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ISOCRATIC MIXED MODE LIQUID CHROMATOGRAPHIC SEPARATION OF PHOSPHOLIPIDS WITH OCTADECYLSILANE-SILICA STATIONARY PHASES

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Summary—Molecular species of phosphatidylcholine, phosphatidylethanolamine and phosphatadic acid were resolved by isocratic reversed phase high performance liquid chromatography (HPLC) using mobile phases of methanol–isopropanol containing *para*-toluenesulfonic acid (*p*-tsa). Separation by both non-polar fatty acid chain length and by polar head group functionality was achieved concurrently upon a commercially available octadecylsilane (C18) column endcapped with trimethylsilane (C1) groups. Using a mobile phase of 97.5:2.5 methanol:isopropanol with 70m*M* *para*-toluenesulfonic acid (*p*-tsa) at a pH of approximately 1, twelve phospholipid species comprised of four tail group classes (dilauroyl-, dimyristoyl-, dipamitoyl- and distearoyl-) and three head group speciations (phosphatidylcholine, phosphatidylethanolamine and phosphatadic acid) were separated. The column was then exposed to the acidic mobile phase for 48 hours continuously during which the bound phase underwent severe acid-induced hydrolysis, after which the separation of the twelve analytes resulted in the separation of the phospholipid species by non-polar tail group alone. The experimental results are discussed in terms of potential separation mechanisms including dependency of the separation on adsorption of the counter ion into the stationary phase, residual acidic silanol group interactions, and potential interactions of the surface active phospholipids with C1 groups.

Separation of charged and neutral phospholipid species is traditionally achieved through the use of a complicated multistep gradient elution process, and typically results in a characterization of either the non-polar fatty acids chains (tail group) or the polar functionality bonded to the phosphate group (head group).[1–13] With the development and characterization[14–27] of reversed phase ion pairing chromatography (RP–IPC) and the selection of an appropriate ion pairing reagent, separations of phospholipid species by a limited range of head and tail groups has been performed.[2,11–13] These separations normally focus upon a limited range of tail groups of a specific head group, or involve the normal phase separation of a number of head groups without any separation of analytes by non-polar nature. The retention characteristics of these tail and head group separations have been shown to depend upon the concentrations of the organic modifier and of the ion pairing agent, respectively, in the mobile phase.[25–27] The retention mechanism for reversed phase separations has been debated, but is well characterized[16,28,29] However, the nature of the mechanism by which the polar head group functionality of these species is separated on C18 is not clearly described. Classical descriptions of anion exchange mechanisms suggest that the separation of phospholipid species by ionic nature upon silica-based stationary phases are a function of the interaction of the ionic moiety (choline, ethanolamine or phosphatadic acid) with residual acidic silanol groups which result from incomplete derivatization of the silica. Theoretical surface coverage of the silica stationary phase material is in the order of 8.1 μmol/m^2. Derivatization of the silica with alkyl groups, such as C18 and C8 for reversed phase chromatography, normally results in a surface coverage of 2.0–3.5 μmol/m^2. Subsequent endcapping of the residual silanol groups, using trimethylchlorosilane or a similar material, is normally performed to further increase the net surface coverage and therefore minimize the effects of residual acidic silanol groups. It has been suggested by Kohler that at surface coverage of 3.0–3.5 μmol/m^2, acidic silanol interactions are effectively shielded by the derivatized groups.[30] As such, the separation of

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phospholipid species by the basis of their acid-base properties can be minimized with a properly derivatized column.

According to reversed phase-ion pairing chromatography theory as presented by Stahlberg and co-workers, separation of ionic species upon a C18 stationary phase material is dependent upon the adsorption of a counter ion by the stationary phase and the formation of an electrostatic layer.[27] Ionic analytes interact with the counter ion in this generated electronic double layer and are separated on the basis of differences in electrostatic surface potential. As such, the separation of phospholipid species by their ionic nature can be modified by changes in the formation of the electrostatic double layer formed as a result of adsorption of the counter ion into the stationary phase.

