and chemical stability, and potential applications of the new SP. The choice of polymer is based on the significant presence and wide application of octadecyl (C18) groups with commercially available SP.

2. Experimental

2.1. Materials and methods

Methanol (99.9%), chloroform (99.8%), acetonitrile (99.9%) and n-hexane (95%) were from Tedia (Fairfield, USA). Toluene (99.9%) was from J.T. Baker (Phillipsburg, USA). The mobile phases were prepared with ultrapure water from a Millipore Direct-Q™ system (Billerica, USA). The reagents used to prepare the mobile phases: KH_2PO_4 (98%), K_2HPO_4 (99%), H_3PO_4 (85%) and $KHCO_3$ (99.7–100.5%) were from Synth (Diadema, Brazil). Trifluoroacetic acid (99.5%) (TFA) was from Vetec (Duque de Caxias, Brazil), and K_2CO_3 (>99.0%) was from Merck (Darmstadt, Germany).

Kromasil silica, from Akzo Nobel (Bohus, Sweden), with a mean particle size of 5 μm, mean-pore diameter of 11.1 nm and a specific surface area of 310 m²/g was used as support for preparation of the SP. Zirconium tetrabutoxide (80%, w/w) and powdered ZrO_2 (>99%) were purchased from Aldrich (Steinheim, Germany). The PMODS polymer, average molar mass (Mw) 11 000, was obtained from United Chemical Technologies (Bristol, USA).

The test solutes were acetophenone (>99%) and naphthalene (>98.5%) from Vetec, benzene (99%) from Synth, uracil (98%), acenaftene (99%), amitriptyline hydrochloride (99%),

phenylbenzylimidazol sulfonic acid (96%), butylbenzene (>99%), benzophenone-3 (2-hydroxy-4-methoxy-benzophenone) (98%) and octocrylene (2-ethylhexyl-2-cyano-3,3-diphenylacrylate) (97%) from Aldrich (Milwakee, USA), benzylamine (>99%), pentyl-benzene (>98%) and o-terphenyl (>99%) from Merck-Schuchardt (Hohenbrunn, Germany), ethylbenzene (99%), nortriptyline hydrochloride (98%) and quinizarin (96%) from Aldrich (Steinheim, Germany), caffeine (>98.5%), theobromine (99%) and theophylline (99%) from Acros Organics (Geel, Belgium), phenol (>99.5%), N,N-dimethylaniline (>98%) and triphenylene (>98.0%) from Fluka (Buchs, Switzerland), aniline (99.5%) from Merck (Gernsheim, Germany), 4-methylbenzylidene camphor and octylmethoxycin-namate from AccuStandard (New Haven, USA). Fluoxetine and diazepam were kindly donated by Dr. Paulo César Pires Rosa of EMS (Hortolândia, Brazil). The commercial column (150 mm × 3.9 mm) used for comparisons was a bonded and end capped Nova-Pak C18 phase from Waters (Milford, USA). The SP characteristics are: particle size: 4 μm, 7% carbon content and 120 m²/g of specific surface area.

2.2. Synthesis of porous zirconized silica particles (Si–Zr)

Porous zirconized silica particles were synthesized using the optimized procedure described by Faria et al. [13,14]. For each synthesis 2.5 g of chromatographic silica was added to a solution containing 7.1 g of zirconizing reagent, zirconium (IV) tetrabutox-ide, dissolved in 20 mL of anhydrous toluene. This mixture was placed in a thermostated bath for 5 h 30 min at 40 °C. After this period, the solution was centrifuged for 15 min, the supernatant was discarded and the resulting solid washed with anhydrous toluene and again centrifuged. This washing was repeated five times. The material was hydrolyzed with 15.0 mL of 10^{-3} mol/L aqueous HNO_3 and centrifuged for 15 min. The solid was washed with 15.0 mL of deionized water, centrifuged for 10 min, and then dried in an oven at 120 °C for 12 h. The material obtained after one zirconization procedure was submitted to a second zirconization, using the same procedure, which improved the % Zr by approximately 2–3%.

2.3. Preparation of the stationary phases

Stationary phases were prepared with a PMODS loading of 1.25 g PMODS/g Si–Zr. A 10% (w/v) solution of PMODS in n-hexane was added to the appropriate quantity of zirconized silica, previously dried at 140 °C for 12 h. This mixture was slowly stirred at room temperature for 3 h, and then placed in a fume hood for evaporation of the solvent at room temperature (at least 6 days). Si(PMODS)

stationary phase was prepared by the same procedure, replacing zirconized silica with a bare silica support.

2.4. Optimization of thermal immobilization

To optimize the immobilization of the poly(methyloctadecylsiloxane)-coated zirconized silica stationary phases, Si–Zr(PMODS), a central composite design (CCD) with response surface methodology (RSM) was used. A full 2^2 factorial design evaluated efficiencies and asymmetry factors, as functions of the variation of time and temperature. Portions of 2.5 g of SP obtained after solvent evaporation were placed individually in an oven at the specified conditions as summarized in Table 1. The immobilizations were performed under an air atmosphere, in duplicate. Immobilization of PMODS onto bare silica support used the same procedure, with a temperature of 120 °C for 16 h.

2.5. Solvent extraction of excess polymer

The excess of PMODS was extracted after each thermal immobilization. The tubes containing the SP were connected to a Waters 510 pump (Milford, USA) for extraction of non-immobilized PMODS by passing hexane at 0.5 mL/min for 3 h at 50 °C. The SP was dried with nitrogen for 3 h. An illustration of the synthesis process of the Si–Zr(PMODS) SP is shown in Fig. S1 of the Supplementary Material.

2.6. Column packing

Columns (60 mm × 3.9 mm id) were made from type 316 stainless steel tubing. The internal surface was polished using a technique developed in our laboratory [21]. The columns were slurry packed using 5% (w/v) slurries of the SP in chloroform. A constant packing pressure of 38 MPa (Haskel packing pump, Burbank, USA) was used with methanol as propulsion solvent. The pressure was maintained until the passage of 200 mL methanol to assure good packing and removal of excess polymer. Columns were conditioned for 3 h with 80:20 (v/v) methanol–water mobile phase at 0.5 mL/min prior to the chromatographic tests.

