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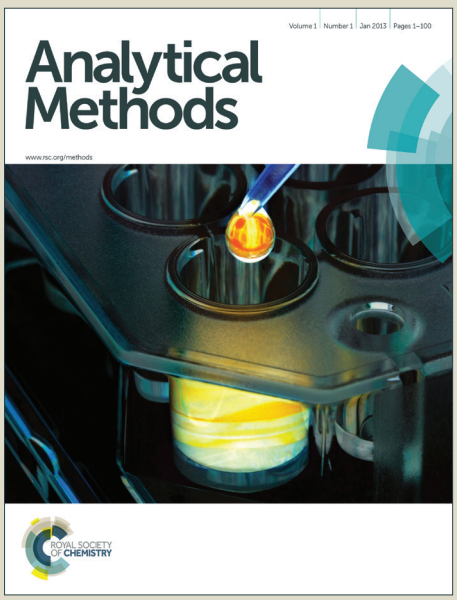

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Hydride-based HPLC stationary phases: A rapidly evolving technology for the development of new bio-analytical methods

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Abstract

This review focuses on the application of various silica hydride stationary phases under conditions that enable the advantages of reversed-phase, aqueous normal phase and organic normal phase selectivity to be gained. Here, an overview is given related to the characteristics of the most frequently used silica hydride stationary phases, including those involving short *n*-alkyl chain (Diamond hydride), phenyl, octyl, octadecyl and cholesterol phases. Explanations are provided to account for their unique retention properties, which enable their versatile application in the analysis of biological and pharmaceutical samples under conditions significantly different to those employed with conventional silica-based reversed phase or hydrophilic interaction stationary phase materials.

Introduction

Modern bioanalysis often entails the separation of complex samples that contain a wide variety of compound types spanning a broad range of polarities, structures and molecular sizes. In many cases, only a few compounds, sometimes just one, represent the target analyte(s) in the mixture. In this regard, silica-hydride-based stationary phases offer unique selectivities with the breadth of their separation capabilities accommodating many of the needs of analysis of biological, medical, physiological and pharmaceutically-related samples. Increasingly, these new types of stationary phases have been shown to provide access to regions of separation space not attainable by the traditional modes of chromatographic separation such as reversed-phase or normal phase high performance liquid chromatography, thus permitting applications across diverse fields of chemical analysis. Compared to previous reviews, which have focused on the chemistry of silica

hydride materials[1] or on their specific use in the separation of different classes of analytes[2], in this minireview the emphasis has been placed on recent developments with silica hydride stationary phases, related to their selectivity characteristics under different elution conditions, their retention mechanisms and the potential for their use in various other application fields within the life and chemical sciences..

1. Characteristics of silica hydride stationary phases

Microparticulate silica hydride is now becoming a recognized alternative to the traditional types of chemically modified silicas for the fabrication of HPLC stationary phases[1-7]. The essential difference between these materials is that the surface of ordinary silica is populated with silanol groups (Si-OH), whilst with silica hydride the predominant surface group (at least 95%) is Si-H. Figure 1A shows two possible pathways for the creation of a hydride layer on the surface of an unmodified Type A or Type B silica. These procedures involve either chlorination of the silanol groups, using a reagent such as thionyl chloride, followed by reduction with a reducing agent, typically $LiAlH_4$, or alternatively via a silanisation reaction with, *e.g*. triethoxysilane. These chemical modifications result in fundamentally different chromatographic support materials with surfaces that are weakly hydrophobic in comparison to the highly polar nature of silica itself. Moreover, the Si-H moiety can be readily modified *via* hydrosilation reactions (Figure 1B), so that it is possible to chemically immobilize a large variety of organic groups onto the surface and thus control chromatographic selectivity. The overall synthetic scheme is summarized in Figure 1C including some of the most common silica hydride phases now available. The lower series of modifications referred to as Diamond Hydride (DH) phases are based on surfaces where a large fraction of the species are Si-H with relatively few organic moieties attached via hydrosilation. Tables 1 and 2 show a more complete list of the compounds that have been successfully bonded

to a silica hydride surface by either metal complex or free radical catalysis methods[8]. Alk-1-enes and alk-1-ynes, having the unsaturated bond in the terminal position, can be readily attached to a hydride surface. This versatility results in new avenues to make a wide range of stationary phases having chromatographic selectivities suitable for the demanding separations required for biological samples as well as those from other complex matrices.

