On-Column Quantification of Amino Functionalities Bonded to Solid Porous Matrices Packed within HPLC Columns.

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KEYWORDS Amino-modified silica; solids for CO_2 capture; UV retro-titration method for dosage of amino groups; characterization of basic stationary phases; on-column dosage of amino groups bonded to porous solids packed within HPLC columns.

ABSTRACT: Stationary phases (**SP**) based on silica matrices functionalized with amino groups linked to the surface through alkyl chains of various length have found remarkable success in performing HILIC separations, showing really effective resolution towards a wide typology of compounds of biological interest, such as carbohydrates, nucleosides, purine and pyrimidine bases. Recently, we developed an operationally simple procedure, named **DNBA-M**, non-destructive for the analysed **SP**, designed to quantify the density of basic groups (typically amino groups) chemically bonded to the surface of porous solids. In the present study the **DNBA-M** procedure has been suitably modified to allow the quantification of any typology of amino groups present on silica matrices packed into HPLC columns. The new approach,

OC-DNBA-M, has been successfully validated through analysis of two HPLC columns packed with aminopropyl-silica matrices. Afterwards, it was also demonstrated as the **OC-DNBA-M** procedure may allow the effective and in-depth analysis of the structural composition characterizing **SP*s*** packed inside HPLC columns, in which amino-groups have been differently and only partially involved in following ureidic functionalizations. It was also proved how the analysed columns can be readily re-employed for the chromatographic applications for which they have been designed, without appreciable deterioration of the respective discrimination abilities.

1. INTRODUCTION

Siliceous stationary phases characterized by amino groups stably bonded on their surface find a lot of important applications, such as CO_2 capture, storage preparation of stationary phases for liquid chromatography and synthesis of solid promoters for heterogeneous catalysis.

In such a context, the development of siliceous porous solids containing amines covalently bonded to the surface through alkyl chain spacers, has become a primary goal for the design of new and more efficient molecular recognitors, especially for Hydrophilic Interaction Liquid Chromatography (HILIC) applications, with particular attention to characterize the obtained final structure from a qualitative and quantitative point of view [6-13]. Indeed, it is of fundamental importance not only to determine in which extent the chosen selector is linked to the phase, but also in which percentage the individual basic/nucleophilic items introduced through chemical linking are really and readily available to establish the desired interaction with the guest molecule. In thes~~e~~ amino-derivatized solid matrices the basic-nitrogen can be part of various structural forms, such as -NH_2, -NHR, -NR_2, amino alcohols or amino-terminated dendrimers [14-17]. In particular, NH_2-silica phases, especially those obtained by synthetic processes involving (3-aminopropyl)-trimethoxysilane as electrophilic precursor, are among the most common stationary phases, **SPs,** exploited as such, or after further suitable derivatization. Indeed, this typology of selectors find large use in HILIC applications, which has been proven to be really effective in promoting the resolution of a wide typology of compounds of biological interest, such as carbohydrates, nucleosides, purine and pyrimidine bases [28-46]. In addition, 3-aminopropyl-silica materials can also profitably act as good nucleophiles, able to link on the

silica surface molecular structures with improved discriminating properties [18-27], and as frameworks effective in the coordination of metal ions. Accordingly to this latter property, amino-coordination of chromium and arsenic have found relevant applications in the treatment of waste water, while complexes with copper, rhodium and other transition metal ions have been fruitfully employed in catalysis [1-5].

Moreover, the primary -NH_2 groups linked on silica matrices, thanks to their minimal steric hindrance, are also suitable to favour a stable deposition on the surface of wide molecular selectors able to act as effective acceptors and donors hydrogen bonds. A relevant example is represented by amino-alkyl-silica matrices used for the preparation of polysaccharide-derived chiral stationary phases, **CSPs**, based on cellulose or amylose, which are characterized by a very high resolving power, and are particularly effective for the enantio-discrimination of racemates. Such polysaccharide structures can be simply adsorbed or chemically bonded to the surface of H_2N-modified silica. In the first case, the interactions established between selector and solid support are essentially due to the formation of a large network of hydrogen bonds between carbohydrate -OH groups and -NH_2 alkyl-silica groups. In this case, however, the resulting composite structures are not stable enough to be used as **SP** with mobile phases containing chlorinated solvents, as under these conditions, the polymer is inevitably dissolved and eluted from the column. Such a restriction is however surmounted if the polymer is chemically anchored to the matrix by formation of covalent bonds [39-46]. Nevertheless, the functionalization of amino-propyl-silicagel matrices with a polysaccharide structure does not guarantee the total involvement of the underlying amino functions in the establishment of the quoted network of H-bonds or covalent links with the selector. As undesirable consequence, the non-engaged NH_2 groups, confer to the **SP** a latent basicity which, in some circumstances, could represent an obstacle to the successful outcome of the separation. For example, it has been demonstrated that the presence of free NH_2 groups, can activate dynamic chemical processes concerning solutes particularly susceptible to isomerize through base-promoted mechanisms, giving rise to the generation of plateau zones between the peaks of the discriminated isomeric forms. A relevant example is represented by the chromatographic study performed on 2-[2-(1-methyl-1H-pyrrol-2-yl)-2-oxo-1-phenylethyl]-isoindole-1,3-dione, a chiral molecule characterized by high anti-MAO activity type-A [47]. This molecule, when subjected to discrimination of its enantiomeric forms by means of the commercial **CSP** Chiralpak AD (which

is based on an amylose derivative adsorbed on amino-silica gel), shows, in fact, a clear tendency to enantiomerize. This was put in evidence by the formation of a plateau zone between the peaks of the resolved enantiomers in the registered chromatograms, which can be attributed to the basicity of residual -NH_2 groups present on the siliceous matrix, free from any interaction with the polysaccharide selector. Chromatographic effects of residual amino groups on achiral **SP** derived from amino-propyl-silica gel can also be represented by strong variation of retention times, as in the case of phenylazo-8-quinolinol-silica gel (QSG) **SPs**, where wide changes of elution order were registered for a set of test molecules [48]. For the above-mentioned reasons, the quantification of the density of amino groups not involved in any kind of chemical interactions with the selector would represent an important information to obtain, in order to define the whole real properties possessed by **SP** prepared starting from amino-derivatized silica matrices. This, in fact, can allow a rational and in-depth comparison between columns packed with selectors of this typology.

In the field of heterogeneous catalysis, continuous flow reactors find application in various branches of chemistry, including photochemistry and processes that require the use of Supercritical Fluids [49]. In this scenario, propyl-aminated silica materials are used, for example, as solid catalyst in Michael's reactions and Knoevenagel condensations, which can be carried out in continuous flow conditions. Studies performed on this last kind of reactions have suggested that residual silanol groups, not involved in the derivatization process with the propylamine framework, can play a key role in the catalytic mechanism [50-52]. The acid-base cooperation between residual silanol groups and amino groups in the catalytic processes was more in depth investigated through the promotion of the Henry's reaction, which forms the α,β-unsaturated product via quasi-equilibrated iminium intermediate. The obtained results afforded key details about the structural requirements that make possible a good performance by this typology of solid catalysts [53]. Nevertheless, in the above-considered materials, an acid-base reaction may also occur between amino groups and residual silanols, which will be dependent from the geometric proximity existing on the silica surface among such functionalities [54, 55] (see **Figure 1**). In this case it is expected that the heterogeneous catalysis performed by means of continuous flow reactor will necessarily lead to lower yields. Therefore, it is important to characterize the catalyst structure not only about the total amount of amino functionalities

covalently bonded on the surface, but also to the amount that preserves the basic-nucleophilic activity.

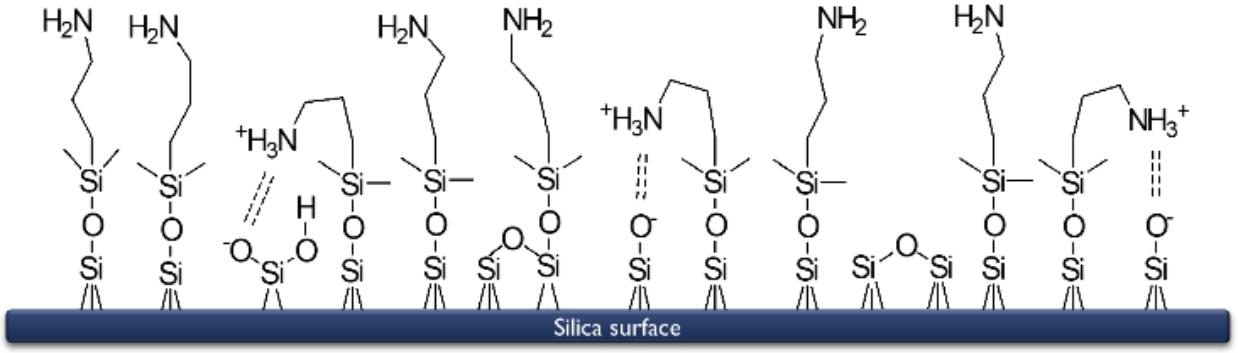

Figure 1. Possible acid-base reaction involving alkyl-amino groups bound on the surface of silica matrix and acid silanols placed in favourable proximity.

In a previous paper, we have reported about a simple and fast analytical procedure (**DNBA-M**), non-destructive for the analysed sample, which allows to quantify the density of amino groups (henceforth mentioned with the acronym **AGD**) covalently bonded through an alkyl-spacer on the surface of siliceous solid supports [56]. As a significant aspect characterizing the approach, in the obtained data are excluded the amino functionalities that have lost their basic-nucleophilic properties because of involvement in previous nucleophilic-electrofilic or acid-base reactions, including any chemical interaction that may have occurred with silanols at the surface. Interestingly, such a method could be, in principle, extendable to any other type of basic group of suitable strength (pKa ≥ 5).