2.7. Physical–chemical characterization of zirconized silica support and SP

2.7.1. X-ray fluorescence (XRF)
The quantity of zirconium incorporated onto silica was determined by XRF using a Shimadzu model XRF-700 spectrometer (Kyoto, Japan) with micro sample holders. The analytical curve was obtained using mechanical mixtures of pure silica (5 μm) and powdered ZrO_2, containing from 4 to 22% (w/w) Zr.

2.7.2. Fourier transform infrared (FTIR) spectroscopy
Chemical bonding of the oxides on the zirconized silica support and the presence of PMODS on SP were investigated with a Bomem MB-102 FTIR spectrometer (St. Laurent, Canada). The samples were prepared using potassium bromide (KBr) pellets with a ratio of 1:10. The FTIR spectrum background was corrected using a freshly prepared pure KBr pellet. The IR spectral range was evaluated from 400 to 4000 cm^{-1}, scan rate of 20 scans/min and resolution of 4 cm^{-1}.

2.7.3. Surface area and porosity—BET/BJH
Full adsorption–desorption isotherms of nitrogen at −195.8 °C on dried samples were measured at various partial pressures in a Micromeritics model ASAP-2010 apparatus (Norcross, USA). Specific surface areas (S_{BET}) and pore-size distributions (PSD) were determined with the Brunauer–Emmett–Teller (BET) and Barret–Joyner–Hallenda (BJH) methods, respectively. BET surface areas were obtained from the adsorption data points whereas the PSD were derived from the desorption isotherms. For BET/BJH measurements, the samples were previously degassed for 3 h at 120 °C in an out-gassing station of the adsorption apparatus.

2.7.4. Thermogravimetric analysis
The thermal stability of the zirconized silica support and Si–Zr(PMODS) SP were studied using samples of approximately 5 mg, heating from 25 to 1000 °C at 10 °C/min in an air atmosphere, using a TA model TGA-2050 instrument (New Castle, USA).

2.7.5. Solid-state ^{29}Si CP-MAS NMR spectroscopy
Solid-state ^{29}Si NMR measurements, using cross polarization and magic angle spinning (CP-MAS), were performed on a Bruker AC 300 NMR (Rheinstetten, Germany). A frequency of 59.6 MHz was used and the contact time and pulse interval time were 5 ms and 1.5 s, respectively. Approximately 300 mg were spun at 4 kHz using 7 mm double ZrO rotors. Typically, 1.5 k free induction decays (FIDs) with an acquisition time of 35 ms were accumulated in 1 kb data points, zero-filling to 8 kb prior to Fourier transformation. The line broadening was 30 Hz and the spectral width for all spectra was about 25 kHz.

2.7.6. Elemental analysis
The Si–Zr(PMODS) SP (after the immobilization procedure) were submitted to elemental analysis for determination of % C. Approximately 10 mg of each extracted stationary phase sample were analyzed on a Model CHN-2400 Perkin-Elmer Analyzer (Shelton, USA). From these data, the mass of PMODS per gram of zirconized silica, representing the specific mass of polymer, was calculated using the formula: m PMODS = %C/(63.2 − %C), since 63.2% of the PMODS refers to carbon. For the calculation of layer thickness (τ) the equation: $\tau = -(\sqrt{d^2 - Fd^2} - d/2)$, was used, where F is the immobilized fraction ($F = m_{immobilized}/m_{fullpores}$) and d is the mean pore diameter. For this calculation it is assumed that the pores of the silica have a constant diameter and that the immobilized PMODS is characterized by a layer of constant thickness on the pore walls [22]. The specific pore volume (v_p) of zirconized silica support is 0.72 mL/g and the density of PMODS is 0.89 g/mL. From this pore volume, the mass of polymer ($m_{fullpores} = v_p \times \rho_{PMODS}$) that fills the pore system is 0.64 gPMODS/gSi–Zr.

2.8. Chromatographic evaluation

The chromatographic evaluations were performed using a modular HPLC system from Shimadzu (Kyoto, Japan) equipped with a LC-10AD liquid chromatographic pump, a SPD-10A UV–VIS (254 nm) detector with 8 μL cell, a CTO-10A column oven and a Rheodyne 8125 injector (Cotati, USA) with 5 μL loop. All data were acquired and processed using Chrom Perfect for Windows, version 3.52 and Report-Write Plus software from Justice Innovations (Mountain View, USA). The separations were carried out at room temperature with flow rates of 0.3 mL/min, the optimal flow rate determined from a van Deemter curve.

To evaluate the best immobilization conditions (based on temperature and time), two test mixtures were used. The first was prepared with neutral compounds with different polarities: acetophenone (200 mg/L), benzene (1700 mg/L), toluene (1700 mg/L) and naphthalene (129 mg/L), and the second was prepared with acidic, basic and neutral compounds: phenol (220 mg/L), N,N-dimethylaniline (40 mg/L), naphthalene (129 mg/L) and acenaphthene (200 mg/L). The column dead time, t_M, was determined using uracil (12 mg/L) as the unretained compound. The mobile phase was methanol/water (70:30, v/v) and was prepared volumetrically from previously filtered individually measured amounts of each solvent and degassed before use. Chromatographic

performance was evaluated by means of efficiency (N/m), retention factor (k), resolution (Rs) and asymmetry factor (As) measured at 10% of the peak height.

The optimized SP was also chromatographically characterized with the Engelhardt [23,24], Tanaka [25] and SRM 870 [26,27] tests.

2.8.1. Engelhardt test

The Engelhardt test [23] is used to evaluate three important parameters in stationary phases: hydrophobic interaction (measured by the retention factors for ethylbenzene (k_E) and toluene (k_T), hydrophobic selectivity (measured by the retention factor ratio between ethylbenzene (k_E) and toluene (k_T)) and silanol activity (measured by the asymmetry factors of basic solutes (aniline, As_A and N,N-dimethylaniline, As_N) and the asymmetry factor ratio between aniline and phenol (As_A/As_P)). The test was carried out at a column temperature of 40 °C with a 55:45 (v/v) methanol–water mobile phase and detection at 254 nm.

