Separation of urea, hexamethylenetetramine, and their reaction products in sol–gel feed solution by zwitterionic HILIC

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ABSTRACT

Studies were carried out to develop a chromatographic methodology based on hydrophilic interaction liquid chromatography (HILIC) for the separation of various reaction products of hexamethylenetetramine (HMTA) –urea used in the sol–gel process for the preparation of ceramic microspheres. Different chromatographic parameters such as organic modifiers, pH of mobile phase, buffer concentration, column temperature, etc. were studied to arrive at the optimum conditions for separation. Compounds such as urea, monomethylolurea (MMU), dimethylolurea (DMU), and HMTA were separated from a synthetic mixture using a mobile phase consisting of acetonitrile and acetate buffer of pH 6. The methodology developed based on HILIC stationary phase is simple and amicable for integration with electrospray ionization mass spectrometry (ESI-MS) to identify the unknown reaction products. The methodology was applied for the separation of reaction products in pre-boiled and untreated urea–HMTA mixtures used as feed in the sol–gel process.

KEYWORDS

ESI-MS; HILIC; HMTA; reaction products; sol–gel; urea

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Sol–gel process-based methods are extensively used for the preparation of ceramics, glasses, catalysts, thin films, etc. at considerably lower temperatures.[1,2] Sol–gel process, being a wet chemical route, is dust-free and is ideally suitable for the remote and automated manufacturing of highly radiotoxic plutonium and $^{233}$U bearing mixed-oxide nuclear fuels.[3,4] In this process, acid-deficient metal nitrate solutions are mixed with gelation agents, viz. hexamethylenetetramine (HMTA) and urea, in the required proportions under cold conditions. The resultant solution is dispersed in the form of droplets into a hot organic medium at $\sim 70^\circ$C causing homogenous gelation and solidification of these droplets as microspheres. These microspheres are washed, dried, calcined, and sintered to produce ceramic microspheres of the required density. Characteristics of the microspheres produced are found to be dependent on many parameters such as (i) HMTA/metal mole ratio, (ii) nitrate/metal mole ratio, (iii) the concentration of metal in the broth, (iv) the gel-forming temperature, (v) aging of the formed microspheres,[5,6] and (vi) use of pre-boiled HMTA–urea solution.[7,8]

HMTA undergoes hydrolytic decomposition to form ammonia and formaldehyde. In neutral or moderately alkaline conditions, urea reacts with HCHO to form mono-, di-, and trimethylolurea.[9] Monomethylolurea can also react with urea to form methylendiamine and its homologs.[10] The pre-boiled HMTA–urea solution used as a feed in the sol–gel process is therefore expected to be a complex mixture consisting of HMTA, urea, NH$_4$OH, HCHO, monomethylolurea (MMU), dimethylolurea (DMU), trimethylolurea (TMU), methylendiamine homologues, etc. in varying proportions. Collins et al. reported that HMTA–urea solutions boiled under controlled conditions provide the required range of sphere densities in a controlled manner and also affect the gelation time.[11] It is postulated that the presence of urea-formaldehyde reaction products in pre-boiled HMTA–urea solution may influence the gelation chemistry, although no systematic study in this regard is reported in the literature.

