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Design and evaluation of hydrolytically stable bidentate urea-type stationary phases for hydrophilic interaction chromatography

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ABSTRACT

We have developed conceptually new stationary phases containing two bidentate urea-type functions suitable for the separation of a wide variety of polar compounds by hydrophilic interaction chromatography (HILIC) through a facile one-pot two-step procedure with the aim of obtaining high hydrolytic stability in a variety of elution conditions. The preparation of the new phases involves a first reaction of 1,2-ethylendiamine with (3-isocyanatopropyl)triethoxysilane to give an intermediate bis-urea with two pendant triethoxysilane functions, followed by anchoring on the silica surface. Two stationary phases were prepared, namely an urea-type stationary phase (USP-HILIC) and an urea-type phase bearing free amino groups (USP-HILIC-NH_2), whereas silanization with 1,2-bis(trichlorosilyl)ethane yielded USP-HILIC-sil and USP-HILIC-NH_2-sil phases, respectively. The silanization step aimed at forming a hydrophilic stable coating through cross-linking between adjacent silanols which prevents silica dissolution at alkaline pH. A full chemical characterization of the new materials has been obtained through solid-state NMR (both ^{29}Si and ^{13}C CPMAS) spectroscopy. A major application field of the bidentate urea-type stationary phase with free amino groups USP-HILIC-NH_2-sil was sugars analysis, usually hampered by α/β anomer peak splitting and instability of the stationary phases under conditions normally employed to suppress it. Complex mixtures of mono-, di- and oligosaccharides were successfully resolved under mild chromatographic conditions, which also allowed an easy interface with mass spectrometry. The potential of such materials was shown in the separation of other highly polar compounds, including polyols, hydroxybenzoic acids, nucleobases, and vitamins.

1. Introduction

The first HPLC separations of polar compounds such as carbohydrates on polar stationary phases were published in the 1970s [1], using mobile phases containing water and a higher percentage of an organic solvent (typically acetonitrile). However, it was not until the early 1990s that new phases started emerging and Alpert [2] gave the practice a name by calling it "hydrophilic interaction chromatography" (HILIC) to emphasize the presence of water in the mobile phase as the stronger eluting member, and the partition mechanism involved in the retention. Following twenty years of continuous development, HILIC is nowadays accepted as a common separation mode [3,4], essentially dedicated to the separation of very polar compounds, such as glycopeptides [5–8] amino acids [2,9] oligonucleotides [10,11] and highly polar natural products [12–15].

A major advantage of HILIC seems to be its easy coupling with mass spectrometry (MS) which extends its applicability to impurity detection [16]. In fact, the use of a low aqueous/high acetonitrile mobile phase significantly improves detection sensitivity for compounds analyzed by LC/electrospray ionization (ESI) MS, thus overcoming the mismatch between normal-phase LC and ESI-MS [16–19].

The exact retention mechanism for HILIC is still open to considerable debate. The partitioning mechanism arises from the preferential adsorption of water on the polar stationary phase, which results in a relatively higher water content in the stagnant liquid phase on the stationary phase support than in the mobile phase [3,20]. Others reported that the separation in the HILIC mode is mainly governed by polar–polar interactions (*i.e.*, hydrogen bonding, dipole–dipole, charge–dipole interactions) because of the strong dependence of the elution order on the number of polar functional groups involved [3,16,21,22]. In some cases, a combination of both partitioning and surface adsorption can take

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place, depending on the nature of the stationary phase (*i.e.*, the hydration degree and charge), the properties of the solutes (*i.e.*, the kind and number of polar functional groups) and the mobile phase composition [23]. A comprehensive study of both retention and selectivity effects in HILIC elution mode has just been published [24], showing the pivotal role of adsorptive solute–silanol interactions in the separation features of polar urea-type bonded phases.

Although the number of commercially available columns designed specially for HILIC is dramatically growing [25], there still is not a substantial variety in stationary phases composition. Since HILIC was considered as the aqueous normal-phase mode, it turns out that the first separations were performed using conventional unmodified silica gels, and actually a large fraction of the recently published works is still using such materials [3,4,16]. One of the properties that make underivatized silica inherently attractive in LC/MS analyses is the lack of ligands that may detach and show up as spurious peaks in the mass spectra. However, severe irreversible adsorption has been observed on bare silica in HILIC mode. Recent bonded phase materials for HILIC seem to have addressed this issue, and various functionalized silica gels are today starting to become as common as unmodified silica in HILIC/MS applications [17–19]. A serious concern with bonded phases is the limited stability in basic aqueous eluents frequently used in HILIC that leads to fast bleeding and the concomitant exposure of free silanols. Earlier studies report the use of a "bidentate approach" in order to overcome hydrolytic instability of silica-based materials in reversed phase separations [26–28]. However, to our knowledge, there are no reports on the development of hydrolytically stable bidentate HILIC stationary phases.

Herein, we report the preparation of a conceptually new stationary phase containing two bidentate urea-type functions suitable for the separation of a wide variety of polar compounds by HILIC (polyols, sugars, hydroxybenzoic acids, nucleobases, vitamins). Bidentate urea-type stationary phases (USP-HILIC) were envisioned and designed through a facile one-pot procedure with the aim of obtaining high stability in a variety of elution conditions, as well as a good selectivity towards the tested compounds. Moreover, a variant of the bidentate urea-type stationary phase was realized in which free amino groups were intentionally left on the silica surface to act as α/β sugar anomerization catalysts, a feature that proved beneficial to overcome the unwanted peak splitting observed when α and β anomers interconvert slowly on the chromatographic time scale.

2. Experimental

2.1. Apparatus

All HILIC separations were performed on a chromatographic system consisting of a Waters Alliance 2695 pump (Waters, Milford, MA, USA) and an autosampler equipped with a model 996 PDA detector. A model Sedex-55 evaporative light scattering detection (ELSD) system (S.E.D.E.R.E., France) was also used. Chromatographic data were collected and processed using the Empower 2.0 software (Waters).

LC–MS was performed using a Thermo Fischer Scientific HPLC separation module (San Jose, CA, USA) consisting of an Accela 1250 Pump, an autosampler and a PDA detector, coupled to a Thermo Finnigan LXQ linear ion trap mass spectrometer, equipped with an ESI ion source bearing a steel needle. Chromatographic and mass spectrometric data were collected and processed using the Thermo Xcalibur Chromatography Manager software, version 2.1.

FT-IR spectra (KBr pellets) were recorded on a Jasco 430 Fourier transform IR spectrometer (Jasco Europe, Cremella, Italy) at a resolution of 4 cm^{-1}.

The mobile phase pH was measured with a Metrohm Model 632 pH meter (Metrohm, Heriscan, Switzerland).

^{1}H NMR and ^{13}C NMR solution spectra were recorded on a Varian INOVA spectrometer operating at a field of 14.4 T (600 MHz for ^{1}H, 150.8 MHz for ^{13}C) with an indirect-triple resonance probe.

Solid state ^{29}Si and ^{13}C CPMAS NMR spectra were measured on a Bruker Avance III spectrometer at 79.49 MHz and 100.63 MHz, respectively. Samples were packed into 4 mm zirconia rotors, and sealed with Kel-F caps. The spin rate was 8 kHz. The cross-polarization (CP) was performed by applying the variable spin-lock sequence RAMP-CPMAS [29]; the RAMP was applied on the ^{1}H channel and during the contact time the amplitude of the RAMP was increased from 50 to 100% of the maximum value. ^{29}Si CPMAS spectra were obtained with a contact time optimized at 3 ms, the recycle delay being 4 s. ^{13}C CPMAS spectra were obtained with a contact time optimized at 6 ms, the recycle delay being 3 s. All spectra were acquired with a time domain of 1024 data points, zero filled and Fourier transformed with a size of 4096 data points, applying an exponential multiplication with a line broadening of 4 Hz. ^{29}Si and ^{13}C chemical shifts were referenced to tetramethylsilane used as an external reference. The deconvolution of ^{29}Si CPMAS spectra was performed by using the dm2006 software package [30]. Each resonance was modelled by a Gaussian lineshape, and characterized by the amplitude, the chemical shift and the width at half height.

Thermogravimetric analysis (TGA) was carried out and certified by REDOX snc laboratories (Monza, Italy). The percent values of weight losses are extrapolated from the TGA curves by tangential-mode integration.

2.2. Chemicals and reagents

Spherical Kromasil Si 100 silica gel (pore size 100 Å, particle size 5.0 μm, specific surface area 340 $m^2\,g^{-1}$) was purchased from Eka Nobel (Bohus, Sweden). Ethylenediamine, (3-isocyanatopropyl)triethoxysilane, 4-(dimethylamino)pyridine (or 4-DMAP), dry toluene, 1,2-bis(trichlorosilyl)ethane, toluene-d_8, methanol-d_4, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose as well as 1,3,5-*cis*,*cis*-cyclohexanetriol, xylitol, *chiro*-inositol, *myo*-inositol, thymine, uracil, cytosine, adenine, cytidine, adenosine, acetylsalicylic acid, salicylic acid, 4-hydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 4-hydroxybenzenesulfonic acid, and gallic acid were from Sigma–Aldrich (St. Louis, MO, USA). Mannitol, maltose, saccharose, ascorbic and isoascorbic acids, and triethylamine (TEA) for HPLC were from Fluka (Buchs, Switzerland). Ammonium acetate (NH_4OAc) was supplied from Baker (Division of Mallinckrodt Baker Inc., Phillipsburg, NJ). HPLC gradient grade water, acetonitrile, hexane, tetrahydrofuran, chloroform stabilized with ethanol were purchased from Sigma–Aldrich. Other polar analytes were available from earlier studies.

2.3. Preparation of the bidentate urea-type USP-HILIC stationary phase

A solution of 1,2-ethylenediamine (0.29 ml; 4.3 mmol) in dry toluene (70 ml) was heated at 110 °C under a nitrogen atmosphere. After distillation of 5 ml of solvent, a solution of (3-isocyanatopropyl)triethoxysilane (2.13 ml; 8.6 mmol) in dry toluene (10 ml) was added and the solution was stirred under nitrogen for 4 h at 100 °C. Six grams of dried silica (Kromasil Si 100, 5.0 μm, specific surface area 340 $m^2\,g^{-1}$) were suspended in ethanol 95% by volume (250 ml) and kept overnight under stirring. Hydrated silica gel, washed and dispersed in 40 ml of dry toluene,

was added to the above reaction flask and the mixture was heated at 90 °C under continuous stirring for 3 h. Finally, 4-DMAP (0.64 g; 5.2 mmol) was added and the slurry was kept under continuous stirring for 16 h at 90 °C. After cooling at room temperature, the modified silica gel (urea-type stationary phase or USP-HILIC) was isolated by filtration, washed with methanol and dichloromethane, and dried *in vacuo* (0.1 mbar) at $T = 60$ °C to constant weight (7.1 g; weight increment = 19%). FT-IR (KBr): 3329, 2978, 2933, 1876, 1645, 1560, 1076, 800 cm^{-1}. Elemental analysis: C 7.04%, H 1.43%, N 2.71%, corresponding to 574 μmol of substrate per gram of silica or 1.71 μmol/m^2 (based on nitrogen). Calculation based on carbon content yielded similar values, *i.e.*, 580 μmol and 1.71 μmol/m^2, respectively.

Two grams of USP-HILIC were dispersed in a 10 mM NH_4OAc solution in water-acetonitrile 70:30 (v/v) (60 ml). The slurry was kept overnight under continuous stirring at 30 °C, then filtered, washed with water, acetone, methanol and dichloromethane and dried *in vacuo* (0.1 mbar) at $T = 60$ °C. After the washing procedure, the dried silica was added to a reaction flask containing imidazole (0.10 g; 1.45 mmol) dissolved in dry toluene (20 ml). A solution of 1,2-bis(trichlorosilyl)ethane (0.5 ml; 2.5 mmol) in dry toluene (5 ml) was added dropwise to the slurry and was allowed to react for 2 h under a nitrogen atmosphere and continuous stirring at room temperature. Finally, the silanized silica gel (USP-HILIC-sil) was filtered, washed with toluene, chloroform, methanol and dichloromethane, and dried *in vacuo* (0.1 mbar) at $T = 60$ °C. FT-IR (KBr): 3352, 2943, 2887, 1869, 1635, 1566, 1053, 806 cm^{-1}. Elemental analysis: C 6.96%, H 1.37%, N 2.60%.

