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# Towards multimodal HPLC separations on humic acid-bonded aminopropyl silica: RPLC and HILIC behavior

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ABSTRACT

The stationary phase characteristics of the material obtained through immobilization of humic acid (HA) to aminopropyl silica (APS) via amide-bond formation were investigated. The material was characterized in terms of elemental analysis, FTIR, thermogravimetric analyses, pH point of zero charge measurements, potentiometric titrations, and contact angle measurements. Amount of HA bonded to APS was determined from the elemental analysis results, and found as 170 mgHA/gAPS. Stability of the material was studied in aqueous media at different pH values, and amount of HA released at pH = 8 did not exceed 2% of the total immobilized HA. Stationary phase characteristics of the well-characterized material were investigated in an HPLC system by using some low-molecular weight polar compounds (i.e. some nucleosides and nucleobases) as test solutes. Effect of some experimental variables such as column conditioning, composition of mobile phase, and temperature on the chromatographic behavior of the studied compounds was studied. Role of ammonium solutions at different pH values on retentive properties of the species was also studied. Retention factors ($k'$) versus volume percentage of organic modifier exhibited a U-curve, which was evaluated as an indication for RPLC/HILIC mixed-mode behavior of the stationary phase. Orthogonality between RPLC and HILIC modes was analyzed through geometric approach, and found as 48.5%. Base-line separation for the studied groups of compounds was achieved under each studied mode, and some differentiations were observed in elution order of the compounds depending on the HPLC mode applied. Chromatograms recorded under RPLC and HILIC modes were compared with those recorded on APS under similar conditions, and thus the influence/importance of HA immobilization process was evaluated in detail. In light of the obtained results, immobilized HA is represented as a useful stationary phase for HPLC separations.



## 1. Introduction

High performance liquid chromatography (HPLC) is one of the most popular techniques being used for qualitative and quantitative analyses of a wide range of chemical species. The performance of the technique is highly related with the properties of the stationary phase used [1,2]. So, the chromatographers attach special importance to the design of efficient stationary phases for HPLC.

Reducing the analysis time and increasing the selectivity at the same time are the main criteria in method development for HPLC [3] as well as in design of new stationary phases. In designing new stationary phases, the surface of a suitable solid support is, generally, modified by inclusion of proper functional groups and/or molecules. Such a surface modification process usually requires complicated reactions where high volume of chemicals is used both in synthesis and purification steps. As the stationary phases are usually obtained by immobilization of uni-type and/or uni-character ligands to solid supports, this type of stationary phases sometimes exhibit poor efficiency in HPLC separations, and it is, generally, difficult to increase the efficiency by applying a different mode of HPLC on the same stationary phase.

To increase the efficiency of HPLC separations, the idea "*applying more than one HPLC modes on the same stationary phase*" is deemed important, especially, by the chromatographers dealing with 2D-LC separations. The feasibility of multimodal HPLC separations is directly related to the physical and chemical properties of the stationary phase. So, the stationary phase to be used in multimodal separations must be designed carefully. Recent trends in design of new stationary phases are towards inclusion of (*i*) hydrophobic chains containing hydrophilic groups, and (*ii*) mixed phases containing various functional groups to the surface of solid support [1].

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Humic acid (HA) is the term used for definition of naturally occurring bio-macromolecules bearing hydrophobic, hydrophilic, aromatic, ionizable and electron-donor functionalities in the same structure [4]. So, HA exhibits a multifunctional character, and this special character was thought to be useful in multimodal HPLC separations of various types of compounds by immobilizing it to a suitable solid support. Through immobilization, not only HA is turned into a less-soluble form, but also mechanical properties of HA can be improved. This point of view may lead design of efficient stationary phases with multifunctional character.

Considering the experimental processes followed in immobilization of HA to solid supports, main goal of the proposed methodologies seems to be towards obtaining a stable material. So, the researchers dealing with this topic have mainly concentrated on chemical-bond formation between HA and solid support and therefore different methodologies have been proposed in the literature. In the method proposed by Bulman et al. [5], HA is immobilized via chemical bond formation between diazonium-functionalized solid support and aromatic structures present in HA. In a different method proposed by Bulman et al. [5], HA is immobilized to a glutaraldehyde-modified solid support through amide-bond formation between aldehyde groups of solid support and amine groups of HA. In the following years, Koopal et al. [6] have suggested a more efficient method that yields a stable material with low isoelectric point value. In this method, immobilization is done through amide-bond formation between carboxyl groups of HA and amine groups of aminopropyl silica. Recently, a variation of this method has been suggested by Luo et al. as well [7]. Klavins and Eglite [8] have reported immobilization of HA to different epoxy-functionalized solid supports. In the mentioned methodologies, immobilization is, generally, done in a non-aqueous media. On the other hand, there are some studies that report immobilization of HA in aqueous media, where HA macromolecules are immobilized to solid support through adsorption and/or electrostatic interactions, and in presence of a coupling-reagent [6,8–10]. However, immobilization via covalent-bond formation seems to be more efficient to obtain a stable material. According to the study of Koopal et al. [6], the method based on amide-bond formation between HA and aminopropyl silica seems to be preferable. The stability of the product obtained via this method has been improved through a supplementary process, called *end-capping*, by which residual-amino groups on the surface of the solid support are acetylated.