In a similar vein, surfactant molecules have been used to modify chromatographic columns to create a hydrophobic shield upon the stationary phase in order to facilitate the separation of small polar species in a large protein matrix.[31] A characterization of the adsorption of various surfactants to bonded silica-based stationary phases reveals a preference of these surface active species for C1 groups.[32,33] The adsorption of surfactants upon C1 bonded silica-based stationary phases was greater than on phases bonded with various alkyl chains (C18, C8, *etc.*), cyano groups, and much greater than observed for unbonded silica.[32,33]

Phospholipids are surface active species, consisting of a large non-polar fatty acid region and a small polar moiety. A characterization of the interactions of various surface active species with the silica-based C18 stationary phase reveals a preferential interaction with C1 groups that are commonly used to endcap residual silanol groups in commercially available columns. A characterization of silica-based stationary phases in general reveals that under acidic conditions (pH < 2), and to a lesser extent even in the pH stable region of the silica-based stationary phase (pH 2–7), the bonded material upon the stationary phase is hydrolyzed.[34–39] It has been shown that the extent of this hydrolysis depends upon the nature of the bonded phase. In particular, C1 groups are particularly susceptible to hydrolysis and can result in a loss of up to 96% of the original bonding concentration.[34]

A definitive description of the mechanism by which polar head group separation is achieved on a C18 column is not present in literature. We present a separation of phospholipid species by both polar head and non-polar tail groups upon a silica-based C18 column. Traditionally, separation of the phospholipids by head group requires the use of gradient techniques and chloroform or one of its analogs, and yields no information about the length of the alkyl tail groups. The presented separation is novel in its ability to separate the phospholipid species by both polar and non-polar characteristics of the analytes without the use of a complicated gradient system or the use of chlorinated solvents. Phospholipids consisting of three differing head groups, phosphatidylcholines, phophatidylethanolamines, and phosphatadic acids, and 4 differing tail groups, dilauroyl-, dimyristroyl-, dipamitoyl-, and distearoyl-, are separated using isocratic methods upon a commercially available silica-based C18 column endcapped with C1. The C1 endcapping was then selectively removed using acidic media, and resulted in a complete loss in the ability of the column to separate the phospholipid species by the polar head group. As the C18 alkyl chains were not completely hydrolyzed, the separation of the phospholipids by fatty acid chain length (non-polar tail group) was retained. Column efficiency decreased on average by only 30% for the peaks examined, which agrees with the values expected for the degradation of a C18 packing material by exposure to acidic media. The separation of the same species performed using a poly(vinyl alcohol) (PVA) based C18 column, which contained no C1 groups, demonstrated no polar head group selectivity. Potential mechanisms that describe the nature of the complete separation, subsequent degradation of the separation after exposure of the C1 endcapped column to acidic media, and absence of a separation upon the PVA column are discussed.

EXPERIMENTAL

Materials

Dilauroyl-(C12), dimyristoyl-(C14), dipamitoyl-(C16), and distearoyl-(C18) phosphatidylcholines, phosphatidylethanolamines, and phosphatadic acids and *para*-toluenesulfonic acid were purchased from Sigma Chemicals (St Louis, MO, U.S.A.). HPLC grade methanol and 2-propanol were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Analyte abbreviations are presented in Table 1.

HPLC

The apparatus used in this study is similar to that used in previous reports.[40-45] Refractive index gradient (RIG) detection was used to detect the analytes, but conventional refractive index detection would have been adequate. The peaks will appear as the derivative of the typical Gaussian shaped peaks. Integration of the peaks was performed to clarify any potential ambiguity resulting from RIG detection. Column efficiency calculations were performed using the Foley-Dorsey method for asymmetric peaks.[46] Column efficiency for the combined head and tail group separations was determined using only the leading (ethanolamine) peak in each tail group cluster, as the lack of baseline resolution made determination of $W_{0.1}$ as required by the Foley-Dorsey method burdensome. Based upon the treatment of reduced plate height and reduced velocity presented by Giddings,[47] and using a 5-μm particle diameter, an average flow velocity of 0.15 cm/sec, and an average analyte diffusion coefficient of 10^{-6} cm^2/sec, the column is found to possess a reduced plate height of 13 at the reduced flow velocity of 75. According to Giddings,[48] the optimal reduced plate height of 2 occurs at reduced flow velocities of 1 or 2. With increased reduced flow velocity, the band of reduced plate heights that describes high performance columns increases in value and broadens in range. As such, a reduced plate height of 13 is therefore predicted to be within the band of high performance, thus the initial efficiency of the column, prior to acid-induced degradation, is acceptable for a separation of this nature.