2. Silica hydride under Aqueous Normal Phase Chromatographic Conditions

Silica hydride materials have some properties in common with alternative types of stationary phases fabricated from microparticulate silicas, but they also possesses other features that are quite different. One feature that distinguishes silica hydride materials from most other conventional types of silica-based normal phase, reversed-phase or hydrophilic interaction stationary phases is their ability to retain compounds in both the reversed-phase and the normal-phase modes[1-4,9,10]. Because water is the common mobile phase component in these two modes with silica hydride materials, when normal phase selectivity is observed it is referred to as "aqueous normal phase" (ANP). Thus, when the water content of the mobile phase is high, then reversed-phase behavior is generally observed, whilst when a high organic solvent content is present normal phase selectivity is dominant. This leads to a variety of opportunities with silica hydride materials to achieve selectivity orders and extent of chromatographic resolution, depending on the polarity and size of the molecule(s) being analyzed. It also means that the resolution of these analytes is very dependent on how the zeta potential of the silica hydride stationary phase surface changes with the organic solvent content and pH of the mobile phase. For samples containing hydrophobic analytes, these compounds will be retained when the water content of the mobile phase is high, whilst for hydrophilic compounds retention will be favored with mobile phases of high organic solvent content. If the sample contains a mixture of both

classes of compounds then the two selectivity options explicit to silica hydride stationary phases can be simultaneously exploited. For example, when compounds in a mixture separate into two discrete retention regions, then two separate isocratic chromatographic runs can be carried out with mobile phases of widely differing elutropicities using the same stationary phase in order to achieve a more comprehensive analysis. Alternatively, if their retention regions overlap as may be the case with mobile phases of intermediate compositions, both polar and nonpolar compounds can be retained simultaneously and eluted under gradient conditions. Another scenario occurs for those compounds that have both hydrophobic and hydrophilic functionalities in their molecular structures. In this case these compounds can be retained by either a reversed-phase or a normal phase mechanism depending on whether water or a miscible organic solvent is predominant in the mobile phase.

3. Retention Mechanism in Aqueous Normal Phase Chromatography

The compositional features of chemically modified silica hydride stationary phase materials are different to alternative, commonly used silica-based stationary phases, which leads to differences in the retention mechanism for silica hydride phases, and particularly their unusual characteristics to strongly retain both hydrophobic and hydrophilic compounds. In the reversed-phase mode, hydrophobic interactions between the bonded organic moiety and the analytes appear to dominate the mechanism with silica hydride materials. However, the surface, being approximately 95% Si-H groups, interacts with analytes differently to that occurring with *n*-alkylsilicas with some residual silanols present, particularly with basic compounds. Thus, retention times, selectivities and peak shapes vary when comparing stationary phases derived from an octadecyl (C18) group that has been bonded onto a silica hydride or directly onto a type B silica material. These differences can prove to be very advantageous for the design of

orthogonal methods involving the sequential analysis of complex mixtures with these two types of stationary phases operating in tandem initially in a reversed-phase mode and then in an aqueous normal phase mode[11] Thus, a very high degree of orthogonality has been observed between RP C18 and Diamond Hydride columns as evidenced from a low Pearson correlation coefficient obtained from retention time data of > 100 metabolite standards.