2.4. Preparation of the bidentate urea-type USP-HILIC-NH_2 stationary phase

A bidentate urea-type stationary phase bearing some free amino groups (USP-HILIC-NH_2) was prepared as reported for USP-HILIC (see Section 2.3) starting from 3 g of hydrated Kromasil Si 100 silica gel and using a different molar ratio between (3-isocyanatopropyl)triethoxysilane (1.08 ml; 4.4 mmol) and ethylenediamine (0.16 ml; 2.4 mmol). After identical work-up, the modified silica gel (USP-HILIC-NH_2) was washed with 50-ml portions of methanol and dichloromethane, and dried *in vacuo* (0.1 mbar) at $T = 60$ °C to constant weight (3.58 g; weight increment = 12.7%). FT-IR (KBr): 3329, 2938, 2880, 1867, 1635, 1560, 1053, 800 cm^{-1}. Elemental analysis: C 7.42%, H 1.37%, N 3.17% corresponding approximately to 619 μmol of substrate per gram of silica or 1.82 μmol/m^2 (based on carbon), calculated assuming $-(CH_2CH_2CH_2NH{-}CO{-}NH{-}CH_3)_2$ as the ligated fragment.

Two grams of USP-HILIC-NH_2 silica gel were silanized as reported for USP-HILIC (see Section 2.3). After identical work-up, the silanized silica gel (USP-HILIC-NH_2-sil) was filtered, washed with 50-ml portions of toluene, chloroform, methanol and dichloromethane and dried *in vacuo* (0.1 mbar) at $T = 60$ °C. FT-IR (KBr): 3334, 2935, 2882, 1876, 1633, 1567, 1095, 804 cm^{-1}. Elemental analysis: C 7.26%, H 1.42%, N 2.73%.

2.5. Preparation of the diol-modified silica gel

Three grams of dried silica (Kromasil Si 100; 5 μm, specific surface area: 340 $m^2\,g^{-1}$) was suspended in methanol (35 ml) containing H_2SO_4 80% (0.1 ml). A solution of (3-glycidoxypropyl)trimethoxysilane (0.5 ml; 2.3 mmol) in methanol (10 ml) was added dropwise in an argon atmosphere. The slurry was heated to 70 °C for 2 h with continuous mechanical stirring. After cooling to room temperature, the diol-modified silica was isolated by filtration; washed with 30-ml portions of methanol, water, methanol, and acetone; and dried under reduced pressure (0.1 mbar) at $T = 60$ °C. Elemental analysis: C 3.18%, H 0.77%, corresponding to 474 μmol of substrate per gram of silica or 1.40 μmol/m^2.

2.6. Chromatographic set-up

Stainless steel (150 mm × 4.0 mm I.D.) columns were packed with the four bidentate urea-type USP-HILIC, USP-HILIC-sil, USP-HILIC-NH_2 and USP-HILIC-NH_2-sil stationary phases using a slurry packing procedure already described [31]. A Kromasil Si 100, 5 μm diol-modified silica gel was *ad hoc* prepared (see Section 2.5) and packed as well into a stainless steel (150 mm × 4.0 mm, I.D.) column. For comparative purposes, two more commercial columns (150 mm × 4.0 mm, I.D.) were used, namely Hypersil APS-2 (Si 120, 5 μm; specific surface area: 170 $m^2\,g^{-1}$) from Thermo Fischer Scientific and ZIC-HILIC (Si 200, 5 μm; specific surface area 135 $m^2\,g^{-1}$) from SeQuant (Sweden).

All chromatographic runs were carried out under isocratic or gradient elution with the flow rate set to 1.0 ml/min. Eluents were composed of acetonitrile and water or aqueous buffers in a volume ratio of 90:10 or 85:15. The aqueous buffers were prepared by dissolving appropriate amounts of NH_4OAc, KH_2PO_4 or TEA in water so that the final concentration in the hydro-organic mixture amounted to 5–30 mM of the desired additive.

The pH was measured both in aqueous buffer prior mixing with acetonitrile (namely, ${}^{w}_{w}pH$) and in the final hydro-organic solvent mixture (${}^{s}_{w}pH$). Sample solutions were prepared in the mobile phase (0.5–2 mg/ml) and allowed to equilibrate at $T = 25$ °C prior to injection into the HPLC system (2–5 μl aliquots).

The column hold-up volumes (V_0) were determined by static pycnometry (pyc) using the following equation:

$$V_0^{pyc} = \frac{w_{CHCL_3} - w_{THF}}{\rho_{CHCL_3} - \rho_{THF}}$$

where w and ρ are the masses of the columns filled with solvents and solvent density, respectively [32,33]. The number of theoretical plates per meter was calculated by built-in functions in the Empower software using 50% peak width. Resolution values were calculated by built-in functions in the Empower software using the equation $(t_2 - t_1) \times 1.18/(w_2 + w_1)$ where t_2 and t_1 are retention times and w_2 and w_1 are peak widths at 50% of peak height.

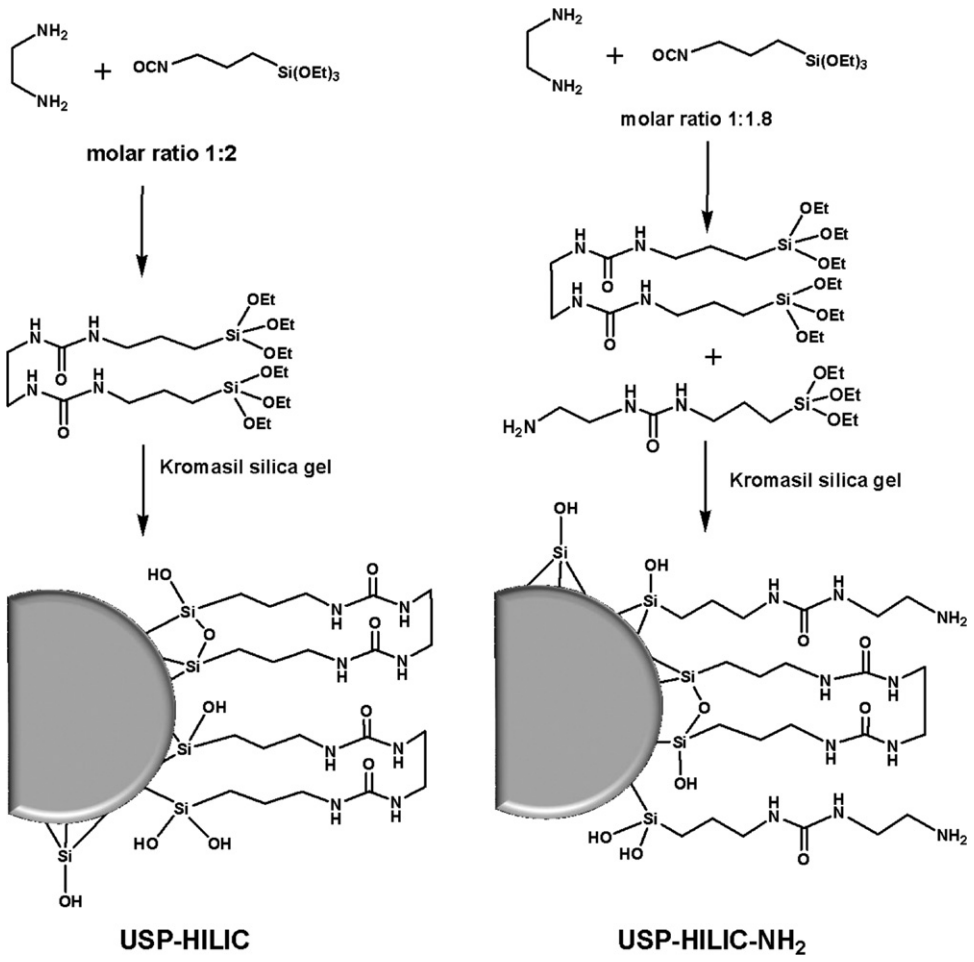
2.7. HILIC/ESI-MS analysis

HILIC/MS was performed by electrospray ionization (ESI) using an ion trap analyzer operated under the following conditions: ion polarity, negative; capillary temperature, 275 °C; source voltage, 4.0 kV; capillary voltage, −44.5 V; tube lens offset, 44.0 V; sheath gas, 10 arbitrary units; auxiliary gas, 5 arbitrary units. Total ion current (TIC) from 220 to 1500 amu was monitored.

3. Results and discussion

3.1. Preparation of new bidentate urea-type stationary phases

When we first started designing the preparation of these new stationary phases, we had already been working on derivatizing silica gel through a facile procedure to obtain chemically and thermally stable glycopeptide-based chiral stationary phases that would prove useful under different separation modes [34]. These derivatization strategies rely on the use of di-isocyanate terminated spacers that react sequentially with surface anchored aminopropyl groups and with amino groups of the glycopeptides to yield a final stationary phase where the chiral selector is secured to the silica surface through the spacer by two stable, polar ureidic bonds. Drawing inspiration from these synthetic schemes, we envisioned



Scheme 1. Synthetic strategy for the preparation of the bidentate USP-HILIC and USP-HILIC-NH_2 stationary phases.

a new route to silica-based stationary phases, based on the one-pot reaction of a simple 1,2-diamine, an isocyanate-terminated organosilane and silica gel. The reaction sequence, sketched in Scheme 1, generates a highly polar, silica-based stationary phase that can be operated under HILIC conditions in the separation of polar compounds.

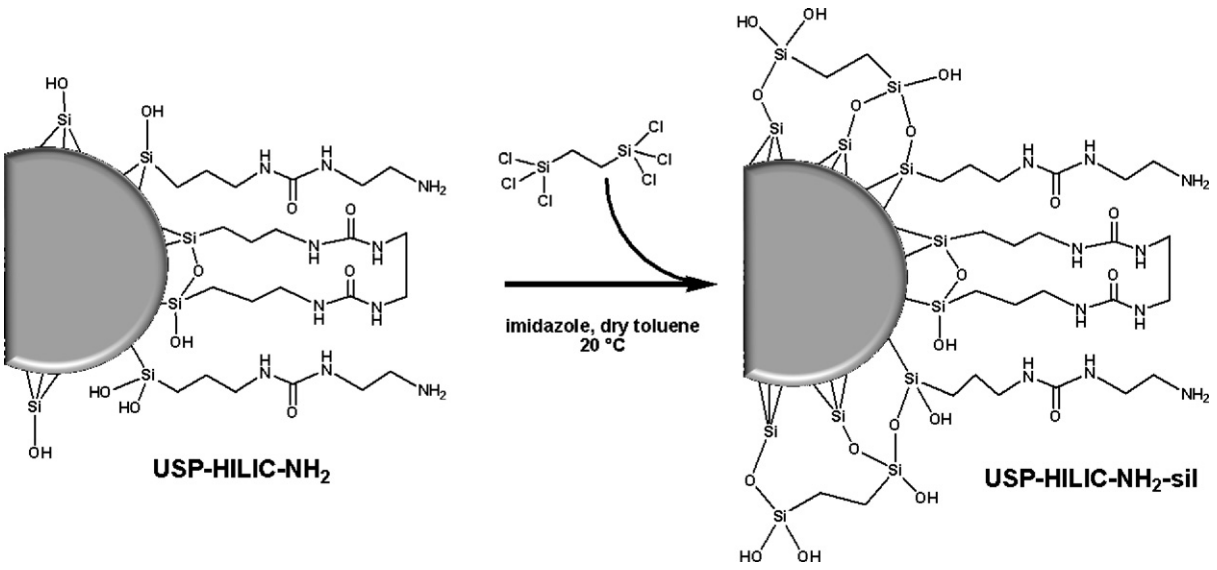
The new procedure is highly attractive as it is easy to realize in a reproducible manner and yields a final material that is very stable from the chemical and thermal points of view as a result of the two bidentate urea-type functions that represent the organic fragment anchored to the silica surface. Indeed, the bidentate technology was applied to a series of stationary phases to provide good hydrolytic stability in both low and high pH of the mobile phase [27].