In the present study our attention has been focused on the extension of the **DNBA-M** method to the on-column analysis of amino-alkyl-silica matrices (**SiO_2-R-ND_2**, with **D** representing hydrogens, alkyls or derivatizing groups), already packed into HPLC columns or continuous flow reactors. Taking advantage by the mild operating conditions required by the procedure, both chemical and structural properties are expected to be unchanged at the end of the determination, so preserving the related chromatographic discrimination ability or catalytic activity.

2. EXPERIMENTAL SECTION.

2.1 MATERIALS AND METHODS

2.1.1 Materials

LiChrosorb Si 100 NH_2 (pore size 100Å, particle size 10.0 µm, Art. 9331) was provided by Merck Millipore (Darmstadt, Germany). Spherical Kromasil Si 100 silica gel (pore size 100Å, particle size 5.0 µm, specific surface area 340 m^2/g) was purchased from Eka Nobel (Bohus, Sweden). Thermo Syncronis (pore size 190Å, particle size 1.7 µm, specific surface area 220 m^2/g) was from Thermo Scientific (Waltham, MA, US).

LC Column Luna® 5 µm C18 100 Å, 150 x 4.6 mm ID was provided by Phenomenex (Torrance, California, US).

(3-Aminopropyl)trimethoxysilane (**APTMS**), (3-isocyanatopropyl)triethoxysilane, ethylenediamine, tetraethylenpentamine (**TEPA**), 3,5-dinitrobenzoic acid (**DNBA**) at 99% of purity, potassium bromide FTIR grade (KBr), ammonium acetate, ammonium formate, (R,R)-1,2-diaminocyclohexane (**DACH**), 4-(dimethylamino)pyridine (**4-DMAP**), (R,R)-1,2-diphenyl-1,2-ethylenediamine (**DPEDA**), 1,2-Bis(trichlorosilyl)ethane, N-(Trimethylsilyl)imidazole, naphthalene, uracil, adenosine, acetonitrile (**ACN**), dichloromethane (**DCM**), methanol (MeOH), chloroform, water HPLC grade, n-hexane, dry toluene, hydrochloric acid reagent grade 37% (**HCl**), trifluoroacetic acid (**TFA**) were purchased from Sigma Aldrich. **HCl** solutions 1.0 and 0.1 M were prepared by dilution of **HCl** 37% with MilliQ water.

2.1.2 Instruments.

A Jasco 430 Fourier transform FTIR spectrometer with a resolution of 4 cm_1, Jasco Europe, Milan, Italy. A Jasco V570 UV/Vis/NIR spectrometer, Mary's Ct, Easton, MD 21601, US. The

chromatographic method was performed on a high-performance liquid chromatograph type Thermo Fisher Surveyor, equipped with PDA (Photodiode Array Detector) Plus Detector, Surveyor MS Pump and Rheodyne Model 7725 manual injector, Thermo Fisher Scientific, San Jose, CA, US. The elemental analysis was carried out using an elemental analyzer EA 1100 Carlo Erba, Milan, Italy.

The synthesis reactions of the **SPs** shown below were carried out by using a rotating evaporator (Buchi, Flawil, Switzerland), suitably modified, connected to a reflux condenser, a collector and a fitting for the introduction of Argon. This reactor allows the addition of reagents in an inert atmosphere. It was possible to carry out reactions at a controlled temperature by immersing the reaction flask in a thermostated oil bath. The agitation is obtained by rotating the reaction flask around its vertical axis.

2.1.3 Synthesis of 2-aminopropyl silica gel packed into column Col^2-PrNH$_2$.

The 3-aminopropyl silica, subsequently packed into column **Col^2-PrNH$_2$**, was prepared according to the methods described in literature [57]. Commonly, a slurry of 3.0 g of previously dried (high vacuum pump, T=120°, 1 h; P= 0.1 mbar) Kromasil Si 100 silica gel (pore size 100 Å, particle size 5.0 µm, specific surface area 340 m^2/g) in 60 ml of dry toluene was carried out in inert atmosphere. The (3-aminopropyl)triethoxysilane was then added (1.5 ml, 6.6 mmol) and the mixture was heated to reflux for 4 h. The silica gels thus obtained were isolated by filtration, washed with 30 ml portions of toluene, MeOH, and DCM and dried until constant weigh (90 ◦C, 0.1 mbar, 1 h). Modified amino-silica were characterized by FT-IR (KBr pellet and ATR) and (C,H,N) elemental analysis. FT-IR (KBr): 2931, 2854 cm^{-1}.

2.1.4 Synthesis of bidentate urea-type USP-HILIC SP (packed into column Col-Pr-Ur-ED)

The bidentate urea-type USP-HILIC **SP**, subsequently packed into column **Col-Pr-Ur-ED**, was prepared according to the methods described in literature [58]. In this case, a Thermo Syncronis (pore size 190Å, particle size 1.7 µm, specific surface area 220 m^2/g) was used. In the reaction flask, 0.16 ml of ethylenediamine (0.146 ml; 2.43 mmol) are dissolved in 25 ml of dry toluene and heated to a temperature of 110 ° C in an inert environment and under mechanical stirring. After distillation of 3 ml of solvent, 1.08 ml of (3-isocyanatopropyl)triethoxysilane (1.08 ml; 4.37 mmol) dissolved in 10 ml of toluene are added drop by drop. The reaction is maintained in an inert environment, under mechanical stirring for about 4 hours at 90-110 ° C. After, 3.18 g of silica (Syncronis 190Å; 1.7 µm), activated with ethanol 96° (275 ml), is added to the solution containing the product (activated ethylenediamine), and suspended in 25 ml of dry toluene. It is left to react at 80-90 ° C, under mechanical stirring and in an inert environment for 8h. At this point, 0.352 g of 4-(dimethylamino)pyridine (4-DMAP) dissolved in 10 ml of dry toluene are added and it is left to react for about 4 hours at 100 ° C. After work-up, the modified silica gel was washed with 50-ml portions of methanol and dichloromethane and dried in vacuo (0.1 mbar) at T = 60 ◦C to constant weight. At this point we proceed with "end-capping". A part of modified silica (1.6285 g), previously treated with a 10mM ammonium acetate solution, is suspended in 30 ml of dry toluene in the reaction flask. 0.086 g of imidazole are added to the suspension and 0.5ml (0.646 g) of 1,2-Bis(trichlorosilyl)ethane. It is left to react under reflux at 40-50 ° C under mechanical stirring for 2h. The silica thus obtained is filtered, washed with dichloromethane and dried a 50 ° C up to constant weight. Final weight: 1.8554gr Weight gain: 13.9%. Modified bidentate urea-type silica, after end-capping, were characterized by FT-IR (KBr pellet and ATR) and (C,H,N) elemental analysis. FTIR (KBr): 3367, 2978, 1645, 1559, 1079, 968, 789 cm^{-1}. Elemental analysis: % C = 6.275; % H = 1.36; % N = 2.221.

2.1.5 Preparation of the DPEDA-Crab-like CSPs (packed into columns Col[1]-Pr-Ur-DFED and Col[2]-Pr-Ur-DFED)

The DPEDA-Crab-like **CSP** was prepared following a one-pot procedure as already reported in a previous paper [59]. In this case, a Spherical Kromasil Si 100 silica gel (pore size 100 Å, particle size 5.0 µm, specific surface area 340 m^2 g^{-1}) was used for the preparation of the **CSP** packed into **Col[1]-Pr-Ur-DFED**. A solution of (R,R)-**DPEDA** (0.285 g; 1.34 mmol) in 20 mL of dry toluene was heated at 110 °C under a nitrogen atmosphere. After distillation of 3 mL of solvent, a solution of (3-isocyanatopropyl)triethoxysilane (0.70 mL; 2.68 mmol) in 5 mL of anhydrous toluene was added. The solution was kept under continuous stirring for 4 h at 110 ◦C. Kromasil Si-100 silica gel, previously hydrated with ethanol 96% (v/v) and thus washed and dispersed in 30 mL of toluene, was then added (2.512 g; 5 µm) and the reaction was kept for 8 h under continuous stirring at 80 °C. The derivatized silica was then treated with 4-DMAP (0.140 g; 1.13 mmol) for 4 h at 110 ◦C. The reaction was quenched by filtration. The silica gel was washed with toluene and dichloromethane and dried in vacuo (0.1 mbar, T = 80 ◦C). The derivatized and dried silica is dispersed in 60 ml of anhydrous toluene and 1 ml of N-(Trimethylsilyl)imidazole is added, it is left to react for 12 h at 80 ° C and under mechanical stirring. Modified silica, after end-capping, was characterized by FTIR (KBr): 3629, 3361, 2978, 2933, 1867, 1643, 1564, 1454, 1076, 953, 793 cm^{-1}, and elemental analysis: % C = 9.985; % H = 1.331; % N = 1.598. A second batch (packed into column **Col[2]-Pr-Ur-DFED**) was prepared with similar procedure. FTIR ? and elemental analysis data (% C = 8.809; % H = 0.984; % N = 1.532).

2.1.6 Preparation of the DACH-Crab-like CSP (Col-Pr-Ur-DACH)

The DACH-Crab-like **CSP** was prepared according to the methods described in literature [59]. In this case, a Spherical Kromasil Si 100 silica gel (pore size 100A˚, particle size 5.0 µm, specific surface area 340 m2 g−1) was used for the preparation of the **CSP** afterwards packed into column **Col[1]-Pr-Ur-DFED**. A solution of (R,R)-DACH (0.530 g; 4.60 mmol) in 60 mL of dry toluene was heated at 110 ◦C under a nitrogen atmosphere. After distillation of 3 mL of solvent, a solution of (3-propyl-isocyanate)triethoxysilane (2.18 mL; 9.20 mmol) in 5 mL of anhydrous toluene was added drop-wise. The solution was kept under continuous stirring for 4 h at 110 ◦C. Silica (Kromasil Si 100, 5 µm) previously hydrated with ethanol 96% (v/v) and later washed and dispersed in 40 mL of toluene, was then added (3.05 g) and the reaction was kept for 14 h under continuous stirring at 80 ◦C. The derivatized silica was then treated with 4-dimethylaminopyridine (4-DMAP; 0.187 g; 1.13 mmol) for 8 h at 110 ◦C. The reaction was quenched by filtration. The silica gel was washed with dichloromethane and dried at reduced pressure (0.1 mbar, T = 80 ◦C). Subsequently, the end-capping process is carried out in the same way as for DPEDA-Crab-like CSP. FT-IR (KBr): 3892, 3589, 3355, 2978, 2937, 2864, 1876, 1637, 1558, 1448, 1086, 960, 891, 795 cm−1. Elemental analysis: % C = 10.22; % H = 1.79; % N = 2.80.