At present, retention processes in the normal phase mode appear to be much more complex, and mechanistically more difficult to interpret than the better understood hydrophobic interactions mode observed with *n*-alkylsilica reversed-phase adsorbents. While a coherent mechanism has been proposed for hydrophilic interaction liquid chromatography (HILIC) that also displays normal phase properties, *i.e*. retention increases as the non- or less polar component of the mobile phase is increased, a general mechanism has not yet been fully established for aqueous normal phase chromatography (ANPC) using silica hydride stationary phases. With silica-based HILIC materials, it has also been proposed that retention is a result of a partition mechanism whereby a layer of water forms on the stationary phase surface and this water layer has a higher affinity for polar analytes than the more nonpolar mobile phase[12,13]. This same scenario does not seem likely for silica hydride-based materials[14]. Soukup *et al*. have shown a relatively low level of preferential adsorption of water from aqueous acetonitrile onto silica hydride materials[15] and determined that the water layer on silica hydride particles to be approximately 0.5 of a monolayer[16]. With such a thin and probably non-uniform water layer on the surface, partitioning to any great extent would not be favored. Thus, it is more likely that retention of analytes is based on adsorption mechanisms. In this case, the solute would displace either adsorbed water molecules or a mobile phase derived component from the surface. This type of behavior would explain the difference between ANPC and HILIC for the retention of polar compounds and

would be consistent with the observed reversed-phase properties, particularly when an organic moiety is bonded to the silica hydride surface. Moreover, this behavior would be consistent with the observed trends in negative zeta potential values for silica hydride materials as the organic solvent content of the mobile phase is varied.

4. Applications of modified silica hydride phases involving reversed-phase-like selectivity

While a wide variety of hydrophobic organic moieties can be attached to silica hydride surfaces that are suitable for reversed-phase (RP) applications as shown in Table 1, most applications reported to date have utilized C18, C8, phenyl and cholesterol bonded phases. Stationary phases derived from C18- and C8-bonded silica hydrides can be readily made from the corresponding *n*-alk-1-ene, and compliment the *n*-alkylsilica materials conventionally produced by organosilanization of Type A or Type B silica. The cholesterol bonded Si-H phase is a relatively unique bonded material. Both chromatographic and solid-state NMR spectroscopy studies have been used to characterize the shape selectivity of the cholesterol bonded Si-H phase, especially for compounds such as steroids[17-20]. End-capping of the silica hydride phases following hydrosilanisation is generally considered not to be crucial since the unreacted sites on the surface are Si-H groups rather than the silanols found with conventional *n*-alkylsilicas.

The first bonded phase synthesized on a hydride surface was a C18 moiety. Early studies verified the typical reversed-phase properties for hydrophobic organic compounds with this material [1-4]. These silica hydride-based C18 phase materials have subsequently been used for the analysis of various drugs and pharmaceuticals. For example, the analysis of 10 synthetic tetracyclines, semisynthetic derivatives of naturally occurring tetracyclines as well as tetracyclines degradation

products has been compared on three types of silica hydride stationary phases[21]. Because of the proliferation of drugs of abuse, new methods for their analysis are of significance in forensic science. Both C18 and phenyl silica hydride phases have been evaluated using a mixture of 17 common drugs of abuse[22]. As shown in Figure 2, the phenyl silica hydride phase was able to separate this mixture, including two pairs of isobaric compounds, in less than eight minutes. Also illustrated in Figure 2B is a feature that is common to all types of silica-hydride stationary phases, *i.e.* a high degree of repeatability in consecutive runs (in most cases an RSD of 0.5% or less). To demonstrate that silica hydride-based separation materials can function in a broad range of reversed-phase applications, a general method for the analysis of analgesic drugs has been developed with a C18 silica hydride stationary phase[101]. Furthermore the cough suppressant benzonatate as well as its degradants and excipients have been analyzed on a silica hydride C18 column by gradient elution[102]. The analysis of this compound is often problematic with many conventional *n*-alkylsilica reversed-phase columns because its secondary amine functionality can result in poor peak shape. A fluorinated phase was synthesized on a silica hydride matrix and as expected it displayed reversed-phase properties when tested with the hydrophobic solute cellobiose octaacetate[23]. An unusual alkyl C5 bonded silica hydride stationary phase has also been utilized for the separation of proteins and peptides[24] and a SiH material containing C30 groups has been used in a reversed-phase selectivity mode for the preparative purification of carotenoids from biological samples[25].

