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Development of an accelerated low-pH reversed-phase liquid chromatography column stability test

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Abstract

The important experimental design criteria for an accelerated low-pH RPLC column stability test are discussed. The influence of method variables on the amount and rate of retention-loss and the final optimized parameters for the accelerated low-pH RPLC stability test are presented. The retention-loss curves for selected C_8 and C_{18} stationary phases are compared. These studies indicate that ligand chain length, functionality and bonding density play an important role in determining the low-pH stability of a stationary phase. Additionally, elemental analysis data are used to infer the mechanism responsible for the observed retention-loss under low-pH conditions.

Keywords: Stationary phases, LC; Column stability; Reversed-phase materials; Bonded phases; Stability test

1. Introduction

Acidic mobile phases have found widespread application in the reversed-phase HPLC separation of many important pharmaceutical and environmental compounds. Solutes such as pharmaceuticals and biomolecules often show peak shape, retention and selectivity changes when the mobile-phase pH is changed from neutral to 2 or 3. Lowering the pH helps suppress silanol interactions between basic solutes and the residual surface silanols, thus resulting in less tailing, lower retention and potentially different selectivity. Additionally, lower-pH mobile phases will protonate certain classes of acidic solutes, thus increasing retention and altering selectivity. It is important to keep in mind that low-pH mobile phases are essential to the success of some biomolecule separations. For example, hydrophobic peptide and protein separations use trifluoroacetic acid for solubility. Additionally, basic analytes are preferably positively charged in the HPLC eluent for positive-mode electrospray LC–MS applications, thus requiring low-pH mobile phases.

The vast majority of RPLC applications are performed with silica-based bonded phases [1]. For these materials, low-pH mobile phase use is limited by the stability of the siloxane bond between the reversed-phase organosilane ligand and the silica surface. When the pH of the mobile phase is less than 3, acid-catalyzed hydrolysis of the siloxane bond between the organosilane and the silica surface becomes significant [2–6]. This results in continuous loss of the bonded phase and concomitant loss of chromatographic retention. Because of the peak shape, retention and selectivity benefits of low-pH mobile phases, many column manufacturers have developed RPLC stationary phases that are resistant to acid-catalyzed hydrolysis and loss of the reversed-phase ligand.

The synthetic approaches designed to improve the hydrolytic stability of bonded phase ligands under low-pH conditions are the subject of many excellent articles and reviews [4,5,7–16]. Although this synthetic literature outlines the methods used to probe ligand stability under low-pH conditions, very little explanation is provided regarding the choice of low-pH test conditions. The goals of this work are to discuss the important criteria for designing an accelerated low-pH RPLC column stability test, to show how experimental variables such as column temperature and the organic composition of the low-pH mobile phase affect retention-loss, to demonstrate the effect of organosilane type and bonding density on low-pH stability and to use elemental analysis to better understand the mechanism responsible for retention-loss under low-pH conditions.

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Table 1
Summary of stationary phase characteristics

Stationary phase	Starting silane	APD (Å)	Total C (%)	Ligand density (μmol/m^2)
MH1-C_8	Dimethyloctylchlorosilane	92	12.1	3.52
MH1-C_{18}	Dimethyloctadecylchlorosilane	94	19.6	3.25
MH2-C_{18}	Dimethyloctadecylchlorosilane	99	20.7	3.72
D2M2-C_{18}	Methyloctadecyldichlorosilane	99	15.9	3.06
DL1-C_{18}	Methyloctadecyldichlorosilane	86	12.9	1.65
DM1-C_{18}	Methyloctadecyldichlorosilane	86	15.1	2.08
DH1-C_{18}	Methyloctadecyldichlorosilane	90	19.3	3.22
TM1-C_{18}	Octadecyltrichlorosilane	86	15.9	2.40
TM1-C_{18}-NE[a]	Octadecyltrichlorosilane	86	14.3	2.40
TH1-C_{18}	Octadecyltrichlorosilane	90	19.2	3.32

[a] Not endcapped (NE).

2. Experimental

2.1. Columns and stationary phases

All stationary phases were packed in-house and tested shortly thereafter. Stainless steel column configurations were 2.1 mm × 20 mm, 3.0 mm × 50 mm, 3.9 mm × 100 mm, 4.6 mm × 75 mm and 4.6 mm × 100 mm. The characteristics of the stationary phases under investigation are given in Table 1 where M, D and T designate monofunctional, difunctional and trifunctional silanes; H, M and L designate high (≥3.2 μmol/m^2), medium (2.0–3.1 μmol/m^2) and low (<2.0 μmol/m^2) bonding densities; and 1 and 2 designate different high-purity base silicas. All stationary phases were endcapped using trimethylchlorosilane unless otherwise noted. A "2" after the functionality code indicates that two phases were bonded with only minor differences.

Silica-1 has particle properties similar to Waters Symmetry silica. Silica-2 is not manufactured by Waters but has particle characteristics—purity (<10 ppm Fe), specific surface area (SSA), and average pore diameter (APD)—similar to Waters Symmetry silica. The SSA and APD were measured using the multipoint nitrogen sorption method (Micromeritics ASAP 2405) as follows: the SSA was calculated using the Brunauer, Emmett, and Teller (BET) multipoint method and the APD was calculated from the desorption branch of the nitrogen sorption isotherm using the Barrett, Joyner, and Halenda (BJH) method. All silicas used in this work have specific surface areas from 340 to 349 m^2/g. Their APD are listed in Table 1.

The carbon data reported in Table 1 were obtained using an Exeter Analytical Inc. combustion analyzer model CE-440. The ligand densities reported in Table 1 were calculated using the Berendsen–de Galan equation [17]:

$$\text{ligand density } (\mu\text{mol/m}^2) = \frac{10^6 \times P_{cS1}}{\text{SSA} \times (100 \times 12n_1 - P_{cS1}M_{E1})} \quad (1)$$

where P_{cS1} is the percentage carbon on the step 1 silica, SSA the specific surface area (m^2/g) of the underivatized silica, 12 the atomic weight (g per atom) of carbon, n_1 the number of carbon atoms in the step 1 ligand, and M_{E1} is the effective molecular weight (g/mol) of the step 1 ligand.

The effective molecular weight of the step 1 ligand is the molecular weight of the ligand on the silica surface minus the molecular weight of the surface leaving group (i.e. H for mono-chlorosilane bondings). For multifunctional silanes, the relative concentration of groups bonded to the surface versus a neighboring ligand was not known. This introduces uncertainty in the effective molecular weight for these bonded phases. Ligand densities for difunctional and trifunctional silanes were calculated assuming 100% bi-dentation: one bond to the surface and the other to a nearby ligand.