The one-pot synthesis includes two steps: a first one where 1,2-ethylendiamine reacts with (3-isocyanatopropyl)triethoxysilane to yield an intermediate bis-urea with two pendant triethoxysilane functions. This step is easily performed by simply heating the two reacting partners in dry toluene and does not require any purification as no by product is generated during the urea bonds formation. In the second step, performed in the same reaction flask, a slurry of hydrated silica is added to the bifunctional silane solution and the slurry is heated for 3 h to obtain the desired surface chemical modification. A weight ratio of 1:2.5 between the dimeric ethylenediamine–isocyanate reagent and the previously hydrated silica gel was chosen in order to obtain a maximum degree of derivatization. Hydrating the silica gel prior to the derivatizing procedure by treatment with ethanol 95% (by volume) aimed at creating a monomolecular water layer on the silica surface. In fact, during silanization, water plays a role in later stages, when hydrolysis of unreacted ethoxysilyl groups becomes crucial for further polymerization [35]. In such a way, hydration with a defined amount of water allows mainly horizontal polymerization, and the resulting silica shows enhanced surface density of silanes than the non-hydrated silica gel.

Four new bidentate stationary phases were prepared, namely an urea-type stationary phase (USP-HILIC) and an urea-type phase bearing free amino groups (USP-HILIC-NH_2), as well as the corresponding silanized USP-HILIC-sil and USP-HILIC-NH_2-sil phases (Schemes 1 and 2).

The urea-type phase bearing free amino groups was specifically designed for applications to HILIC sugars analysis. Free amino groups on the silica surface have a potential catalytic effect on the α/β anomer interconversion of reducing sugars, and this effect in turn translates in α/β anomer peaks averaging and collapse with concomitant simplification of the chromatographic pattern.

For the synthesis of the USP-HILIC phase and the corresponding silanized version, a molar ratio of 1:2.0 was used between 1,2-ethylenediamine and (3-isocyanatopropyl)triethoxysilane. In the preparation of USP-HILIC-NH_2 phase and the corresponding silanized version, a lower amount of (3-isocyanatopropyl)triethoxysilane was used (*i.e.*, a 1:1.8 molar ratio, see Scheme 1).



Scheme 2. Silanization reaction of the USP-HILIC-NH_2 stationary phase with 1,2-bis(trichlorosilyl)ethane yielding USP-HILIC-NH_2-sil.

In order to get a higher stability of the stationary phase, silanization with 1,2-bis(trichlorosilyl)ethane was used in the final stages of the synthesis. The silanized stationary phases showed good results in terms of both stability and hydrophilicity, resulting from the formation of a hydrophilic stable coating through cross-linking between adjacent silanols. We believe that multiple attachments formed between ligands, the silica surface and the adjacent ligands produce a thin film on the surface which prevents silica dissolution at alkaline pH. In Scheme 2 is reported the silanization of USP-HILIC-NH_2 stationary phase with 1,2-bis(trichlorosilyl)ethane yielding USP-HILIC-NH_2-sil. As for Scheme 1, the depicted structures aim at representing the stationary phases from a qualitative point of view, and are coherent with the data obtained through solid state NMR spectroscopy (see Section 3.2).

3.1.1. NMR study of step 1

A complete characterization of the derivatizing bis- and mono-urea alkoxysilanes was performed using ^{1}H NMR, ^{13}C NMR spectroscopy as well as homo- and heterocorrelated 2D NMR experiments.

Two different ureido silanization reagents were prepared using, in the first case, 1 equiv. of ethylenediamine with 2 equiv. of (3-isocyanatopropyl)triethoxysilane in deuterated toluene (as reported for the synthesis of the USP-HILC phase), whereas, in the second case, the mixture was obtained by reacting 1 equiv. of diamine with 1.5 equiv. of silane in the same solvent. Although the USP-HILIC-NH_2 was prepared using a 1:1.8 molar ratio (see Section 3.1), in this experiment a slightly higher ratio was found useful to detect the desired mono-substituted compound. Structural assignment by NMR spectroscopy confirms that only the bis-substituted urea is formed in the first case, while two compounds are present in the second mixture.

In detail, the ^{1}H NMR spectrum of the bi-component mixture in deuterated methanol showed a triplet at 2.68 ppm that was not present in the ^{1}H spectrum of the bis-substituted urea (see Fig. 1). A second triplet of the same intensity is visible at 3.16 ppm, albeit partially overlapped by the singlet corresponding to the four isochronous CH_2—N hydrogen atoms of the bis-substituted urea (3.17 ppm). These two triplets can be assigned to the two different CH_2N of the mono-substituted urea (scalar coupling with the NH_2 is removed by the exchange of the NH_2 hydrogen atoms with the solvent). Also the multiplet at 3.07 ppm shows that two triplets with different intensity are present. These signals correspond to the CH_2—N of the propyl chain, and the small triplet corresponds to the mono-substituted urea (for full NMR spectra, see Figs. S1–S3 of Supplementary data).

Two 2D NMR spectra (see Figs. S4 and S5 of Supplementary data) were recorded to confirm this assignment. In particular, ^{1}H–^{1}H COSY confirmed that the two signals at 2.68 and 3.17 ppm are scalarly coupled, as expected for the mono-substituted compound. ^{1}H–^{13}C edited-HSQC confirmed that both the triplets correspond to secondary carbons, with chemical shift very similar to that of the bis-substituted compound.

3.2. Characterization of derivatized silica gels by solid state NMR and TGA

The outcome of surface modification was studied for each chemical step, including washing with a 10 mM NH_4OAc solution, by means of solid state ^{29}Si and ^{13}C CPMAS NMR spectroscopy.

After each step of chemical modification (introduction of ureido functions, washing with NH_4OAc solution, silanization with bis-silyl ethane bridge), the silica samples were characterized by solid state ^{29}Si and ^{13}C CPMAS NMR spectroscopy.

^{29}Si CPMAS NMR spectra of the investigated samples are shown in Fig. 2. All spectra show the resonances of structural elements of silica consisting of Q^2, Q^3, and Q^4 units, the superscripts indicating the number of Si—O—Si bonds. The resonance of bulk siloxane Q^4 units is observed at −110 ppm, the resonance of Q^3 units of silanol groups and that of Q^2 units due to geminal silanol groups are observed at −101 and −90 ppm, respectively. Spectra of modified silica samples also show the typical signals of trifunctional silanes T [36]. In particular, resonances are observed at −49, −56, and −65 ppm due to silicon atoms of the silane molecules in which one (T^1), two (T^2), or three (T^3) hydroxyl groups are involved into reactions with hydroxyl moieties of silica surface. In accordance with these findings, T^2 and T^3 units of the modified silica samples are easily observed in the structures reported in Scheme 1. T^1 units are possibly ascribed to silane silicon atoms having two hydroxyl groups or one or two unreacted ethoxyl groups as substituents.

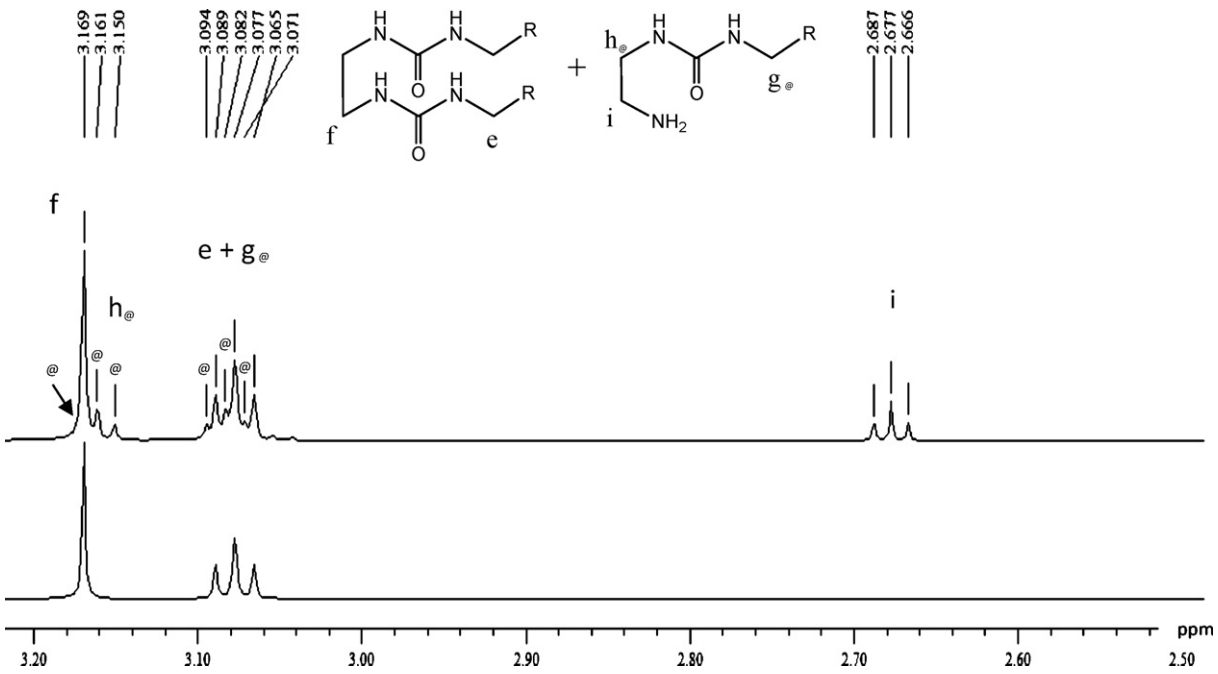


Fig. 1. ^{1}H NMR spectra in methanol-d_4 of reaction products obtained using a 1:1.5 molar ratio (*top*) and 1:2.0 molar ratio (*bottom*) of 1,2-ethylendiamine and (3-isocyanatopropyl)triethoxysilane. R = $(CH_2)_2$—Si$(OCH_2CH_3)_3$.

Even if spectra acquired with cross-polarization are not directly quantitative, it is evident that the highest amount of T units with respect to Q units is found in USP-HILIC-sil (D) and USP-HILIC-NH_2-sil (G) silica gels, besides in these two samples, the highest amount of T^1 units is also found.

^{13}C CPMAS NMR spectra are shown in Fig. 3. The spectrum of the USP-HILIC silica gel (B) shows a resonance at 10.1 ppm ascribed to the methylene carbon atoms having the silane silicon atom of T^2 and T^3 units as a first neighbour. The resonance at 24.3 ppm is due to the methylene carbon atoms having the silane silicon atom as a second neighbour, the resonances at 42.7 ppm is due to the methylene carbon atoms having the NH group as a first neighbour, and the resonance at 161 ppm is due to the carbonyl carbon (see also Scheme 1). Resonances at 16 and 58 ppm are ascribed to methyl and methylene carbon atoms of unreacted ethoxyl groups.

These signals match the corresponding signals of the bis-urea silane recorded in solution (see Section 3.1.1), which are found at 7.41, 17.66, 23.64, 40.42, 42.63, 58.31 and 160.12 ppm (see Fig. S2 of Supplementary data). Signals of the nonequivalent carbon atoms close to the two different ureido nitrogens are seen in solution at 40.42 and 42.63 ppm, whereas a single unresolved signal is observed in the solid state at 42.7 ppm.

The spectrum of USP-HILIC silica washed (C) shows a reduction of the intensity of the resonances of unreacted ethoxyl groups. Spectra of USP-HILIC-sil (D) and USP-HILIC-NH_2-sil (G) silica gels show an additional resonance at 4.4 ppm of methylene carbon atoms belonging to the bridge responsible of the cross-linking between adjacent silanols [37] (see also Scheme 2). Spectra of USP-HILIC-NH_2 (E) and USP-HILIC-NH_2 (F) washed silica gels are very similar to that of HILIC silica (B), as the methylene carbon having the free amino group as a first neighbour may resonate at a chemical shift very close to that of the methylene carbon atoms having the NH group as a first neighbour [38]. All samples but USP-HILIC-NH_2-sil show a variable amount of unreacted ethoxyl groups.

Stability of the bidentate urea-type USP-HILIC-sil and USP-HILIC-NH_2-sil phases was attested by thermogravimetric analysis (TGA) in the 30–800 °C temperature range and both phases are stable up to 250 °C (Fig. 4). The overall losses of organic matrix amount to 10.0% and 8.4% for USP-HILIC-sil and USP-HILIC-NH_2-sil respectively, while water desorption for both samples accounts for the first step of weight loss up to around 150 °C (about 2.5%).