5. Applications of modified silica hydride phases involving normal-phase selectivity

As shown in Table 2, several hydrophilic organic moieties, which are suitable for normal-phase applications, can be bonded to a silica hydride surface. As noted earlier, even stationary phases with a hydrophobic group, such as *n*-octadecyl (C18), when immobilized onto the silica hydride

surface can result in retention in the normal phase mode. However, to date the vast majority of normal-phase applications have utilized either a completely unmodified silica hydride or a minimally modified (~2% carbon loading) silica hydride surface. The former has been used mainly for organic normal phase applications [26,27] such as the separation of substituted phenols and phenolic acids. The latter, usually referred to as the Diamond Hydride™ (DH) phase, has proven very successful for the retention and separation of polar compounds. Kulsing *et al*. have verified that the surfaces of silica hydride stationary phases, including the DH phase, have negative zeta potentials that account for their polar nature and hence their chromatographic properties for the retention of basic and other hydrophilic compounds[28].

A large variety of small polar molecules, not readily analyzable by reversed-phase chromatography with *n*-alkylsilicas, are readily retained in the ANP mode on silica hydride phases, particularly the DH stationary phase[1-4,29]. These hydrophilic compounds are frequently found in clinical, pharmaceutical, food, forensic, natural product and metabolic samples. Because there are fewer choices for the separation of these types of compounds than for hydrophobic analytes, most applications have been developed in the normal phase mode. For example, metabolites such as amino acids, carbohydrates and small organic acids, either as standard compounds or when present in urine or saliva samples, can be analyzed with the DH stationary phase[30,31]. Similarly, the analysis of nucleotides has also been successfully carried out[32,33]. Figure 3 shows the separation of a 19-component mixture of underivatized amino acids on the DH phase. Because mass spectrometry (MS) was used in this case for detection, it was only necessary to optimize the separation for those compounds that were isobaric. The peaks for the isobaric pair, leucine (**1**) and isoleucine (**2**), are shown in the insert. Another study has focused on the sulfur containing amino acids as a predictor of cardiovascular disease risk[34]. Since a large

number of pharmaceutical compounds are polar (often nitrogen containing), ANP in conjunction with SiH phases has proven to be useful for the analysis of these compounds. For example, the drug cycloserine, an antibiotic that is used for the treatment of tuberculosis, was analyzed on the DH for its purity and the concentration of its dimer and degradation products[35]. A variety of columns operating in the ANP/HILIC modes were evaluated for the determination of zanamivir, an anti-viral flu drug. The DH provided good retention with a lower level of matrix effects evident amongst all of the columns tested[36]. In the field of food and beverage analysis, the DH phase has been used to successfully analyze folic acid in cereals and juices[37] and histamine in tuna, cheese and wine[38]. Weisenberg *et al.* have investigated the intercellular metabolomes of the bacteria *Enterococcus faecium* and *Staphylococcus aureus*[39], whilst de Carvalho *et al.* have isolated the *Mycobacterium tuberculosis* protein Rv1248c[40,41]. These investigators demonstrated that with the DH phase operated in the ANPC mode, the retention of known metabolites was unaffected by the presence of intracellular chemical matrix components in the sample. Thus, a combination of accurate mass-retention time (AMRT) analysis is a viable approach for untargeted metabolite profiling. With the information obtained using the DH stationary phase, it was possible to differentiate bacteria according to their phylogenetic constituents additionally to their antibiotic susceptibility. Callahan and coworkers have studied two other complex biological matrices using ANP and the DH phases: urine and plant extracts[42]. Approximately 1000 compounds were reproducibly detected in the urine samples and 400 compounds in a soybean fluid. The combination of reliable chromatographic measurements and advanced data analysis methods provides a dependable protocol for complex mixtures of polar metabolites. Several other studies have confirmed the usefulness of this approach, such as the metabolic profiling of *Leishmania mexicana*[43], *Plasmodium falciparum*[44] and *Mycobacterium tuberculosis*[45].