2.1.7 Salification reaction of SiO_2-R-NH_2 amino groups with 3,5-dinitrobenzoic acid (preparation of SiO-R-NH^+ DNB^- silicas) and subsequent detachment.

Two different, and comparative, approaches have been developed for the quantification of the basic sites present on the silica surface. The first, already reported in the previous work, consists in the salification of silica in batches. The second, and innovative method, allows the quantification of the silica present in pre-packed HPLC columns.

- **Off column approach.**

In accordance with the procedure described in the previous paper [56], an amount ranging from 100 to 900 mg of commercial amino-silica LiChrosorb Si 100 NH_2, subsequently packed in the column **Col^1-PrNH$_2$**, is reacted with DNBA by dispersing it in a acetonitrile solution of 3,5-dinitrobenzoic acid 1.0 x 10^{-2} M (molar mass: 212,12 g/mol; 0.4221 g dissolved in 200 ml of ACN) under constant stirring, at room temperature, for 30 minutes, respecting a silica-mass/DNBA-solution ratio of 1 mg/ml. The so-treated silica powder (SiO_2-R-NH_3^+ DNB^-) is recovered by filtration, and then washed with acetonitrile. Finally, the SiO_2-R-NH_3^+ DNB^- silica is exhaustively dried, under reduced pressure (40°C, 0.1 mmHg), until constant weight. FTIR (KBr), shows new characteristic absorbing bands close to 1635, 1547, 1385 and 1356 cm^{-1}. In the second step of the procedure, the conjugated base of ADNB is detached from the amino-silica by an 5 ml of a 0.1 M solution of tetraethylenpentamine (**TEPA**) in acetonitrile (ACN) (molar mass: 189.31 g/mol; 4.735 g dissolved in 250 ml of ACN), which allows the formation of the salt between the polyamine and the ADNB, which goes into solution. Next, the supernatant, containing the **H:TEPA$^+$DNB$^-$** salt, is recovered by centrifugation and transferred into a 20 ml graduated flask, while the isolated silica is again dispersed into 5 ml of the 0.1M **TEPA** solution. This procedure is carried out for three times, collecting all the recovered supernatants into the same 20 ml graduated flask.

The same procedure was used for the salification of the synthesized silica Kromasil Si 100 (see paragraph 2.1.3), subsequently packed in the column **Col^2-PrNH$_2$**.

- **On column approach.**

Taking as an example from the off column analytical procedure described in the previous subsections, a methodology was developed for the quantification of basic amino-type sites present on **SP*s*** of prepackaged chromatographic columns. The treatment, to which all six

columns examined in this study were subjected (**Table S2 of Supplementary Material, SM**), can be summarized in the following four phases:

1) salification of the amino groups present on the phase to be characterized, obtained by flushing in the column 110 ml of 1.0 x 10^{-2} M solution (molar mass: 212.12 g / mol; 0.4221 g dissolved in 200 ml of ACN) of DNBA, a volume such as to ensure that the protonation process of the basic sites is quantitative;

2) extensive washing of the column from the residual DNBA solution obtained by flushing a large excess of acetonitrile (110 ml) into it;

3) quantitative deprotonation of ammonium sites, previously salified with DNBA, obtained by fluxing 45 ml of a 0.1 M solution of **TEPA** (molar mass: 189.31 g/mol; 4.735 g dissolved in 250 ml of ACN) in the column. These are then brought to the final volume of 50 ml in a calibrated flask to be subjected to UV or HPLC dosing of the disconnected DNBA (see next paragraph);

4) extensive washing of the column from the residual DNBA solution obtained by flushing a large excess of acetonitrile into it.

The salification operation of the **SP** with DNBA and the subsequent detachment with **TEPA** were carried out by loading a reservoir, directly connected to the chromatographic column under examination, with the solution containing the acid or polyamine and pushing this, through the use of hexane, into the head of the column.

2.1.8 Quantification of the dinitrobenzoic acid detached from the silica.

Two possible and independent approaches have been developed for make possible such retrotitration. The first one plans to perform a UV spectrophotometric analysis of the displaced ADNB in its ionized or neutral form. The second one implies, instead, the ADNB quantitation

performed by measurement of the area subtended by its relevant and well resolved chromatographic peak obtained by performing a HPLC analysis in reverse phase conditions.

- **UV-visible quantitation of the obtained H:TEPA^{+}DNB^{-} species.**

The content of the graduated flask is brought to volume with 0.1M **TEPA** solution, and the absorbance at 250 nm of the **H:TEPA^{+}DNB^{-}** species is registered by UV visible spectrophotometry, after dilution with methanol (ACN:MeOH 1:2). The quantitation of the released **H:TEPA^{+}DNB^{-}** salt (**Table S1 and S2 of SM**) is then performed by referring the recorded absorbance to the relevant calibration curve obtained from a standard solution of **TEPA** in ACN, that was used for the preparation of six diluted solutions with concentrations ranging from 8.3×10^{-5}M to 8.3×10^{-6} M, respecting the solvent ratio ACN:MeOH 1:2 (**Figure S1 of SM**).

- **RP-HPLC quantitation of the DNBA species.**

A quantification method of the DNBA displaced from salified silica samples of type SiO_2-R-NH_3^+ DNB^-, alternative to the one based on the UV-spectrophotometric measurements described in the subsections above, has been developed by resorting to a High Performance Liquid Chromatography (HPLC) procedure. For this reason, reverse phase chromatographic operating conditions were used which proved useful to ensure a satisfactory retention of DNBA in its totally undissociated form and its elution as a well isolated peak. The adopted chromatographic conditions are outlined below:

Instrument - Surveyor PDA Plus Detector - Thermo Fisher;

column - Luna C18 5µm (150 x 4.6 mm ID);

eluent - acetonitrile–water 45:55 (v/v) added with 0.1% of TFA;

flow rate - 1.00 ml/min; detector: UV 229 nm;

injection – 5 µL.

Then, with the intent to force the DNBA species to exclusively assume in solution its protonated form, the samples, used for the UV spectrophotometric analysis, undergo a first dilution step in the ratio of 1: 5 (v / v) using acetonitrile as solvent . Then a second dilution step takes place, corresponding to the ratio of 1: 9 (v / v), obtained by adding an aqueous solution of 1M HCl. The sample thus obtained is injected in reverse phase according to the chromatographic conditions described above. By extrapolating the area present under the chromatographic peak curve corresponding to ADNB, it is possible to obtain the quantitative data of interest (**Table S1 and S2 of SM**) by entering the value in the appropriate calibration curve obtained from a standard acqueos solution of 1M HCl, that was used for the preparation of six diluted solutions with concentrations ranging from 2.2×10^{-4} M to 8.8×10^{-6} M (**Figure S2 of SM**).

2.1.9 Chromatographic set-up.

HPLC columns: Stainless steel (250 mm × 4.6 mm I.D.) columns were packed with the DPEDA-Crab-like CSP; stainless steel (150 mm × 4.6 mm I.D.) columns were packed with and 2-aminopropyl silica gel; stainless steel (150 mm × 3.2 mm I.D.) column was packed with DACH-Crab-like CSP; stainless steel (100 mm × 4.6 mm I.D.) column was packed with the bidentate urea-type USP-HILIC **SP**. The solvent used for the packing step was acetonitrile as packing solvent and a backpressure value of 800 bar.

All chromatographic runs were carried out under isocratic elution at T = 25°C.

Col^1-$PrNH_2$, **Col^2-$PrNH_2$** and **Col-Pr-Ur-ED** have been tested in hydrophilic interaction chromatography (HILIC) mode with the flow rate set at 1.0 mL/min.

Col^{1}-Pr-Ur-DFED and **Col^{2}-Pr-Ur-DFED** have been tested in RP mode with the flow rate set at 1 mL/min.

Col-Pr-Ur-ED, **Col^{1}-Pr-Ur-DFED**, **Col^{2}-Pr-Ur-DFED** and **Col-Pr-Ur-DACH** have been tested in hydrophilic interaction chromatography (HILIC) mode with the flow rate set at 0.7 mL/min.

Col-Pr-Ur-DACH has been tested in Polar Organic Mode (POM-HPLC) for chiral application with the flow rate at 0.8 mL/min.

In RP mode, the mobile phase consisted of water/acetonitrile, 50/50 (v/v); HILIC mobile phase consisted of acetonitrile/10mM ammonium acetate in water 90/10 (v/v); POM mobile phase consisted of 10mM ammonium formate in acetonitrile/methanol 85/15 (v/v) 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 (1–5 µL aliquots).

In all RP and HILIC chromatographic runs, a test mixture containing naphthalene, uracil and adenosine.

In all POM chromatographic runs, a test mixture containing scalemic mixture of FMOC-aminoacids (FMOC-Leucine, FMOC-Phenylalanine and FMOC-Methionine).

3. RESULTS AND DISCUSSION

In order to develop a reliable procedure to determine the **AGD** values of amino-silica materials **SiO$_2$-R-ND$_2$** packed inside HPLC columns or flow-reactors, we referred to the analytical method **DNBA-M** described in literature [56]. The reported procedure consisted of the following, essential, four steps:

1) quantitative salification of the basic amino groups by fluxing trough the column a water solution of dinitrobenzoic acid (**DNBA**), with formation of **SiO_2-R-$ND_2H^+DNB^-$**;

2) extensive washing of the column with water, to eliminate the not-reacted DNBA aqueous solution;

3) quantitative displacement of the **DNB^-** anion from the salified amino groups, by fluxing an acetonitrile solution of tetraethylene-pentamine (**TEPA**), which allows the formation of the new salt **$TEPAH^+DNB^-$**, compound that, in turn, going into solution, will be next dosed by suitable approach;

4) extensive washing of the column with acetonitrile, so that the column would be ready to be used in the chromatographic or catalytic applications for which it was designed.