In addition to the above limitations, it is important to note that the ligand density equation is based on several general assumptions. It assumes that the washing and drying steps have removed all unreacted silane, reaction by-products, and residual solvents prior to carbon analysis. It also assumes that endcapping and/or subsequent washes do not remove the step 1 ligand.

2.2. HPLC instrument parameters

All flow rates were scaled such that the same mobile phase linear velocity is used for the different column configurations. This allows for a fair comparison of the low-pH stability of columns with different dimensions. The flow rates for 2.1, 3.0, 3.9 and 4.6 mm i.d. columns were 0.3, 0.6, 1.0 and 1.4 mL/min, respectively.

The low-pH stability test was performed using the following Waters systems: an Alliance 2690 for solvent delivery and sample injection, a 486 or 2487 UV detector monitoring absorbance at 230 nm for TFA and 254 nm for all other analytes, and a column heater box with temperature control module (TCM) set to 80 °C for controlling column temperature. A six-port switching valve from Rheodyne was used to divert the flow to waste during the hydrolyzed ligand removal or "strip" step.

2.3. Temperature parameters

All column heater temperature control modules were set to 80 °C.

2.4. Initial column characterization

The column was equilibrated in 100% acetonitrile for 120 min to fully wet the column bed and to allow the column to reach temperature. After column equilibration, acetone was injected to determine the void volume. Following void volume determination with acetone, the column was equilibrated in 50/50 acetonitrile/water for 45 min. After this equilibration step, uracil and acenaphthene were injected to further monitor void volume and non-polar solute retention in a mobile phase capable of maintaining a solvated stationary phase (a "wetting solvent"). This 50/50 acetonitrile/water sequence was repeated every 37 h to confirm retention-loss under wetting conditions and to eliminate dewetting as a possible reason for retention-loss.

2.5. Accelerated low-pH aging conditions

Once initial column characterization was completed, the column was equilibrated in 1% trifluoroacetic acid (TFA) in H_2O for 60 min followed by an injection of 5% TFA in H_2O with a 2 min run which was intended to monitor void volume changes. However, retention times for the TFA peak decrease with increasing exposure to the low-pH aging mobile phase for reasons not fully understood at this time. Therefore, retention times and not retention factors are reported. This was followed by injection of a sample containing methyl and ethylparaben with a run time of 88 min to monitor retention time loss for parabens. Next, a sample containing benzene and toluene was injected with a run time of 90 min to monitor retention-loss for alkylbenzenes. Unexplained retention behavior was occasionally observed for the alkylbenzenes. In contrast, the retention of the alkylparabens consistently behaved in a systematic, reproducible manner. For this reason, only retention data for the alkylparabens are reported. The 1% TFA in water exposure time was 4 h. These injections were followed by a purge step with 1% TFA-in-acetonitrile mobile phase used to "strip" or desorb any residual hydrolyzed phase not removed in the previous step. This step also assures that the phase is fully solvated for the next exposure cycle. The time for this step was 40 min per 100 mm of column length (minimum strip time of 30 min). The 1% TFA-in-water exposure and 1% TFA-in-acetonitrile steps were repeated until the ethylparaben loss was >50% of its initial retention time or had reached at least 37 h of exposure time.

All retention times were system-volume corrected. The system volumes range between 0.191 and 0.247 mL. In order to avoid stationary phase dewetting during the 1% TFA-in-water exposure step, backpressure was added after the detector. A backpressure of approximately 900 psi (no column, 0.6 mL/min, 1.0% TFA in H_2O) was achieved by adding a combination of 5 μm HPLC columns after the detector. Narrow-bore tubing and pressure valves were not used after the detector due to problems with plugging.

2.6. Post-aging procedure

Once the low-pH stability test was completed, the material was removed from the column and washed sequentially with 250 mL each of methanol, Milli-Q water, and methanol prior to drying at 80 °C overnight under full vacuum. The phases were submitted for CHN elemental analysis. Carbon results were recorded on an Exeter Analytical Inc. Model CE-440 combustion analyzer before and after the low-pH testing procedure to monitor the percent carbon loss.

3. Results and discussion

3.1. The low-pH stability of silica-based C_{18} stationary phases

Many different types of silica-based C_{18} stationary phases are currently available [7–9,18–20]. Although these phases are produced in nominally similar ways, there are pronounced chromatographic performance differences between brands. In addition to peak shape and chromatographic selectivity differences, different brands of silica-based C_{18} phases show marked differences in low-pH stability [2,5,10,12]. The differences in low-pH stability are due to differences in the type of C_{18} ligand used for the bonding, the presence or absence of an endcap, the bonding density of the phase and the characteristics of the underlying silica. [2,5,21–24].

3.2. Designing a low-pH stability test for RPLC columns

3.2.1. Overview of the accelerated low-pH stability test for RPLC columns

A general outline of our accelerated low-pH stability test steps is given below with an explanation of each step.

A. Initial column characterization
   - 0.1. The column is equilibrated in 100% acetonitrile for 120 min at 80 °C to fully wet the column bed and allow the column to reach the set temperature.
   - 0.2. The column is equilibrated in 50/50 acetonitrile/water for 45 min. After this equilibration step, uracil and acenaphthene are injected to monitor void volume and non-polar solute retention in a wetting solvent. This set is repeated after every 37 h of 1% TFA in H_2O exposure to confirm retention-loss under wetting conditions.

B. Accelerated low-pH aging conditions
   - 0.1. The column is equilibrated in 1% TFA in H_2O for 60 min followed by a 2 min injection of 5% TFA in H_2O to further monitor void volume changes.

0.2. While continuing to purge the column with 1% TFA in H_2O, a sample containing methyl and ethylparaben is injected. A run time of 88 min is used and the retention changes for the parabens are monitored.
0.3. While continuing to purge the column with 1% TFA in H_2O, a sample containing benzene and toluene is injected. A run time of 90 min is used and the retention changes for the alkylbenzenes are monitored.
0.4. These injections are followed by exposure to 1% TFA in acetonitrile used to "strip" or desorb any residual hydrolyzed phase not removed in the previous step. The duration of this step is 40 min per 100 mm of column length (minimum strip time of 30 min). The influence of the "strip" step and the choice of solvent to desorb the hydrolyzed phase are currently under investigation.
0.5. This sequence is repeated until the retention time of ethylparaben is <50% of its initial value.