An increasing number of methods have been developed based on the use of the DH stationary phase on a variety of polar compounds. For example, the potent neurotoxin, anatoxin-A, was easily analyzed in fresh water samples in order to prevent both animal and human poisonings[103] and the biomarkers UDP-glucose and UDP-galactose were separated along with ATP in red blood cell extracts[104].

Another potentially valuable use of the DH phase when employed in the ANPC mode is the analysis of peptides. It has been demonstrated that with these chromatographic methods it is possible to analyze both hydrophobic and hydrophilic peptides in a single run[46,47], *e.g.* peptides with the amino acid sequences of H-Tyr-Tyr-Tyr-Tyr-Tyr-Tyr-OH, H-Tyr-Gly-Gly-Phe-Leu-OH, H-Arg-Gly-Asp-OH and H-Gly-Arg-Ala-Asp-Ser-Pro-Lys-OH. Subsequently, this same approach was further validated for the separation of hydrophilic and hydrophobic bases[48]. In typical proteomic analyses, either most of the hydrophilic peptides are not detected because they elute at or near the void volume from an *n*-alkylsilica reversed phase column, or a two dimensional method is need to analyze for the presence of both polar and nonpolar peptides. The ANPC approach can provide a simpler and more comprehensive method for proteomic and other types of peptide analyses. Recently, it has also been demonstrated that ANPC can be used to separate physiologically relevant peptides from various interfering compounds such as phosphatidylcholines, lysophosphatidylcholines, sphingomyelin and cholesterol in physiological samples[49]. Clearly, applications in the various areas of "omics" research, based on these new opportunities for the use of ANPC, will grow in the future. Finally an unusual application of ANP was demonstrated for the analysis of PEG 400 isomers in perfusate samples that were separated using the DH column and quantified by evaporative light scattering detection[50].

Another interesting feature of silica hydride materials is that they can also function in the organic normal phase chromatographic (ONPC) mode, corresponding to the traditional use of normal phase chromatographic materials where only organic solvents are used to retain and elute compounds with some degree of polarity. This type of application with silica hydride phases takes advantage of the reduced affinity for water, and hence the mobile phase does not have to be extensively dried before use. The presence of a relatively small amount of water on the surface (0.5 of a monolayer) leads to better reproducibility in retention times from run-to-run. The best stationary phase materials for the ONP mode are either the unmodified silica hydride or the DH phase, but all phases with a hydride surface can function in this format. An early demonstration of the ONPC capabilities was for a series of substituted phenols on both the unmodified silica hydride and the C18 hydride phase[26]. Such compounds with small structural differences were easily separated in the ONPC mode with two different hydride-based columns. For example, the separation of isomers of nonylphenol, a starting material for various surfactants, was accomplished by ONPC methods with a silica hydride column and a hexane/ethyl acetate gradient[105].

6. Conclusions and future trends

The use of silica hydride materials is a rapidly expanding and developing area of chromatographic separations. Because these materials involve surface chemistries that are different to those found with Type A or Type B *n*-alkylsilicas, silica hydride-based stationary phases provide an additional avenue to investigate separation capabilities and to develop new approaches to meet the challenging analytical problems encountered with complex biological samples. The versatility offered through the ability to modify silica hydride via hydrosilation

reactions enables a wide choice of organic moieties that can be attached to the surface and used in the RPC, ANPC and ONPC modes. These differences in surface properties lead to the ability to tailor-make new chromatographic materials with characteristics that can be exploited to improve compound resolution, and to facilitate shorter analysis times due to rapid equilibration when mobile phase compositions are changed or after gradient runs. Excellent reproducibility between successive chromatographic analyses and between different lots of the same stationary phase has also been demonstrated.

In the future, it is likely there will be two areas of further significant development, namely (1) the fabrication of new types of silica hydride-based stationary phases, and (2) the expansion of their application to other classes of compounds, samples and matrices. For example, stationary phases generated with pentafluorophenyl, chiral, amino or ion-exchange ligands are just a few exemplars, where surface modifications of silica hydride stationary phases could produce different or enhanced separation capabilities compared to conventionally modified silica-based materials. Deployment of these new silica hydride stationary phases for protein, proteomic, metabolomic, biomedical, nutraceutical, forensic and environmental analyses represent some of the exciting but as yet untapped areas of application.