2.8.2. Tanaka test

The Tanaka test [25] evaluates six parameters, using four different chromatographic conditions. The retention factor for pentylbenzene (k_{PeB}) reflects the ligand density. The hydrophobicity or hydrophobic selectivity (α_{CH_2}), measured by the retention factor ratio between pentylbenzene and butylbenzene, reflects the surface coverage of the phase, since these alkylbenzenes are differentiated by one methylene group. The shape selectivity ($\alpha_{T/O}$) is measured by the retention factor ratio between triphenylene and o-terphenyl, which is influenced by the relative rigidity of their structures. The hydrogen bonding capacity ($\alpha_{C/P}$) was evaluated by the retention factor ratio between caffeine and phenol, which is a measure of the number of available silanol groups and the degree of end capping. The total ion-exchange capacity ($\alpha_{B/P(pH\ 7.6)}$) is measured by the retention factor ratio between benzylamine and phenol at pH 7.6, which is an estimate of the total silanol activity. The ion-exchange capacity in acidic media ($\alpha_{B/P(pH\ 2.7)}$), measured by the retention factor ratio between benzylamine and phenol at pH 2.7, is an estimate of the acid activity of the silanol groups. Other parameters were also considered based on the asymmetry factors of caffeine in an unbuffered mobile phase and of benzylamine at pH 2.7 and 7.6 (As_C, $As_{B2.7}$ and $As_{B7.6}$). For the evaluations, four different conditions were applied: k_{PeB}, α_{CH_2}, $\alpha_{T/O}$ and N_{PeB} were evaluated using a 80:20 (v/v) methanol–water mobile phase; $\alpha_{C/P}$ and As_C were evaluated using a 70:30 (v/v) methanol–water mobile phase; $\alpha_{B/P}$ (pH 7.6) and $As_{B7.6}$ were evaluated using a 70:30 (v/v) methanol–phosphate buffer (pH 7.6; 20 mmol/L) mobile phase; $\alpha_{B/P}$ (pH 2.7) and $As_{B2.7}$ were evaluated using a 70:30 (v/v) methanol–phosphate buffer (pH 2.7; 20 mmol/L) mobile phase. Detection was at 254 nm and column temperature was 40 °C.

2.8.3. SRM 870 test

The SRM 870 test [26] evaluates four parameters: retention factors for toluene (k_T) and ethylbenzene (k_E), which reflect hydrophobic retention, methylene selectivity ($\alpha_{E/T}$) and efficiency (N_E and N_T), the asymmetry and tailing factors of quinizarin (As_Q and Tf_Q), which indicate activity toward metal chelators, and the asymmetry and tailing factors of amitriptyline (As_{am} and Tf_{am}), which indicate the silanol activity of the stationary phase. The mobile phase used for this test was 80:20 (v/v) methanol–phosphate buffer (pH 7; 20 mmol/L), with detection at 254 nm at a column temperature of 23 °C.

2.9. Stability testing

The optimized SP based on PMODS thermally immobilized onto a zirconized silica support was submitted to stability testing with basic (pH 10) and acidic (pH 1.7) mobile phases, to simulate the performance of SP under drastic conditions of pH. The stability was compared to that of a phase using PMODS thermally immobilized onto unmodified silica.
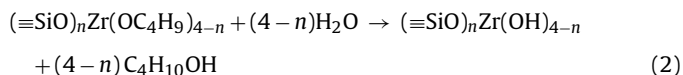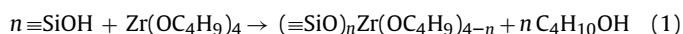
For the stability test in an alkaline mobile phase methanol:0.05 mol/L carbonate buffer (70:30, v/v) was used. The pH was adjusted to 10 using a calibrated pH meter before addition of methanol. The flow rate through the column was 0.5 mL/min at 50 °C. The column was periodically tested for the separation of a mixture consisting of uracil, phenol, N,N-dimethylaniline, naphthalene and acenaphthene. The conditions used in this test accelerate column deterioration and it is possible to obtain faster evaluations using considerably less mobile phase and instrument time [14,28].

For the stability test in an acidic mobile phase 50:50 (v/v) methanol–0.2% trifluoroacetic acid was used. The flow rate through the column was 0.5 mL/min at 50 °C. The column was periodically tested for the separation of a mixture of ethylbenzene, quinizarin and amitriptyline to evaluate column deterioration in acidic conditions.

3. Results and discussion

3.1. Synthesis and characterization of porous zirconized silica particles (Si–Zr)

The silanol groups on the surface react with zirconium(IV) tetrabutoxide, as represented by reactions (1) and (2) [13]:

$$n \equiv SiOH + Zr(OC_4H_9)_4 \rightarrow (\equiv SiO)_n Zr(OC_4H_9)_{4-n} + n\,C_4H_{10}OH \quad (1)$$

$$(\equiv SiO)_n Zr(OC_4H_9)_{4-n} + (4-n)H_2O \rightarrow (\equiv SiO)_n Zr(OH)_{4-n}$$
$$+ (4-n)C_4H_{10}OH \quad (2)$$

where $n \equiv SiOH$ refers to silanol groups on the silica surface.

The XRF spectrum of zirconized silica particles indicates the incorporation of Zr onto the silica support by the appearance of peaks at 15.76 keV and at 17.64 keV, corresponding, respectively, to the Kα and Kβ peaks of the metal. The analytical curve, constructed using the intensities of the X-ray fluorescence peak of zirconium (15.7 keV) versus its concentration (in percentage) for each standard, is represented by: $I_{K\alpha} = 21.8\%$ Zr−29.3 with $R = 0.9934$ and was used to determine the concentration of Zr incorporated on the support. The concentration of Zr on the silica support was 21.5%. The XRF spectrum of zirconized silica is shown in Fig. S2 of the Supplementary Material.