Analyte mixtures were prepared by dissolving the crystalline phospholipid species in mixtures of methanol–isopropanol. Isopropanol levels varied form 1 to 10% (volume ratio) in the initial tests, but for the final reproducible experiments all sample mixtures were prepared in a solution of the mobile phase used to perform the separation. *Para*-toluenesulfonic acid concentrations ranged from 2.5 to 70 m*M*, which is the same range of concentrations used in similar analyses that use ion pairing reagents to solubilize the phospholipid species. For the column degradation analysis discussed in this text, and including the complete separation of the phospholipid species by the head and tail group, the same mobile phase batch was used, ensuring that the ionic strength and pH of the system remained constant for the experiments shown. Samples were sonicated thoroughly to remove solubilized gases, as were the prepared mobile phases. A sodium hydroxide solution, prepared in methanol, was used to modify the pH of the chromatographic system. pH Levels were approximated using both pH paper and a pH meter used in comparison with the methanol prepared pH modifying solution.

Table 1. Analyte identificaton key

Phospholipid name	Abbreviation
Dilauroyl- (C12)	
phosphatidylcholine	LC
phosphatidylethanloamine	LE
phosphatadic acid	LA
Dimyristoyl- (C14)	
phosphatidylcholine	MC
phosphatidylethanloamine	ME
phosphatadic acid	MA
Dipamitoyl- (C16)	
phosphatidylcholine	PC
phosphatidylethanloamine	PE
phosphatadic acid	PA
Distearoyl- (C18)	
phosphatidylcholine	SC
phosphatidylethanloamine	SE
phosphatadic acid	SA

Samples were introduced to the system using an injection value (Rheodyne, CA, U.S.A.) fitted with a 1-μl injection disk. All connecting tubing consisted of 1/16 in. o.d. by 0.005 in. i.d. poly(ether etherketone) (PEEK) tubing. A syringe pump (ISCO, NE, U.S.A.) was used to deliver the mobile phase to the system, which was then passed through a 250-psig pressure restrictor (Upchurch, WA, USA) and to waste.

A variety of microbore HPLC columns were examined. In the final study, a microbore, 1.0 mm i.d. by 25.0 cm length, Adsorbosphere HS, 5 μm, C18 silica-based column, endcapped with hexamethyldisilazane (HMDS), was used (Alltech, IL, U.S.A.). HMDS, like trimethylchlorosilane, endcaps free silanol groups with C1 groups. Secondary analyses were performed upon a microbore, 1.0 mm i.d. by 25.0-cm length, Asahipak, 5 μm, C18 poly(vinyl alcohol) based column, which contained on C1 groups upon the stationary phase. Analyses were performed at a volumetric flow rate of 50 μl/min.

RESULTS AND DISCUSSION

A separation of a limited group of phospholipid species (LE, LC, ME, MC) is presented as Fig. 1. This separation demonstrates that the phospholipid species were separated by both non-polar head group isocratically, and without the use of chlorinated solvents. In addition, as

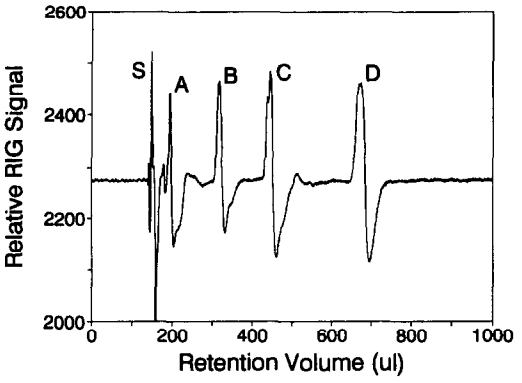

Fig. 1. Separation of A) LE, B) LC, C) ME, and D) MC with a solvent peak (S) using a mobile phase of 95:5 methanol:isopropanol with 70m*M* *p*-tsa, at a pH of 5, upon an Adsorbosphere HS C18 column endcapped with trimethyl groups with refractive index gradient detection.