Techniques such as nuclear magnetic resonance, infrared (NMR-IR) spectroscopy, thermal analysis techniques (thromogravimetry and differential thermal analysis (TG, DTA)), and chromatography have been reported to be used for the characterization of urea–formaldehyde system.[11–13] Tsuge and Senba have reported the separation of urea, MMU, DMU, and methylendiamine in urea–formaldehyde resins by using a column packed with polystyrene with the hydroxyl end group.[14] LC methods are reported for the analysis of urea in urea–formaldehyde fertilizers and in aqueous urea solutions using amine column.[15] In addition to normal phase chromatography and ion pair chromatography, hydrophilic interaction liquid chromatography (HILIC) has become an established technique for the separation of polar compounds.[16,17] HILIC chromatography employing a porous monolithic stationary phase containing zwitterionic sulfobetaine groups led to a new mode of separating biomacromolecules using only aqueous buffers as the mobile phase.[18] Use of zwitterionic monoliths in electrophromatography demonstrated that the optimization of HILIC behavior is possible by changing the nature of the grafted selector.[19] HILIC offers several advantages over the normal phase such as improved solubility of the polar solutes, enhanced mass transfer as a result of low viscosity, and relatively more environmentally friendly solvents as the mobile phase. Another advantage of HILIC solvents is the improved sensitivity when coupled to electrospay ionization mass spectrometry (ESI-MS) due to its ease of desolvation.[20] Mass-spectrometry-based methods can provide the selectivity and sensitivity needed for the detection and identification of unknown compounds or co-eluting species.

In the present work, a systematic study was undertaken to develop a HILIC method for the separation of compounds present in the pre-boiled HMTA–urea solution used in the sol–gel process. Identification of various constituents in the feed solution would help in understanding the influence of process conditions, which is important for the development of process flow-sheets. Initial experiments were carried out using a UV-Vis detector for developing the separation method. Two stationary phases, namely a silica-based zwitterionic and an unmodified monolith silica, were employed in the study. Commonly used protic and aprotic solvents were compared for their use as organic modifier in the HILIC mode of separation of the analytes. The influence of chromatographic parameters such as buffer pH and concentration of the electrolyte in the mobile phase were also studied for arriving at the suitable isocratic conditions for the separation. Efforts were also made to understand the mechanism of separation of the compounds under investigation on the zwitterionic stationary phase. The optimized LC method was then applied for analyzing sol–gel feed solutions that showed the presence of unknown compounds also. The developed method was then coupled to ESI-MS with the objective of identifying the unknown compounds present in the pre-boiled feed solution.

**Materials and methods**

**Materials**

Formaldehyde, urea, and HMTA of AR grade were used in the present work. Water purified by Milli-Q system (Millipore, Bengaluru, India), acetonitrile, methanol, isopropanol, and acetone (Chromasolv grade, Sigma-Aldrich) were used for the dilutions and also as the mobile phase. Acetic acid (HPLC Grade, J.T. Baker) and NH$_4$OH (Suprapure Grade, Merck) were used for the preparation of buffer.

Eutech-make pH meter was used for measuring the pH of the buffer solutions. A Hitachi HPLC system consisting of an L-2130 (Elite, LaChrom) low-pressure quaternary gradient pump, an L-2450 (Elite, LaChrom) diode array detector (DAD), and an L-7360 (LaChrom) column oven were used in the work. A silica-based ZIC-HILIC column (Merck) of dimensions 50 mm $\times$ 4.6 mm, 5 $\mu$m particle size, and an unmodified monolith silica column (Merck) of similar dimensions were used as the stationary phases. Samples were injected into the column using a Rheodyne injector (Model 9725i) with a 20 $\mu$L sample loop. The eluted species were monitored in the wavelength region 200–300 nm using the DAD.

An electrospray ionization mass spectrometer (MicroTOFq-II, Bruker Daltonik GmbH) equipped with a hybrid quadrupole time-of-flight analyzer was employed. Hystar 2.1 software was used to control the LC and the MS. Compass Data Analysis
software (Bruker Daltonics GmbH) was used to process the data obtained in the MS as well as in the MS/MS modes. Compass Isotope Pattern software (Bruker Daltonics GmbH) was used to obtain theoretically predicted spectra. Nitrogen gas was used as both the auxiliary gas and the sheath gas.

**Methods**

MMU and DMU were synthesized as reported in the literature. Pre-boiled HMTA - urea solution was prepared by mixing HMTA and urea solution (3 M in each) and then refluxing for 1 hr. The solution was then allowed to cool to room temperature by keeping the vessel in running water.