3.3. Chromatographic evaluation of the bidentate urea-type columns

To investigate the separation abilities of the bidentate urea-type columns in HILIC mode, chromatographic studies were performed with polar analytes as probes. A comparison was made with an aminopropyl-bonded silica (APS) column, which is the first bonded stationary phase to be routinely used for carbohydrate separations in HILIC mode [21]. The APS column used was a 150 mm × 4.0 mm I.D. Hypersil APS-2 silica (Si 120, 5 μm, surface area 170 $m^2\,g^{-1}$). A column packed with a diol-modified silica gel was also included in the comparative study, as the diol-modified silica has long been considered as nearly the ideal phase for HILIC applications owing to its high polarity and hydrogen-bonding properties. The diol-modified silica gel was prepared for this purpose from (3-glycidoxypropyl)trimethoxysilane and silica gel under acidic conditions using a one-pot procedure (see Section 2.5). Acid-catalyzed ring-opening reaction of the oxirane group gave the diol-modified silane which was grafted onto the silica surface to form a siloxane-linked 2,3-dihydroxypropyl ligand. Finally, a 150 mm × 4.0 mm I.D. SeQuant ZIC-HILIC column (Si 200, 5 μm; specific surface area 135 $m^2\,g^{-1}$) was added to the study. To evaluate the retention of highly polar compounds, a mixture of uracil, adenosine, and cytosine (see Fig. 5 for structures) was eluted with a mobile phase consisting of acetonitrile/water = 90:10 (v/v) through the different columns in order to perform a HILIC mode test (see Table 1). Naphthalene was added as an unretained marker of dead volume ($k = 0$ in all cases), and was always eluted before uracil, which is a common void volume marker in reversed phase HPLC. The number of theoretical plates per meter for the bidentate urea-type columns was calculated on uracil ($0.82 < k < 1.24$) and ranged

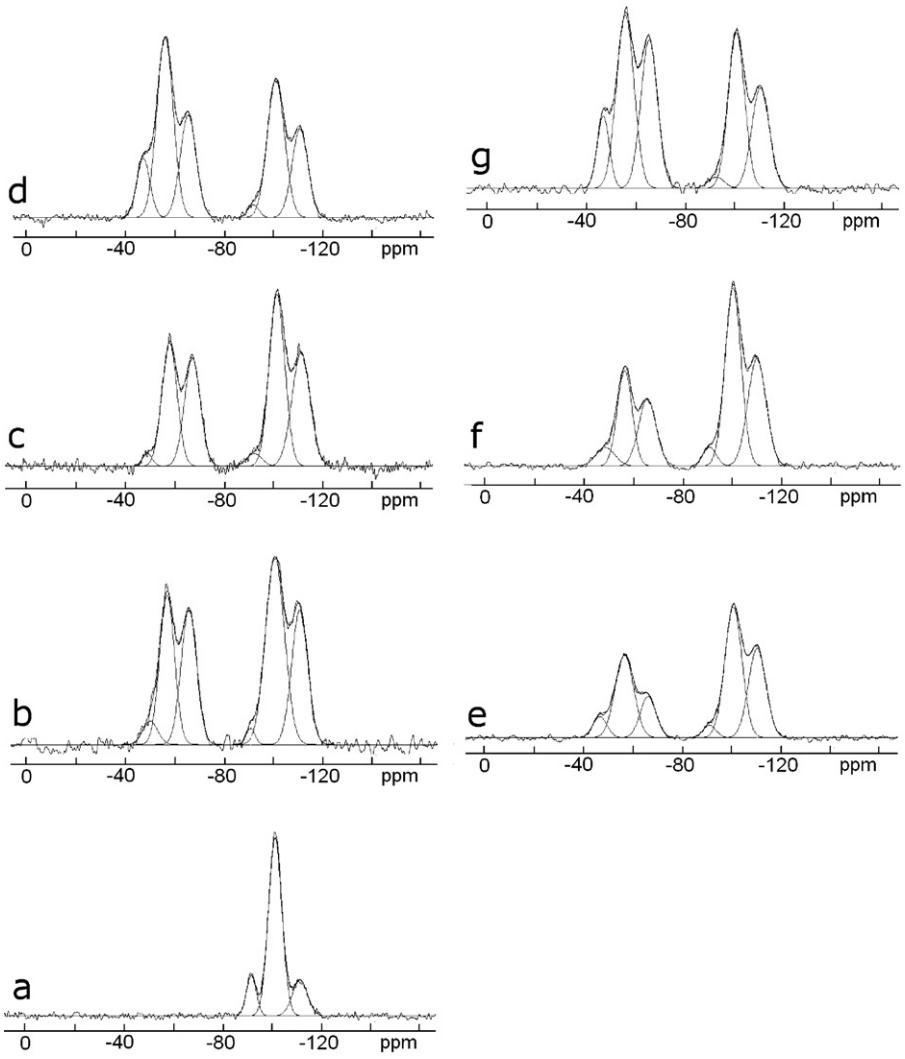


Fig. 2. ^{29}Si CPMAS NMR spectra at 79.49 MHz of Kromasil (A), USP-HILIC (B), USP-HILIC washed (C), USP-HILIC-sil (D), USP-HILIC-NH_2 (E), USP-HILIC-NH_2 washed (F), and USP-HILIC-NH_2-sil (G) silica gels. "Washed" refers to modified silica washed with a 10 mM NH_4OAc solution (see Section 2.3). Deconvoluted spectra are superimposed to the experimental ones.

from 36,000 to 80,000. The APS-2 and the DIOL columns, which have long been considered ideal under HILIC conditions, gave the lowest retention values, while the ZIC-HILIC column showed the highest ones. With regard to the bidentate urea-type columns, the USP-HILIC performed very well in terms of retention (always higher than the APS-2 and the DIOL columns) and selectivity (higher than APS-2 for the adenosine/uracil and cytosine/adenosine couples, but lower than the DIOL column). A sizeable increase in retention (9–19%) was observed for the polar compounds moving to the USP-HILIC-NH_2 column, while selectivity and resolution remained almost unchanged. The effect of silanization with 1,2-bis(trichlorosilyl)ethane on the USP-HILIC-NH_2 column led to a slight increment of retention (k) for adenosine (from 2.57 to 2.77) and cytosine (from 3.99 to 4.40), without significant influence on selectivity and resolution.

Since it is known that salts can promote the retention of "non-charged" compounds under HILIC mode [11,39], we added NH_4OAc to the above described mobile phase (final concentration 10 mM; ${}^{w}_{w}pH = 6.51$; ${}^{s}_{w}pH = 8.05$) and found for the USP-HILIC-sil column even better retention and selectivity values with respect to the non-silanized homologue phase. In fact, in the presence of NH_4OAc, the stagnant water-rich layer on the silica gel tends to thicken as the buffer attracts more water; thus, the liquid–liquid partitioning phenomena taking place become more consistent, leading to higher elution times. Moreover, in our case, hydrogen bonding formation between the urea functionality of the stationary phase and the acetate anion can be invoked (see Scheme 3), which indeed contribute to increase retention of the analytes. Salt addition proved useful also for the USP-HILIC-NH_2 columns, yielding increased retention and selectivity. The APS-2 showed a similar behaviour, while the DIOL column presented a decrement in the retention and selectivity values, probably due to a minor extent of the above-described hydrogen bonding interactions.

3.4. Analysis of polyols

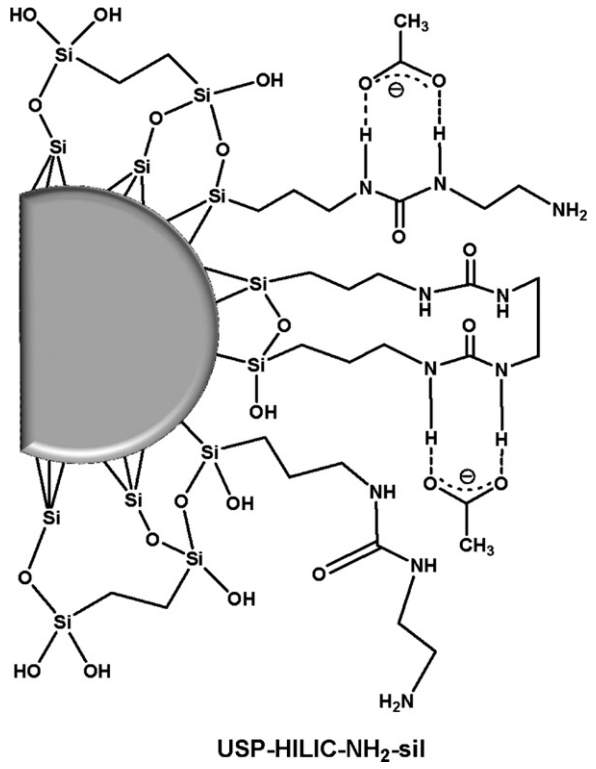
In order to evaluate the "sugarophilicity" of the bidentate urea-type columns, a preliminary test was devised, apt to determine their suitability in sugars analysis. We started our investigation by analyzing a mixture of four sugar alcohols and a cyclic polyol, namely 1,3,5-*cis*,*cis*-cyclohexanetriol (see Fig. 5 for structures) with a mobile phase composed of acetonitrile/water 85:15 (v/v). Since sugars alcohols and sugars in general do not have any significant UV

Table 1
Chromatographic data of the HILIC tests obtained on the bidentate urea-type columns in comparison with Hypersil APS-2, DIOL, and ZIC-HILIC columns.[a]

Sample	USP-HILIC			USP-HILIC-sil			USP-HILIC-NH_2			USP-HILIC-NH_2-sil			Hypersil APS-2			DIOL			ZIC-HILIC		
	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]
No buffer added																					
Naphthalene	0.00			0.00			0.00			0.00			0.00			0.00			0.00		
		–	8.68		–	14.26		–	10.34		–	8.76		–	9.20		–	5.61		–	1.72
Uracil	1.04			0.82			1.24			1.16			0.91			0.57			0.64		
		2.26	8.59		2.17	8.55		2.07	8.29		2.39	7.46		2.01	6.00		3.03	9.58		2.03	–
Adenosine	2.35			1.78			2.57			2.77			1.83			1.73			1.30		
		1.49	5.55		1.58	5.95		1.55	6.55		1.59	5.32		1.25	2.46		1.59	6.02		2.41	–
Cytosine	3.50			2.81			3.99			4.40			2.29			2.76			3.13		
10 mM NH_4OAc added																					
Naphthalene	0.00			0.00			0.00			0.00			0.00			0.00			n.d.	n.d.	n.d.
		–	9.39		–	9.44		–	10.31		–	9.48		–	6.80		–	5.30			
Uracil	1.14			1.04			1.20			1.27			0.65			0.57			n.d.	n.d.	n.d.
		2.35	9.57		2.57	9.69		2.33	9.67		2.61	8.71		2.58	8.28		2.47	7.08			
Adenosine	2.68			2.67			2.80			3.31			1.68			1.41			n.d.	n.d.	n.d.
		1.47	5.93		1.54	6.08		1.61	7.61		1.56	5.70		1.54	5.59		1.59	5.58			
Cytosine	3.94			4.11			4.51			5.16_5			2.58			2.24			n.d.	n.d.	n.d.
ε_T[e]	0.61	0.65	0.59	0.61	–	–	–														
K_F[f]	2.46	3.33	3.08	2.46	3.29	2.61	–														

[a] Columns geometry: 150 mm × 4.0 mm I.D.; eluent: acetonitrile/water = 90:10 (v/v); flow rate: 1.0 ml/min; T = 25 °C; UV detection at 254 nm.
[b] Retention factor.
[c] Selectivity factor.
[d] Resolution, calculated as described in Section 2.6.
[e] Porosity.
[f] Permeability [10^{-14} m^2], calculated according to Ref. [42].

chromophore group, evaporative light-scattering detection (ELSD) was employed. Pulsed amperometric detection (PAD) coupled to anion exchange chromatography is an interesting alternative for the analysis of mono- and oligosaccharides; however, the main drawback is the predictable hydrolysis of methylated and acetylated oligosaccharides due to the high pH values of the eluent used for separation and detection [40].



Scheme 3. Hydrogen bonding interactions between the urea functionality of the stationary phase and the acetate anion occurring during the HILIC retention mechanism.