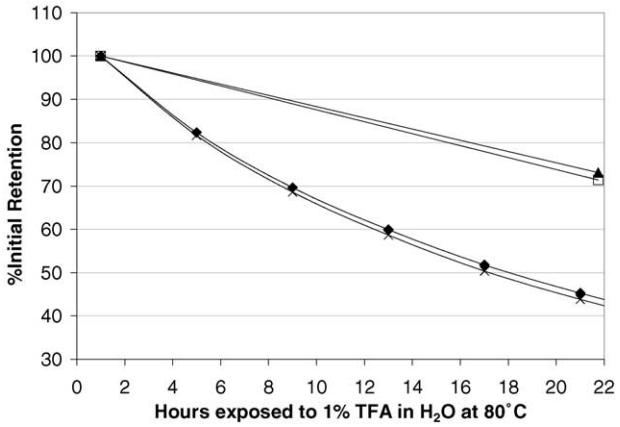

Fig. 1. The retention-loss curves for a monofunctional C_8 (MH1-C_8) stationary phase aged under static and dynamic low-pH aging conditions at 80 °C. All columns aged under static conditions were re-equilibrated under acetonitrile flow for 10 min prior to retention measurement: (▲) methylparaben retention-static, (□) ethylparaben retention-static, (◆) methylparaben retention-dynamic, (×) ethylparaben retention-dynamic.

3.2.2. General considerations for low-pH stability test methods

RPLC columns are regularly used under a wide variety of low-pH conditions. The column temperature, mobile-phase organic composition and the type and concentration of acid or acidic buffer all play an important role in the lifetime of a column. As a result of the enormous variety of possible separation conditions, the design of a low-pH stability test method requires the analyst to make judicious choices and compromises.

There are four main guidelines for designing an accelerated low-pH stability test for RPLC columns. First, as pointed out by others as well [25], the test must mimic typical chromatographic conditions as closely as possible (i.e. a constantly replenished source of an acid typically used in RPLC and removal of any hydrolyzed ligand). We refer to this type of low-pH stability test as a "constant-flow" or a dynamic test. Conversely, simply aging the stationary phase under "no-flow" conditions is referred to as a static test. In a static test, dewetting and/or the development of an equilibrium between ligands with broken bonds to the surface and surface silanols will slow ligand hydrolysis. Alternatively, a dynamic test continuously replenishes the low-pH and "strip" mobile phases and removes the hydrolyzed ligands from the stationary phase. Second, the test conditions must allow for a fair comparison of columns with different configurations. This is simply accomplished by scaling the flow rate such that an equivalent linear velocity of low-pH aging mobile phase is used for all of the different column configurations. Third, the method should show a significant amount of retention-loss in a reasonably short period of time (<5 days). This requires the use of higher than normal HPLC-compatible acid/acidic buffer concentrations and high column operating temperatures. The extensive use of TFA in biomolecule separations and its low pK_a (pK_a = 0.5) value make it the obvious choice for an accelerated low-pH stability test. The effect of pH on the retention-loss curves is discussed in detail below. Finally, the accelerated low-pH stability test conditions must only probe the hydrolytic stability of the bonded ligand under acidic conditions. This relatively simple assertion is critically important to understanding the retention-loss trends for a given column. For example, column dewetting and/or channeling of the column bed due to silica dissolution during the test leads to incorrect retention-loss curves. All of the guidelines are discussed in more detail below.

3.2.3. Static versus dynamic low-pH stability testing

Dynamic low-pH stability testing is essential simply because it mimics how the column will be routinely used. However, this type of testing is also needed to accelerate the test cycle. This is clearly shown in Fig. 1. Under otherwise identical conditions (1% TFA in H_2O, 80 °C), the dynamic low-pH stability test degrades the column nearly 2.5 times faster than the static test. This is, at least in part, due to dewetting of the stationary phase in the static test and is discussed in detail below. Additionally, a constantly replenished source of mobile phase to remove hydrolyzed ligand from the stationary phase is critical to accelerating the low-pH stability testing cycle. The relative importance of each of these effects requires further investigation.

3.2.4. The relationship between retention time loss and exposure times and/or volumes

Retention-loss due to low-pH exposure is frequently plotted against column volumes. Column volumes (CV) are frequently calculated using two different equations—one with the interstitial porosity in the denominator and the other without this term (i.e. empty column volumes)—or empirically determined as the void volume of the column. In Fig. 2,

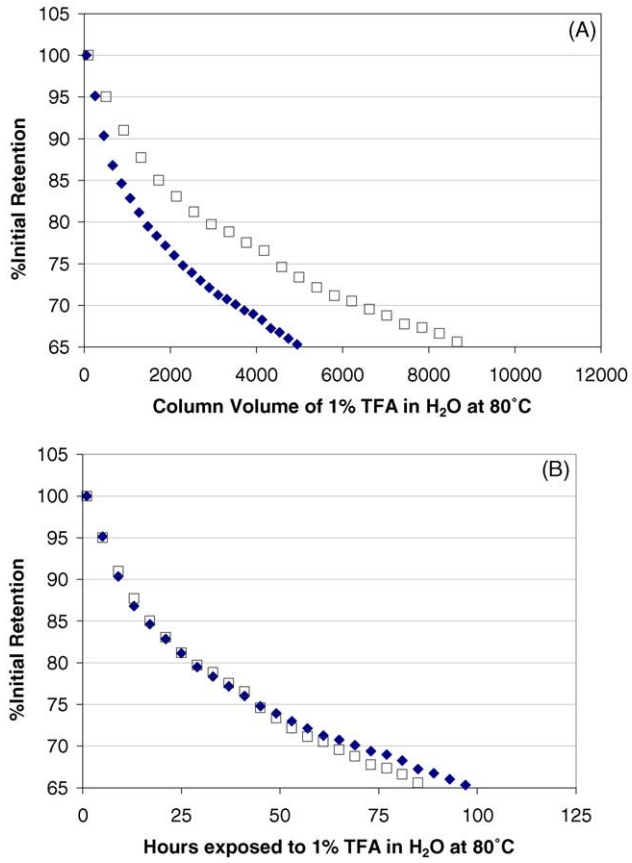

Fig. 2. Loss of methylparaben retention curves on a difunctional C_{18} (D2M2-C_{18}) stationary phase plotted against two different *x*-axes: (A) retention-loss as a function of column volumes, (B) retention-loss as a function of hours of exposure; (□) 3.0 mm × 50 mm, (◆) 3.0 mm × 100 mm.