The three inlets of the quaternary gradient system were used for feeding acetonitrile, water, and buffer. While studying the influence of composition of the mobile phase, the concentration of the buffer was maintained at 5 mM. Similarly while studying the influence of composition of the mobile phase, the concentration of the buffer was fixed at 90:10 (v/v). During the pH study, the aqueous portion of the mobile phase was kept fixed at pH 5. Both the aprotic solvents tested, viz. acetone and acet- onitrile, have similar properties such as density, viscosity, and dielectric constant values. However, the dielectric constant values for the mobile phase consisting of acetonitrile and acetate buffer of 90% organic and 10% aqueous proportion in the mobile phase was kept fixed at 90:10 (v/v). The pH study, the aqueous portion of the acetate buffer was adjusted to the desired pH level by adding NH₄OH solution.

Mobile phase consisting of acetonitrile and acetate buffer of 5 mM concentration and at pH 5 was passed through the column at a flow rate of 0.5 mL/min. Initial dilutions of the compounds were carried out with acetonitrile, whereas the final dilutions for injections into the LC system were made using the mobile phase. In the case of the samples, the initial dilutions were performed with a 1:1 (water:acetonitrile) mixture, whereas subsequent dilutions were made using the mobile phase. Solution of one standard at a time was injected in the isocratic mode to determine its retention time.

For MS coupling, the outlet of the column was connected to the nebulizer of the ESI-MS system. ESI source in positive ion mode was employed for all the MS analyses. Using a syringe pump for infusion, the ESI-MS was tuned by passing about 0.1 ppm each of the individual compounds at a flow rate of 4 µL/min. Capillary potentials of −4500 and an end-plate offset voltage of −500 V were employed. ESI-MS interface settings used while it is connected to the LC were nebulizer gas flow: 1.5 bar; dry gas flow: 12 L/min; and dry gas temperature: 200°C.

**Results and discussion**

Preliminary studies for optimizing the separation conditions were carried out employing a UV-VIS detector and by injecting individual compounds.

**Effect of organic modifier**

Figure 1 shows the effect of different modifiers, viz. acetonitrile, acetone, methanol, and isopropyl alcohol, on the retentions of urea, MMU, DMU, and HMTA on the zwitterionic stationary phase. The mobile phase composition was 85% (v/v) organic solvent and 15% (v/v) water containing 5 mM of acetate buffer at pH 5. Both the aprotic solvents tested, viz. acetone and acetonitrile, have similar properties such as density, viscosity, and solubility in water. Compared to acetonitrile, acetone has the advantage of better volatility, which is in favor of its compatibility with MS interfacing. Many workers have reported the use of alcohols in place of acetonitrile for the HILIC separation of small molecules. Hence, methanol and isopropyl alcohol were also included in the comparative study of organic modifiers. The operating back-pressures observed for different organic modifiers containing mobile phase combinations were 9, 8, 12, and 33 bar for acetonitrile, acetone, methanol, and isopropyl alcohol, respectively, which are in agreement with the viscosities of the solvents. The dielectric constants of the solvents might also be playing an important role in the retention of solutes and it is worth noting that this parameter changes substantially with the change in the nature of the organic modifier. The dielectric constants of the pure solvent increase in the sequence: isopropyl alcohol < acetone < methanol < acetonitrile < water. However, the dielectric constant values (ε) for the mobile phase consisting of 90% modifier – 10% water are reported to be 43.6 and 41.2 for the organic modifiers methanol and acetonitrile, respectively, at 288 K. The retention of all the analytes on zwitterionic column was found to increase with the nature of the organic modifier in the sequence: methanol < isopropyl alcohol < acetone < acetonitrile.