However, it is worth noting that **DNBA**, due to its aromatic framework, is poorly soluble in water so that a maximum operative concentration of 2.3×10^{-3} M can be reached. Therefore, in order to guaranty the contact of all the amino groups bonded on silica with a large excess of **DNBA** (not less than a factor 2.5 in moles), the volume of water solution of **DNBA** to be fluxed through the column should amount to about 500 ml. Thus, with a flow rate in the range of 0.70÷1.00 ml/min, the first step of the procedure would require from 12 to 8 hours, a certainly too long time to be managed, during which hydrolytic degradation reactions can occur on the structure of silica, due to the long exposure to the acidic solution.

For these reasons, in the first step of the present procedure acetonitrile was selected as a more convenient solvent than water to dissolve **DNBA**.

Indeed, in acetonitrile, the solubility of **DNBA** increased of about a factor 4 (reached operative concentration of **DNBA** = 1.0×10^{-2} M) and, at the same time, hydrolytic degradation reactions of the silica structure is avoided.

In order to optimize the mentioned analytical approach, we first tested the ability of the on-column procedure (from now on symbolized as **OC-DNBA-M**) to provide reliable **AGD** values for aminoalky-silica stationary phases (**A-SP*s***) packed into HPLC columns. For this purpose two aminopropyl-silica matrices have been selected, one of commercial production, which was packed inside a column that will be denoted **Col^1-PrNH$_2$**, the other one specifically prepared in our laboratory and next packed into the column that will be reported with the acronym **Col^2-PrNH$_2$**. The respective **AGD** values have been determined by both the **OC-DNBA-M** and the **DNBA-M** procedures, the latter applied on the relative unpacked materials. In this way it was possible to verify the consistence between the results obtained from the off- and on-column determination.

With regard to the third step of the **OC-DNBA-M** procedure, the retro-titration of the **DNB$^-$** anion displaced from the **SP** by **TEPA** has been performed by means of two independent approaches, the same adopted within the **DNBA-M** method. The first of these consists in an UV-spectrophotometric analysis in which the absorbance at 250 nm has been used to estimate the **DNB$^-$** concentration, referring to an appropriate calibration curve, from which the final ^{UV}**AGD** value can be obtained by the known amount of **SP** packed into the HPLC column. The second approach consists in the acidification of the displaced **DNB$^-$** anions with an aqueous HCl solution into a graduated flask. Then, the obtained **DNBA** has been quantified by HPLC analysis in reverse phase conditions, measuring the area subtended by its well resolved chromatographic

peak. In this way, the amino groups density (^{HPLC}**AGD)** can be determined taking into account the amount of **SP** packed into the HPLC column.

In order to confer a greater statistical significance to the performed determinations, for each of the two analyzed HPLC columns the whole **OC-DNBA-M** procedure has been repeated two times. The final **AGD** quantity was then obtained as the average of the found ^{UV}**AGD** and ^{HPLC}**AGD** values.

Finally, the resolution ability of both the analyzed columns, employed in HILIC conditions, towards naphthalene, uracil and adenosine (used as selectands test), has been registered before and after the on-column **AGD** determination. In this way, it was also possible to verify the absence of any degradation reaction triggered on the structure of silica matrix, which would manifest itself in terms of reduction of both efficiency and resolution of the analyzed column.

3.1 Application of the OC-DNBA-M procedure to the analysis of the A-SP packed into the HPLC column Col^{1}-PrNH$_2$.

Commercial aminopropyl-silica Lichrosorb Si 100 NH_2, 10 µm (0.814 g) have been packed into a HPLC column (150 × 4.6mm I.D.), whose chromatographic discrimination ability under HILIC conditions has been tested toward naphthalene, uracil and adenosine in mixture (red chromatographic profile of **Figure 2**). The **AGD** value of the packed **A-SP** was then determined as average of the found ^{UV}**AGD** and ^{HPLC}**AGD** values from the **OC-DNBA-M** on-column procedure (^{UV}**AGD-Col$^{1}_{on\text{-}column}$** and ^{HPLC}**AGD-Col$^{1}_{on\text{-}column}$** in **Table 1**). The **AGD** value can be compared with that obtained on the same **A-SP**, in the un-packed form, by the **DNBA-M** off-column method (i.e. the average value from the ^{UV}**AGD-Col$^{1}_{off\text{-}column}$** and

^{HPLC}**AGD-Col$^{1}_{off\text{-}column}$** values of **Table 1**). The analysis of the data reported in **Table 1** clearly shows that there is a full consistency between **AGD** values estimated with the two distinct procedures **OC-DNBA-M** and **DNBA-M**, as they differ by no more than 3%. Therefore, this result suggests the good reliability of the proposed on-column analysis in determining **AGD** values for **SP*s*** packed in HPLC columns. In addition, all the original chromatographic characteristics of **Col^{1}-PrNH$_2$** are completely preserved, as suggested by the comparison between the chromatographic profiles registered before and after the analytical treatment (red trace against green trace in **Figure 2**).

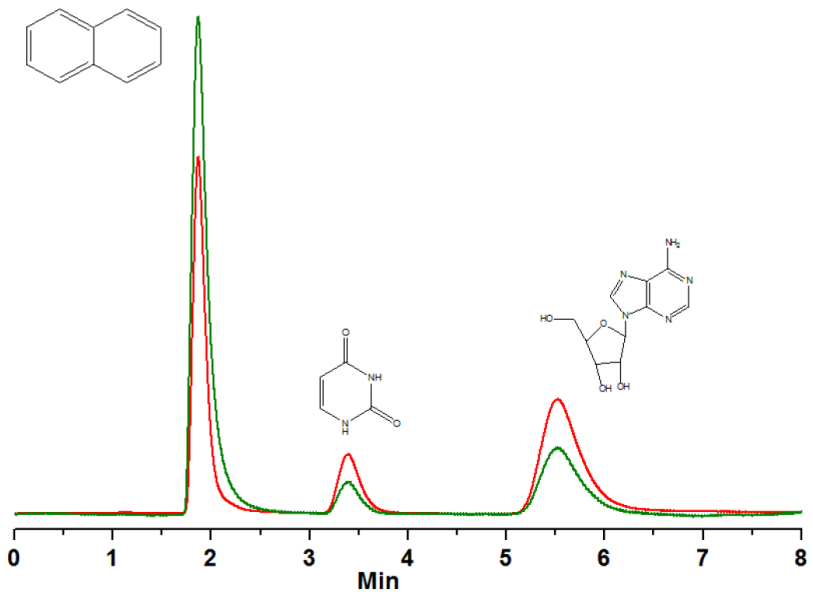

Figure 2. Test of the recognition ability of Col^{1}-PrNH$_2$ column towards the molecules of naphthalene, uracil, and adenosine in mixture. In red the chromatographic profile recorded before the treatment with the OC-DNBA-M procedure, in green the one recorded after the treatment.

Table 1. Off- and On-column AGD analysis of the amino-silica SP packed into column Col^{1}-PrNH$_2$

Kind of **AGD** determination	Single determinations of **AGD**	Average **AGD**	Average **AGD: off-column and on-column**	% difference between **AGD$_{off\text{-}column}$** and **AGD$_{on\text{-}column}$**
	(mmol ADNB/g A-SP)			
^{UV}**AGD-Col$^{1}_{off\text{-}column}$**	0.52 0.53 0.53 0.56	0.54	0.55	±3.0
^{HPLC}**AGD-Col$^{1}_{off\text{-}column}$**	0.63 0.52 0.56 0.52	0.56		
^{UV}**AGD-Col$^{1}_{on\text{-}column}$**	0.59 0.60	0.59	0.58	
^{HPLC}**AGD-Col$^{1}_{on\text{-}column}$**	0.66 0.56 0.62 0.53 0.48	0.57		

3.2 Application of the OC-DNBA-M procedure to the analysis of the A-SP packed into the HPLC column Col^{2}-PrNH$_2$.

The determination of **AGD** for the **SP** by the **OC-DNBA-M** procedure was carried out also for the **A-SP** (1.086 g of home-made aminopropyl-silica obtained by reaction between Kromasil 100-5-SIL, 5 µm, and APTMS) packed into the HPLC column **Col^{2}-PrNH$_2$** (150 × 4.6mm I.D.).

The obtained results are collected in **Table 2**. As observed in the case of **Col[1]-PrNH$_2$**, the analysis of the data confirms the existence of a very good correspondence between the **AGD** values determined with the two analytical off- and on-column procedures, which differ by less than 7%.

Table 2. Off- and On-column AGD analysis of the amino-silica SP packed into column Col[2]-PrNH$_2$

Kind of **AGD** determination	Single determinations of **AGD**	Average **AGD**	Average **AGD: off-column and on-column**	% difference between **AGD$_{off\text{-}column}$** and **AGD$_{on\text{-}column}$**
	(mmol ADNB/g A-SP)			
^{UV}AGD-Col$^{2}_{off\text{-}column}$	0.14 0.13	0.14	0.13	±6.7
^{HPLC}AGD-Col$^{2}_{off\text{-}column}$	0.12 0.15 0.09 0.10	0.11		
^{UV}AGD-Col$^{2}_{on\text{-}column}$	0.15 0.14	0.14	0.14	
^{HPLC}AGD-Col$^{2}_{on\text{-}column}$	0.15 0.14 0.14 0.14	0.14		

Moreover, the chromatographic discrimination ability under HILIC conditions possessed by the column toward naphthalene, uracil and adenosine has been tested before and after the performed **OC-DNBA-M** procedure also in this case (**Figure 3**).