for simplicity, the CV was calculated as follows:

$$\mathrm{CV} = \frac{FT}{\pi r^2 L} = \frac{T}{\mu L} \quad (2)$$

where F is the flow rate, T the exposure time, r the internal radius of the column, L the length of the column and μ is the superficial linear velocity. To study the relationship between retention-loss and CV, the linear velocity was held constant. The retention-loss curves for the D2M2-C_{18} phase with two different column lengths are shown in Fig. 2. The lengths are different so that the column volumes are different for a given period of exposure time. The low-pH stability tests were performed identically on both columns (see Experimental for details) and differ only in the unit of measure for the *x*-axis—CV for Fig. 2A and exposure time for Fig. 2B. It is clear from Fig. 2A that the retention-loss curves for the different column configurations do not overlap when plotted as a function of column volumes of the aging mobile phase. If retention-loss under low-pH conditions were dependent on the number of column volumes of the aging mobile phase alone, these curves should overlay. When the data are plotted in terms of exposure time to the aging mobile phase (Fig. 2B), the retention-loss curves overlay. This behavior indicates that retention-loss at a given linear velocity under low-pH conditions is dependent on exposure time and not column volumes. Fig. 2B also gives some indication as to the reproducibility of our accelerated, low-pH stability test. To ensure an accurate and fair comparison of columns with different configurations, the same linear velocity was used during the low-pH test and all data were plotted against the exposure time.

The linear velocity chosen for the test was based on early data suggesting that 1.4 mL/min led to faster retention-loss than 1.0 mL/min on a 4.6 mm i.d. column. However, these flow rate experiments were performed before stationary phase dewetting was identified as a problem. This topic is discussed in detail in a section below. The effect of linear velocity under wetting conditions on the low-pH retention-loss curves has not yet been investigated.

3.2.5. The effect of pH on retention-loss

As discussed above, the widespread use and favorable pK_a of TFA make it an ideal acid choice for the low-pH stability testing of RPLC phases. Several concentrations of TFA in H_2O were evaluated as aging mobile phases for the low-pH stability test. No organic modifier was used in the aging mobile phases. The reason for using a purely aqueous aging mobile phase and some important considerations for using this type of aging mobile phase are discussed in detail below. The retention-loss curves for a monofunctional C_{18} phase aged with different low-pH mobile phases are shown in Fig. 3. It is clear that dropping the pH to approximately 1.1 greatly accelerates the low-pH stability test relative to pH = 2.5 and 3.5. The 1% TFA containing mobile phase used in our accelerated low-pH stability test is sufficiently acidic (pH = 1.0) to rapidly degrade phases without being too harsh to the HPLC equipment.

3.2.6. The effect of column temperature

Preliminary accelerated low-pH stability tests were conducted using column temperatures in the range of

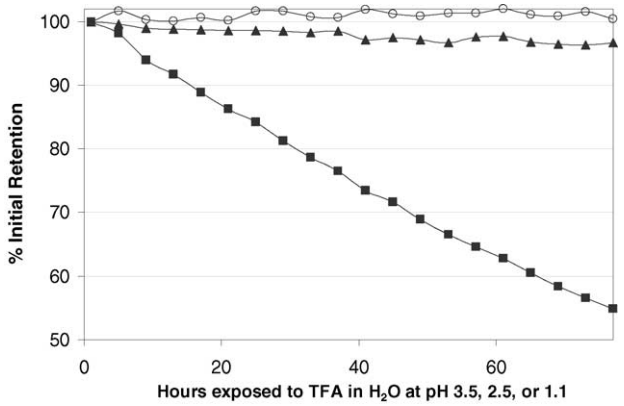

Fig. 3. The effect of aging mobile-phase pH on the loss of methylparaben retention on a monofunctional C_{18} (MH1-C_{18}) stationary phase: (○) pH = 3.5, (▲) pH = 2.5, (■) pH = 1.1.

65–150 °C. Control experiments were conducted by exposing a C_8-silica column to water (no TFA) at different column temperatures. This allowed us to monitor any undesired changes in retention due to changes in bed stability or non-acid catalyzed phase loss at a given temperature. For example, operating the column at 150 °C leads to rapid retention-loss, but it also *dissolved* a large fraction of the packing material (76 mm void) in a 100 mm column. Obviously, this temperature is not suitable for probing ligand stability. Our experiments show that column temperatures higher than 80 °C lead to instability of the chromatographic bed, thus giving erratic retention-loss curves that are very difficult to interpret.

The column temperature in our accelerated low-pH stability test is 80 °C for several reasons. First, this temperature maximizes the amount and rate of retention-loss in a given time without affecting the stability of the column bed itself. Second, an 80 °C eluent that contains 1% TFA does not lead to excessive maintenance and repair of the HPLC systems being used for the testing. Finally, an operating temperature of 80 °C does not require oil baths or specialized column heaters for precise temperature control.

3.2.7. The effect of backpressure and organic composition during accelerated low-pH aging

Our goal is to assess the low-pH stability as rapidly as possible. By aging the columns with a 100% aqueous acidic mobile phase, the test is reasonably fast as long as post-detector backpressure is added. The literature describing the wetting characteristics of C_8 and C_{18} stationary phases shows that column wetting or dewetting must be considered under these conditions [26–29]. The dewetting of alkyl stationary phases in 100% aqueous mobile phases is a function of the backpressure on the column, the ligand density, the alkyl chain length, and the pore size of the base silica. Dewetting can be minimized or eliminated if the column can be maintained at sufficiently high pressures. The Young–Laplace equation [30] describes the relationship between the pressure required to force a non-wetting liquid into a capillary (or pore) of a given diameter size and the surface tension of the liquid and the contact angle at the liquid–solid interface. Since there is no organic component in the aging mobile phase to wet the stationary phase, pressure must be used to ensure that the phase does not dewet during the test. Experiments have shown that many stationary phases dewet under the test conditions (100% aqueous mobile phase, 80 °C) without additional, post-detector backpressure.

Table 2 contains the initial methylparaben retention times per column length (normalized retention times) and the conditions under which they were obtained. On any RPLC stationary phase, a shorter column will have the same retention factor as a longer column if the phases are completely wet. However, this is not true if the phases are dewet to varying degrees. The *degree* to which a given column dewets depends on the column backpressure, which is a function of the flow rate, particle size and the column length. As the length of the column increases, the backpressure produced by the column increases. In a non-wetting mobile phase, a shorter column with lower backpressure, as predicted by the Young–Laplace equation, will show a higher percentage of dewetting than a longer column that produces a higher backpressure. Lower retention factors (or normalized retention times) on smaller columns are indicative of a higher percentage of stationary phase dewetting. Equivalent retention factors on columns of different lengths indicate that the stationary phase in both is completely solvated or "wet" with mobile phase. Complete dewetting of a stationary is indicated by a complete lack of retention—analytes will appear in the void.