Retention times of all the compounds, especially of HMTA, shortened significantly when alcohols were used in the mobile phase. This might be due to the existence of long-range hydrogen bonding between surface silanol groups and alcohol hydroxyl groups at room-temperature conditions, which has been reported. Under HILIC separation conditions, a thin layer of water is sorbed onto the polar stationary phase and the partitioning of the analytes occurs between the mobile phase and the layer of water. Relative to methanol, isopropyl alcohol is more hydrophobic in nature and hence is expected to favor the partitioning of the solutes into the aqueous layer on the stationary phase. Hence, between the two alcohols tested, isopropyl alcohol gave longer retentions for all the analytes. However, the peaks corresponding to urea, MMU, and DMU could not be resolved by either of the alcohols used. Compared to aprotic organic modifiers, a change in the retention profile of urea was observed when alcohol-based solvents were used. The change in the elution pattern of urea with the change of protic solvents could be due to the interactions offered by the solvents by way of H-bonding.

Although acetone offered relatively better retention for HMTA, the early eluting compounds such as urea, MMU, and DMU were found to be co-eluting when acetone was used as the organic modifier. The poor separation obtained by acetone is in contrast with the pattern reported for the separation of sugar derivatives by Hutchinson et al. Acetonitrile alone was found to provide satisfactory separation for all the compounds and hence was selected as the organic modifier for further studies. The following chromatographic parameters were studied with the objective of optimizing the separation conditions.

**Effect of acetonitrile content in the mobile phase**

The performance of ZIC-HILIC column was compared with a monolith unmodified silica column under identical experimental conditions. ZIC-HILIC column is a silica-based stationary phase containing both the quaternary ammonium
and the sulfonic acid groups. Figure 2a and b shows the effect of acetonitrile content in the mobile phase on the retentions of the four analytes onto the stationary phases ZIC-HILIC and unmodified silica, respectively. During this experiment, the acetonitrile composition was changed from 65 to 95% (v/v) while maintaining the buffer concentration at 5 mM. The order of retention on the ZIC-HILIC stationary phase was found to be HCHO < urea < MMU < DMU < HMTA. The number of -OH functional groups present in a given analyte seems to influence their retention on the ZIC-HILIC stationary phase. In the case of unmodified silica stationary phase, the order of retention was found to be HCHO < DMU < MMU < urea < HMTA. The different pattern observed in the elution of urea and MMU could not be explained based on H-bonding or electrostatic interactions. HMTA was found to have the highest retention on unmodified silica as well as on ZIC-HILIC stationary phases. The long retention and tailing observed for HMTA on both the columns must be due to the presence of four tertiary amine-like groups in this molecule. The silanol groups in the stationary phase are known to interact strongly with the basic amine groups.

All the analytes used in the present study showed increased retentions at a higher percentage of acetonitrile for both the stationary phases. This is in accordance with the elution strength expected for water under normal-phase chromatographic conditions. Both the columns showed maximum retention at 90% of acetonitrile and further increase in the organic content (95%) in the mobile phase led to a decrease in the retention times. This deviation from the typical HILIC behavior might be due to the corresponding depletion in the dynamic aqueous layer formed close to the stationary phase.

Compounds such as urea, MMU, and DMU were observed to elute as sharp peaks, especially at a higher percentage of acetonitrile. This is attributed to the overall decrease in the mobile phase viscosity, which translates to efficient mass transfer of these compounds between the stationary phase and the mobile phase. HMTA was observed to elute as a broad peak even when 90% of the acetonitrile was used in the mobile phase. It is also seen that HMTA exhibited longer retentions on the unmodified silica relative to ZIC-HILIC at a given organic modifier content. This indicates that the retention of HMTA is mostly due to the interaction with the silanol groups.

Table 1 shows the comparison of the resolutions as well as the number of theoretical plates achieved by the two types of columns as a function of acetonitrile content in the mobile phase. Resolutions among the pairs, urea–MMU and MMU–DMU,
electrostatic interaction in addition to the H-bonding interaction typical of the silica backbone structure. Again, the sulfobetain group helps absorb water by hydrogen bonding and the bulk layer of water, which forms a part of the stationary phase, might be contributing to the retention and separation mechanisms. In view of the better resolutions among all the analytes, the ZIC-HILIC stationary phase was selected for further studies.