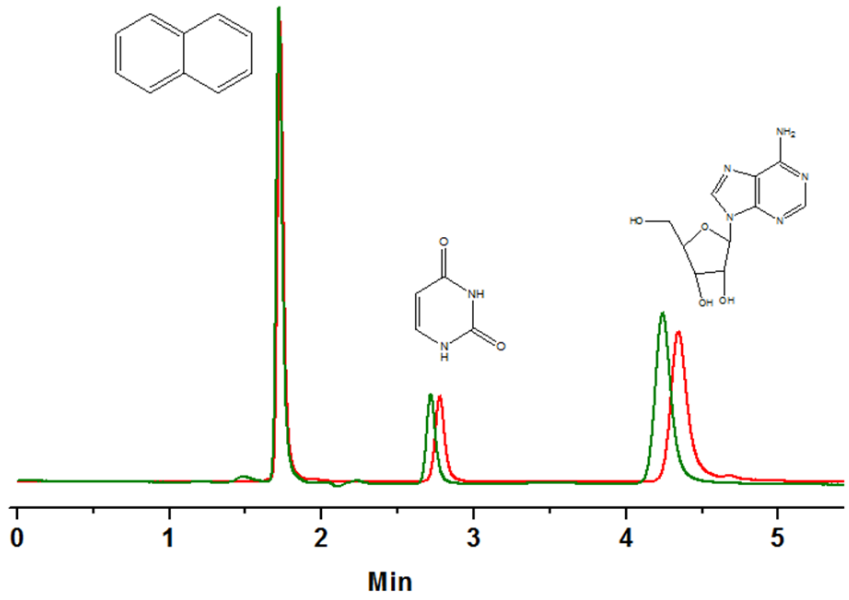

Figure 3. Test of the recognition ability of Col^2-PrNH$_2$ column towards the molecules of naphthalene, uracil and adenosine in mixture. In red the chromatographic profile recorded before the treatment with the OC-DNBA-M procedure, in green the one recorded after the treatment.

The comparison between the relevant chromatograms registered before and after the analysis shows that also in this case the recognition test is positive. Thus, the good coherence between **AGD** data obtained by the off- and on-column methods for both **Col^1-PrNH$_2$** and **Col^2-PrNH$_2$**, as well as the observation that the **A-SP*s*** completely retain their chromatographic discrimination

ability after the on-column treatment, allows us to consider validated the new **OC-DNBA-M** procedure proposed in the present study.

3.3 Application of the OC-DNBA-M procedure to the analysis of the structural compositions of SP*s* packed into HPLC columns containing amino groups partially reacted to form new groups endowed with negligible basic/nucleophilic activity.

The good results obtained about the ability of the **OC-DNBA-M** method to afford reliable **AGD** values of **A-SP**s packed into HPLC columns prompted us to consider the possibility of a further extension of its application. In particular, we analyzed if it could give quantitative indications about the final structural composition of a **SP** containing both free amino groups and amino groups chemically derivatized to form new functionalities endowed with negligible basicity/nucleophilicity, such as ureidic or amidic frameworks. For this purpose, the **OC-DNBA-M** treatment has been applied to the **AGD** analysis of four HPLC columns typically employed in HILIC conditions, equipped with amino-ureidic chromatographic selectors. Such **SP*s*** were prepared in our laboratory, following a common synthetic procedure according to which isocyanate functionalities are linked to the surface of silica matrices (silica hereafter symbolized as **(SiO_2)-C_3H_6-N=C=O**) by reaction between these and 3-(trimethoxysilyl)propyl isocyanate. Then, the **(SiO_2)-C_3H_6-N=C=O** silicas were submitted to nucleophilic acyl substitution reactions promoted by one among three different alkyl diamines: 1) 1,2-ethylenediamine, **ED**; 2) (R,R)-1,2-diphenyl ethylenediamine, **DFED**, from which were prepared two independent lots of **CSP*s***; 3) (R,R)-1,2-diaminocyclohexane, **DACH**. In this way, they were generated three possible functionalities of ureidic type, schematized in **Figure 4.** The

so derivatized silicas were submitted to elemental analysis, and then packed into the following four HPLC columns:

1) the phase derived by amine **ED** into a column (100 mm × 4.6 mm ID), denoted **Col-Pr-Ur-ED**;

2) the phases derived by amine **DFED**, into two distinct columns (250 mm × 4.6 mm ID), denoted **Col^1-Pr-Ur-DFED** and **Col^2-Pr-Ur-DFED**, respectively;

3) the phase derived by amine **DACH** into a column (150 mm × 3.2 mm ID), denoted **Col-Pr-Ur-DACH**.

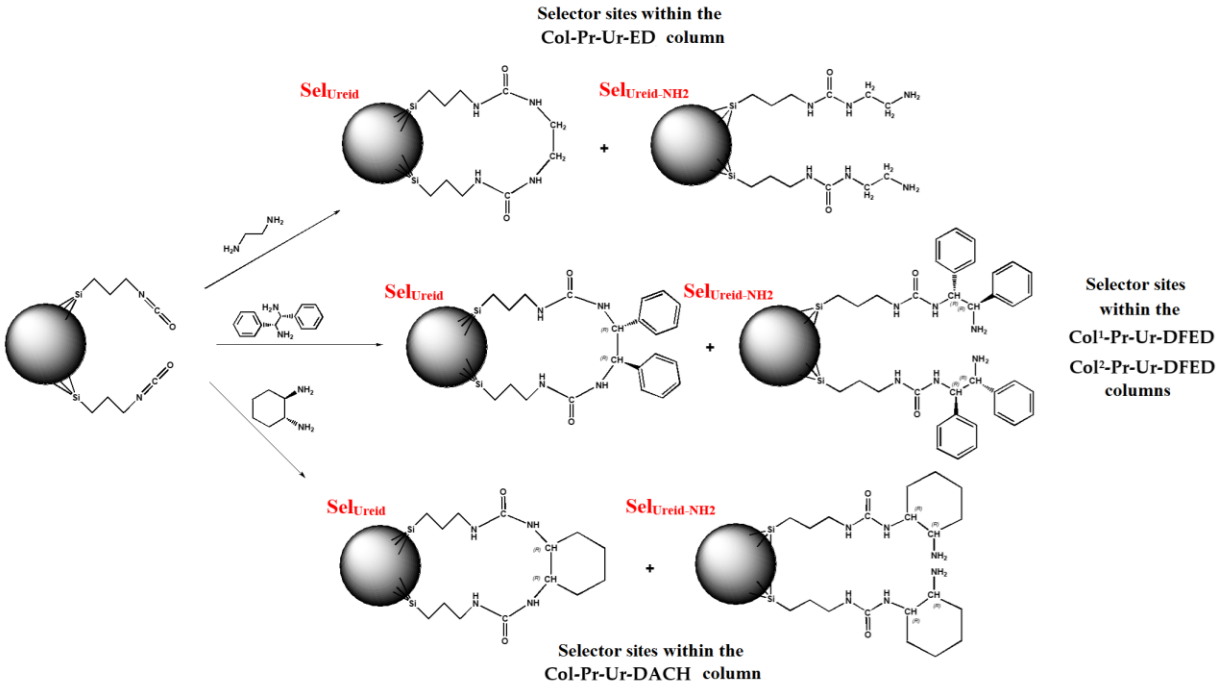

Figure 4. Structures possessed by the portions of selectors contained inside the Col-Pr-Ur-ED, Col^1-Pr-Ur-DFED, Col^2-Pr-Ur-DFED e Col-Pr-Ur-DACH columns.

As shown in **Figure 4**, in all the three kind of synthesized **SP*s*** there are two typologies of ureidic structures. One typology is derived from the reaction of both the amino groups of the employed diamine with two isocyanate groups sufficiently close one to each other, so that in this ureidic selector (**Sel_{Ureid}**) all the original nitrogen atoms of the diamine have lost their good ability to act as basic/nucleophilic sites. In the other typology (**$Sel_{Ureid\text{-}NH_2}$**), only one of the two amino groups of the diamine is involved in the substitution reaction, so that the unreacted amino groups retain their typical basicity and nucleophilicity. In this way, if for a given derivatized **SP** of the above type the total moles of N atoms per gram of derivatized silica is known by suitable and independent determination (e.g by elemental analysis), the evaluation of the **AGD** values by the **OC-DNBA-M** approach would allow to quantitatively distinguish the fraction of N atoms of the non-reacted amino groups from the ones making part of the ureidic frameworks. This is just what we have done in the last part of the study, with the intent to finely characterize the structure of the **SP*s*** packed into the four columns **Col-Pr-Ur-ED**, **Col^1-Pr-Ur-DFED**, **Col^2-Pr-Ur-DFED** and **Col-Pr-Ur-DACH**.

3.3.1 Evaluation of the fractions of selectors type Sel_{Ureid} and $Sel_{Ureid\text{-}NH_2}$ constituting the SP of column Col-Pr-Ur-ED.

The **SP** of column **Col-Pr-Ur-ED** has been analyzed by means of the **OC-DNBA-M** procedure (a single determination) in order to quantify its **AGD**. This was done by resorting to both spectrophotometric (**$^{UV}AGD_{Col\text{-}Pr\text{-}Ur\text{-}ED}$**) and HPLC (**$^{HPLC}AGD_{Col\text{-}Pr\text{-}Ur\text{-}ED}$**) approach. The obtained data, together with the related elemental analysis results, have been collected in **Table 3**.