As shown in Table 2, for phases tested without additional post-detector backpressure dewetting was confirmed by the observed differences in the normalized retention times for methylparaben on identical phases in different column configurations. The percentage of dewetting in Table 2 was estimated using the following equation:

$$\text{dewet}\,(\%) = \frac{(t_R/L)_A - (t_R/L)_B}{(t_R/L)_A} \times 100 \quad (3)$$

where t_R is the system-volume corrected retention time, L the column length, A the normalized retention time with post-detector backpressure added and B is the normalized retention time with no additional pressure added after the detector.

Table 2
Comparison of initial retention times for selected stationary phases in different column configurations under various conditions

Stationary phase	Methyl paraben (t_R/L)[a]	Column (mm × mm)	Aqueous 1% TFA[b]	Pressure added	Dewet (%)
MH1-C_8	0.01	3.9 × 20	0% MeCN	No	96
	0.18	4.6 × 75	0% MeCN	No	25
	0.25	4.6 × 75	0% MeCN	Yes	0
MH1-C_8	0.14	3.9 × 20	3% MeCN	No	–
	0.19	4.6 × 75	3% MeCN	No	–
MH1-C_{18}	0.25	3.9 × 20	0% MeCN	Yes	9
	0.16	4.6 × 75	0% MeCN	No	44
	0.28	4.6 × 75	0% MeCN	Yes	0

[a] System-volume-corrected retention time per column length (mm) for methylparaben used for calculations.
[b] Acetonitrile composition of the 1% TFA aging mobile phase.

As illustrated by the MH1-C_8 phase, the normalized retention times are dramatically different for the 3.9 mm × 20 mm and 4.6 mm × 75 mm columns if post-detector pressure is not added to prevent dewetting. The dewet (%) values for the MH1-C_8 phase in the different column configurations using the 100% aqueous mobile phases are consistent with expectations. The data indicate that the 20 mm length column is close to completely dewet (96%). This also suggests that the last 20 mm of the 75 mm column is close to completely dewet. The ratio of 20 mm:75 mm is 27%, which is very close to the estimate of the dewetting (%) for the 75 mm column. The actual value measured for the dewet (%) value on the 75 mm column is expected to be lower than that predicted by the 20 mm column because the backpressure generated from the HPLC tubing and flow cell is higher for the 4.6 mm i.d. column due to the higher flow rates required to maintain the same linear velocity.

In contrast to the MH1-C_8 phase, the MH1-C_{18} phase in 3.9 mm × 20 mm and 4.6 mm × 75 mm columns has very similar normalized retention times due to the addition of pressure after the detector. The normalized retention time on the MH1-C_{18} phase in the 4.6 mm × 75 mm column, without the addition of backpressure, confirms that the phase does dewet (44%) and under these test conditions it does so to a greater extent than the MH1-C_8 phase (25%) in the same column configuration. Therefore, the data in Table 2 also indicate that dewetting is prevented by the addition of an appropriate amount of backpressure after the detector.

Based upon the Young–Laplace equation [30] and conservative estimates of surface tension and contact angle, a minimum *total* backpressure of approximately 650 psi (4.5 MPa) is required to prevent dewetting of a phase with an average pore diameter of 100 Å. Experiments in our lab have shown that very little change in the retention-loss curves occurs for a wide variety of phases when a minimum additional, post-detector backpressure of 870 psi (6 MPa) is used. Our accelerated low-pH stability test uses an additional, post-detector backpressure of approximately 900 psi (6.2 MPa) to ensure that phase dewetting is avoided.

As shown in Fig. 4, additional, post-detector backpressure substantially affects the retention-loss curves for MH1-C_{18} and MH1-C_8 stationary phases. The obvious solution to the dewetting problem in 100% aqueous low-pH aging mobile phases is to add a small amount of organic modifier. However, as shown in Fig. 4, it is clear that as little as 3% acetonitrile drastically lowers the magnitude and rate of retention-loss for MH1-C_{18} and MH1-C_8 phases during the low-pH stability test.

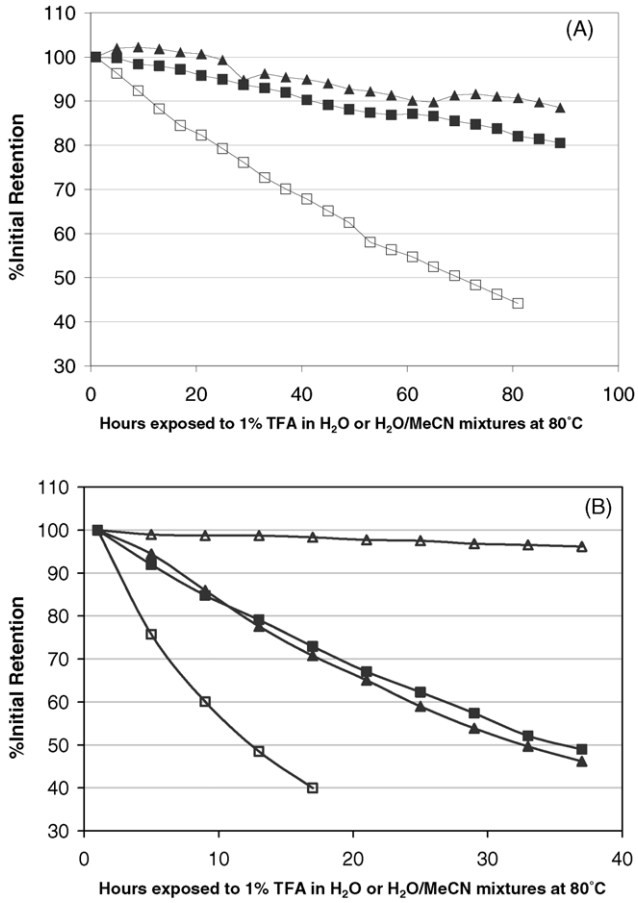

Fig. 4. The effect of post-detector backpressure and organic composition on the loss of ethylparaben retention under accelerated low-pH aging conditions: (A) MH1-C_{18}, (B) MH1-C_8; (▲) aged in 100%, 1.0% TFA in H_2O without additional backpressure, (□) aged in 100%, 1.0% TFA in H_2O with additional backpressure, (■) aged in 97/3 1.0% TFA in H_2O/acetonitrile without additional backpressure, (△) aged in 70/30 1.0% TFA in H_2O/acetonitrile without additional backpressure.