It is seen from Table 1 that resolution among the analytes deteriorates when acetonitrile content is increased from 90% to 95%. A small percentage of aqueous content in the mobile phase is essential for the formation of a water-enriched layer on the polar stationary phase and this layer helps in the partitioning of the solutes. Thus neat acetonitrile containing bare minimum buffer would not be providing the best separation although it is preferred for interfacing the LC with MS.

**Effect of concentration of acetate buffer in the mobile phase**

The effect of concentration of acetate buffer in the mobile phase on the retentions of urea, formaldehyde, MMU, DMU, and HMTA was studied (Figure S1 in Supplemental material). During this experiment the mobile phase composition consisted of 5% (v/v) of acetate buffer of a given concentration, 90% (v/v) of acetonitrile, and 5% (v/v) of water. pH of the buffer was kept at 5.0. The concentration of the buffer stream was adjusted such that the overall concentration of ammonium acetate in the mobile phase is changed from 0 to 24 mM. The retentions of all the compounds decreased with the increase in theionicstrengthfrom0to5mM. Further increase in the concentration of the electrolyte showed no significant change in the retentions.

As discussed earlier, the ZIC-HILIC stationary phase provides electrostatic interaction with the polar analytes through the quaternary ammonium and sulfonate groups of the sulfobetain moiety. At higher salt concentrations, the electrolyte ions surround the charged groups and this can lead to a reduction in the electrostatic interaction and a corresponding decrease in retentions. The extent of decrease of retention is quite noticeable for HMTA, due to the number of functionalities present in it. However, even at an electrolyte concentration of 24 mM, HMTA exhibited appreciable retention (~17 min) and this is indicative of the dominance of hydrophilic interaction in the separation. Based on the observations, an acetate buffer concentration of 0.25 mM (i.e., 5% of 5 mM acetate buffer) was selected for further studies.

| Table 1 | Comparison of resolution and number of theoretical plates of ZIC-HILIC column and unmodified silica column as a function of acetonitrile content in the mobile phase. |
|---------|-------------------------------------------------------------------------------------------------------------|
| **ACN content** | **ZIC-HILIC column** | **Unmodified silica column** |
| | **Res. (Rt/W1/2)** | **No. of theoretical plates** | **Res. (Rt/W1/2)** | **No. of theoretical plates** |
| 95% | 1.72 ± 0.08 | 3102 ± 153 | 8212 ± 201 | 9861 ± 632 | 8619 ± 750 |
| 90% | 2.1 ± 0.4 | 3010 ± 347 | 10,362 ± 496 | 10,865 ± 568 | 7947 ± 835 |
| 85% | 1.52 ± 0.08 | 3261 ± 235 | 6435 ± 832 | 5749 ± 952 | 7386 ± 680 |
| 75% | 0.22 ± 0.02 | 2769 ± 483 | 6042 ± 468 | 5988 ± 649 | 8775 ± 817 |
| 60% | 0.07 ± 0.02 | 2578 ± 122 | 7832 ± 217 | 4163 ± 560 | 7749 ± 296 |

Resolution Rs = 1.18[Rt(W1/2)]/[W1/2(W1/2)], number of theoretical plates, N = 5.54[Rt(W1/2)]², where Rt is the retention time and W1/2 = peak width measured at half the peak height.
**Effect of pH of acetate buffer**

The effect of pH of the buffer on the retention was studied in the pH range of 3–7 and the data are presented in Figure 3. This study was performed with a mobile phase consisting of acetonitrile/water/buffer in the ratio 90/5/5 (v/v%), respectively. It is seen that with the increase in pH, the retention of HMTA showed significant change, whereas those of other compounds showed only a slight increase. The zwitterionic stationary phase is reported to contain equal amounts of oppositely charged groups independent of the pH of the environment and hence capable of offering ionic and weak electrostatic interactions.[30] It may also be noted that ZIC-HILIC is a silica-based stationary phase and silica is expected to carry negative charge at pH > 5. Different silanol groups on silica have different activity with pKₐ ranging from 5 to 7.[31] Thus ZIC-HILIC, despite containing both sulfonic and quaternary amine functionalities, would be dominated by negative charge on its surface at higher pH conditions. This explains the slight increasing retention of urea, MMU, and DMU with the rise in pH of the buffer used in the mobile phase. No noticeable change in the retention was observed for formaldehyde as its pKₐ is 13.3 and thus it is not expected to get charged in the pH range studied. Increased silanol activity at high pH is responsible for the increased retention shown by HMTA.