Table 3. On-column AGD analysis of the amino-ureidic SP packed into column Col-Pr-Ur-ED

Kind of **AGD** determination	Single **AGD** determinations	Average of ^{UV}**AGD** and ^{HPLC}**AGD** values	Final **AGD**
	(mmol ADNB/g A-SP)		
^{UV}**AGD**$_{Col\text{-}Pr\text{-}Ur\text{-}ED}$	0.36 0.38	0.37	0.33
^{HPLC}**AGD**$_{Col\text{-}Pr\text{-}Ur\text{-}ED}$	0.25 0.35 0.23	0.28	
Elemental analysis **% C = 6.275; % H = 1.36; % N = 2.221.**			1.59

From these data, the ratio between the **Sel**$_{Ureid}$ and **Sel**$_{Ureid\text{-}NH_2}$ molar fractions present on the surface of the **SP** can be calculated. Indeed, since within each arm of **Sel**$_{Ureid\text{-}NH_2}$ selector it is present just one basic N atom making part of a NH_2 group, the measured **AGD** value (that is, 0.33 mmol/g) will coincide precisely with the density of the **Sel**$_{Ureid\text{-}NH_2}$ selector component of the **SP**. In addition, since within each arm of **Sel**$_{Ureid\text{-}NH_2}$ selector there are also two not basic ureidic N atoms, in this framework the overall density of N atoms is 3 times the determined value of **AGD** (N^{D}-^{ED}**Sel**$_{Ureid\text{-}NH_2}$ = 0.99 mmol/g). Consequently, the density of the just ureidic N atoms, with no appreciable basicity and belonging to the second type of selector **Sel**$_{Ureid}$ (N^{D}-^{ED}**Sel**$_{Ureid}$), can be obtained by subtracting N^{D}-^{ED}**Sel**$_{Ureid\text{-}NH_2}$ to the absolute N density value coming from elemental analysis, that is, 1.59 mmol/g. Therefore, the achieved N^{D}-^{ED}**Sel**$_{Ureid}$

value corresponds to 0.60 mmol/g. Starting from these last information, the densities of the $^{ED}Sel_{Ureid\text{-}NH_2}$ and $^{ED}Sel_{Ureid}$ selector frameworks on the **SP** surface can be obtained through the ratios $N^{D}\text{-}^{ED}Sel_{Ureid\text{-}NH_2}/3$ and $N^{D}\text{-}^{ED}Sel_{Ureid}/4$ (where 3 an 4 refer to the number of N atoms present in the $^{ED}Sel_{Ureid\text{-}NH_2}$ and $^{ED}Sel_{Ureid}$ selector portions, respectively).

Thus, structure of **SP** active for molecular recognition in the column **Col-Pr-Ur-ED** must contain 0.33 mmol/g of sites $^{ED}Sel_{Ureid\text{-}NH_2}$ and 0.15 mmol/g of sites $^{ED}Sel_{Ureid}$, which correspond to 69% of sites $^{ED}Sel_{Ureid\text{-}NH_2}$, and 31% of sites $^{ED}Sel_{Ureid}$ (**Figure 5**). Finally, the chromatographic resolution test of the column performed on the molecules of naphthalene, uracil and adenosine before and after the **OC-DNBA-M** analysis confirmed the absence of any degradation reaction suffered by the selector, which, in fact, completely retains its chromatographic discrimination ability (**Figure 5**). This observation provides a further indication in favor of the mild operative conditions that characterize the **OC-DNBA-M** procedure.

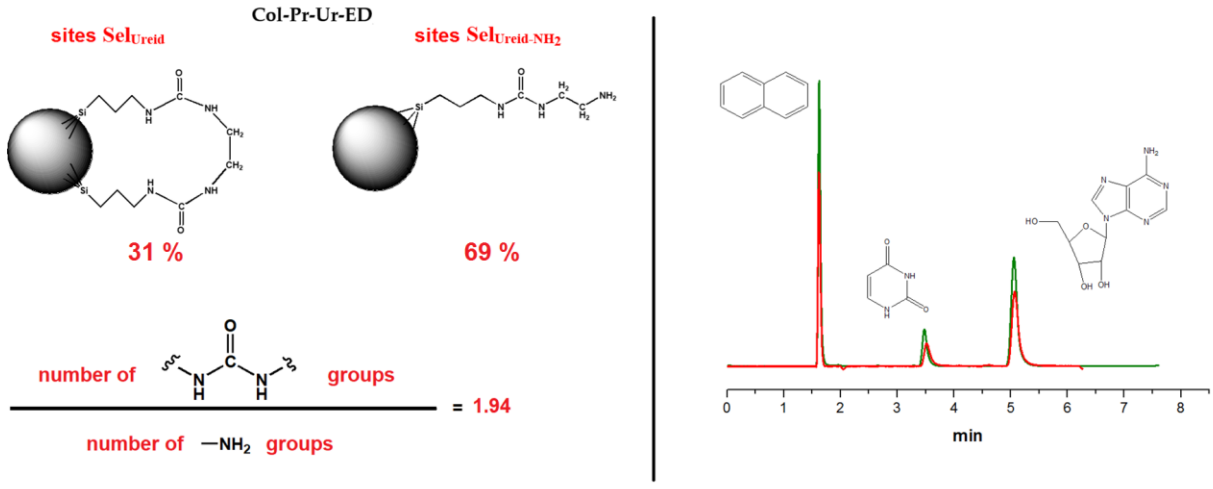

Figure 5. On the left: estimated percentage of the sites $^{ED}Sel_{Ureid}$ and $^{ED}Sel_{Ureid\text{-}NH_2}$ constituting the selector of column Col-Pr-Ur-ED. On the right: chromatographic resolution of naphthalene, uracil and adenosine performed before (red trace) and after

(green trace) the OC-DNBA-M analysis. Chromatographic conditions: column 100 mm × 4.6 mm ID; mobile phase CH_3CN/CH_3COONH_4 90/10, 10 mM; flow rate 0.7 ml/min; T=25°C.

3.3.2 Evaluation of the fractions of selectors type $^{DFED}Sel_{Ureid}$ and $^{DFED}Sel_{Ureid\text{-}NH_2}$ constituting the CSPs of columns Col^1-Pr-Ur-DFED and Col^2-Pr-Ur-DFED.

In aim to obtain quantitative information about the ratio with which the two typologies of selector sites $^{DFED}Sel_{Ureid}$ and $^{DFED}Sel_{Ureid\text{-}NH_2}$ participate to the constitution of the amino-ureidic **CSPs** obtained by reaction between isocyanate and DFED amine, the whole procedure described in the previous paragraph **3.3.1** has been repeated for the couple of columns **Col^1-Pr-Ur-DFED** and **Col^2-Pr-Ur-DFED**. Also in this case, in the third step of the procedure it was done resort to both spectrophotometric ($^{UV}AGD_{Col1\text{-}Pr\text{-}Ur\text{-}DFED}$ and $^{UV}AGD_{Col2\text{-}Pr\text{-}Ur\text{-}DFED}$) and HPLC ($^{HPLC}AGD_{Col1\text{-}Pr\text{-}Ur\text{-}DFED}$ and $^{HPLC}AGD_{Col2\text{-}Pr\text{-}Ur\text{-}DFED}$) determinations of the displaced **DNBA**, taking the average of their values as the final amount of $AGD_{Col1\text{-}Pr\text{-}Ur\text{-}DFED}$ and $AGD_{Col2\text{-}Pr\text{-}Ur\text{-}DFED}$. All such data, together with the respective determinations of elemental analysis, have been collected in **Table 4** for column **Col^1-Pr-Ur-DFED** and **Table 5** for column **Col^2-Pr-Ur-DFED**, respectively.

Table 4. On-column AGD analysis of the amino-ureidic CSP packed into column Col[1]-Pr-Ur-DFED

Kind of **AGD** determination	Single **AGD** determinations	Average of ^{UV}**AGD** and ^{HPLC}**AGD** values	Final **AGD** values
	(mmol ADNB/g A-SP)		
$^{UV}AGD_{Col^1\text{-}Pr\text{-}Ur\text{-}DFED}$	0.05 0.06	0.06	0.08
$^{HPLC}AGD_{Col^1\text{-}Pr\text{-}Ur\text{-}DFED}$	0.11 0.08 0.07	0.09	
Elemental analysis **% C = 9.985; % H = 1.331; % N = 1.598**			1.14

Table 5. On-column AGD analysis of the amino-ureidic CSP packed into column Col[2]-Pr-Ur-DFED

Kind of **AGD** determination	Single **AGD** determinations	Average of ^{UV}**AGD** and ^{HPLC}**AGD** values	Final **AGD** values
	(mmol ADNB/g A-SP)		
$^{UV}AGD_{Col^2\text{-}Pr\text{-}Ur\text{-}DFED}$	0.08 0.09	0.09	0.11
$^{HPLC}AGD_{Col^2\text{-}Pr\text{-}Ur\text{-}DFED}$	0.14 0.11 0.10	0.12	
Elemental analysis **% C = 8.809; % H = 0.984; % N = 1.532**			1.09

In accordance with the same considerations already discussed for column **Col-Pr-Ur-ED**, starting from the total density of N atoms afforded by elemental analysis and from the obtained $\mathbf{AGD}_{\mathbf{Col_1\text{-}Pr\text{-}Ur\text{-}DFED}}$ and $\mathbf{AGD}_{\mathbf{Col_2\text{-}Pr\text{-}Ur\text{-}DFED}}$ values, it was possible to estimate the structure active in the molecular recognition of both column **Col^1-Pr-Ur-DFED** and column **Col^2-Pr-Ur-DFED**. For the first of these, the results indicate that the selector is constituted for 23% from sites of type $^{\mathbf{DFED}}\mathbf{Sel}_{\mathbf{Ureid\text{-}NH_2}}$, while for 77% from sites $^{\mathbf{DFED}}\mathbf{Sel}_{\mathbf{Ureid}}$. For the second column the equivalent percentages were instead 34% of sites $^{\mathbf{DFED}}\mathbf{Sel}_{\mathbf{Ureid\text{-}NH_2}}$, and 66% of sites $^{\mathbf{DFED}}\mathbf{Sel}_{\mathbf{Ureid}}$ (**Figure 6**). Thus, it is possible to affirm that exist a marked similarity between the amino-ureidic phases packed into the two here analyzed columns, and that, at the same time, it can, instead, be observed a significant difference in the percentage composition of the sites $^{\mathbf{DFED}}\mathbf{Sel}_{\mathbf{Ureid}}$ and $^{\mathbf{DFED}}\mathbf{Sel}_{\mathbf{Ureid\text{-}NH_2}}$ among these phases and the one found for column **Col-Pr-Ur-ED**.

