EXPLORING THE POTENTIAL ROLE OF MOLECULAR DYNAMICS SIMULATIONS FOR RATIONALIZING RP-HPLC METHOD DEVELOPMENT: TENTATIVE CORRELATION OF SOLVATION AND RETENTION FACTOR

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I would also like to thank Assoc. Prof. Dr. Gülay Ertaş for planting the seed of my interest in analytical chemistry.

ABSTRACT

Molecular simulations are an important research tool used to explore and provide insights to a variety of phenomena in molecular systems. There has been a seldom application of these tools in High Performance liquid chromatography. Whilst the preferred techniques for method development in HPLC have been successful, they solely depend on guides proposed in different texts which in turn have limitations and consume a considerable amount of time as they are based on a trial and error approach. In this work we will utilize the molecular modelling principle tools from molecular dynamics to consider molecular simulations as an alternative approach towards method development in reversed phase-high performance chromatography. The analysis obtained from the molecular simulations is examined to extract information on the mobile phase-analyte interactions, effects of ions on those interactions and the effects of change in mobile phase composition on the interaction. The results of the analysis will shine a light on the significance of molecular simulations in method development and its possible application in mobile phase modelling.

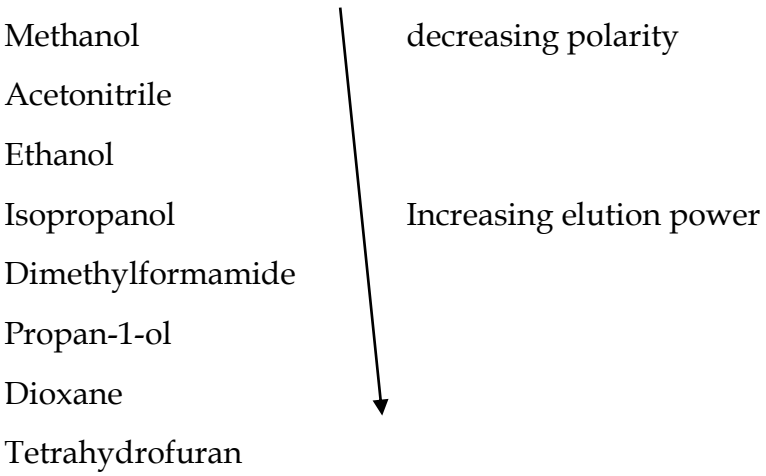
1. INTRODUCTION

1.1. Reversed-Phase High Perfomance Liquid Chromatography(RP-HPLC)

Is a type of HPLC in which the stationary phase is less polar than the mobile phase. Chemically bonded Octadecylsilane (ODS), C_{18} and C_8 are the most commonly used stationary phases. The common mobile phases for RP-HPLC are methanol, acetonitrile and tetrahydrofuran [1].

1.1.1. Mobile Phase

The mobile phase usually consists of either water, an aqueous buffer or various polar solvents used to elute the analyte [2].



However, for very non-polar analytes the mobile phase must be mixed with water because the non-aqueous version is not ideal for separation to be observed [2]. Mobile phases are required to be extremely pure thus the water added must undergo extensive purification before use. The need for use of pure substances in mobile phase is to avoid tailing and broadening of the peaks caused by impurities which then affects the resolution and separation of analytes [3].

1.1.2. Characteristics of Common Mobile Phases

Every mobile phase has its unique properties that make it suitable for a specific separation. The following are common mobile phases and their characteristics in RP-HPLC:

I. Methanol: It is favorable to use methanol if buffer salts or ion-pair reagents are to be added to the eluent. Such additions have a relatively better solubility in methanol than in acetonitrile or tetrahydrofuran. The disadvantages that arise from using methanol as a mobile phase are; i) volume contraction when mixed with water is substantial leading to different retention times for the same analyte and ii) the methanol-water mixture does not give the same results when pre-mixed and manually prepared by a high pressure gradient system [3].

II. Acetonitrile: It causes no viscosity related problems but is very expensive.

III. Tetrahydrofuran: It is useful in separation selectivity but has a high UV cut off of 220nm and once its seal is broken it creates peroxides, which can react with the analyte and are a safety risk [4].