Single sharp peaks were obtained for each analyte on the four bidentate urea-type columns (Fig. 6A–D, solid lines), with good retention and selectivity data. The DIOL column showed very low "sugarophilicity" (Fig. 6F, solid lines) and the ZIC-HILIC column proved unstable after being used under basic (*vide infra*) conditions (data not shown). The APS-2 (Fig. 6E, solid lines) column retained slightly more the polyols, with respect to the lab-made columns.

As already checked in the HILIC test (see Section 3.3), addition of NH_4OAc to the above-described mobile phase (final concentration 10 mM) increased retention on the four bidentate urea-type columns, although the increment was more consistent for the USP-HILIC-NH_2 columns (Fig. 6C and D, dot lines). In particular, k values of *myo*-inositol (the most retained analyte) were duplicated, passing from 6.18 to 11.46 on the USP-HILIC-NH_2 column and from 4.99 to 10.07 on the USP-HILIC-NH_2-sil column (see Table 2). Selectivity values also showed a slight increment. The APS-2 column showed a similar behaviour (Fig. 6E, dot lines), with analysis time increasing from the initial 15 min up to 30 min. However, the high noise level was particularly high with this column, due to its high instability in the presence of mobile phases at $^{s}_{w}$pH = 7.65. Such instability became more evident when increasing the $^{s}_{w}$pH value to approximately 10 (data not shown). Finally, the DIOL column showed both a better retention and a baseline separation of the five polyols (Fig. 6F, dot lines), when compared to the previous analysis where no buffer was used. Nevertheless, peak shape was not satisfying, especially when compared to the bidentate urea-type columns.

3.5. Analysis of sugars

After evaluating the "sugarophilicity" of the bidentate urea-type columns, we examined their selectivity towards a complex mixture of nine sugars comprising two monosaccharides (namely, fructose and glucose), two disaccharides (namely saccharose and maltose) and a series of five maltose oligomers (see Fig. 7 for their structures). Since the diverse sugars of the test mixture show a broad range of adsorption affinities, the only practical method for their separation was gradient elution. Therefore, a 25 min linear gradient elution from 10% to 52% of water in acetonitrile was used in combination with ELSD detection, which indeed gave signal-to-noise

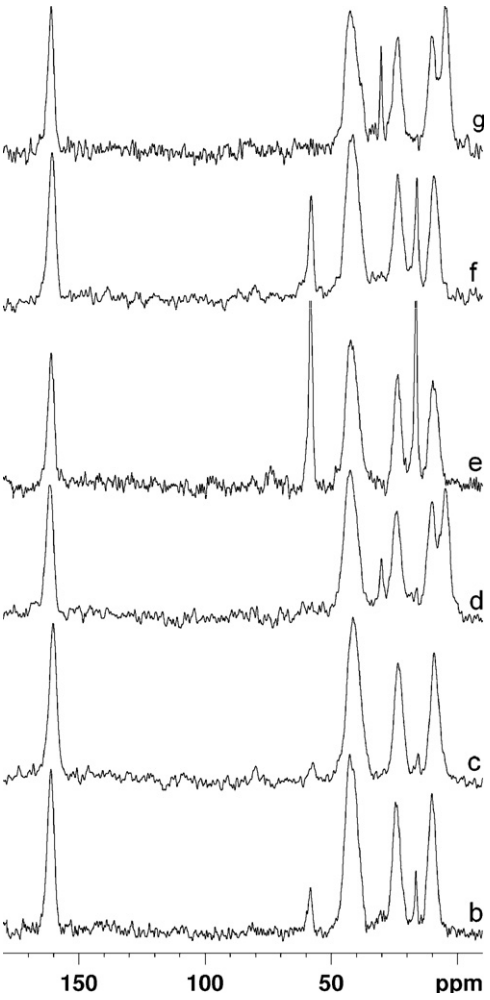


Fig. 3. ^{13}C CPMAS NMR spectra at 100.63 MHz of USP-HILIC (B), USP-HILIC washed (C), USP-HILIC-sil (D), USP-HILIC-NH_2 (E), USP-HILIC-NH_2 washed (F), USP-HILIC-NH_2-sil (G) silica gels. "Washed" refers to modified silica washed with a 10 mM NH_4OAc solution (see Section 2.3).

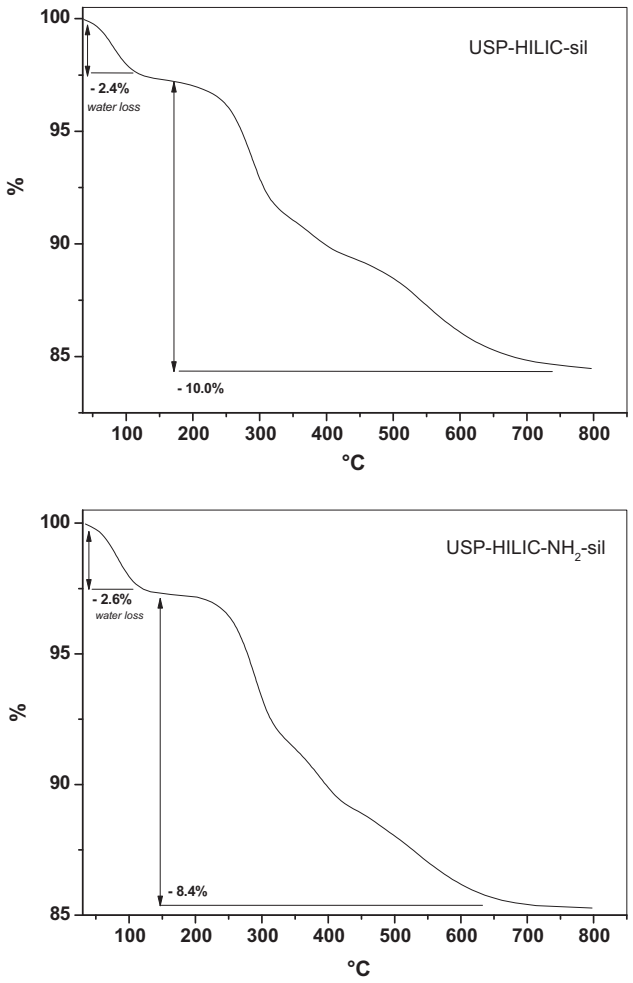


Fig. 4. TGA curves of USP-HILIC-sil and USP-HILIC-NH_2-sil phases.

ratios always much larger than UV detection, since ELSD response is only marginally affected by changes in the eluent composition (see Fig. 8 for representative chromatograms). By comparing the results obtained for the four bidentate urea-type columns with the APS-2, the DIOL, and the ZIC-HILIC columns (see Table 3), the USP-HILIC-NH_2-sil column proved the best for sugar analysis, in terms of both stability and selectivity. In fact, drift-free baselines were obtained under gradient elution conditions (see Fig. 8B), and satisfactory selectivity among couples of sugars belonging to homologue series: the monosaccharides were separated by a Δt_r of 2.26 min from the disaccharides, and the disaccharides were splitted from the series of maltose oligomers by Δt_r of 2.32 min. Retention increases roughly with the number of available hydroxyl groups in the saccharide (*i.e.*, maltooligosaccharides > disaccharides > monosaccharides), in accordance with the chromatographic behaviour of the overall HILIC mode. Notably, each analyte gave a single chromatographic peak (for a total of nine) for at least two reasons: (*i*) on-column anomerization (see Section 3.5.1) occurs faster than the chromatographic separation process and (*ii*) the USP-HILIC-NH_2-sil column shows a low selectivity in discriminating among anomers. This did not happen on the USP-HILIC-sil column, where anomeric peaks were observed for fructose, glucose and maltose (Fig. 8A, *vide infra*).

With regard to the columns used as reference, the analysis on the APS-2 column was seriously hampered by the high noise level observed (Fig. 8C), despite the comparable retention (retention time ranging from 6.47 min to 18.75) and selectivity achieved among homologue series, with the individual sugars appearing as a single peak with no splitting due to α/β anomers. On the DIOL column (Fig. 8D), elution was too fast (retention time ranging from 3.87 to 11.65) and separation not complete, while on the ZIC-HILIC column anomerization occurred during the chromatographic run for at least seven of the nine sugars analyzed (Fig. 8E). For this reason, chromatographic data reported for the ZIC-HILIC column in Table 3 were obtained by considering the averaged retention time of the two anomers. Starting from these results, we decided to perform further investigations on the on-column anomerization phenomena, in order to find suitable chromatographic conditions for controlling their effect on the final chromatographic results.

3.5.1. On-column anomerization of sugars

A major problem in the chromatographic separation of sugars, beside the low UV detectability, is that sugars exist in two anomeric forms that can interconvert slowly on the chromatographic time scale at neutral pH values, thus giving two peaks for each solute that can lead to overcrowding in the chromatogram. Such phenomena are observed when bare silica columns are used, whereas with amino-silica columns they are normally not observed, due to the basicity of such phases [21,41]. At high pH values, in fact, the interconversion of sugar anomers (*i.e.*, anomerization) is rapid and only a single peak is detected. When analysis of reducing sugars is carried out on diol-modified silica, diisopropylethylamine (DIPEA) or

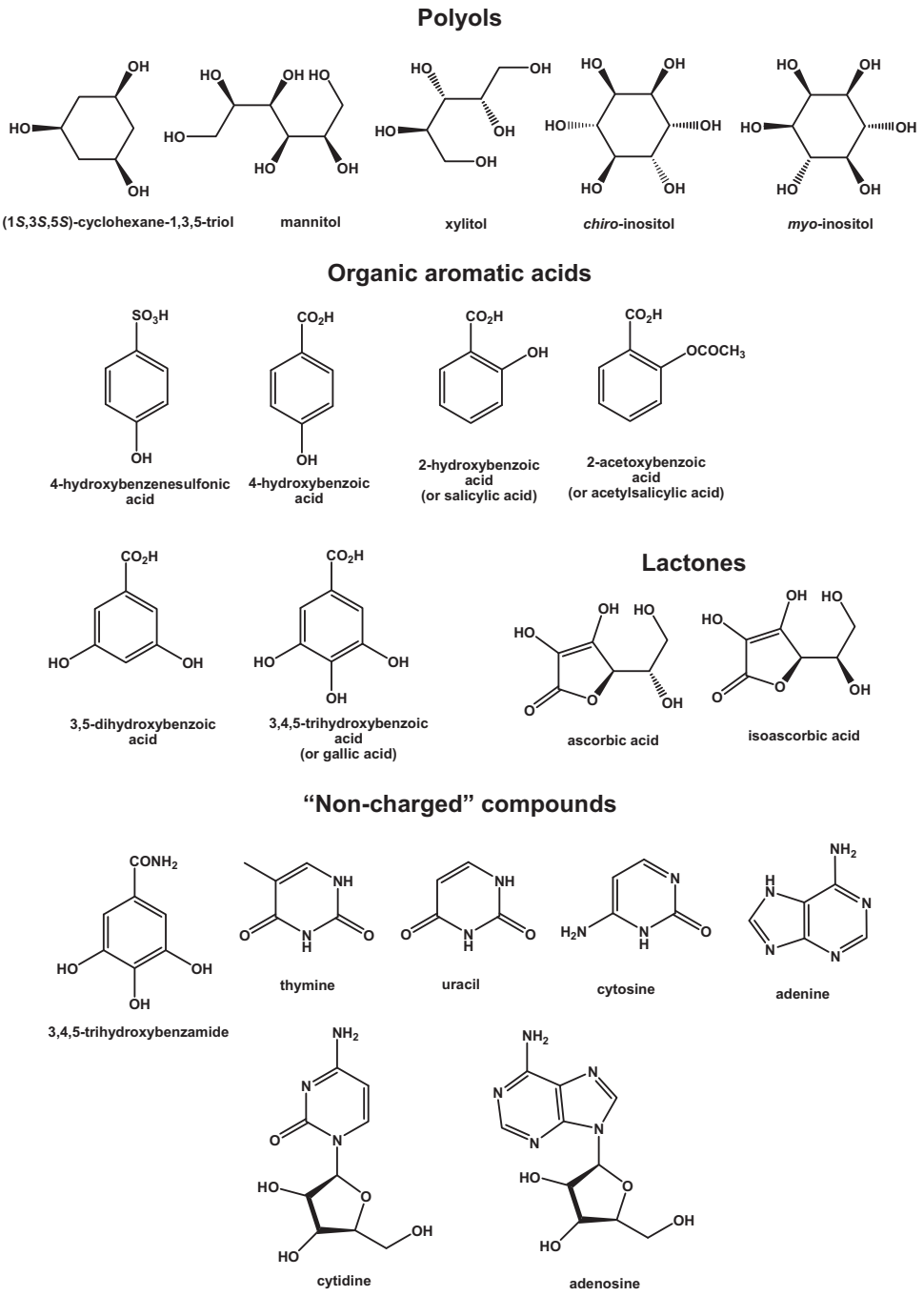


Fig. 5. Structural formulas of the samples analyzed on the bidentate urea-type columns. "Non-charged" refers to the charge state under the HILIC elution conditions used in most experiments.