para-toluenesulfonic acid is not commonly used as an ion pairing reagent in RP-IPC separations, the ability of *p*-tsa to function as an adequate ion pairing reagent for the separation of phospholipid species by polar head group is demonstrated. The use of *p*-tsa as an ion-pairing reagent allows for the use of indirect UV detection as a measurement technique. Indirect detection using photometric techniques applied to the analysis of surfactants in a similar separation to our phospholipid separation, and with the use of alkyl sulfonates instead of *p*-tsa, have demonstrated detection limits in the order of sub-ppm.[47] Compared to traditional RI detection this limit would be an improvement, but RIG detection has demonstrated similar, or improved, detection limits in the order of 0.9 ppm.[40] In addition, RIG detection is insensitive to thermal gradients which are problematic for traditional RI detection.[45]

Separation of the non-polar tail groups by reversed phase mechanisms is well characterized. However, the mechanism for retention of the polar head groups is not clearly understood. In order to begin to characterize this retention mechanism a more complete understanding of the interactions of these ionic, surface active species with acidic free silanol groups, with the adsorbed counter-ion complex, with various bonded silica-based stationary phases, and a characterization of the chemical behavior of silica phases in general was needed.

An experiment was designed to allow for the preliminary examination of these factors. Using a mobile phase with an approximate pH of 1 the separation was attempted upon a silica-based stationary phase that had been derivatized with C18 groups, and then fully endcapped with C1 groups. Under these conditions, the bonded material on silica-based stationary phases will undergo severe acid-induced hydrolysis. C1 groups are almost completely hydrolyzed, while only up to 40% of the C18 groups are lost.[34] Initially, under these conditions and prior to any significant degradation, a complete separation of the phospholipid species by each of 3 polar head groups and 4 non-polar tail groups was obtained, and presented as Fig. 2. The separation of the distearoyl-cluster is not shown for the sake of clarity, due to the fairly large retention volumes of SE, SC, and SA were 1504.0, 1630.0 and 1926.0 μl, respectively. An examination of the retention characteristics of this separation as a function of the concentration of isopropanol (tail group separation modifier) and *p*-tsa (head group separation modifier) followed the same trends as reported by Stahlberg and Hagglund on the separation of similar species.[24] Retention times increased with increased *p*-tsa concentrations even though the solubility in the mobile phase also increased for the phosphatidylethanolamines and phosphatidylcholines.

The same mobile phase, with a constant ionic strength, used to achieve the separation in Fig. 2, was then passed through the column for 48 hours continuously, after which the separation appeared as presented in Fig. 3. Only four peaks (the distearoyl-peak is not shown for clarity, but possessed a retention volume of 1244.0 μl), corresponding to the four tail groups, were observed. A chromatogram at the

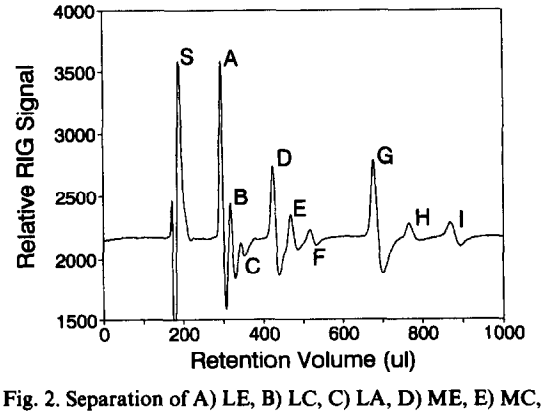

Fig. 2. Separation of A) LE, B) LC, C) LA, D) ME, E) MC, F) MA, G) PE, H) PC, and I) PA with solvent peak (S) using a mobile phase of 97.5:2.5 methanol:isopropanol with 70m*M* *p*-tsa, at pH of 1, upon an Adsorbosphere HS C18 column endcapped with trimethyl groups prior to the acid-induced hydrolysis of the endcapping material. Detection method as in Fig. 1.