Mobile phases which have a high water content (less than 10% organic solvent) are not recommended as they cause the C_{18} alkyl chains to fold and it is not easy to straighten them when in contact with organic solvents [4].

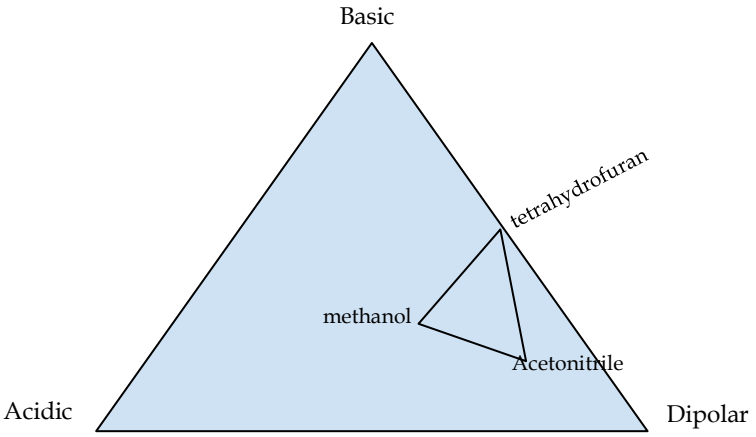


Fig 1. Mobile phase selectivity triangle for RP-HLC.

The figure above describes the selectivity of common mobile phases. The eluent selectivity is directly related to this triangle where by the localization effects are not affecting the solvents.

1.1.3. Retention Mechanism in RP-HPLC and Selected Theories

1.1.3.1. Retention Mechanism

In RP-HPLC the packing of the column is hydrophobic and the most popular packing are silica based bonded phases where the ligands are C_{18} and C_8 [5].

This hydrophobic surface interacts with the hydrophobic part of the analyte. It is found that the larger the hydrophobic part the longer the retention. When plotting the log of the retention factor of a homologous series of analytes a linear relationship is obtained [5].

Polar functional groups reduce in RP-HPLC in a predictable way but their incremental contribution depends on their position on the molecule and on the solvent. Aldehydes reduce retention weakly compared to Alcohols [6]. Carboxylic acids when ionized reduce retention drastically pointing out the role of pH of a mobile phase on controlling retention times in RP-HPLC [6].

Amino functions play a specific role in RP-HPLC because they interact with the silanols on the surface of the column. That is to say as the pH of the mobile phase increases so does the retention of amines or amino functional groups when they are positively charged. If the pH is sufficiently high such that the positive charge is removed from the amino functional group, then the retention and interaction with the stationary phase decreases. These silanophilic interactions play a much broader role in RP-HPLC retention times with other polar functional groups [6].

A linear relationship is also obtained when a graph of the log of K (retention factor) is plotted against the mobile phase composition in methanol-water mixtures. In tetrahydrofuran (THF) and aqueous acetonitrile a curvature is observed. This curvature and deviation from linearity is very small such that the assumption of linearity adopted from methanol-water mixtures is usable for all common mobile phases in actual practice [7].

Factors affecting the retention factor log K

1. The higher the energy of interaction between solute and stationary phase in the gas phase the higher the retention factor.

2. High surface area of contact between the hydrophobic moiety of the solute and stationary phase increases retention.
3. High polarity and surface tension of the mobile phase increases the retention factor
4. Decrease in retention time is due to increase in interaction between solute and mobile phase
5. The higher the dipole moment of the analyte the lower the retention factor

Mathematical Representations of the Retention and Solute-Solvent Interactions

Retention

j+ L ↔ jL solvent has a major role in displacing the equilibrium on either direction [8]. $\ln K = \frac{-\Delta A}{RT} + \ln\psi$ [8]. K= retention factor/capacity factor

j - solute L- solvent A°= free energy for the equilibrium

Solute-Solvent Interactions

$E(r) = E_j(r) + E_L(r)$ r= vector indicating position of each atom in the molecule

$E_L(r) = \sum_{i=1}^{n} ZiAi$ Z_i= Atomic solvation parameter

A_i = solvent accessible area of the atom i

1.1.3.2. Selected Theories

In this study we will focus on only two theories of retention in RP-HPLC. The first theory is the theory of Jaroniec and the second theory is the solvophobic theory.