Stationary phase dewetting minimizes the exposure of the phase to the aqueous TFA and leads to misleadingly lower retention-loss. This assertion is supported by the data in Table 3 that summarizes retention-loss data obtained in a wetting mobile phase (50/50 MeCN/H_2O) for acenaph-

Table 3
Retention-loss data for a monofunctional C_8 phase obtained under selected conditions

Exposed (h)	ACE[a] (%)	MP[a] (%)	Difference (%)	Column (mm × mm)	Aqueous 1% TFA[b]	Pressure added
17	53	58	−10	4.6 × 75	0% MeCN	Yes
37	44	47	−8	4.6 × 75	3% MeCN	No
37	5	53 gain	1168	3.9 × 20	0% MeCN	No
49	6	5	15	4.6 × 75	30% MeCN	No

[a] Percent of retention-loss (unless otherwise noted) for acenaphthene (ACE) in 50/50 acetonitrile/water (v/v) and methylparaben (MP) in the 1% TFA mobile phases.
[b] Acetonitrile composition of the 1% TFA aging mobile phase.

thene and in the aging mobile phase for methylparaben. With pressure added post-detector the retention-losses after 17 h for both acenaphthene and methylparaben are greater than the losses observed after 37 h for the same stationary phase in the same column configuration but tested without the post-detector pressure. This is even more dramatically illustrated by the almost total lack of retention-loss (5%) for acenaphthene on the 3.9 mm × 20 mm column (no additional post-detector backpressure) after 37 h of exposure to the aging mobile phase. The complete dewetting of the phase in the non-wetting aging mobile phase has limited its exposure to the conditions responsible for phase hydrolysis. The concomitant increase in retention for methylparaben in the non-wetting aging mobile phase is most likely due to the increased wettability of the phase over the course of testing. The degree of dewetting, as pointed out above, depends partly on the ligand density of stationary phase and as ligand is hydrolyzed and removed during the test, more of the stationary phase becomes available for retention. Even though the increase in retention appears dramatic, the dewet (%) for the phase in the 3.9 mm × 20 mm column at the end of the 37 h was found to be 94% compared to its initial value of 96%.

Although the columns tested using 3% acetonitrile did not have post-detector backpressure added, the data in Table 2 for the MH1-C_8 phase allows us to assume that a very small portion of the 4.6 mm × 75 mm column is dewetted (∼10%), yet retention-loss is drastically lower than that of an identical column tested using 100% aqueous TFA with backpressure added. This indicates that even a small amount of organic modifier substantially increases column lifetime under routine low-pH conditions. This assertion is further supported by the retention-loss curves for the MH1-C_8 phase using 1% TFA in 30% acetonitrile, which showed only a 4% retention-loss after 37 h of exposure. We rationalize this behavior as follows. At the stationary phase surface, the concentration of acetonitrile is enriched due to favorable interactions with the ligand [31]. The acetonitrile-enriched mobile phase at the surface helps shield the polar siloxane bond from the acid in the mobile phase, thus slowing hydrolysis.

Because even small amounts of organic modifier substantially increase column stability, aging the columns using 100% aqueous mobile phases with additional post-detector backpressure was selected for our stability test. Although appropriate backpressure amounts have been established for high ligand density C_{18} phases with pore sizes of 86 Å or larger, any new phase, particularly on smaller pore size silicas, should be checked according to the protocol used in Table 2. The monitoring of retention-loss for acenaphthene in a wetting mobile phase serves as confirmation that the retention-loss for the parabens in the aging mobile phase is consistent with expectations. The data in Table 3 indicate that comparable retention-losses are observed for acenaphthene and methylparaben as long as the stationary phases remain reasonably well solvated during testing.

3.3. Stationary-phase characteristics and low-pH stability

3.3.1. The effect of bonded-phase chain length and functionality on the low-pH stability of RPLC stationary phases

As reported by others, the low-pH stability of various stationary phases is dependent on ligand type [2,5,21,22], density [21], and functionality [21,22]. A series of phases were tested using our stability test to confirm that the test shows differences in the low-pH stability of phases that are commensurate with their synthetic differences.

The retention-loss curves for several C_8- and C_{18}-silica stationary phases are shown in Fig. 5A. The MH1-C_8 phase is much less resistant to ligand loss than the MH1-C_{18} silica phase. This qualitatively agrees with previously published results [2,5,21,22]. The increased hydrophobicity provided by the bulky C_{18} ligand helps to better shield the siloxane bond between the silane and the silica surface.

The impact of the bonded-phase ligand functionality on the low-pH stability of C_{18}-silica phases is also shown in Fig. 5A. The low-pH stability of the phases increases as the functionality of the starting silane increases for simi-

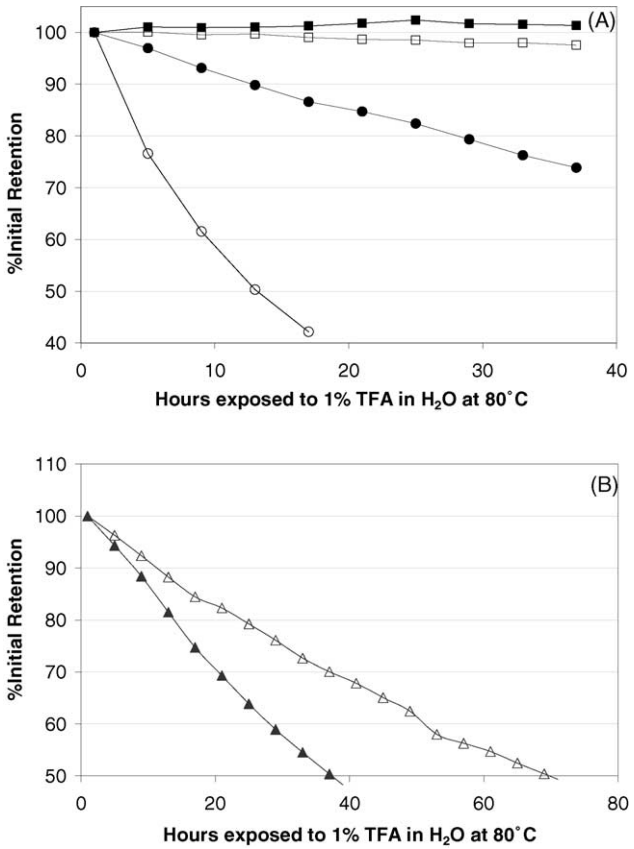

Fig. 5. The effect of aliphatic chain length, ligand functionality and underlying silica substrate on the loss of methylparaben retention under accelerated low-pH aging conditions: (A) C_8- and C_{18}-silica stationary phases with different chain lengths and ligand functionality, (B) C_{18} Stationary phases on different silica substrates; (■) TH1-C_{18}, (□) DH1-C_{18}, (●) MH1-C_{18}, (○) MH1-C_8, (▲) MH2-C_{18}, (△) MH1-C_{18}.

lar bonding densities of 3.25, 3.22, and 3.32 μmol/m^2 for the monofunctional (M), difunctional (D) and trifunctional (T) C_{18} phases, respectively. The greatest enhancement in low-pH stability of high-ligand density C_{18}-silica phases occurs when the functionality of the starting silane is increased from one to two and agrees with previously published work [21,22].