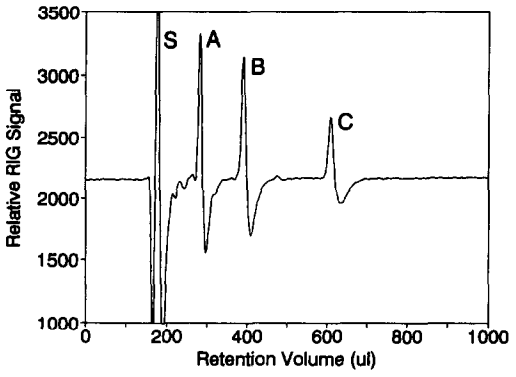

Fig. 3. Separation of the A) dilauroyl-, B) dimyristoyl, and C) dipamitoyl-phospholipid species, with solvent peak (S), using a mobile phase of 97.5:2.5 methanol:isopropanol with 70m*M* *p*-tsa, at a pH of 1, upon an Adsorbosphere HS C18 column after the hydrolysis of the trimethyl group endcapping. Note that no polar head group separation is observed. Detection method as in Fig. 1.

intermediate time of 24 hours, not shown for brevity, appeared as a poorly resolved combination of Figs. 2 and 3. Each of the 12 analyte species examined was then introduced as a single species and it was confirmed that the four peaks observed in Fig. 3 correspond to the separation of tail group species by tail group alone. Head group separation was no longer observed as the three species (choline, ethanolamine, and acid) all co-elute. Figures 4(A) and 5(A) show the results of integrating the dimyristoyl- and dipamitoyl-series from Fig. 2, respectively. It is observed that three distinct peaks are present in each case. Similar results were obtained for the dilauroyl- and distearoyl-groupings. Figures 4(B) and 5(B) show the result of integrating the dimyristoyl- and dipamitoyl-series from Fig. 3, respectively. In these cases, only one peak is observed. An analysis of column efficiency, presented as Table 2, demonstrates that only a 15–30% loss in overall column efficiency occurred during the acid-induced degradation of the column. The nature of this degradation, and its implications, will be discussed shortly. Quantification of the peaks was not performed and the observed difference in peak heights is due to differing concentrations of the phospholipid species used in the sample mixture. From these observations, however, it is clear that the separation of the polar species has been lost, but the separation of the same species by the primary reversed phase mechanism remains intact.

As a last experimental test in this preliminary examination, the separation was attempted upon a poly(vinyl alcohol) (PVA) based column that contained no C1 groups. In this case, the reversed phase separation of the phospholipid species by tail group was achieved, and appeared as it does in Fig. 3, but no head group separation was achieved.

In order to explain the experimental results, and to begin to elucidate a potential mechanism for the separation of the polar head group species for a common tail group, a discussion of the various interactions between phospholipids, *p*-tsa, bonded silica phase materials, and a characterization of silica-based stationary phases in general is needed. A characterization of silica-based stationary phases was reported by Kohler *et al.*[35] and Glajch *et al.*[36] Of key importance is the preferential degradation of these stationary phases in the presence of highly acidic media. Most bonded silica-based stationary phases are reported to be stable from pH 2–7. At lower pH, and to some degree in the pH stable region, bonded material is hydrolyzed. Specifically, it has been observed that the C1 groups, which are customarily used to endcap