The Theory of Jaroniec

This theory represents the most comprehensive explanation of how retention occurs. It treats the retention mechanism as a mixture of partitioning and displacement. According to this treatment, there comes two stages of the process of retention in RP-HPLC: i) The formation of combined solvent surface stationary phase and i) the partitioning of solute(analyte) between the mobile phase and its stationary phase. In the first stage the hydrophobic ligand attached to the silica surface incorporates solvent molecules (adsorption) to form the stationary bonded phase [9]. This stationary bonded phase is enriched to different degrees depending on the mobile phase used i.e. methanol is adsorbed less than acetonitrile which is in turn adsorbed less than tetrahydrofuran [9].

In the second stage the solute partitions between the mobile phase and the stationary phase. In this process the solute displaces the solvents from the stationary bonded phase depending on the molecular size 'r' of the solute 's' in solvent 'i' [9].

$$K_{s,i} = (a_{s,s}/a_{s,m})^{ri}(a_{',m}/a_{i,s})^{rs}$$

The Solvophobic Theory

In order to dissolve a non-polar molecule in a polar solvent, a nonpolar-polar interaction must replace a polar-polar interaction between solvent molecules. This is not favored and requires a substantial amount of energy, A^{cav} , in order to create a cavity for the solute[10].

$A^{sol}_{j,L} = A^{cav}_{j,L} + A^{Vdw}_{j,L} + RTln(RT/P_oV_s)$ P_o= atmospheric pressure

V_s= molar volume of solvent/molecular mass divided by density

1.1.4. Method Development in RP-HPLC

This refers to the proposed steps in determining the best approach towards determining the proper composition of the mobile phase and the equally proper column for best separation results to be obtained [11].

Proposal for non-ionic samples [11].

1. Adjust the percentage of organic solvent or gradient range for retention factors between 1 and 10
2. Change the organic solvent
3. Use a mixture of organic solvents
4. Change Temperature

Proposal for ionic samples [12].

1. Adjust the percentage of organic solvent or gradient range for retention factors between 1 and 10
2. Change the pH
3. Adjust the percentage organic solvent
4. Change Temperature

If the separation does not work with the above proposals other methods are used apart from RP-HPLC.

If the analyte is basic and charged as well as the still present and accessible silanol groups, then tailing and unstable separation will be observed due to mixed retention times. The solution is always to use a buffered mobile phase or to work in acidic conditions [12].

When choosing a mobile phase, the first characteristic considered is the ionizability of the analyte or parts of it. If the analyte is ionizable then a buffer solution must be used to ensure reproducible results. A properly used buffer allows easy control of the pH. The pH affects the rate of silonaphilic interactions which are the main causes of tailing in RP-HPLC [12].

1.1.5. The Elution Problem

In mixtures containing more than 20 components, the separation presents numerous problems when done under isocratic conditions such as poor peak resolution, broad peaks, and background noise [13].
This is termed as the general elution problem which is usually tackled by changing:

a) Solvent gradient
b) Column switching
c) Temperature and flow gradients

This general elution problem is not a matter of concern in this study as our mixture will have a total of less than 20 components [13].

1.2. Molecular Modelling

Molecular modelling is the description of a molecular system in mathematical terms devised to understand the properties of known systems and to eventually predict those unknown ones. Molecular modelling uses methods of molecular mechanics, minimization, simulations, conformational analysis and other computer based methods for understanding and predicting the behavior of a molecular system.

Molecular modelling puts emphasis on the representation of the structures of molecules and properties that are dependent upon those three dimensional structures.

1.2.1. Molecular Dynamics

In molecular dynamics (MD) successive configurations of the system are generated by integrating Newton's laws of motion. The result is a trajectory that specifies how the positions and velocities of the particles in a system vary with time, with respect to a predefined potential.

1.2.2. Molecular Dynamics Simulations

MDs are computer simulations done under the governing conditions from equations integrated and derived from molecular dynamics. The information that can be obtained from molecular dynamics is vast and includes important aspects of a molecular system such as the diffusion constant of a solvent or solute, radial distribution function of any molecule in the system, solvation

shell radius and the type of interactions taking place in the system at each frame of the simulation.