**Effect of temperature**

The influence of temperature of the mobile phase on the retention of the analytes was studied using a ZIC HILIC column. A mobile phase consisting of 90% (v/v) acetonitrile, 5% (v/v) of 5 mM acetate buffer, and 5% (v/v) of water was used. The temperature of the column was changed in the range 20–55°C. van’t Hoff plots, i.e., change in retention factor as a function of inverse of column temperature, were obtained for the analytes (Figure S2). Retention behavior of HCHO was omitted from the plot as it eluted at close to column void volume (dead-time) and no noticeable change in its retention time was observed. The retention factors of all the other compounds showed a decreasing trend, indicating that the process of transferring the solutes from the mobile phase to the stationary phase is exothermic in nature. The retention of the solutes is dominated by enthalpic contribution, although it is reported that in the HILIC mode retentions are controlled by entropy contribution.[32,33] Retention factors of urea, MMU, and DMU were found to decrease slightly with the increase in temperature of the column. This trend suggests that these solutes are electrostatically attracted by the stationary phase. HMTA showed a significant decrease in the retention factor due to the presence of a relatively large number of amino functional groups that can interact with the stationary phase through H-bonding. However, no change was observed in the elution order and hence in the selectivity among these compounds over the temperature range studied.

Table 2 shows the data on the resolution and number of theoretical plates obtained for urea, MMU, and DMU as a function of temperature. It is seen that both the resolutions as well as the number of theoretical plates initially increase with temperature and then decrease at temperatures above ~35°C. The mass transfer kinetics are expected to increase with temperature due to the decrease in viscosity and this should result in better column performance. However, another factor influencing the retention could be the change in the dielectric constant of mobile phase as a function of temperature. It is reported that the dielectric constant of 90% ACN–10% water mixture changes from 40.39 to 36.29 when the temperature is increased from 20 to 55°C.[25] Thus a temperature of 25°C was selected for performing the separation of the analytes in view of obviating the use of column oven.

**Analytical performance**

Based on the above experiments, a mobile phase consisting of acetonitrile, 5 mM acetate buffer pH 5, and water in the proportion 90% (v/v), 5% (v/v), and 5% (v/v), respectively, was selected for the separation. Chromatogram obtained by the injection of a synthetic mixture of urea, MMU, DMU, and HMTA at a concentration of 6 ppm each shows adequate separation among the compounds (Figure S3). Although urea, MMU, and DMU elute in the same pattern as in Figure 1, under the present conditions a void volume peak was observed and this might be due to the small change in the composition of the mobile phase. Linear UV-Vis detector response was obtained for all the compounds except for formaldehyde in the concentration range of 1–50 ppm in the synthetic mixture (Figure S4). As formaldehyde elutes very closely with a void peak, its peak response could not be determined reliably. An attempt was made to derivatize formaldehyde by treating with acetylacetone in the presence of ammonium acetate to form diacetylhydrolutidine as per the procedure given elsewhere.[34]

![Figure 3](image-url)  
**Figure 3.** Effect of pH of the acetate buffer on the retentions of formaldehyde, urea, MMU, DMU, and HMTA using ZIC-HILIC column.