3.2. Optimization of the thermal immobilization of the SP

Thermal immobilization of the SP was carried out using the conditions indicated in Table 1. The intervals of time and temperature were based after the execution of univariate tests and on the thermal stability of the PMODS, up to 175 °C, determined by thermogravimetric analysis. Chromatographic parameters for the Si–Zr(PMODS) immobilized under different conditions are summarized in Table 1. Better chromatographic performances were obtained for the SP produced in experiments 3 and 4, where columns with high efficiencies (higher than 85 000 N/m) and symmetrical peaks were obtained for naphthalene, a neutral compound. For the basic compound, N,N-dimethylaniline, better asymmetry was obtained in experiment 4 (see Fig. S3 of the Supplementary Material). The highest carbon percentage was obtained in the experiments with higher immobilization temperatures. As a consequence of the percent carbon, the polymeric film thickness (τ) of these SP was approximately 1.0 nm, which corresponds to a monolayer coating of the support by the polymer. These results

suggest that the coating of the support is uniform and homogeneous, probably due a better distribution of the polymer onto the chromatographic support, which results in reduced interactions between the basic compound and the residual silanol groups. On the other hand, experiments in which lower immobilization temperatures and/or shorter immobilization times were used resulted in SP with poorer chromatographic performances, some without complete separation of the compounds, suggesting that the immobilization temperatures and times employed were not sufficient to distribute or immobilize the PMODS onto the chromatographic support. This results in a lower percent carbon on these SP and a polymer layer with less than 1.0 nm thickness. The repeatability of the procedure of immobilization was evaluated from the triplicate experiments performed at the center point (experiments 5, 6 and 7). The estimated RSD was 2.0% for column efficiency and 0.1% for polymer coating (in terms of percent carbon), results that are very satisfactory. Fig. 1 shows the response surface diagrams of efficiency and asymmetry factor for the naphthalene peak, used to determine the optimal region of both time and temperature to be used for the subsequent preparation of the optimized Si–Zr(PMODS). In order to represent the surface responses, quadratic models for both the efficiency and asymmetry factor were used, as given by:

$$\text{Efficiency}\,(N\ m^{-1} \times 1000) = 52.1(\pm 0.10) + 9.9x_1(\pm 0.07)$$
$$- 0.5x_2(\pm 0.07) - 1.9x_1^2(\pm 0.08) + 4.7x_2^2(\pm 0.08) + 0.5x_1x_2(\pm 0.09)$$

$$\text{Asymmetry}\,(As) = 1.76(\pm 0.03) + 0.10x_1(\pm 0.02) + 0.04x_2(\pm 0.02)$$
$$- 0.34x_1^2(\pm 0.02) - 0.14x_2^2(\pm 0.03) - 0.03x_1x_2(\pm 0.01)$$

The darkest zone of Fig. 1A corresponds to column efficiencies higher than 75 000 N/m. Higher column efficiencies were obtained when the immobilization step was carried out at higher temperatures and with longer times. By plotting a Pareto chart (Fig. 1C) for efficiency, it is possible to see that the immobilization process is more affected by variations in temperature. The same region in Fig. 1B corresponds to asymmetry factors between 0.9 and 1.5. These values of asymmetry factors were obtained when the immobilization was carried out at intermediate to high temperature. Again, these results are in agreement that temperature is more significant than time. The optimal region was determined by superposition of these two response surfaces, and the result obtained shows the optimal region for both immobilization time and temperature, which should result in SP with column efficiencies above 75 000 N/m and asymmetry factors for the naphthalene peak between 1.0 and 1.2. Thus, the thermal immobilization conditions were defined as heating at 120 °C for 16 h, which also gives good symmetry for the basic compound, N,N-dimethylaniline.

3.3. Physical–chemical characterization of the zirconized silica support and the optimized stationary phase

3.3.1. Surface area and porosity—BET/BJH
The surface area of the zirconized support (Si–Zr) presented specific surface area (S_{BET}) of 333 m^2/g, indicating that the optimal incorporation of Zr appears to slightly increase the surface area from that of the original silica that presented surface area (S_{BET}) of 306 m^2/g. The incorporation of the PMODS on the zirconized silica particles significantly decreased the surface area of the SP particles, which showed a value of 119 m^2/g. The process of zirconization and thermal immobilization of the polymer also reduced the pore volume, as represented by the values of 0.86 cm^3/g, 0.72 cm^3/g and 0.29 cm^3/g, for bare silica, the zirconized silica and SP, respectively. The average pore diameter showed similar reductions, with
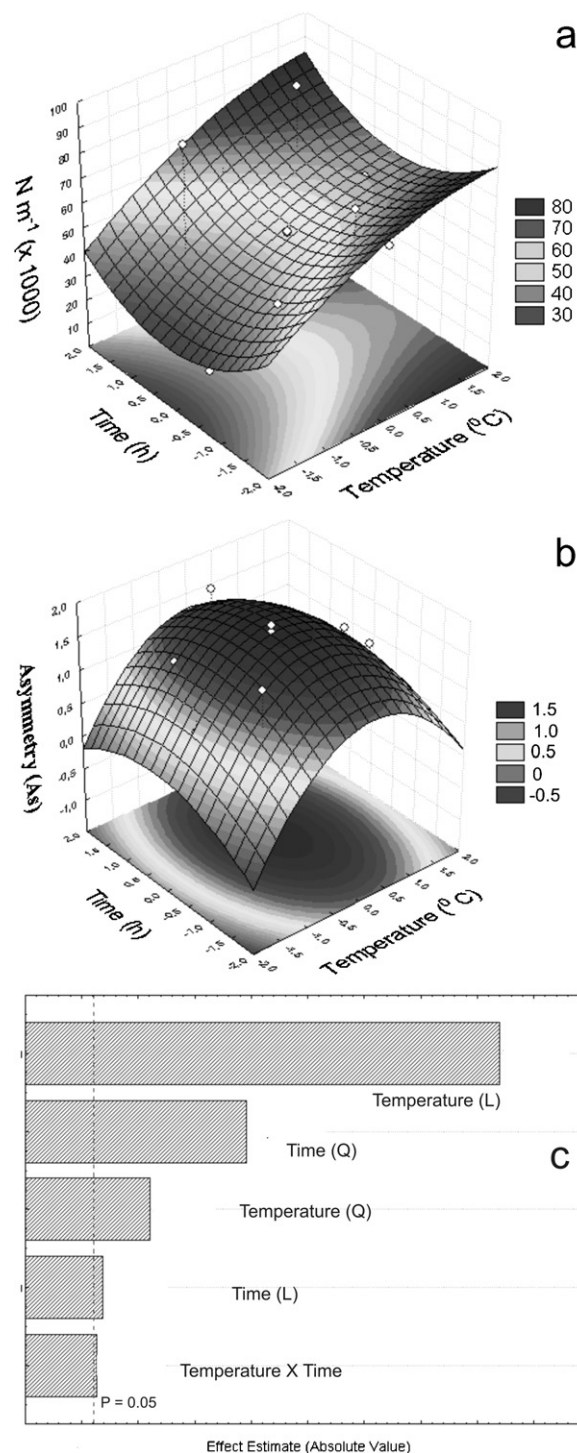