TEA must be added to the mobile phase to enhance the kinetics of anomerization [3]. In general, elevated temperatures (50–90 °C) or increases in the pH level by addition of 0.1% organic amines have been shown to facilitate the collapse of anomer peaks, thus resulting in one distinctive single peak [42]. However, a factor to always keep in mind is the stability of the stationary phase under alkaline pHs. Many commercial columns, in fact, are not adequately stable under basic conditions and the corresponding chromatograms present peak shape deterioration, hampering the quality of analysis. This is exactly what we found after addition of 0.1% TEA (corresponding to a final concentration of 7 mM) in the mobile phase used for the separation of the above-mentioned sugars mixture: the APS-2, the DIOL and the ZIC-HILIC columns proved highly unstable, as it can be seen by the steep drift of the baselines and the noise level resulted in clearly reduced peaks height (see Fig. 9B–D). In particular, the ZIC-HILIC column (Fig. 9D) was seriously damaged after exposure to such alkaline conditions ($^{S}_{W}$pH > 10). The USP-HILIC-NH_2-sil column, instead, yielded very stable and drift-free baselines (Fig. 9A).

The absence of anomeric peaks on the APS-2 column is attributed to a catalytic effect of free surface amino groups on the α/β anomer interconversion process [21]. The accelerated anomerization results in averaged, single peaks for each sugar, thus greatly simplifying the chromatographic profile. This is why we appositely

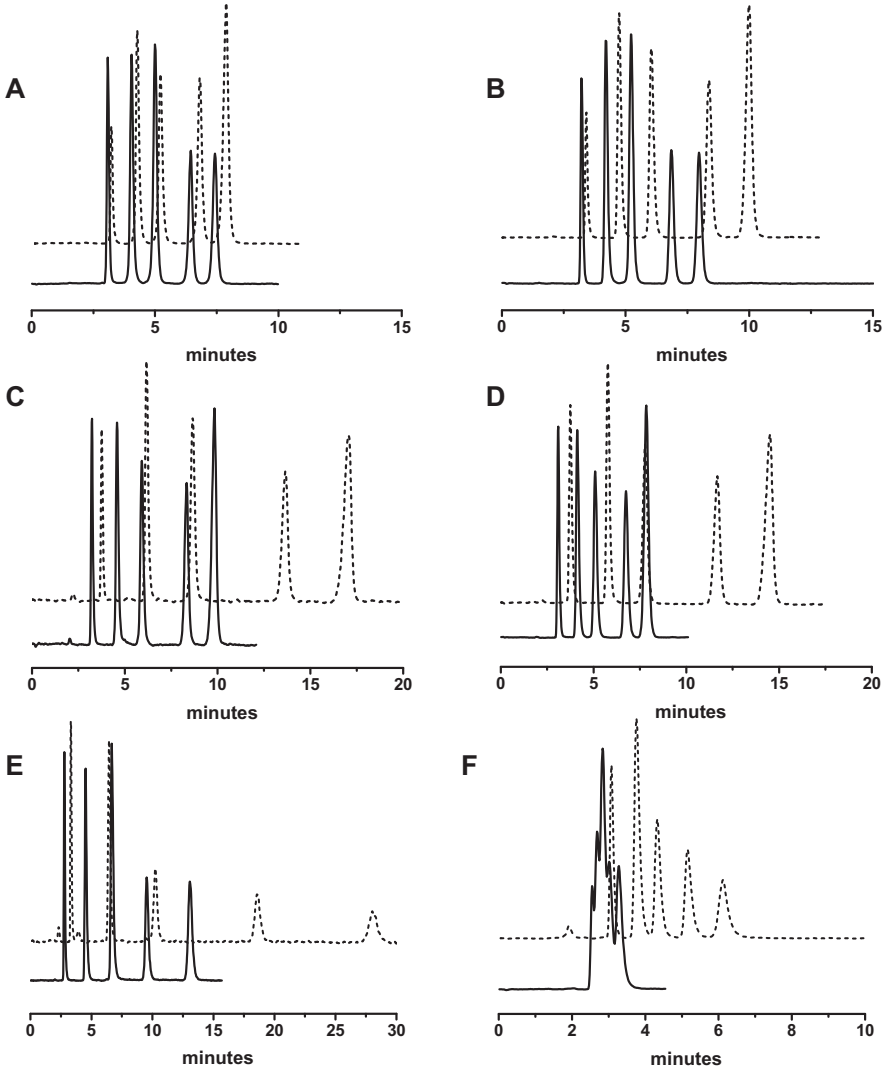


Fig. 6. Analysis of a polyols mixture. Columns: (A) USP-HILIC; (B) USP-HILIC-sil; (C) USP-HILIC-NH_2; (D) USP-HILIC-NH_2-sil; (E) APS-2; (F) DIOL. Eluents: acetonitrile/water 85:15 (v/v) (solid lines) or acetonitrile/water 85:15 (v/v) plus 10 mM NH_4OAc ($^{s}_{w}$pH = 7.65) (dot lines). Flow rate: 1.0 ml/min, T = 25 °C, ELSD detection (T_{neb} = 50 °C). Samples were dissolved in acetonitrile/water 50/50 (v/v) and the solutions (1 mg/ml) were allowed to equilibrate at T = 25 °C prior to injection. Elution order on all columns: (1) 1,3,5-*cis*,*cis*-cyclohexanetriol, (2) xylitol, (3) mannitol, (4) *chiro*-inositol, and (5) *myo*-inositol.

prepared a bidentate urea-type stationary phase bearing some free amino groups (*i.e.*, USP-HILIC-NH_2) in order to exploit such catalytic effect on sugar anomerization. As expected, the USP-HILIC-NH_2 column allowed detection of only nine chromatographic peaks for our sugar mixture as a result of its activating effect on the kinetics of the interconversion process.

3.5.2. Chemical stability of the bidentate urea-type stationary phases

The different chromatographic profiles achieved after addition of 0.1% TEA (corresponding to a final concentration of 7 mM) in the mobile phase used for the analysis of the sugars mixture were taken into account as a comparative stability test of the bidentate urea-type stationary phases at high pHs. Each injection was repeated several times until all phases had reached their minimum noise level and the analysis was reproducible. While the USP-HILIC column showed an excellent stability under basic conditions, the USP-HILIC-NH_2 phase, containing free amino groups, yielded a quite high noise level. Silanization of such phase with 1,2-bis(trichlorosilyl)ethane, however, proved to increase its stability at high pHs, even after a high number of injections (more than 1000 injections were performed on the USP-HILIC-NH_2-sil column, without significant variation of retention and selectivity factors, that was in the range of ≤2%).

3.5.3. Easy interface with mass spectrometry

Carbohydrates are critical components of living systems and mediate a vast number of fundamental biological events—from complex cellular processes required to initiate and sustain life, to recognition phenomena that are responsible for autoimmune diseases, organ rejection, and inflammation. Such functional diversity is reflected in the diversity of carbohydrate structures. Hence, there is an increasing demand for easy access to glycoconjugates which can serve as probes in biochemical studies and also as potential leads for new drugs against carbohydrate-based metabolic disorders.

Since we used ELSD as the detection system in the analysis of sugars on the bidentate urea-type stationary phases (see Section 3.4), we predicted an easy interface with mass spectrometry, allowed by the use of volatile buffers: thus, we coupled an ESI-MS source to our chromatographic system and analyzed the test sugars mixture under the usual conditions. Direct infusion of 100 ppm solutions of maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose in acetonitrile/water 50/50 (v/v)

Table 2
Chromatographic data obtained for a mixture of polyols (see Fig. 5 for structures) on the bidentate urea-type columns in comparison with Hypersil APS-2 and DIOL columns[a].

Sample	USP-HILIC			USP-HILIC-sil			USP-HILIC-NH_2			USP-HILIC-NH_2-sil			HYPERSIL APS-2			DIOL		
	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]
No buffer added																		
1,3,5-*cis*,*cis*-Cyclohexanetriol	1.39			1.41			1.36			1.36			1.02			0.85		
		1.54	4.86		1.52	4.72		1.73	6.22		1.58	4.29		2.07	7.48		1.14	<1
Xylitol	2.88			2.90			3.32			2.89			4.04			1.08_5		
		1.39	5.08		1.42	5.24		1.53	6.91		1.43_5	4.54		1.55	6.77		1.12	<1
Mannitol	2.14			2.14			2.35			2.15			2.11			0.97		
		1.34_5	3.81		1.35_5	3.81		1.41	4.72		1.34	3.17		1.91	6.55		1.12	<1
chiro-Inositol	4.00			4.11			5.08			4.15			6.26			1.22		
		1.17_5	3.10		1.20	3.19		1.22	3.62		1.20	2.62		1.44	6.47		1.17	<1
myo-Inositol	4.70			4.95			6.18			4.99			9.00			1.43		
10 mM NH_4OAc added																		
1,3,5-*cis*,*cis*-Cyclohexanetriol	1.43	1.57	5.25	1.57			1.74_5			1.86			1.21			1.10		
					1.64	6.09		2.01	9.91		1.83	6.92		4.05	11.38		1.43	3.12
Xylitol	2.98			3.47			5.32			4.94			5.97			1.97		
		1.41	5.26		1.49	6.36		1.69	10.05		1.60	7.46		1.96	12.06		1.29	2.70
Mannitol	2.25			2.57			3.50_5			3.40			4.90			1.57		
		1.32	3.65		1.35	4.28		1.52	6.97		1.45	5.16		1.22	8.34		1.25	2.09
chiro-Inositol	4.21			5.17			8.97			7.91			11.69			2.54		
		1.20	3.03		1.23	3.69		1.28	5.17		1.27	4.32		1.56	8.91		1.26	2.53
myo-Inositol	5.04			6.34			11.46			10.07			18.19			3.21		

[a] Columns geometry: 150 mm × 4.0 mm I.D.; eluent: acetonitrile/water = 85:15 (v/v); flow rate: 1.0 ml/min; T = 25 °C; ELSD detection (T_{neb} = 50 °C).
[b] Retention factor.
[c] Selectivity factor.
[d] Resolution, calculated as described in Section 2.6.

containing 10 mM NH_4OAc, as well of mono- and disaccharides, into the ESI-MS instrument provided preliminary data to optimize the experimental mass conditions, which were then used in the HILIC/ESI-MS method. In Fig. 10 is reported the HILIC/ESI-negative chromatogram obtained on the USP-HILIC-NH_2-sil column, by monitoring the total ion current (TIC) from 220 to 1500 amu (molecular weights of maltooligosaccharides range from 504 to 1152 Da).

3.6. Analysis of organic aromatic acids

Encouraged by the results obtained during the analysis of complex mixtures of neutral compounds such as polyols and sugars, we decided to test the ability of the bidentate urea-type columns in separating acid compounds. The analysis of organic acids is very interesting in the pharmaceutical field, but not always chromatographically easy to perform, since poor peak shape is often observed due to deprotonation. To this purpose, we prepared a test mixture containing 4-hydroxybenzenesulphonic acid, 4-hydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid (or gallic acid) and the structurally related 3,4,5-trihydroxybenzamide (see Fig. 5 for their structures), which was isocratically eluted using a 85:15 (v/v) acetonitrile/water plus 30 mM NH_4OAc mixture ($^{s}_{w}$pH = 7.73). The best results in terms of peak shape were obtained with the silanized versions of both the bidentate urea-type columns (see Fig. 11), with a slight retention increment passing from the USP-HILIC-sil to the USP-HILIC-NH_2-sil column.