Increasing the number of covalent bonds between the C_{18} silanes and the silica surface enhances low-pH stability. Acid-catalyzed hydrolysis of difunctional and trifunctional stationary phases and subsequent loss of the ligand require the simultaneous breakage of multiple covalent bonds. This includes bonds to the silica surface as well as bonds to neighboring ligands. This process is not as easy as simply breaking one covalent bond between the ligand and the silica, thus, methylalkyl-difunctional and alkyl-trifunctional phases are more stable than dimethylalkyl-monofunctional phases with similar bonding densities under aggressive, low-pH conditions.

The effect of the underlying silica substrate is shown in Fig. 5B. The phases were bonded and endcapped in the same manner and have similar bonding densities, but the low-pH stability of the C_{18} ligand is clearly much better on MH1-C_{18} despite its lower ligand density. Others have reported differences in the low-pH stability between similarly bonded, less pure silicas [2,21,23]; however, differences even exist amongst similarly bonded high-purity silicas. The cause of this difference is not known at this time.

Ligand functionality is not the only bonded-phase parameter critical to low-pH stability. Another important variable is bonding density. This is illustrated by the retention-loss curves for the three difunctional C_{18} phases shown in Fig. 6. The bonding densities of the difunctional C_{18} phases range from 1.65 to 3.22 μmol/m^2. The difference in low-pH stability of these difunctional C_{18} phases is dramatic. When the bonding density of the ligand is 3.22 μmol/m^2, the phase shows virtually no loss in retention over 37 h whereas the 1.65 μmol/m^2 phase shows approximately 50% retention-loss in 17 h. A lower bonding density permits acidic mobile phases greater access to the hydrolytically labile siloxane bond between the organosilane ligand and the silica surface.

The results of these experiments confirm that our test is able to elucidate low-pH stability differences between synthetically different phases and that these differences are consistent with previously cited papers.

3.3.2. *The effect of endcapping on the low-pH stability of a medium ligand density trifunctional C_{18} phase*

The phases in previous experiments have been endcapped using trimethylchlorosilane. Many commercially available phases are endcapped in order to minimize adverse interaction between residual silanols and basic compounds that would otherwise result in poor peak shape. The loss of the endcapping ligand and its impact on the peak shape for basic compounds has not been probed by our selected test conditions. However, the effect of the presence of a trimethylsilyl endcap on the retention-loss for methylparaben was investigated on a medium ligand density trifunctional C_{18} phase (TM1-C_{18}).

Fig. 7 shows the retention-loss curves for the same C_{18} stationary phase before (TM1-C_{18}-NE) and after endcapping (TM1-C_{18}). Based on previously reported low-pH stability studies [5,32] the initial retention-loss is expected to be associated with the loss of the monofunctional short-chain endcap group. This also suggests that the initial retention-loss would be greatest on the endcapped phase assuming the group contributes to the retention of methylparaben. Comparison of the initial methylparaben retention times (Table 4) on the two phases suggests that the endcapping group does not contribute to its retention and hence the loss of endcap may not be apparent from the retention-loss curves.

As shown in Fig. 7 the unendcapped phase lost retention faster and to a greater extent than the endcapped version of the phase. This result can be rationalized as follows. The overall ligand *plus endcap* coverage of the endcapped phase

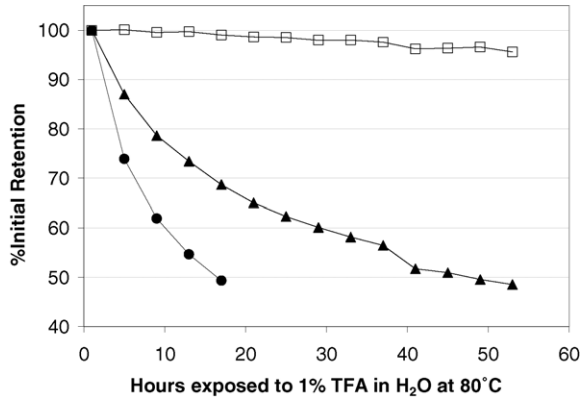

Fig. 6. The effect of ligand bonding density on the loss of methylparaben retention under accelerated low-pH aging conditions for difunctional C_{18} phases: (●) DL1-C_{18} (1.65 μmol/m^2), (▲) DM1-C_{18} (2.08 μmol/m^2), (□) DH1-C_{18} (3.22 μmol/m^2).

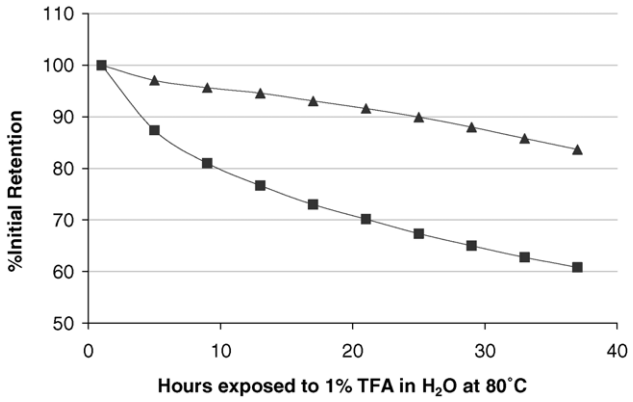

Fig. 7. The effect of trimethylsilyl endcap on the loss of methylparaben retention on a medium ligand density trifunctional C_{18} phase under accelerated low-pH aging conditions: (■) TM1-C_{18}-NE (2.40 μmol/m^2), (▲) TM1-C_{18} (2.40 μmol/m^2).