Fig. 1. Response surfaces for thermal immobilization of the PMODS onto silica: (a) efficiency, (b) asymmetry factor and (c) Pareto chart for efficiency.

values of 11.22 nm, 8.64 nm and 4.90 nm for the bare silica, zirconized silica and Si–Zr(PMODS), respectively. These observations indicate that the presence of zirconium on the silica surface and the posterior immobilization has not modified the overall silica structure.

3.3.2. Fourier transform infrared spectroscopy
Fourier transform infrared spectroscopy (FTIR) of the zirconized silica support and the optimized SP shows bands at 3477 and

1645 cm^{-1} due to OH stretching and to the bending vibrations of water in the samples [13]. The large absorption bands at 1099 cm^{-1} can be assigned to asymmetric stretching vibrations of the Si—O—Si bond [29,30]. The peaks at 790 and 460 cm^{-1} can be attributed to the presence of the ring structure formed by Si—O bonds in the silica network [30,31]. The presence of Zr was shown by the band at 1099 cm^{-1} and the presence of PMODS immobilized on zirconized silica was supported by appearance of a triplet in the region at 2900 cm^{-1} that is attributed to CH_2 and CH_3 stretching [32]. Fig. S4 of the Supplementary Material shows the FTIR spectra of the zirconized silica support and the optimized Si–Zr(PMODS).

3.3.3. Solid-state ^{29}Si CP-MAS NMR spectroscopy

The ^{29}Si CP-MAS NMR spectra for zirconized silica support and the optimized Si–Zr(PMODS) both show the Q^n species, where n is related to the number of Si—O—Si bonds, at −110, −101 and −95 ppm [33,34]. The Q^2 species is diminished in Si–Zr(PMODS) and the ratio of the Q^3/Q^4 species is also reduced, indicating that the total quantity of silanol groups was reduced after immobilization of PMODS. For the SP additional peaks at −20 ppm, belonging to $D^{2''}$, $D^{2'}$ and D_1^H groups indicate the formation of new silicon species, being attributed to PMODS adsorbed and chemically bonded onto the silica particles [14,33]. Fig. S5 of the Supplementary Material shows the ^{29}Si CP-MAS NMR spectra for the zirconized silica support and the optimized Si–Zr(PMODS).

3.4. Chromatographic characterization of the optimized stationary phase

The SP, Si–Zr(PMODS), obtained by optimization of the thermal immobilization, was chromatographically characterized with three mixtures, using the Engelhardt test [23,24], the Tanaka test [25] and the SRM 870 test [26].

3.4.1. Engelhardt test

The hydrophobic selectivity, obtained from the ratio of the retention factor of ethylbenzene (k_E) and toluene (k_T), $\alpha_{CH_2} = 3.2$, indicates that the SP has capacity for the separation of compounds that differ only by a methylene group.

The silanol activity, measured by the asymmetry factors of aniline and N,N-dimethylaniline (As$_A$ = 3.4 and As$_N$ = 5.4), and the elution order and asymmetry factor ratio of aniline and phenol (As$_A$/As$_P$ = 1.2) indicates that the SP also has significant silanol activity [23], although the ratio of the asymmetry factors of aniline and phenol is less than 1.3.

3.4.2. Tanaka test

The shape selectivity ($\alpha_{T/O}$), from the separation factor of non-planar o-terphenyl and highly planar triphenylene and the hydrophobic selectivity, from the separation factor for pentylbenzene and butylbenzene (α_{CH_2}) for Si–Zr(PMODS), shown in Fig. S6A of the Supplementary Material, give values of 1.3 and 1.2, respectively. The methylene selectivity (α_{CH_2}), which indicates that Si–Zr(PMODS) has capacity for the separation of compounds that differ only by a methylene group, is comparable to those of polymeric, chemically bonded and hybrid C18 stationary phases [35–37], while the steric selectivity ($\alpha_{T/O}$) indicates that the Si–Zr(PMODS) has the capacity to differentiate compounds that differ only by their spatial configuration, with elution order typical of octadecylsilica (ODS) polymeric phases, where the non-planar molecule of the polycyclic aromatic hydrocarbon o-terphenyl (O) elutes faster than the planar molecule of triphenylene (T), since polymeric phases have a "slit-like" structure on the surface [38]. On the other hand, the value of $\alpha_{T/O}$ obtained is lower than expected for polymeric C18 SP [39,40]. This result may be a consequence of the

good distribution of the polymer on the zirconized silica support [41].

The residual silanol groups on the silica surface are measured by the hydrogen bonding interaction between Si–Zr(PMODS) and a mixture of caffeine and phenol. The elution of these two compounds (Fig. S6B of the Supplementary Material) with caffeine after phenol ($\alpha_{C/P}$ = 1.4) indicate that there are some residual silanol and/or zirconol groups on the Si–Zr(PMODS). This value is somewhat higher than the values of commercial SP [36,37], suggesting that an endcapping process could reduce residual silanol groups on Si–Zr(PMODS).