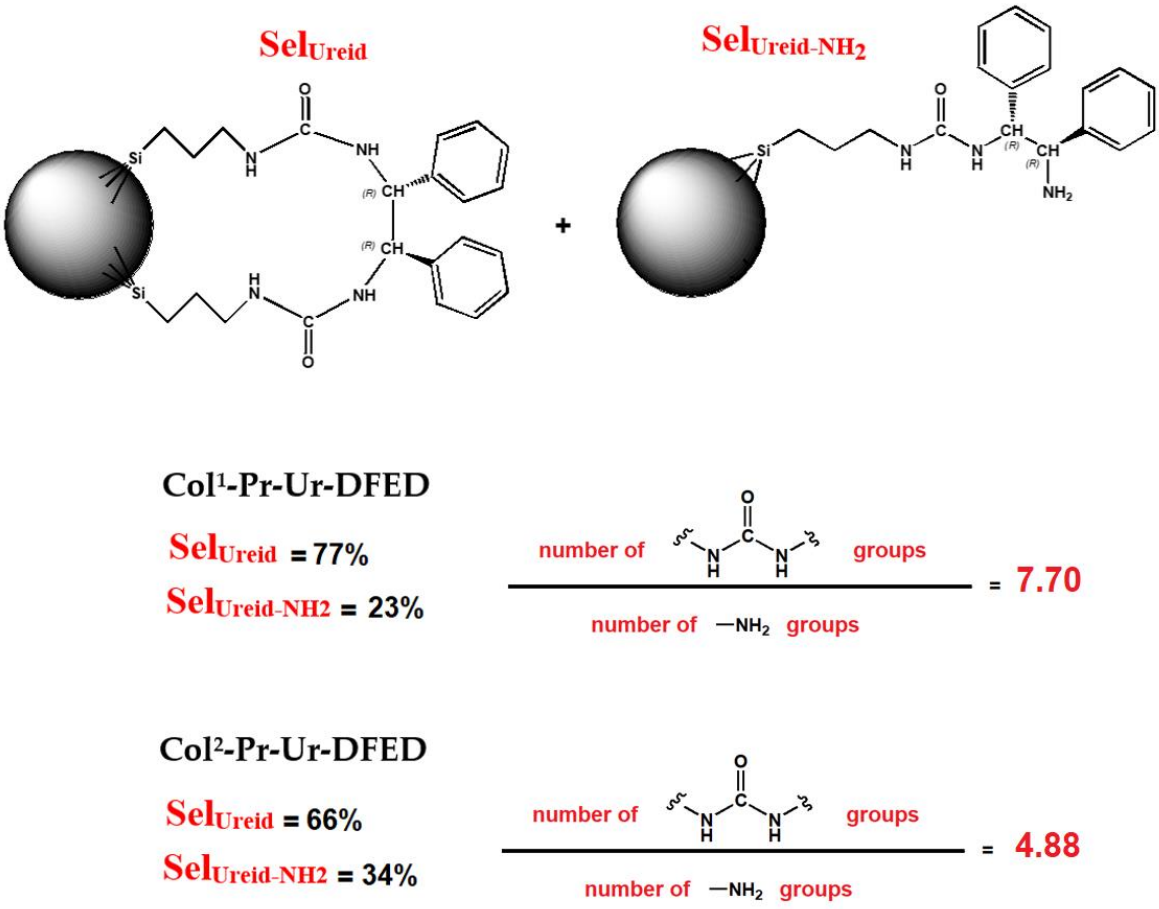

Figure 6. Estimated percentage of sites $^{ED}Sel_{Ureid}$ and $^{ED}Sel_{Ureid\text{-}NH_2}$ constituting the selectors of columns Col¹-Pr-Ur-DFED and Col²-Pr-Ur-DFED.

This difference could take origin by actractive/repulsive interactions that, in the **CSPs** of the two here analyzed columns, the couple of phenyl groups of DFED can establish with the silica matrix in the course of the reaction with the isocyanate groups, so favoring conformations of DFED that promote, by suitable proximity with isocyanate groups, the involvement of both its NH_2 functionalities. The conduction of the chromatographic resolution test on both the columns

before and after their treatment with the **OC-DNBA-M** method also in this case confirmed the absence of appreciable deterioration of the respective selectors, as visible by the comparison between the relevant chromatograms, reported in **Figure 7**.

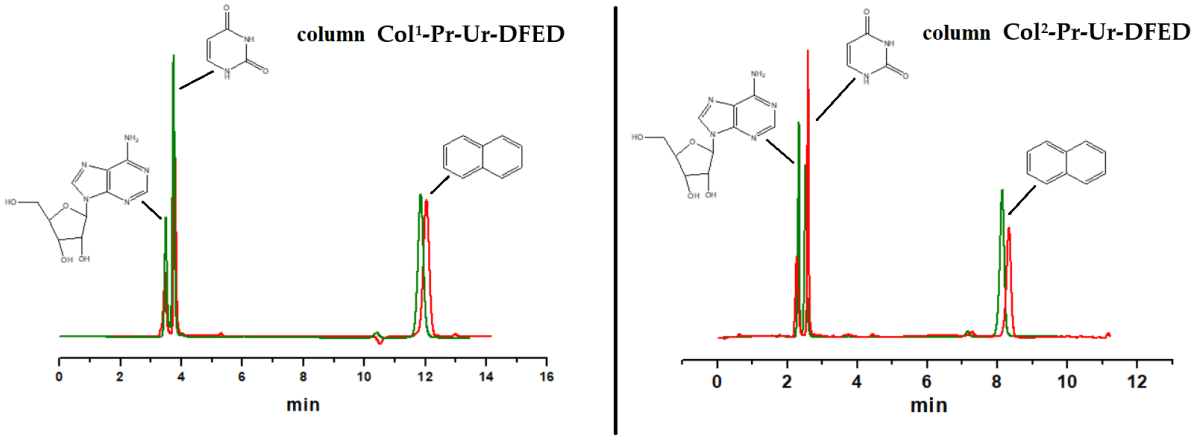

Figure 7. Chromatographic resolution of naphthalene, uracil and adenosine performed before (red trace) and after (green trace) the OC-DNBA-M analysis. Chromatographic conditions: both columns 250 mm × 4.6 mm ID; mobile phase CH_3CN/H_2O 50/50; flow rate 1.0 ml/min; T=25°C.

3.3.3 Evaluation of the fractions of selectors type $^{DACH}Sel_{Ureid}$ and $^{DACH}Sel_{Ureid\text{-}NH_2}$ constituting the CSP of column Col-Pr-Ur-DACH.

As the final step of our study, the on-column analysis of ***SPs*** packed into HPLC columns, based on the **OC-DNBA-M** method, has also been applied to the fourth column

Col-Pr-Ur-DACH, in which the ureidic selector was synthesized starting from the quite rigid structure of the (R,R)-1,2-diaminocyclohexane. Also in this case the determination of the **AGD** of the **CSP** was carried out by means of both spectrophotometric (^{UV}**AGD**$_{\text{Col-Pr-Ur-DACH}}$) and HPLC (^{HPLC}**AGD**$_{\text{Col-Pr-Ur-DACH}}$) approach, whose results were averaged. Such data have been collected in **Table 6**, together with the one coming from the relevant elemental analysis determination.

Table 6. On-column AGD analysis of the amino-ureidic CSP packed into column Col-Pr-Ur-DACH

Kind of **AGD** determination	Single **AGD** determinations	Average of ^{UV}**AGD** and ^{HPLC}**AGD** values	Final **AGD**
	(mmol ADNB/g A-SP)		
^{UV}**AGD**$_{\text{Col-Pr-Ur-DACH}}$	0.53 0.59	0.56	0.58
^{HPLC}**AGD**$_{\text{Col-Pr-Ur- DACH}}$	0.77 0.57 0.42	0.59	
Elemental analysis **% C = 10.22; % H = 1.79; % N = 2.80**			2.00

Afterwards, from their integration, it was possible to assess the percentages of sites ^{DACH}**Sel**$_{\text{Ureid}}$ and ^{DACH}**Sel**$_{\text{Ureid-NH2}}$ constituting the selector bound to the **CSP**, which amount to 11% and 89%, respectively (**Figure 8**).

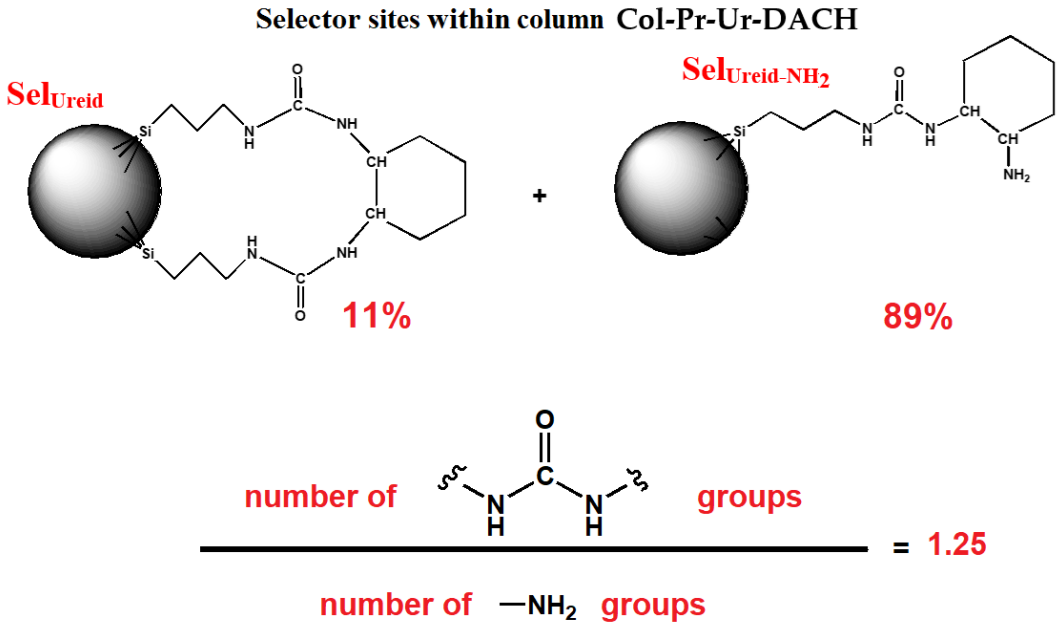

Figure 8. Estimated percentage of the sites $^{ED}Sel_{Ureid}$ and $^{ED}Sel_{Ureid\text{-}NH_2}$ constituting the selector of column Col-Pr-Ur-DACH.