The predictions of these simulations help us to determine the properties of our simulated solvent/mobile phase and solute to an error that depends on the degree of approximation of the phenomena under consideration. In this study these properties will be extensively examined through analysis to extract information on the major interactions between solvent and solute.

1.2.3. Molecular Modelling of the Mobile Phase

This study will conduct simulations of an analyte and a mobile phase to observe the predicted molecular interactions. These interactions will be compared to the theories which have experimentally been applicable in practice of RP-HPLC.

This comparison will shine a spotlight on the correct direction during method development for separation of analytes in RP-HPLC and hopefully provide data that can be used to model a mobile phase with a composition that is best for the given solute.

Aim of this Study

The purpose of this study is to determine the importance of using and incorporating molecular modelling techniques in the method development procedures for RP-HPLC separations. The study will focus on analyzing the properties of the mobile phase and the dissolved analyte.

2. MATERIALS AND METHODS

2.1. Preparation of the Structures

The simulated systems for neutral pyridoxamine, methanol and triethylamine were built using parameters derived specifically for the present study and using parameters from the literature[14] for methanol and NaCl. The system of neutral pyridoxamine was solvated in a square box of solvent using both tleap and packmol modules of AmberTools 18 [14] program package, , with different compositions in terms of methanol, triethylamine, and ions.
Below is a figure displaying some of the boxes built and used in the simulation. The box on the left hand side contains pyridoxamine dissolved in pure methanol, the middle box adds triethylamine to the contents of the previous box and finally the box on the right hand side contains all the components including ions.

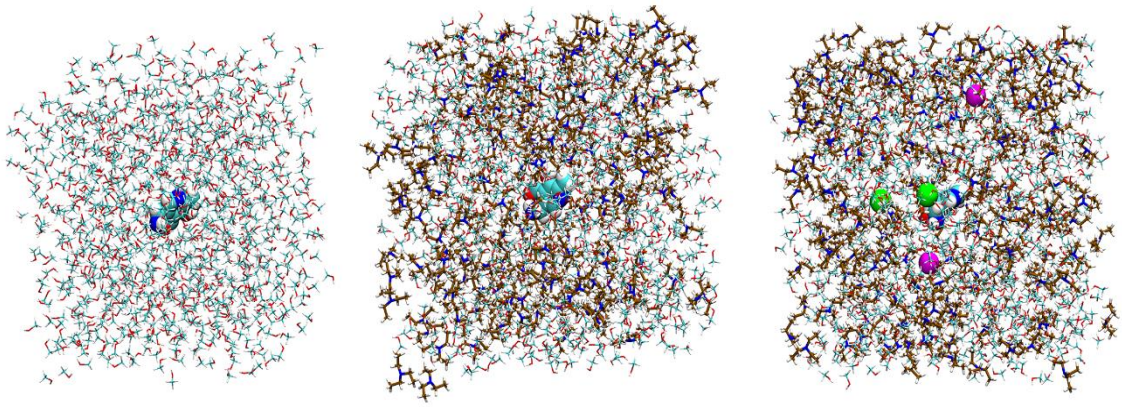


Fig 2. Simulated boxes containing methanol, triethylamine, NaCl ions and Pyridoxamine.

2.2. Molecular Dynamics simulations

All simulations were performed using the ff44SB [15] and gaff [16] force field parameters for the mobile phase and pyridoxamine, respectively. The force field was modified for methanol to include the difference between hydrogen atoms on the oxygen of the methanol and those on the oxygen of the solute. Periodic boundary conditions were applied to all systems and long-range electrostatic conditions were calculated using the particle mesh Ewald method [17]. The sides of the box were made a bit shorter on packmol to avoid clashes due to the periodic boundary conditions.