The presence and acidity of the accessible silanol and zirconol groups was evaluated using buffered mobile phase at two different pH (2.7 and 7.6) with a basic solute, benzylamine (pK 9.4). At pH 7.6 (Fig. S6C of the Supplementary Material), the silanol groups are dissociated while benzylamine is protonated, favoring ion-exchange interactions. The result of $\alpha_{B/P(7.6)}$ = 0.8 shows that the Si–Zr(PMODS) presents low ion exchange properties with this mobile phase and that benzylamine elutes with a symmetrical peak (As = 1.3). At pH 2.7 (Fig. S6D of the Supplementary Material), silanol groups are not dissociated while the benzylamine is protonated with a positive charge. The result ($\alpha_{B/P}$ = 2.1) at this pH is similar to C18 SP without endcapping, that present $\alpha_{B/P}$ of about 2.5 [36]. Since the retention for the basic probe (benzylamine) was not irreversible, strong interactions of this basic solute (with residual silanols or zirconols) do not occur, and Si–Zr(PMODS) should presents low ion exchange capacities in both acidic and alkaline mobile phases. This fact also suggests that even though the presence of zirconium on the silica surface, which increases the acidity of adjacent silanols and consequently increases the interaction intensity between basic compounds (such as benzylamine) and the chromatographic support, did not affected the retention of basic solutes, confirming that the thermal immobilization process resulted in good performance with satisfactory polymer coverage on the SP.

3.4.3. SRM 870 test

The retention factor for ethylbenzene (k_E = 0.8) indicates that Si–Zr(PMODS) has hydrophobicity higher than most commercially available SP [27]. The asymmetry and tailing factors of quinizarin (As$_Q$ = 2.7 and Tf$_Q$ = 2.6) indicate high activity toward metal chelators. The asymmetry and tailing factors of amitriptyline (As$_{am}$ = 1.6 and Tf$_{am}$ = 1.4) indicate, on the other hand, that Si–Zr(PMODS) presents only moderate silanol activity toward this basic compound and, thus, should be able to separate basic pharmaceutical compounds such as antidepressants and their metabolites with structures similar to amitriptyline, as shown by the separation of some psychotropic drugs with better asymmetry than a commercial C-18 SP using a buffered mobile phase (Fig. 2).

3.5. Stability testing

3.5.1. Basic mobile phase

The optimized Si–Zr(PMODS)SP was submitted to continual passing of a pH 10 mobile phase (MeOH:0.05 mol/L K_2CO_3, 70:30, v/v) at 0.5 mL/min at a temperature of 50 °C, with periodic evaluation in the same mobile phase, as shown in Fig. 3. The chromatographic performance was compared to a SP with PMODS thermally immobilized onto silica without zirconization (unmodified silica), using the same immobilization conditions. The column stabilities were evaluated in terms of efficiency, asymmetry and retention factor for the acenaphthene peak. The mobile phase volume that passed through the column is expressed in column volumes and was calculated from the retention time of the unretained compound (uracil), V_C = 0.57 mL. It is clear that the SP with PMODS immobilized onto unmodified silica (Si-PMODS) failed rapidly. It
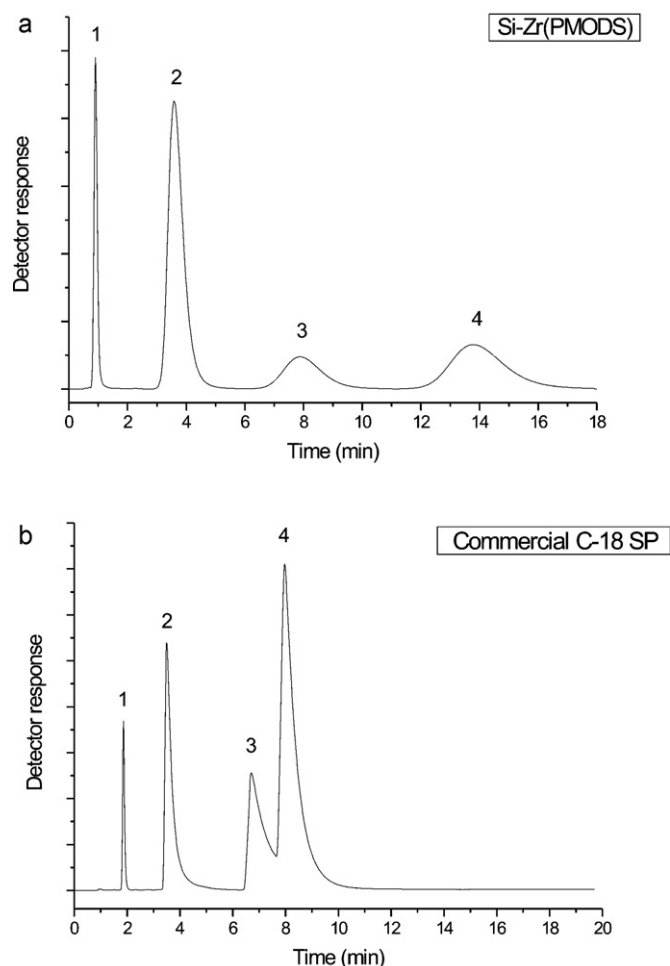
Fig. 2. Chromatogram of four psychotropic drugs on (a) Si–Zr(PMODS) stationary phase (60 mm × 3.9 mm, 5 μm) and (b) commercial (150 mm × 3.9 mm, 4 μm) C18 stationary phase. Mobile phase: methanol: 0.020 mol/L K_2HPO_4/KH_2PO_4 (80:20, v/v) at pH 7.5; flow rates: 0.8 mL/min for (a) and 1.2 mL/min for (b); injection volume: 5 μL; detection at 220 nm, temperature: 23 °C. Peak identifications: (1) diazepam, (2) amitriptyline, (3) fluoxetine, and (4) nortriptyline.

Fig. 3. Stability tests of the PMODS thermally immobilized onto zirconized silica and onto unmodified silica in terms of (a) efficiency and (b) asymmetry factor. Mobile phase: methanol: 0.05 mol/L K_2CO_3/$KHCO_3$ (70:30, v/v) at pH 10 and 50 °C. Test solute: acenaphthene. Detection at 254 nm.

was not possible to observe an efficiency plateau (Fig. 3A). The new Si–Zr(PMODS) phase presented a longer plateau, resisting more than 1215 column volumes (Fig. 3A), a column volume equivalent to or higher than that observed with many commercial SP [28] and equivalent to another SP with a zirconized silica support having an immobilized C_{14} polymer [14]. This indicates that the presence of zirconium significantly increases the column lifetime due to higher protection of the silica support in alkaline mobile phases. Fig. 3B shows that the asymmetry factor increase correlates quite well with the efficiency loss for both columns. The retention factors remained almost unaffected throughout the duration of the tests, indicating that the SP was not hydrolyzed in these basic conditions. In addition, the percentage of carbon before and after the test remained practically constant, implying that the efficiency loss is mainly related to silica dissolution, while the loss of PMODS from the column is minimal.