Table 4
Summary of the elemental analysis[a] and retention-loss data[b] for select trimethylsilane-endcapped stationary phases after accelerated low-pH stability testing

Stationary phase	Ligand density (μmol/m^2)	Initial methyl paraben (t_R/L)[b]	Exposure to 1.0% TFA in H_2O (h)	Total retention time loss (%)	Total relative C loss (%)	Loss in retention time at 13 h (%)
MH1-C_8	3.52	0.25	17	60.1	45.2	49.7
MH1-C_{18}	3.25	0.28	109	68.2	56.5	10.2
DL1-C_{18}	1.65	0.24	17	50.7	14.3	49.1
DM1-C_{18}	2.08	0.32	53	51.5	10.2	26.6
DH1-C_{18}	3.22	0.28	37	6.2	4.9	2.6
TM1-C_{18}	2.4	0.29	37	16.4	8.8	5.4
TM1-C_{18}-NE	2.4	0.31	37	39.2	5.9	23.3
TH1-C_{18}	3.32	0.28	117	12.7	9.1	0.3

[a] C, H, and N were determined for each sample.
[b] System-volume-corrected retention time per column length (mm) for methylparaben used for calculations.

is higher than that for the unendcapped version and, as discussed above, higher ligand density leads to improved stability. It is also likely that during the endcapping process some of the residual silanols associated with the trifunctional ligand are endcapped. This group may contribute to the steric protection of the C_{18} ligand. The low-pH stability improvements associated with sterically protected ligands are well-documented [5,10,12]. The relative importance or validity of these two proposed explanations for this trifunctional C_{18} is not known at this time and merits further investigation.

3.4. Elemental analysis of stationary phases before and after low-pH stability testing and the impact of synthetic choices on low-pH stability

It is important to summarize the elemental-analysis data obtained before and after low-pH stability testing. Reviewing this information is critical to understanding the retention-loss of phases under low-pH conditions.

The elemental-analysis data for select stationary phases are summarized in Table 4. The monofunctional, difunctional and trifunctional high-bonding-density ($\geq$3.2 μmol/m^2) phases show a relative decrease in the percent-carbon content that is similar in magnitude to the percent loss in retention time (i.e. 60% loss in retention, approximately 50% relative loss in carbon). This type of behavior is consistent with *hydrolysis and complete removal* of the bonded ligands from the stationary phases during the accelerated low-pH stability test.

The difunctional and trifunctional C_{18} phases with medium and low bonding densities behave differently than high-bonding-density monofunctional and polyfunctional phases. These C_{18} phases show only minor decreases in carbon content despite a *large* loss in retention. This occurred on both the endcapped (TM1-C_{18}) and non-endcapped (TM1-C_{18}-NE), trifunctional C_{18} phases with a ligand bonding density of 2.4 μmol/m^2. This type of behavior is *not* consistent with *hydrolysis and complete removal* of the bonded ligands. Inconsistencies between the magnitudes of carbon loss and retention-loss have been documented in previous publications [5]. Changes on the surface of trifunctional C_{18} phases during and after accelerated low-pH aging are currently being investigated with ^{29}Si and ^{13}C solid-state NMR spectroscopy.

Table 4 contains the loss of retention data for various C_{18} phases after 13 h of exposure to the aging mobile phase. The data affords the relative comparison of the low-pH stabilities associated with the range of synthetic choices investigated. As expected, the retention-losses for the high ligand density monofunctional, difunctional and trifunctional C_{18} phases reflect the expected relative retention-losses of 10.2, 2.6 and 0.3%, respectively. However, the data also suggests that the greatest absolute improvement is observed between monofunctional and difunctional phases. Only a relatively small absolute improvement was observed between difunctional and trifunctional phases. However, for the medium ligand density difunctional and trifunctional C_{18} phases a significant improvement in stability is afforded by the trifunctional phase (retention-loss of 5.4%) over the difunctional phase (retention-loss of 26.6%). The impact of ligand density is illustrated by the low, medium, and high ligand density series using a difunctional C_{18} ligand with retention-losses of 49.1, 26.6 and 2.6%, respectively. The low ligand density difunctional C_{18} phase (retention-loss of 49.1%) has a low-pH stability comparable to a high ligand density monofunctional C_8 phase (retention-loss of 49.7%).

4. Conclusions

The primary considerations for designing an accelerated low-pH RPLC column stability test are as follows:

1. The test should be a “dynamic” test meaning that mobile phase is flowing throughout the entire test.
2. Retention-loss should be plotted versus exposure time and not column volumes of low-pH aging mobile phase. When testing columns of different internal diameters, the flow rates should be scaled to give a constant linear velocity.
3. The pH and temperature should be chosen to cause a significant amount of degradation in a reasonably short

period of time. However, the temperature should not be so high as to cause dissolution of the particles or disruption to the column bed.

4. For the quickest degradation, the challenge mobile phase should contain no organic modifier. However, the use of 100% aqueous mobile phases requires the addition of backpressure after the detector to prevent dewetting.

The retention-loss curves generated by this test for the different C_8- and C_{18}-silica bonded phases indicate that the organosilane chain length, functionality and the bonding density on the silica surface and endcap strongly influence the low-pH stability of the phase as documented previously [21,22,24]. The paper affords the opportunity to make comparisons between the low-pH stability of the differently synthesized phases investigated. The impact of selected test conditions has been thoroughly (although not exhaustively) investigated and highlights the importance of specific test conditions.

A number of test parameters warrant further investigation, such as the influence of flow rate under wetted conditions and the influence of the "strip" step. The CHN data for low-pH-aged high ligand density ($\geq$3.2 μmol/m^2) monofunctional, difunctional and trifunctional phases are consistent with hydrolysis and complete removal of the ligand. However, the CHN data do not support this mechanism as an explanation for the retention-loss of difunctional and trifunctional C_{18} stationary phases with low to moderate bonding densities. These phases show only small decreases in carbon content despite large decreases in reversed-phase HPLC retention.

In reviewing the data presented in this paper it is important to keep in mind that the selected test conditions are extremely aggressive and are designed to *rapidly* degrade the phases. For example, the retention-loss curve for the MH1-C_8 phase shows substantially faster and larger retention-loss compared to the other phases studied. However, many chromatographers are routinely successful using C_8 phases. The curves presented simply reflect the relative stabilities of the various phases and not necessarily their utility as general-purpose robust stationary phases. Robustness strongly depends on the specific HPLC conditions used for a particular method. For those chromatographers working with low-pH mobile phases under extreme conditions the relative low-pH stabilities provide useful guidance in the selection of the best stationary phase for the desired application.

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