A non-bonded cut off of 12 angstroms, a time step of 2 femtoseconds and an output frequency of 100 frames were used during all simulations for minimization, heat up to 300K, equilibration except for production which had an output frequency of 5 frames. The minimization, heat up, equilibration and production were performed using the SANDER module of Amber 18. The simulation started with minimization then heat up was performed in four stages allowing equilibration to occur. The first stage was heating from 0 up to 100K in the NVT ensemble (constant volume), the second stage was at 100K in the NPT ensemble (constant pressure), the third stage was from 100K to 300K in the NPT ensemble and lastly 300K in the NVT ensemble for 100ps. Production was performed for another 100ps in the same ensemble.

A total of 30 boxes of different compositions of either methanol, triethylamine, NaCl or pyridoxamine were built and simulated using the above procedures and conditions.

Calculations were then made for Radial distribution functions RDFs and diffusion coefficients for different atoms in the box using cpptraj.

3. RESULTS AND DISCUSSION

As a consequence of choosing to simulate 30 different boxes of our solvent and solute, the data obtained from the calculations was a lot. Half of the data obtained was out of the scope of this study therefore it will not be discussed despite its relevance i.e. the data obtained from boxes which did not contain pyridoxamine. All the remaining data will be covered extensively in discussing the analysis and its projections.

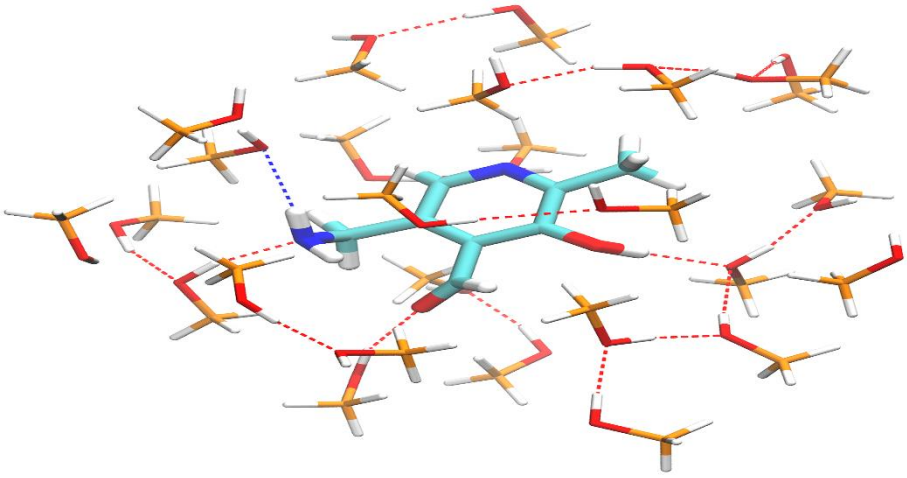


Fig 3. Molecular interactions in a box of pyridoxamine dissolved in methanol

Since the goal of the study was to investigate the properties of the mobile phase as it interacts with the analyte, two types of calculations were performed from the simulated boxes. The first calculation was the radial distribution function of the analyte and the second calculation was the diffusion coefficient of the analyte in the mobile phase. The following were the obtained results:

3.1. RDFs

In order to successfully analyze the RDF data from our boxes, different sites of the analyte molecule were selected as centers due to their electronegativities. This type of selection allowed the analysis to point out the extent of interactions occurring all around the analyte and the effects of the composition of the mobile phase in purr with the presence of ions towards these interactions. Six different types of interactions were selected between atoms of pyridoxamine and those of methanol. The chosen mask for pyridoxamine was 'Pyr' and that for methanol was 'moh', the names and positions of the atoms of interest are displayed on figure below.

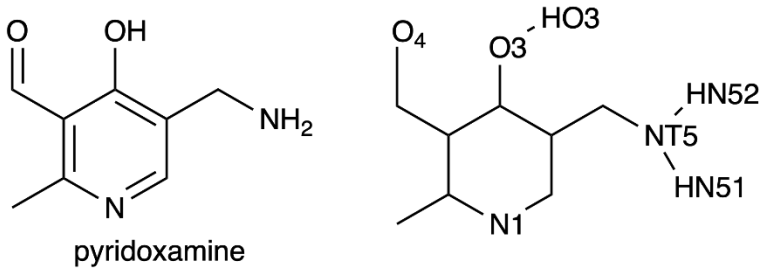


Fig 4. Selected centers for the calculation of RDF of pyridoxamine.