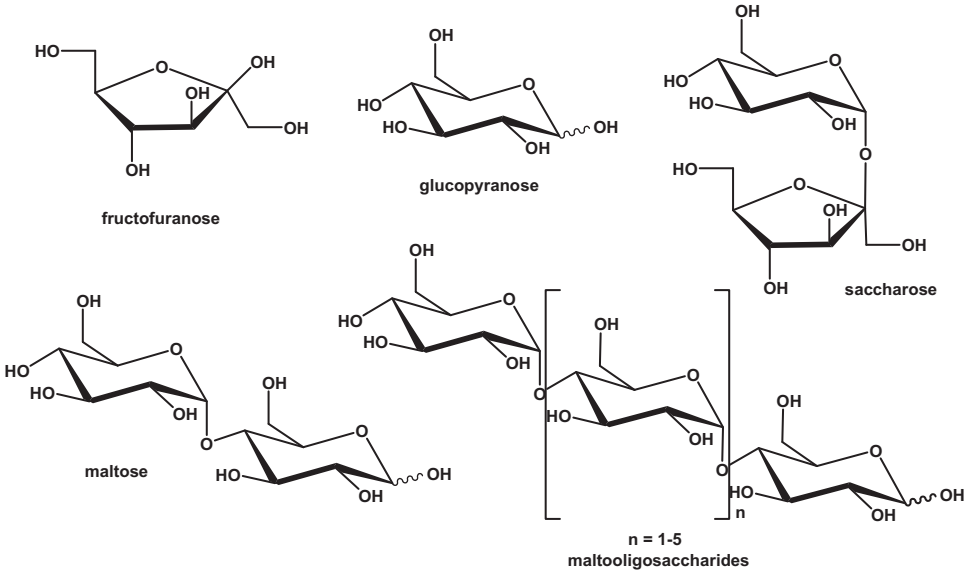


Fig. 7. Structural formulas of the sugars analyzed. Maltose oligomers: n = 1, maltotriose; n = 2, maltotetraose; n = 3, maltopentaose; n = 4, maltohexaose; n = 5, maltoheptaose.

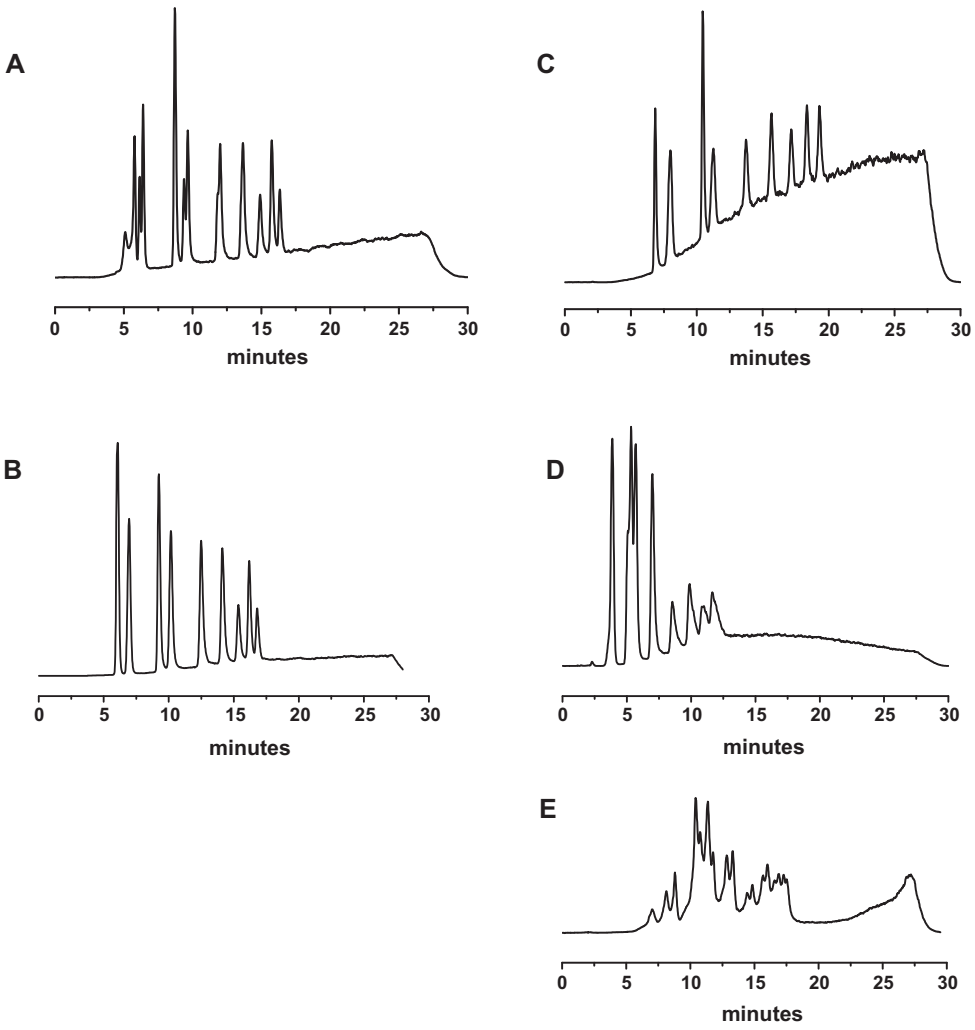


Fig. 8. Analysis of the sugars mixture. Columns: (A) USP-HILIC-sil; (B) USP-HILIC-NH_2-sil; (C) APS-2; (D) DIOL; (E) ZIC-HILIC. Eluent A: acetonitrile/water 90:10 (v/v); eluent B: water/acetonitrile 70:30 (v/v). Gradient elution from 100 to 30% A over 25 min (linear); back to 100% A in 1 min; 10 min re-equilibration time. Flow rate: 1.0 ml/min, T = 25 °C, ELSD detection (T_{neb} = 70 °C). Sugars solutions (0.5 mg/ml) in acetonitrile/water 50/50 (v/v) were freshly prepared and allowed to equilibrate at T = 25 °C prior to injection. For the composition of the sample, see Table 3.

Table 3
Chromatographic data obtained for a mixture of sugars (see Fig. 7 for structures) on the bidentate urea-type columns in comparison with Hypersil APS-2, DIOL, and ZIC-HILIC columns[a].

Sugar	USP-HILIC-sil		USP-HILIC-NH_2-sil		Hypersil APS-2		DIOL		ZIC-HILIC	
	t_r [b]	Δt_r [c]	t_r [b]	Δt_r [b]	t_r [b]	Δt_r [c]	t_r [b]	Δt_r [c]	t_r [b]	Δt_r [c]
Fructose	5.44		5.99		6.47		3.87		7.05	
		0.84		0.89		0.97		0.00		1.07
Glucose	6.28		6.88		7.44		3.87		8.12	
		2.44		2.26		3.32		1.46		0.68
Saccharose	8.72		9.14		10.76		5.33		8.80	
		0.80		0.94		0.57		0.36		2.77
Maltose	9.52		10.08		11.33		5.69		11.57	
		2.49		2.32		1.99		1.30		1.49
Maltotriose	12.01		12.40		13.32		6.99		13.06	
		1.65		1.63		1.91		1.55		1.58
Maltotetraose	13.66		14.03		15.23		8.54		14.64	
		1.25		1.15		1.49		1.34		1.20
Maltopentaose	14.91		15.18		16.72		9.88		15.84	
		0.84		0.80		1.11		0.97		0.89
Maltohexaose	15.75		15.98		17.83		10.85		16.73	
		0.59		0.72		0.92		0.80		0.69
Maltoheptaose	16.34		16.70		18.75		11.65		17.42	

[a] Columns geometry: 150 mm × 4.0 mm I.D.; eluent A: acetonitrile/water 90:10 (v/v); eluent B: water/acetonitrile 70:30 (v/v). Gradient elution from 100 to 30% A over 25 min (linear); back to 100% A in 1 min; 10 min re-equilibration time. Flow rate: 1.0 ml/min, T = 25 °C, ELSD (T_{neb} = 70 °C).
[b] Retention time (min).
[c] Difference in retention time (min).

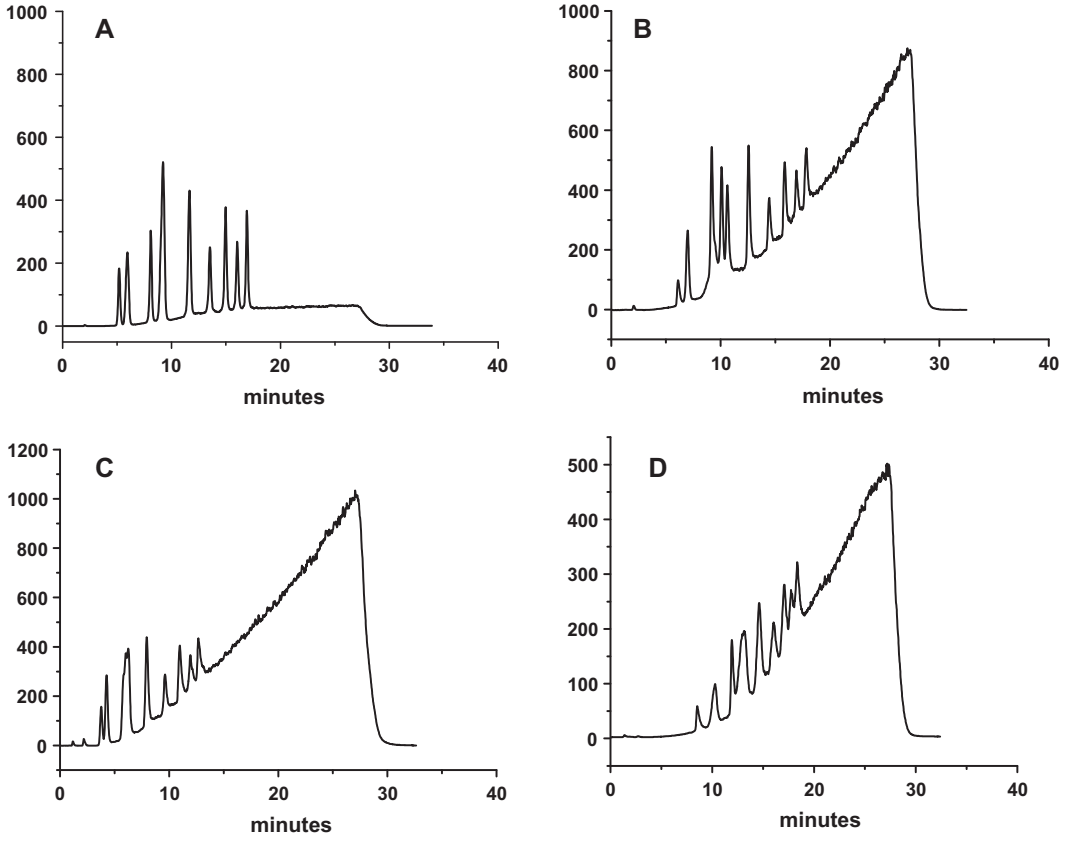


Fig. 9. Effect of the addition of triethylamine (TEA) in the analysis of the sugars mixture. Columns: (A) USP-HILIC-NH_2-sil; (B) APS-2; (C) DIOL; (D) ZIC-HILIC. Eluent A: acetonitrile/water 90:10 (v/v) plus 7 mM TEA (^s_wpH = 10.64); eluent B: water/acetonitrile 70:30 (v/v) plus 7 mM TEA (^s_wpH = 10.88). Gradient elution from 100 to 30% A over 25 min (linear); back to 100% A in 1 min; 10 min re-equilibration time. Flow rate: 1.0 ml/min, T = 25 °C, ELSD detection (T_{neb} = 70 °C). Sugars solutions (0.5 mg/ml) in acetonitrile/water 50/50 (v/v) were freshly prepared and allowed to equilibrate at T = 25 °C prior to injection. For the composition of the sample, see Table 3.