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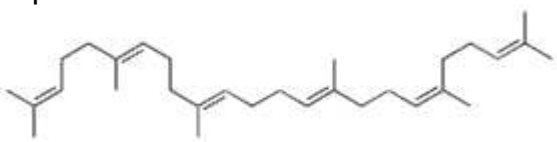
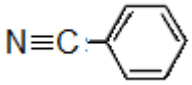
Figure Captions

Figure 1. Synthetic protocol for the fabrication of silica hydride-based stationary phases. A) Conversion of silica to silica hydride via either chlorination/reduction or silanization with a trialkoxysilane. B) Attachment of organic group to silica hydride via hydrosilation. C) Combined production process leading to some commercially available silica hydride stationary phases.

Figure 2. Separation of 17 drugs of abuse using a phenyl hydride column in the reversed-phase mode. Analytes: 1, alpha-hydroxyalprazolam; 2, d-amphetamine; 3, d-Methamphetamine; 4, phentermine; 5, 3,4-methylenedioxyamphetamine; 6, ephedrine; 7, pseudoephedrine; 8, 3,4-methylenedioxymethamphetamine; 9, benzoylecgonine; 10, ecgonine methyl ester; 11, codeine; 12, diazepam; 13, nordiazepam; 14, 6-monoacetylmorphine; 15, oxazepam; 16, temazepam; 17, (l)-9-carboxy-11-nor-delta-9-THC. (adapted from ref. 22)

Figure 3. Separation of 19 underivatized amino acids on the Diamond Hydride column in the ANPC mode. (adapted from ref. 29)

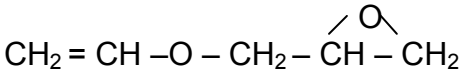
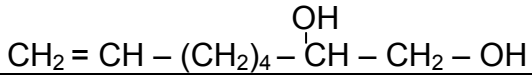
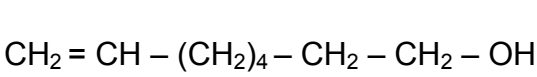
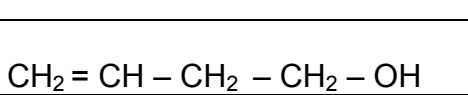
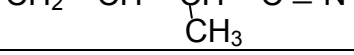
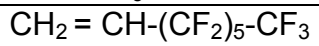
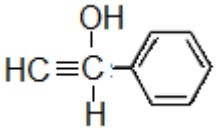
Table 1. *Compounds bonded by hydrosylation. Hydrophobic surface.*

Compound: name and structure		Catalyst	Reference
1-octene	$CH_2 = CH\text{-}(CH_2)_5\text{-}CH_3$	Pt, FR	51
1-octadecene	$CH_2 = CH\text{-}(CH_2)_{15}\text{-}CH_3$	Pt	52
4-phenyl-1-butene	$CH_2 = CH\text{-}CH_2\text{-}CH_2$– 	Pt	53
1-docosene	$CH_2 = CH\text{-}(CH_2)_{19}\text{-}CH_3$	Pt	54
1-triacontene	$CH_2 = CH\text{-}(CH_2)_{27}\text{-}CH_3$	Pt, FR	55
1-Octene, 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro- $CH_2 = CH\text{-}(CF_2)_5\text{-}CF_3$		Pt	56
Squalene		Pt	57
1-octyne	$CH \equiv C\text{-}(CH_2)_5\text{-}CH_3$	Pt	51
1,9-decadiyne	$CH \equiv C\text{-}(CH_2)_6\text{-}C \equiv CH$	Pt	54
1,7-decadiyne	$CH_3\text{-}CH_2\text{-}C \equiv C\text{-}(CH_2)_4\text{-}C \equiv CH$	Pt	54
Benzonitrile		FR	54