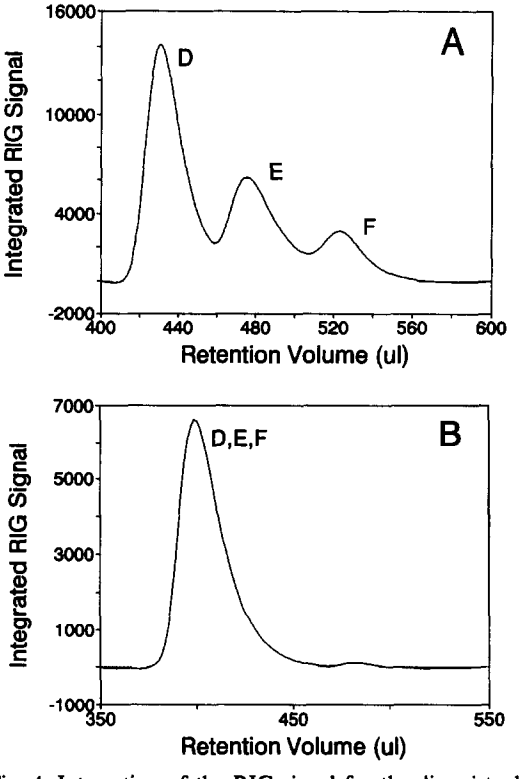

Fig. 4. Integration of the RIG signal for the dimyristoyl-series presented in Fig. 2 illustration the separation of D) ME, E) MC, and F) MA prior to selective degradation of the column. Fig. 4(B) shows the integration of the dimyristoyl-peak found in Fig. 3 which shows no head group separation after acid-induced degradation of the column.

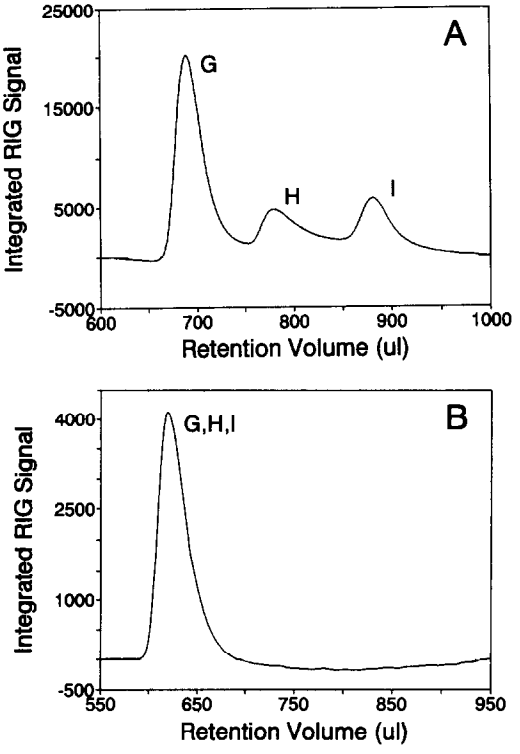

Fig. 5. Integration of the RIG signal for the dimyristoyl-series presented in Fig. 2 illustration the separation of G) PE, H) PC, and I) PA prior to selective degradation of the column. Fig. 5(B) shows the integration of the dimyristoyl-peak in Fig. 3 which shows no head group separation after acid-induced degradation of the column.

free silanol groups that remain after the original bonding process, are preferentially hydrolyzed. Kimata and *et al.*, have reported that up to 96% of the carbon count associated with C1 groups is hydrolyzed, as compared to the 40% associated with C18 and 50% associated with C8 that undergoes hydrolysis, when the silica-based stationary phase was subjected to concentrated hydrochloric acid at 100° for 5 hr.

The end result of the acid-induced degradation is the formation of a large number of acidic silanol groups. The ability of these acidic silanol groups to affect the retention characteristics depends on the surface coverage of the remaining bonded phase. Kohler has reported that for surface coverages of 3.0–3.5 μmol/m^2 the effect of acidic silanol groups is minimal, as the groups are effectively shielded by the remaining bonded phase.[30] In the experiments described in this text, the surface coverage of the bonded phase after acid-induced degradation of the column was not determined, and remains an avenue for further research. However, because the pH of the mobile phase used to obtain Figs. 2 and 3 was quite acidic, interactions between the phospholipid species and acidic free silanol groups was believed to be minimal due to the overwhelming relative concentration of the acidic counter ion additive. In addition, the results clearly demonstrate that the separation of the polar head groups was not evident when the stationary phase possessed an increased concentration of acidic silanol groups. Traditional chromatographic theory suggests that basic groups associated with the phospholipid compounds, such as the ethanolamine and choline moieties, would be retained by an anion exchange method by the acidic silanol groups. Such behavior was not observed for the experiments performed.

Table 2. Examination of column efficiency

Tail group	Efficiency (N/m)* Initial†	Final‡	%Loss
M	3720 ± 60	2640 ± 60	30
P	4920 ± 80	4200 ± 80	15a

*As calculated using the Foley-Dorsey method [46].
†Prior to acid-induced degradation, average for ethanolamine peak, 2 runs.
‡After acid-induced degradation, 2 runs.