| Temperature (°C) | Resolution | No. of theoretical plates |
|-----------------|------------|---------------------------|
|                 | Urea–MMU–DMU | Urea | MMU | DMU |
| 22              | 1.78 ± 0.01  | 1.9 ± 0.2 | 2523 ± 642 | 2384 ± 362 | 2669 ± 208 |
| 25              | 1.95 ± 0.09  | 2.11 ± 0.03 | 2747 ± 392 | 3014 ± 202 | 2651 ± 48 |
| 35              | 2.13 ± 0.02  | 2.3 ± 0.1 | 3723 ± 65 | 3720 ± 390 | 3292 ± 309 |
| 45              | 1.8 ± 0.1    | 2.0 ± 0.1 | 3007 ± 162 | 3017 ± 214 | 3512 ± 130 |
| 55              | 1.52 ± 0.01  | 1.73 ± 0.02 | 2091 ± 215 | 2444 ± 357 | 2819 ± 480 |

Mobile phase: 90% acetonitrile and acetate buffer of 5 mM and pH 5.
The resultant solution was allowed to stand for 5 min and then diluted with mobile phase for injection into LC. However, the peak corresponding to diacetyldihydrolutidine was also found to elute close to the column void volume peak, thereby making the quantitation based on the chromatographic peak area not viable. However, it might be possible to adopt this derivatization methodology using an RP-LC approach.

Limits of detection were obtained for the analytes by successive dilution of the synthetic mixture until the S/N ∼ 3. They were found to be 0.3 ppm, 0.5 ppm, 0.3 ppm, and 0.4 ppm for urea, MMU, DMU, and HMTA, respectively. The precision of the method was determined by analyzing the synthetic mixture. The relative standard deviations were about 2–4% for the retention times when the samples were injected six times. The relative standard deviations on the peak area response were found to be in the range 2–6% when a synthetic mixture of 6 ppm each was injected.

The optimized method was then applied for the analyses of two urea–HMTA mixtures used as the sol–gel feed. Two sample mixtures were analyzed by the method. The first sample was obtained by the direct mixing of urea and HMTA and the chromatogram obtained is shown in Figure 4a. Apart from urea and HMTA, the chromatogram showed the presence of an unknown substance at retention time ∼5 min. The second sample was prepared by boiling a urea–HMTA mixture and the chromatogram obtained is shown in Figure 4b. The retention time of HMTA was found to be slightly shorter in Figure 4b compared to that in Figure 4a. This might be due to the column overloading due to the presence of more number of components present in the pre-boiled mixture. The concentrations of urea and HMTA in the boiled mixture were found to be 2.8 ppm and 7.5 ppm, respectively, in the injected samples.

The chromatogram obtained for the pre-boiled urea–HMTA showed the presence of at least five compounds with retention times different from the standards used for the development of the method. Interestingly, compounds such as MMU and DMU were absent in the reaction mixtures. To identify the unknown compounds, the LC system was interfaced with ESI-MS. For interfacing, the outlet of the HILIC column was connected directly to the nebulizer of the ESI-MS system, bypassing the UV-Vis detector. This resulted in the reduction of extra-column volume and caused a shift in the retention time of peaks observed by the MS detector by ∼0.4 min relative to the UV-Vis detector. The absence of MMU and DMU in the reaction mixture was further confirmed by verifying that the m/z peaks corresponding to 113.19 and 143.21 were not present in the ESI-MS spectra of the mixtures (Figure S5). TMU, if present, is expected to elute after MMU and DMU in accordance with the polar functional groups present in it. The mass spectra obtained for some of the eluted fractions as per the chromatograms shown in Figure 4a and b are presented in Figure 5. Some of the compounds identified in the mixture by ESI-MS were the peak at retention time 4.7 min as m/z 155.05 corresponding to C16H12N4O2; peak at 5.9 min as m/z 196.07 corresponding to C16H10N4O3, and peak at 8.7 min as m/z 268.11 corresponding to C16H14N6O4. The ions were observed in the positive ion mode as [M + Na]⁺-type peaks. C16H8N4O2, C16H10N4O3, and C16H14N6O4 are assigned as methylenediurea, and derivatives of cyclic amidic.