Pt – hexachloroplatinic acid catalyst

FR – free radical catalyst

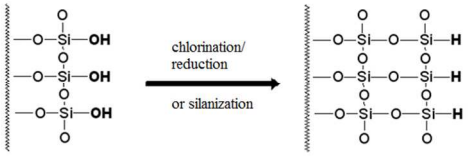
Table 2. *Compounds bonded by hydrosylation. Hydrophilic surface.*

Compound: name and structure	Catalyst	Reference
Allyl Glycidyl Ether 	Pt	58
7-octene-1,2-diol 	Pt, FR	58
1-octene-8-ol 	Pt	59
3-butene-1-ol 	FR	59
2-methyl-3-butenitrile 	FR	54
1H,1H,2H- perfluoro-1-octene 	Pt	56
1-phenyl-2-propyn-1-ol 	Pt	54

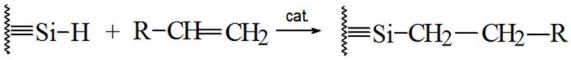
Pt – hexachloroplatinic acid catalyst

FR – free radical catalyst

A **GENERAL SYNTHETIC PATHWAYS FOR MAKING SILICA HYDRIDE**



B **MODIFICATION OF SILICA HYDRIDE HYDROSILATION**



cat = catalyst, metal complex such as hexachloroplatinic acid or free radical initiator such as *t*-butyl peroxide