Current RP–IPC theory suggests that retention occurs as a result of the interactions between the analytes and an electrostatic layer formed on the stationary phase as a result of the adsorption of a suitable counter ion into the bonded material. A number of models exist which attempt to describe the retention characteristics of ionizable solutes in the presence of an ion-pairing reagent,[14–27] but each model depends upon the determination of a number of factors, such as dynamic ion-exchange constant,[18] ion-pair adsorption constants[19], or parameters for an assumed Freudlich-type pairing ion adsorption[20] which are highly dependent upon the exact chromatographic conditions employed. In their simplest form, retention occurs as the result of differences in electrostatic potential between the ionized stationary phase and the bulk mobile phase. Changes in the stationary phase, such as those which result from the acid-induced degradation of bonded silica-based stationary phases, can therefore result in substantial changes in the retention characteristics of ionic materials. This behaviour is observed in the experimental data presented. The data may be interpreted as demonstrating a change in the ability of *p*-tsa to adsorb into the stationary phase with the change in concentration of acidic silanol groups. It is possible

that the loss of the organic C1 group shield, or the relative increase in the net acidity of the stationary phase results in the ability of *p*-tsa to effectively adsorb. As a result, the electronic double layer that is believed to induce the retention of ionic species is no longer formed, and the separation of phospholipid species by polar head group is no longer possible.

One last potential separation mechanism depends upon the interaction of the phospholipid species themselves, or an appropriate phospholipid-*p*-tsa ion pair with the C1 groups traditionally used to endcap reversed phase bonded silica stationary phases. Berthod *et al.*,[32,33] have determined that surface active species interact with differing degrees of preference with various bonded silica-based stationary phases, in an order given as

$$C1 > C18 > C8 > CN > \text{Free Silanols}$$

In typical C18 and C8 silica-based columns, surface coverage of the primary bonded phase is on the order of 31–43%, with the remaining available bonding sites being endcapped with C1 groups.[28,29] It is unclear from reference 16 whether the preference in surface active species interaction as a function of alkyl chain length is due specifically to the hydrophobic nature of the bonded phase, due to a displacement of the C1 group endcapping found on commercially available columns, or due to differences in the carbon load or surface coverage in the bonded phase between the columns examined. However, it is clear that surface active species interact preferentially with C1 groups and significantly less favourably with free silanol groups. By this, it is possible that retention occurs by differences in the adsorption of the ionic phospholipid species, or the associated ion pair, into the bonded material, and subsequent interactions that may depend upon the differences in the polarity of the analyte complexes. Loss of the C1 groups that preferentially occurs as the result of acid-induced hydrolysis results in the loss of this separation mechanism, as surface active species interact less favourably with acidic silanol groups.

CONCLUSION

A separation of a wide range phospholipids with differing polar head groups and non-polar tail groups is presented. The separation is completed without the use of complicated gradient techniques or the use of chlorinated solvents normally associated with separations of this type. A preliminary characterization of the effects of the presence of C1 groups upon the silica-based C18 stationary phase upon the separation of the phospholipids by head group is presented. Further experimentation is required to determine the role of C1 groups in the separation mechanism, but current experimental evidence suggests that the C1 groups aid in the adsorption of either the ion-pairing reagent, the surface active phospholipid species, or the associated ion-pair complex that allows the for subsequent differential retention of the species by differences in ionic character. Subsequent examination of a polymeric stationary phase exhibited no differential retention of the various head group species, and further supports the potential importance of the C1 groups as either a shield for the analytes to the polar silica or as an adsorption site. As a general technique it has been speculated that a multiphase material could be used to achieve mixed mode separations,[25,31,49] however neither a demonstration of this phenomenon on bonded silica-based stationary phases, nor a discussion of the potential separation mechanisms is clearly presented in the literature. It has been suggested that a mixed mode phase can allow for the retention of species by secondary mechanisms, such as is demonstrated in these experimental results. We present that on silica-based C18 stationary phases the C1 groups may behave as a potential secondary phase material, and enable the separation of phospholipid species by both non-polar tail and polar head group.

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