Elution order of hydroxybenzoic acids followed the expected trend of HILIC mechanism of separation, *i.e.*, 4-hydroxybenzoic acid (k ranging from 3.46 to 6.69) < 3,5-dihydroxybenzoic acid (k ranging from 4.49 to 9.18) < 3,4,5-trihydroxybenzoic acid (k ranging from 7.81 to 17.22), the least retained compound being the benzamide (k ranging from 1.23 to 1.80), since it lacks the carboxylic acid function. However, the HILIC-type interaction process cannot be invoked for the relative retention of 4-hydroxybenzoic and 4-hydroxybenzenesulphonic acids: in fact, retention of the former on the bidentate urea-type columns was always higher than that of the latter (k ranging from 2.08 to 3.89), despite its much lower polarity. Such an inverted elution order can be plausibly explained by taking into account superimposed non-HILIC interactions [24].

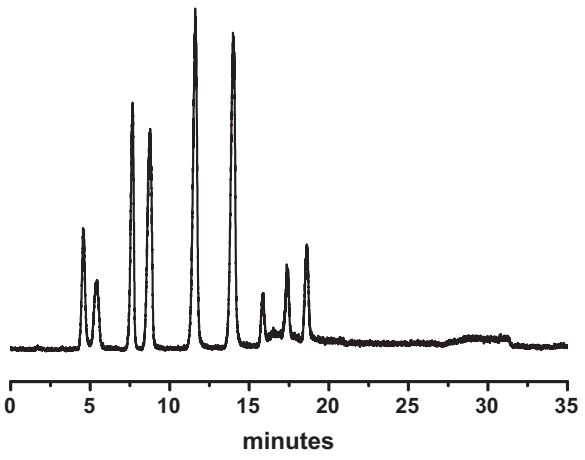


Fig. 10. HILIC/ESI-MS (negative mode) separation of the sugars mixture on the USP-HILIC-NH_2-sil column. Eluent A: acetonitrile/water 90:10 (v/v) plus 10 mM NH_4OAc (^s_wpH = 8.05); eluent B: water/acetonitrile 70:30 (v/v) plus 10 mM NH_4OAc (^s_wpH = 6.80). Gradient elution from 100 to 30% A over 25 min (linear); back to 100% A in 1 min; 10 min re-equilibration time. Flow rate: 1.0 ml/min. T = 25 °C. ESI-MS detection (see Section 2.7). Total ion current (TIC) from 220 to 1500 amu was monitored. Sugars solutions (2 mg/ml) in acetonitrile/water 50/50 (v/v) were freshly prepared and allowed to equilibrate at T = 25 °C prior to injection. For the composition of the sample, see Table 3.

To further investigate the capability of the new columns of separating organic acids, a mixture made up of 2-hydroxybenzoic (or salicylic) and 2-acetoxybenzoic (or acetylsalicylic) acids (see Fig. 5 for their structures), containing also vitamin-like compounds (namely, ascorbic and isoascorbic acids) was separated using a mobile phase consisting of acetonitrile/water 80:20 (v/v) plus 5 mM KH_2PO_4 (^s_wpH = 6.10). Indeed, under the chosen chromatographic conditions, the investigated columns (*i.e.*, USP-HILIC-sil, USP-HILIC-NH_2-sil, APS-2, and DIOL) showed the same elution order (see Table 4), with 2-hydroxybenzoic acid always eluting first (k ranging from 0.42 to 0.48 on the bidentate urea-type columns) or not retained at all (as in the case of APS-2 and DIOL column, k = 0.00). The ascorbic acid (actually featuring a lactone ring rather than a carboxylic acid group) was always eluting last (k ranging from 3.04 on the USP-HILIC-sil to 4.09 on the USP-HILIC-NH_2-sil column), preceded by the isoascorbic acid peak (k ranging from 2.62 on the USP-HILIC-sil to 3.41 on the USP-HILIC-NH_2-sil column), which indeed differs from it only for the stereochemistry of one of the two stereogenic carbons (*i.e.*, the two vitamins are diastereoisomers).

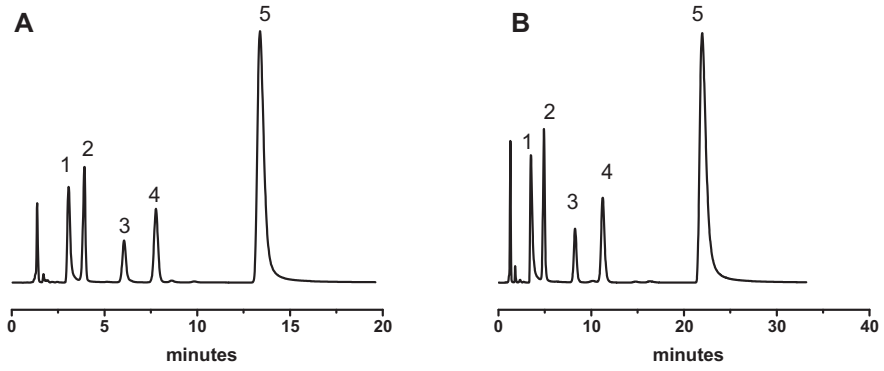


Fig. 11. Analysis of a mixture of organic aromatic acids and a related "non-charged" compound (see Fig. 1 for structures). Columns: (A) USP-HILIC-sil; (B) USP-HILIC-NH_2-sil. Eluent: acetonitrile/water 85:15 (v/v) plus 30 mM NH_4OAc (^s_wpH = 7.73). Flow rate: 1.0 ml/min, T = 25 °C, UV detection at 254 nm. Peak 1: 3,4,5-trihydroxybenzamide; peak 2: 4-hydroxybenzenesulfonic acid; peak 3: 4-hydroxybenzoic acid; peak 4: 3,5-dihydroxybenzoic acid; peak 5: 3,4,5-trihydroxybenzoic acid (or gallic acid).

Table 4
Chromatographic data obtained for a mixture of pharmaceuticals and vitamins-like compounds (see Fig. 5 for structures) on the USP-HILIC-sil and the USP-HILIC-NH_2-sil columns in comparison with Hypersil APS-2 and DIOL columns[a].

Sample	USP-HILIC-sil			USP-HILIC-NH_2-sil			Hypersil APS-2			DIOL		
	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]	k[b]	α[c]	R_s[d]
2-Hydroxybenzoic acid (or salicylic acid)	0.48			0.42			0.00			0.00		
		2.17	3.98		2.29	3.06		–	2.87		–	2.19
2-Acetoxybenzoic acid (or acetylsalicylic acid)	1.04			0.96			0.36			0.25		
		2.52	8.90		3.56	12.8		7.50	16.2		2.64	2.96
Isoascorbic acid	2.62			3.41			2.69			0.66		
		1.16	1.79		1.20	2.27		1.30	3.89		1.24	0.90
Ascorbic acid (or vitamin C)	3.04			4.09			3.49			0.82		

[a] Columns geometry: 150 mm × 4.0 mm I.D.; eluent: acetonitrile/water = 80:20 (v/v) plus 5 mM KH_2PO_4 (^s_wpH = 6.10); flow rate: 1.0 ml/min, T = 25 °C; UV detection at 240 nm.
[b] Retention factor.
[c] Selectivity factor.
[d] Resolution, calculated as described in Section 2.6.

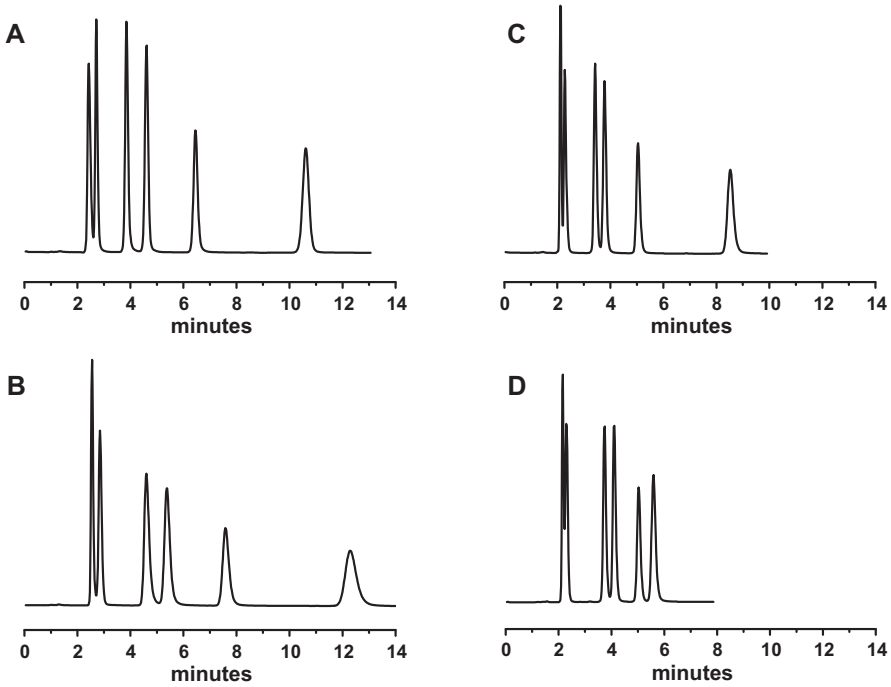


Fig. 12. Analysis of a mixture of nucleobases and nucleosides (see Fig. 1 for structures). Columns: (A) USP-HILIC-NH_2; (B) USP-HILIC-NH_2-sil; (C) APS-2; D) DIOL. Eluent: acetonitrile/water 90:10 (v/v) plus 10 mM NH_4OAc (^s_wpH = 8.05). Flow rate: 1.0 ml/min, T = 25 °C, UV detection at 240 nm. Elution order: thymine, uracil, adenine, adenosine, cytosine, and cytidine.

Furthermore, the USP-HILIC-NH_2-sil column showed higher selectivity values in all cases, when compared to USP-HILIC-sil. Finally, the USP-HILIC-NH_2-sil column behaves in a very similar manner to the APS-2 column, both in terms of peak shapes and retention: they indeed gave the same profiles, with salicylates eluting within the first 3 min of the chromatographic run, and vitamins-like compounds more strongly retained, with a separation between the two elution zones more marked for the APS-2 column (α between acetylsalicylic and isoascorbic acid being 7.50) than the bidentate urea-type column (α = 3.56). Such a simultaneous determination of drugs and vitamins-like compounds is highly desirable in the analysis of food and beverages, as well as of pharmaceutical preparations, and the bidentate urea-type columns proved a suitable alternative to previous approaches for such applications [43].

3.7. Analysis of nucleobases and nucleosides

In order to test the separation abilities of the bidentate urea-type phases over a wider range of analytes, a mixture containing four nucleobases (namely, thymine, uracil, adenine, cytosine) and two nucleosides (adenosine and cytidine) was eluted on the USP-HILIC-sil and USP-HILIC-NH_2-sil columns. The chromatographic profiles were then compared to the ones obtained by eluting the same mixture on the APS-2 and DIOL columns (see Fig. 12). The mobile phase consisted of acetonitrile-water 90:10 (v/v) containing a final 10 mM concentration of NH_4OAc. The elution order on all four columns was the same, with thymine always eluting before uracil. Surprisingly, adenosine was less retained than cytosine, despite the presence of ribose in the structure of the nucleoside. Cytidine was the most retained compound on all four columns. Retention and selectivity were higher on both urea-type columns when compared to the DIOL and the APS-2. A baseline separation of peak 3 (adenine) and peak 4 (adenosine) could only be obtained with the USP-HILIC-NH_2 phases, which also provided a considerably better separation of thymine and uracil. When compared to the non silanized phase, USP-HILIC-NH_2-sil showed an increase in analysis time and similar selectivity values.

4. Conclusions

New liquid chromatography stationary phases suited for HILIC applications have been prepared, packed into HPLC columns and evaluated using a large set of polar test solutes. The original synthetic procedure exploits the preliminary preparation of a bidentate alkoxysilane incorporating urea fragments, and its subsequent immobilization onto silica surface yielding double-tethered, hydrolytically stable urea-type stationary phases. A variant of the urea-type HILIC phase featuring some free amino groups on the silica surface was prepared and shown to be particularly suited for the analysis of sugars, whereby α/β anomer interconversion was accelerated by the residual basic sites and single sharp peaks were obtained for each individual sugar. The new HILIC stationary phases are chemical and thermally stable and showed good selectivities for a broad set of chemically diverse, polar compounds. Chemical inertness of the new urea-type HILIC stationary phases facilitated their use in LC–MS applications, giving drift-free chromatograms as a result of negligible release of bonded ligands during chromatographic elution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.028.

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