C

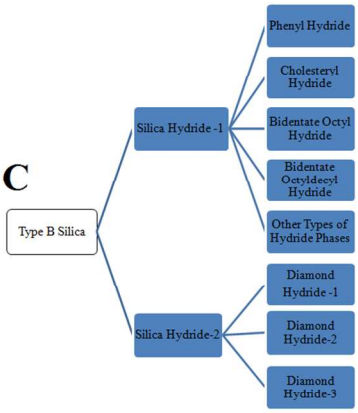
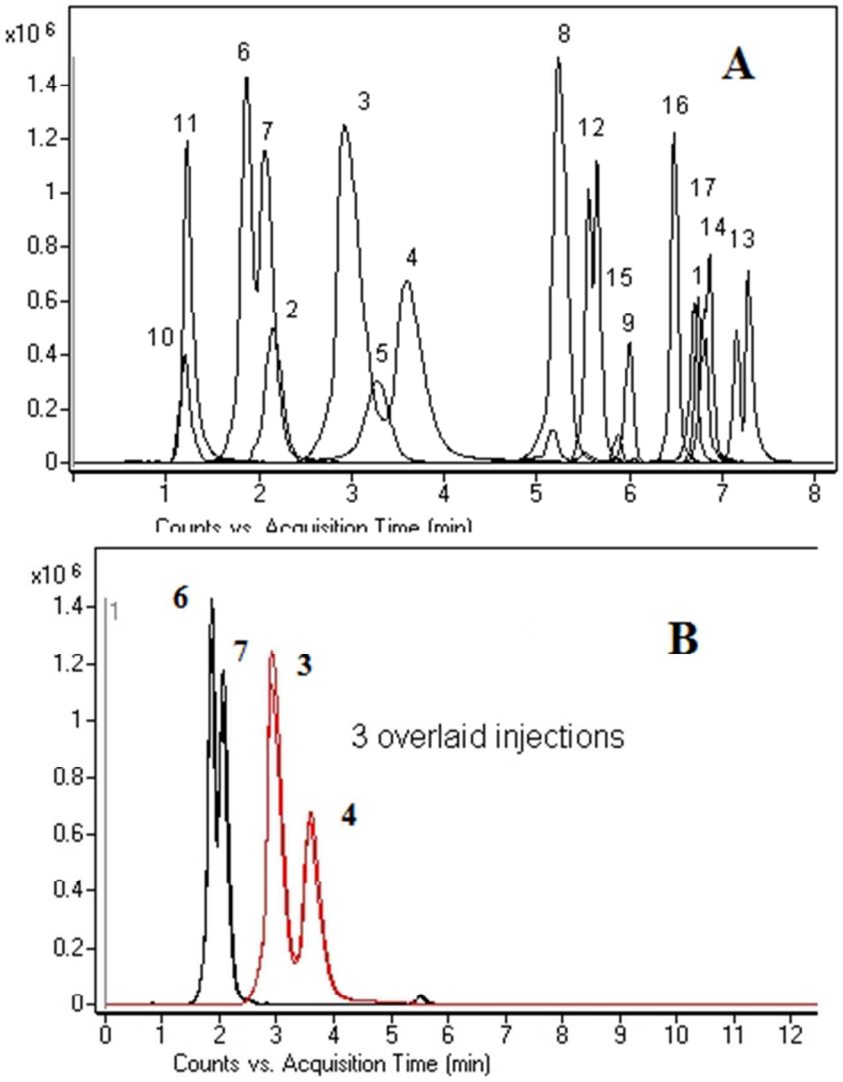
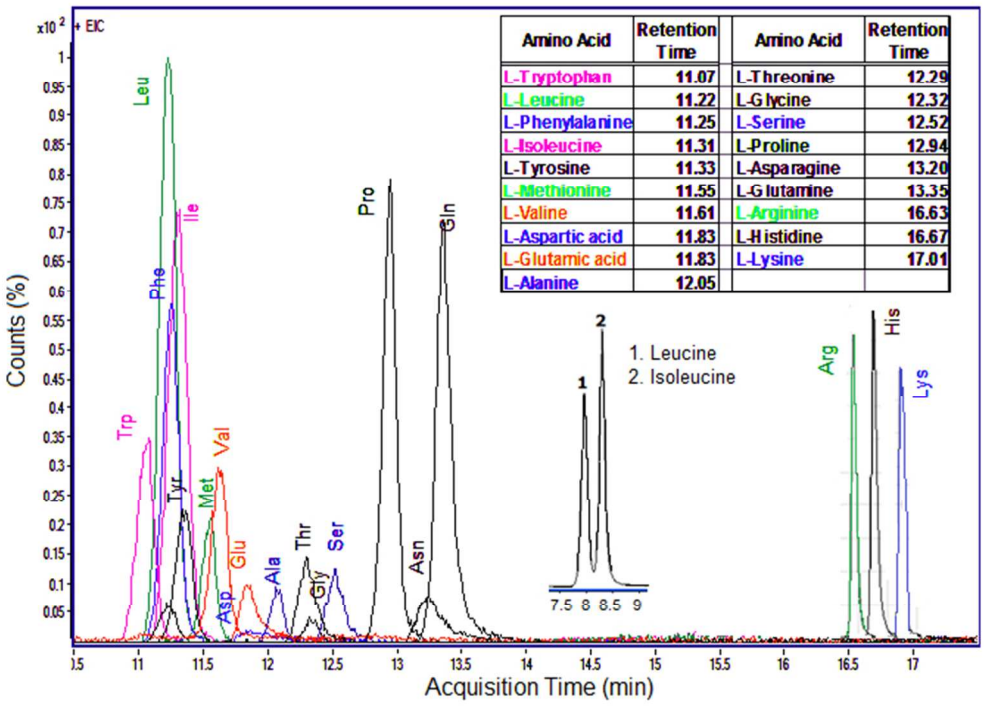


Figure 1





Amino Acid	Retention Time	Amino Acid	Retention Time
L-Tryptophan	11.07	L-Threonine	12.29
L-Leucine	11.22	L-Glycine	12.32
L-Phenylalanine	11.25	L-Serine	12.52
L-Isoleucine	11.31	L-Proline	12.94
L-Tyrosine	11.33	L-Asparagine	13.20
L-Methionine	11.55	L-Glutamine	13.35
L-Valine	11.61	L-Arginine	16.63
L-Aspartic acid	11.83	L-Histidine	16.67
L-Glutamic acid	11.83	L-Lysine	17.01
L-Alanine	12.05		

Figure 3