In the literature, the studies dealing with the usability of HA-immobilized materials have mainly concentrated on sorption behavior of various compounds, such as aminobenzene and metal ions [8], phenols [9], heavy metal ions [11–13], indigo carmin dye [14], aminobenzene, cristal violet, methylene green, flavine mononucleotide and some heavy metal ions [15], while there exists little effort on its usability as a stationary phase in HPLC.

Yu et al. [16] studied the usability of a HA-immobilized material as a hydrophilic interaction chromatography (HILIC) stationary phase for separation of some alkaloids. Kollist-Siigur et al. [17] investigated the effect of some experimental parameters on the retention of some polycyclic aromatic compounds on humic acid-(as well as fulvic acid-) bonded materials, and observed binding characteristics were tried to be related with $K_{oc}$ (organic carbon partition coefficient) of the studied compounds. Casadei et al. [18] intensified on the separation of fullerenes by using a HA-immobilized silica material as a stationary phase. In that study, immobilization of HA was done by pumping HA solution directly to the column pre-packed with solid support. However, according to our knowledge, there is no study that comprehensively intensifies on the characteristics of HA-immobilized materials as an HPLC stationary phase.

Our recent studies have proved the applicability of three modes of HPLC (i.e. RPLC, HILIC and ligand-exchange chromatography) on HA-bonded aminopropyl silica. Moreover, owing to its special character, HA-immobilized materials are believed to be applicable in some other HPLC modes, such as NPLC, adsorption chromatography, ion-exchange chromatography, ion-pairing chromatography, etc. So, HA-immobilized material seems to be useful in multimodal HPLC separations. In the present paper, we have concentrated on RPLC and HILIC behavior of the stationary phase. In the studies, nucleosides and nucleobases, which possess hydrophobic, hydrophilic, aromatic and ionizable functionalities, are thought to be useful to reveal RPLC and HILIC behavior of HA-bonded stationary phase. Thus, the degree of various interactions, such as hydrophobic, hydrophilic, electrostatic, $\pi$–$\pi$ and hydrogen-bond formation, can be evaluated reasonably. So, it is interesting to investigate the chromatographic behavior of these low-molecular weight compounds on this stationary phase.

## 2. Experimental

### 2.1. Chemicals

All the chemicals used were of analytical reagent grade or HPLC grade, and supplied from Merck, Fluka, Sigma and LabScan. Sodium form of Aldrich humic (NaA) acid was purified and converted into its protonated form (HA) before use. Aminopropyl silica (APS; 15-35 μm particle size; ~9 nm pore size) was supplied from Fluka and employed as a solid support in immobilization of HA. Immobilization of HA was done in dimethylformamide (Fluka) which included < 0.01% water and stored on molecular sieves. Aqueous mixtures of ammonia (Merck) and disodium form of EDTA ($Na_2H_2Y$; Merck) were used in solubility tests. Methanol (MeOH; LabScan) and acetonitrile (MeCN; LabScan) were the organic modifiers used in HPLC analyses. Aqueous ammonium solutions having different pH values were used in HPLC studies, and pH of the solutions were adjusted to a desired value by using 0.1 M HCl (Merck) and 0.1 M NaOH (Merck) solutions. The studied nucleosides (i.e. Uridine, Urd, Thymidine, Tyd, Cytidine, Cyd, Adenosine, Ado, and Guanosine, Guo) and nucleobases (i.e. Uracil, Ura, Thymine, Thy, Cytosine, Cyt, Adenine, Ade, and Guanine, Gua), were supplied from Sigma, and their test solutions were prepared in mixture of methanol and water. All the chemicals were used without further purification, and ultra-pure water (UPW; 0.059 μS $cm^{-1}$) was used in the experiments.

### 2.2. Immobilization

Solid humic acid in sodium form was purified according to the method reported in Ref. [6]. Hence, approximately 10 g of solid HA was suspended in 1.0 L of aqueous NaOH solution (pH 11) and stirred overnight. Afterwards, insoluble fractions were removed by centrifugation. Centrifugate was acidified with 1 M HCl to pH = 2 in order to re-precipitate humic macromolecules. The precipitate was separated by centrifugation and washed thoroughly with aqueous 0.05 M HCl solution. Obtained product was dried at 105 °C and stored for further use.

Purified humic acid (HA) was immobilized to APS through the route illustrated in Fig. 1. The illustrated method depends upon amide bond formation between APS and HA, and it was a slightly modified form of the method reported by Koopal et al. [6]. The process was done in DMF at 120 °C over 20 h to immobilize HA (product: HA-APS). Afterwards, residual $-NH_2$ groups on the surface of APS were end-capped in DMF medium by addition of acetyl chloride drop by drop. Obtained mixture was mixed over 5 h at ambient temperature. The product (EC-HA-APS) was rinsed successively with DMF, dichloromethane and acetone till colorless. The dried product was stored for further use.