As mentioned earlier, in the case of the untreated urea–HMTA mixture, there was only one peak in addition to urea and HMTA. This unknown peak having a similar retention time, i.e., 4.7 min, and the same m/z 155.05 was identified as C16H12N4O2, methylenediurea. Based on the species observed, the following scheme has been proposed for the formation of various oligomers observed in the ESI-MS spectra.

\[
(CH_2)_6N_4 + 4H^+ + 6H_2O \rightarrow 6HCHO + 4NH_4^+ \tag{1}
\]

\[
HCHO + NH_2 \rightarrow CO - NH_2 \rightarrow NH_2 - CO - NH - CH_2OH (MMU) \tag{2}
\]
\[ \text{NH}_2\text{–CO – NH – CH}_2\text{OH + NH}_2\text{–CO – NH}_2 \rightarrow \text{NH}_2 \]
\[ \text{–CO – NH – CH}_2\text{–NH – CO – NH}_3\text{(methylenediurea)} \] (3)
\[ \text{HCHO + NH}_2\text{–CO – NH – CH}_2\text{–NH – CO – NH}_2 \]
\[ + \text{HCHO} \rightarrow \text{HOCH}_2\text{–NH – CO – NH – CH}_2 \]
\[ \text{–NH – CO – NH – CH}_2\text{OH \rightarrow –H}_2\text{O \rightarrow C}_5\text{H}_{10}\text{N}_4\text{O}_3 \] (4)
\[ \text{HOCH}_2\text{–NH – CO – NH – CH}_2\text{–NH – CO – NH} \]
\[ \text{–CH}_2\text{OH + NH}_2\text{–CO – NH}_2 \rightarrow \text{HOCH}_2\text{–NH – CO – NH} \]
\[ \text{–CH}_2\text{–NH – CO – NH – CH}_2\text{–NH – CO – NH} \] (5)
\[ \text{HOCH}_2\text{–NH – CO – NH – CH}_2\text{–NH – CO – NH} \]
\[ \text{–CH}_2\text{–NH – CO – NH}_2 + \text{HCHO} \rightarrow \text{HOCH}_2 \]
\[ \text{–NH – CO – NH – CH}_2\text{–NH – CO – NH – CH}_2 \]
\[ \text{–NH – CO – NH – CH}_2\text{OH \rightarrow –H}_2\text{O \rightarrow C}_7\text{H}_{14}\text{N}_6\text{O}_4 \] (6)

Two more peaks were observed with retention times of 6.0 min and 10.5 min and the molecular ion peaks \( m/z \) corresponding to 212.05 and 284.08, respectively. However, the identity of these peaks could not be established so far. Work is in progress to identify the remaining constituents in the pre-boiled urea–HMTA mixture and to establish the link between the formations of various intermediates with the boiling time. It would be of interest to determine how the presence of these oligomer molecules in the sol–gel feed solution influences the characteristics of the final sol–gel microsphere products. It might be possible that during the sol–gel processing, these oligomers might be functioning in a way similar to the polymer additives such as polyvinyl alcohol added in the total gelation method.[35]

**Conclusions**

A chromatographic methodology was developed for the separation of various reaction products in the urea–HMTA mixture used in the sol–gel route. Influence of various chromatographic parameters such as the nature of different organic modifiers, composition and pH of mobile phase, buffer concentration, column temperature, etc. was studied to arrive at the optimum mobile phase conditions. The methodology developed based on HILIC stationary phase promises a simple and efficient method for the separation of urea–HMTA reaction products. The stationary phase provided adequate retention for the small polar analytes and the acetonitrile-rich mobile phase with volatile buffer was suitable for the integration of the separation method with ESI-MS. The present study shows that during boiling, HMTA and urea react to form a number of oligomers. How these oligomers influence
the characteristics of the finished microspheres is still to be established. However, understanding the nature and composition of various reaction products in the sol–gel stream would definitely be playing an important role for fine tuning the process flow-sheets for obtaining the microspheres with the desired characteristics.

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