3.5.2. Acidic mobile phase

The stability of the Si–Zr(PMODS) SP with an acidic mobile phase (0.2% trifluoroacetic acid) was carried out until the passage of 10 000 column volumes (V_c = 0.57 mL) at 50 °C. The retention factor of amitriptyline fell by 27%, with minor changes in efficiency and peak shape at the end of the test. With the Si-PMODS SP the
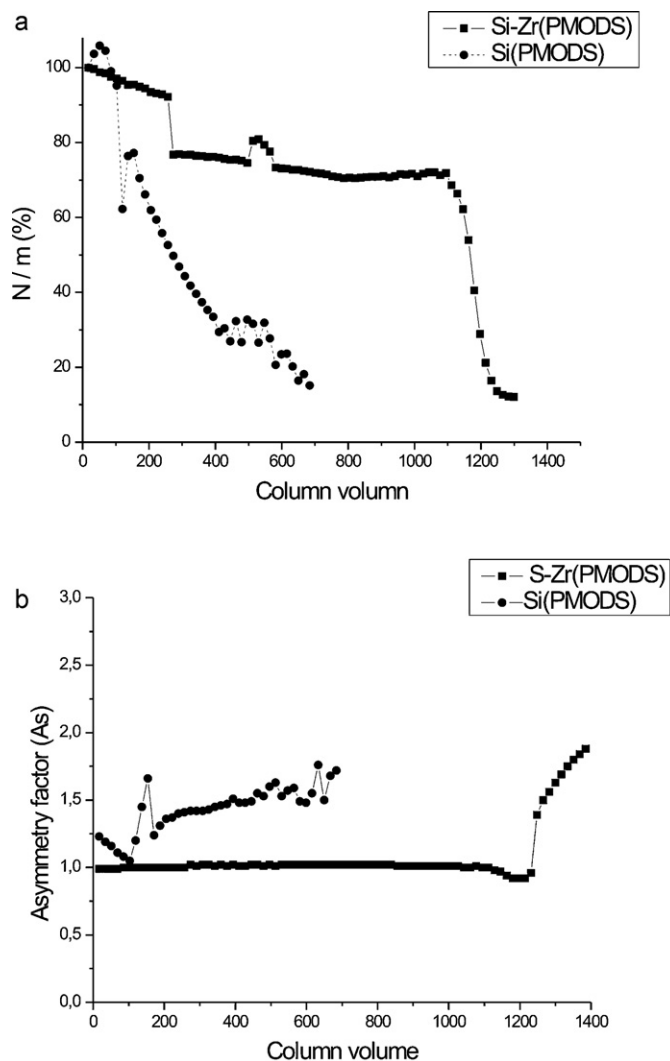
retention factor of amitriptiline fell by 20%. The good stabilities of both stationary phases at low pH, even at high temperature, are probably due to the fact that the cleavage of the Si—C bond is more difficult than with chemically bonded phases due to steric hindrance and that silica is highly stable in most acid media [5].

3.6. Applicability of the new SP

Methylxanthines are considered difficult to separate on many reversed-phase columns and selection of an adequate analytical column for the separation of these compounds is problematic [42]. Their separations have been studied using titania monoliths in nano-liquid chromatography (nano-LC) with buffered mobile phase [43], a bare titania column and aqueous normal phase [44] or zirconia capillary monoliths and hydrophilic-interaction chromatography (HILIC) [45]. Fig. 4 compares the Si–Zr(PMODS) column and a commercial C18 column for the separation of three methylxanthines (theobromine, theophylline and caffeine) found in tea, coffee, cocoa and other foods, using separation with gradient elution. In spite of using of a short column, a suitable separation (Fig. 4A) with good resolution and peak symmetries was obtained.
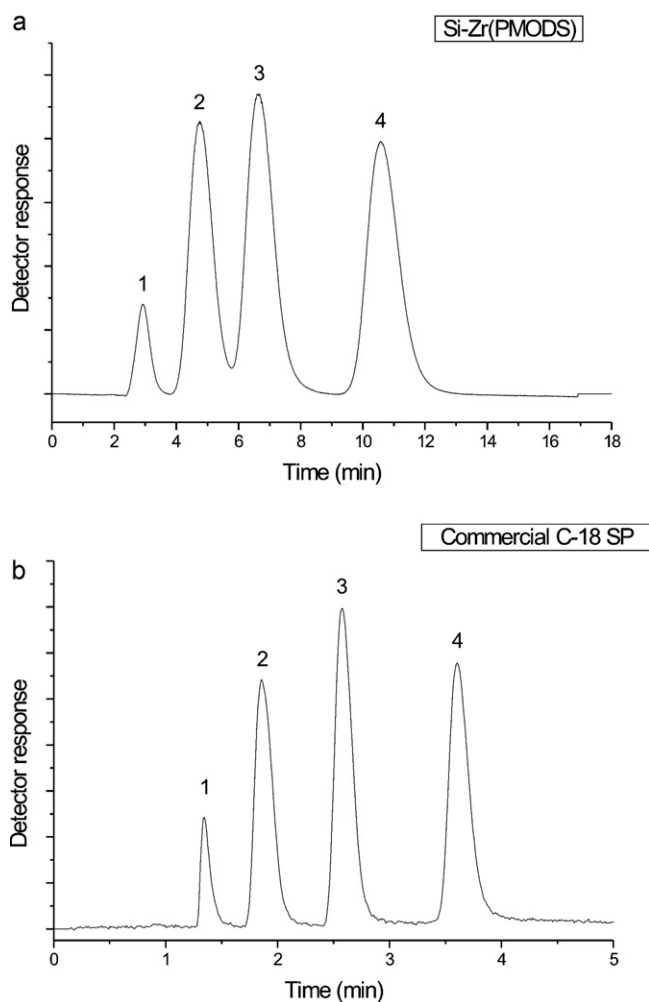
Fig. 4. Chromatogram of methylated xanthines separated on (a) Si–Zr(PMODS) stationary phase (60 mm × 3.9 mm, 5 μm) and (b) commercial (150 mm × 3.9 mm, 4 μm) C18 stationary phase. Mobile phase—A: water and B: methanol:water (70:30, v/v). Solvent B was linearly increased to 30–70% at 0.3 mL/min for Si–Zr(PMODS) and at 1.0 mL/min for the commercial C18 SP; injection volume: 5 μL; detection at 275 nm, temperature: 30 °C. Peak identifications: (1) uracil, (2) theobromine, (3) theophylline, and (4) caffeine. The flow-rate was optimized for both SP using a van Deemter curve for the separation of naphthalene in mobile phase methanol:water (70:30, v/v).