These data clearly highlight the strong dominance of sites of $^{DACH}Sel_{Ureid\text{-}NH_2}$ type, that is, the ones still endowed with basic/nucleophilic activity, a result quite similar to the one found for column **Col-Pr-Ur-ED**, and therefore characterized by a selector-composition trend opposite to that observed for the couple of columns **Col^{1}-Pr-Ur-DFED** and column **Col^{2}-Pr-Ur-DFED**. It is possible to assume that such an experimental evidence may catch its origin from the steric hindrance and rigidity that the cyclohexane fragment imposes to the 1,2-diamino framework of DFED, making difficult the final reactive match between isocyanate and the still free amino group remained after the easy formation of the first ureidic functionality. As the final observation, also in the case of the present column, it was carried out a chromatographic resolution test before and after the analytic treatment with the **OC-DNBA-M** method. However,

unlike the previous cases, the test molecules used for the verification were chosen as pairs of enantiomers mixed in scalemic ratio. In particular, these are represented by the three scalemic mixtures of FMOC-Leucine, FMOC-Phenylalanine and FMOC-Methionine. Thus, column **Col-Pr-Ur-DACH** was analyzed exploiting its homochirality, evaluating before and after the **AGD** determination its ability to discriminate the pairs of the above enantiomers-tests. The obtained results, visible in **Figure 9**, give clear confirmation of the complete retention of all the chromatographic characteristic of column **Col-Pr-Ur-DACH**, so furthermore supporting the already stressed validation of the **OC-DNBA-M** procedure.

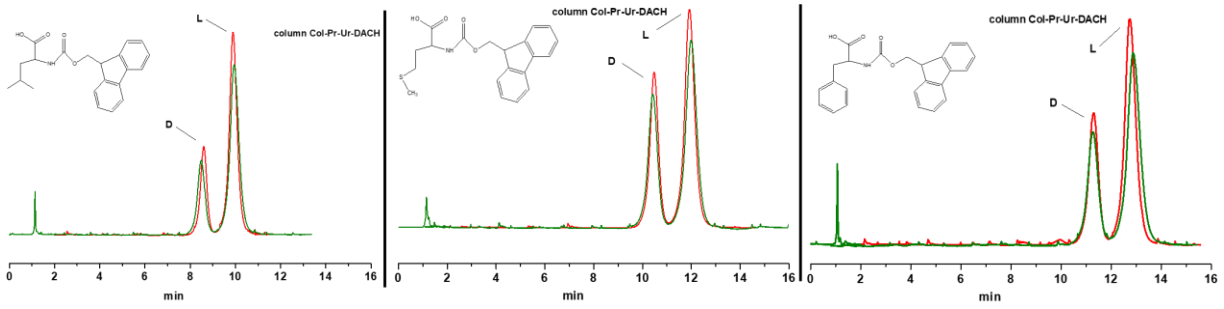

Figure 9. Retention and enantio-selectivity showed by column Col-Pr-Ur-DACH against scalemic mixtures of FMOC-aminoacids (FMOC-Leucine, FMOC-Methionine and FMOC-Phenylalanine) under POM chromatographic operating conditions (mobile phase: 10mM Ammonium Formiate in Acetonitrile/Methanol 85/15 v/v; flow rate: 0.8 ml/min; Detector: UV 254 nm; Temp: 25°C).

3.3.4 Comparison of the retention ability, registered in equal chromatographic operating conditions, among the columns Col-Pr-Ur-ED, Col[1]-Pr-Ur-DFED, Col[2]-Pr-Ur-DFED e Col-Pr-Ur-DACH against test molecules.

After the obtained characterization of the selectors bound on the **SP*s*** of the four analyzed **Col-Pr-Ur-ED**, **Col[1]-Pr-Ur-DFED**, **Col[2]-Pr-Ur-DFED** e **Col-Pr-Ur-DACH** columns, it is seemed interesting try to relate these information with the retention and discrimination ability showed by the same columns against naphthalene, uracil and adenosine, arbitrarily selected as test-molecules, under equal chromatographic operating conditions. The related chromatograms (registered using as common mobile phase a mixture Acetonitrile/10mM Ammonium Acetate in water 90/10 v/v at a flow rate of 0.7 ml/min), are visible in **Figure 10**, while the relevant retention factors, **k'**, and selectivity factors, α, are reported in **Table 7**.

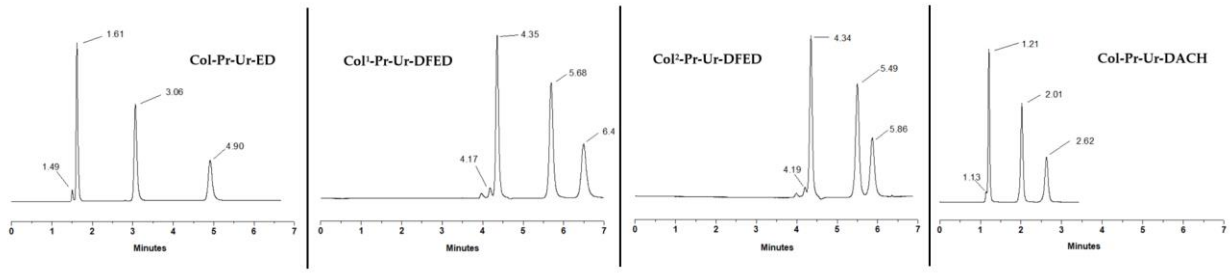

Figure 10. Retention and discrimination ability showed by the Col-Pr-Ur-ED, Col[1]-Pr-Ur-DFED, Col[2]-Pr-Ur-DFED e Col-Pr-Ur-DACH columns against naphthalene, uracil and adenosine under equal chromatographic operating conditions (mobile phase: Acetonitrile/10mM Ammonium Acetate in water 90/10 v/v; flow rate: 0.7 ml/min; Detector: UV 254 nm; Temp: 25°C).

Table 7. Retention factors, k', and selectivity factors, α, showed by the columns Col-Pr-Ur-ED, Col[1]-Pr-Ur-DFED, Col[2]-Pr-Ur-DFED e Col-Pr-Ur-DACH against the test selectand molecules naphthalene (naph), uracil (urac) and adenosine (aden).

Column												
Col^{x}-Pr-Ur-	Sel_{Ureid}	$Sel_{Ureid-NH2}$		naph		urac		aden		aden/urac	aden/naph	urac/naph
	%	%	t_0	t	k'	t	k'	t	k'	α	α	α
x=nul -ED	32	68	1.49	1.61	0.081	3.06	1.054	4.9	2.289	0.47	2.17	4.65
x=nul -DACH	11	89	1.13	1.21	0.071	2.01	0.779	2.62	1.319	0.50	1.69	3.36
x=1 -DFED	77	23	4.17	4.35	0.043	5.68	0.362	6.49	0.556	0.09	1.54	17.92
x=2 -DFED	66	34	4.19	4.34	0.036	5.49	0.310	5.86	0.399	0.07	1.28	18.89

From inspection of such data, it is put in clear evidence as much greater retention factors are evidenced by the columns **Col-Pr-Ur-ED** and **Col-Pr-Ur-DACH**, that is to say, the ones in which the amount of not reacted NH_2 groups in the **SP** is remarkably high (i.e. where the $^{DACH}Sel_{Ureid-NH2}$ sites dominate on the $^{DACH}Sel_{Ureid}$ ones). The same kind of relation is also observed by considering the trend of values assumed by factors **α**, taking into consideration the selectivity found between the couples of molecules adenosine-uracil, adenosine-naphthalene, and uracil-adenosine (**Table 7**). This behaviour seems can be traced back to a consistent number of additive interactions selector-selectand of type Hydrogen-Bond that the significant presence of amino groups of sites $^{DACH}Sel_{Ureid-NH2}$ (just a number a bit smaller than that of ureidic groups, **Figures 5** and **8**) can establish with the structures of the test molecules, reasonably much more effective with the increase in the number of polar frameworks found passing from naphthalene (who has none) to uracil and, next, to adenosine. Also the greater **k'** and **α** values found for column **Col-Pr-Ur-ED** with respect to column **Col-Pr-Ur-DACH** seems can be rationalized, in this case making reference to the scarce flexibility of movement conferred to the free NH_2 groups by the cyclohexane moiety on which they are bound.

4. CONCLUSION

In this paper, the new original on-column **OC-DNBA-M** procedure has been developed in order to allow the quantification of any typology of amino groups present on silica matrices packed into HPLC columns, also demonstrating its particular usefulness in the effective analysis of the structural composition characterizing **SP*s*** in which such kind of functional groups have been only partially involved in following chemical derivatizations. In particular, in the performed study it was demonstrated the possibility to quantify in the investigated **SP*s*** the relative amounts of selector sites endowed with both amino and ureidic functionalities from the ones just containing ureidic groups, and therefore, unable to manifest appreciable basic properties. It was also proved how the procedure is non-destructive for the analysed **SP*s***, so that, at the end of the performed analyses, the related HPLC columns can be readily re-employed for the chromatographic applications for which they have been designed.

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ACKNOWLEDGMENTS

This work was conducted with financial support from Sapienza University of Rome, Italy (DR n 3210/16 of 16/12/2016 and DR n 2936/17 of 20/11/2017).

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at…

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