Fig. 5. Chromatogram of five UV filters on (a) Si–Zr(PMODS) stationary phase (60 mm × 3.9 mm, 5 μm) and (b) commercial (150 mm × 3.9 mm, 4 μm) C18 stationary phase. Mobile phase: methanol:water (80:20, v/v) at 0.3 mL/min for (a) and 1.0 mL/min for (b); injection volume: 5 μL; detection at 288 nm, temperature: 25 °C. Peak identifications: (1) uracil, (2) phenylbenzylimidazol sulfonic acid, (3) benzophenone-3 (2-hydroxy-4-methoxy-benzophenone), (4) 4-methylbenzylidene camphor, (5) octocrylene (2-ethylhexyl-2-cyano-3,3-diphenylacrylate), (6) octylmethoxycinnamate, and (*) degradation product.

However, as shown in Fig. 4B, the commercial C-18 SP can also separate the mixture of methylxanthines with good resolution and a short analysis time. These results allow us to suggest that Si–Zr(PMODS) could be used in the separation of these compounds as an alternative SP.

HPLC is also widely used for the separation of UV filters found in products such as sunscreen, creams, lotions and other cosmetic formulations [46]. Fig. 5 shows the separation of five widely used UV filters on the Si–Zr(PMODS) SP. Once again the use of a short column with an isocratic neutral mobile phase (methanol:water 80:20, v/v) showed a separation with good resolution and a relatively fast analysis time, with better performance than shown with a commercial C-18 SP using the same unbuffered mobile phase. In this case, the acidic characteristics of Si–Zr(PMODS) revealed by the chromatographic characterizations did not affect the separation.

These results suggested that the separation of these types of compounds is a potential application for this stationary phase and encourages us to test the SP in other separations, such as the analysis of other pharmaceuticals and pesticides.
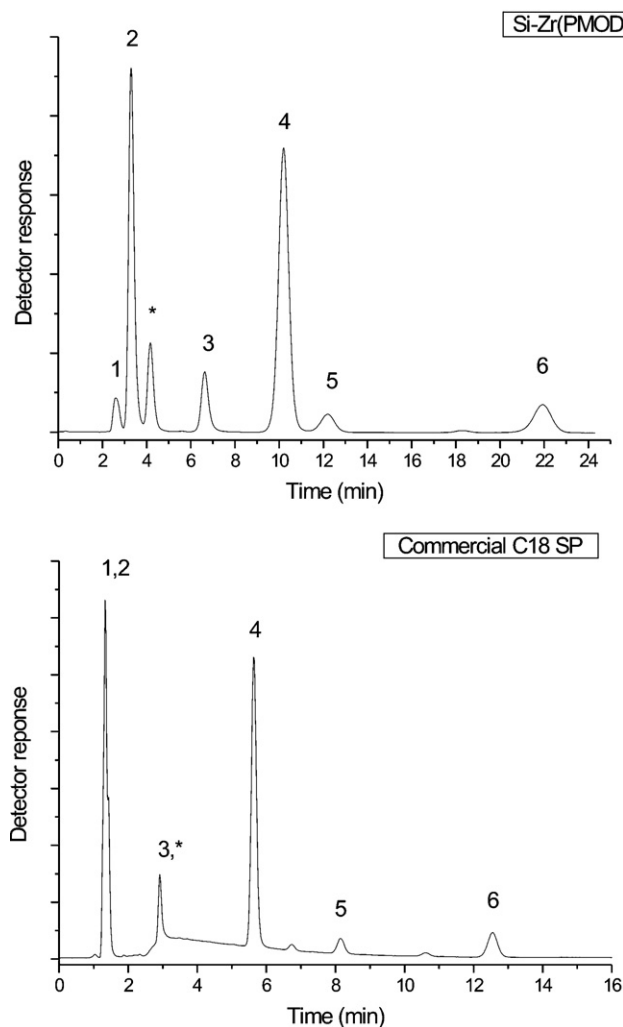
4. Conclusions

The preparation of the metalized support and the thermal immobilization of PMODS onto zirconized silica, optimized by experimental design, produced a SP based on PMODS that showed high efficiency with a low asymmetry factor. The physical–chemical characterization (FTIR and ^{29}Si NMR) confirms that the quantity of residual silanol groups was reduced after thermal immobilization while the chromatographic characterizations (using the Engelhardt, Tanaka and SRM 870 tests) showed performances similar to some commercial bonded phases. On the other hand, these characterizations confirm the high acidity of the residual silanol or zirconol groups, probably not covered by the polymer, suggesting that an endcapping process could improve the performance of Si–Zr(PMODS). The presence of zirconium on the surface significantly decreases silica solubility in severe conditions (alkaline mobile phase at pH 10 and high temperature), increasing column lifetime. The asymmetry values for amitriptyline are within the suggested values and indicate that Si–Zr(PMODS) has good potential for analysis of basic pharmaceutical compounds in acidic mobile

phases. The separation of some methylxanthines was possible using gradient elution without the use of a buffered mobile phase, giving good resolution with a short analysis time while the acidity of SP did not affect the separation of mixtures of psychotropic drugs or UV filters, revealing a promising application for the Si–Zr(PMODS) stationary phase.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.073.

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