3.2. Interaction between Pyr(NT5) and moh(HO1)

3.2.1. Effects of Composition

The RDF from Fig 5. below shows the effects of change in composition of the mobile phase on the specific NT5-HO1 interaction.

It can be observed that the RDF for this NT5-HO1 interaction has a sharp and strong peak just below the distance of 2 angstroms regardless of the composition. This indicates that this interaction is a very strong interaction and

contributes to the solubility of our analyte in methanol. Hindering this interaction or decreasing it may result to lower solubility of our analyte in methanol. This initial peak represents the first solvation shell and the second peak indicates the second solvation shell for the analyte.

The composition of our mobile phase is represented by the amount of methanol molecules present in the phase by percentage that is from 100% to 80% and color coded for better presentation. The interaction is observed to have maxima 85% composition and minima at 100% composition methanol by looking at the amplitude of the RDF peaks at different compositions. This means that our analyte is well solvated in methanol as we decrease the composition of methanol while increasing the composition triethylamine in the mobile phase. But this trend in increased interaction halts when we hit 85% composition because as we move further and reduce the composition to 80% the amplitude of our first peak drops.

3.2.2. Effects of Ions

The RDFs from Fig 6a. and Fig 6b. below shows the effects of ions on the specific NT5-HO1 interaction.

The addition of just a single salt into the solution alters the intensity of the NT5-HO1 interaction and changes the maxima to 80% from the previous 85% resulting into a linear relationship between composition and strength of the specific interaction which

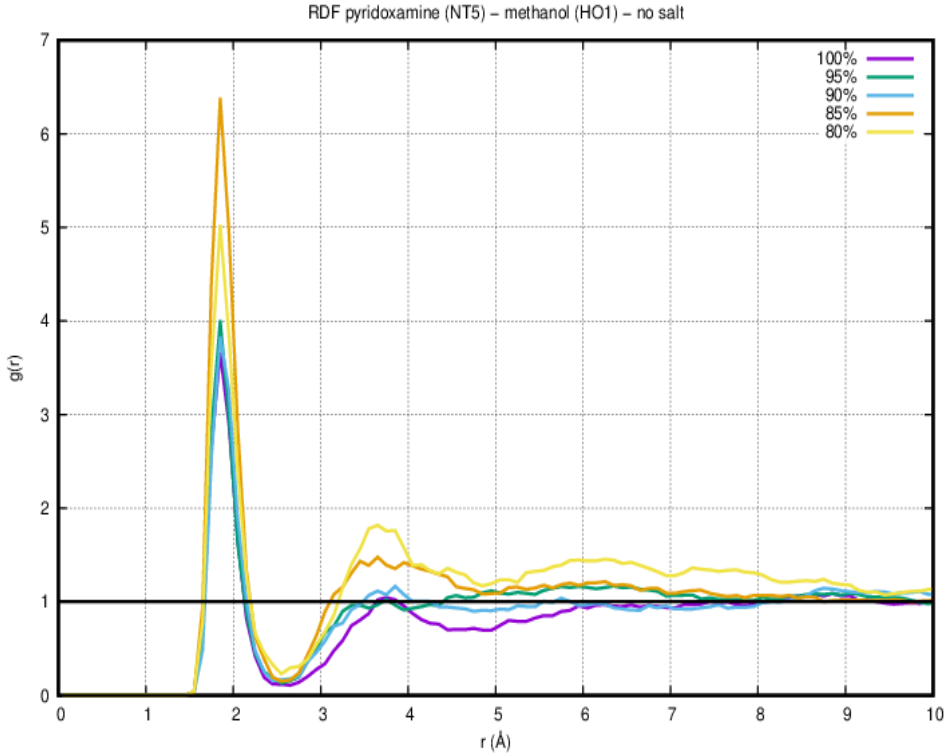


Fig 5. The RDF of pyridoxamine's NT5 atom to Methanol's HO1 atoms.

was not observable before. Consequently, the addition of 2 NaCl ions has the same effect but this time there is a drastic increase in strength of the interaction as the composition is changed from 85% to 80% methanol. This indicates that when adding ions, the concentration of the added species is very important in dictating how the specific interaction will be favored and at what composition of the mobile phase.

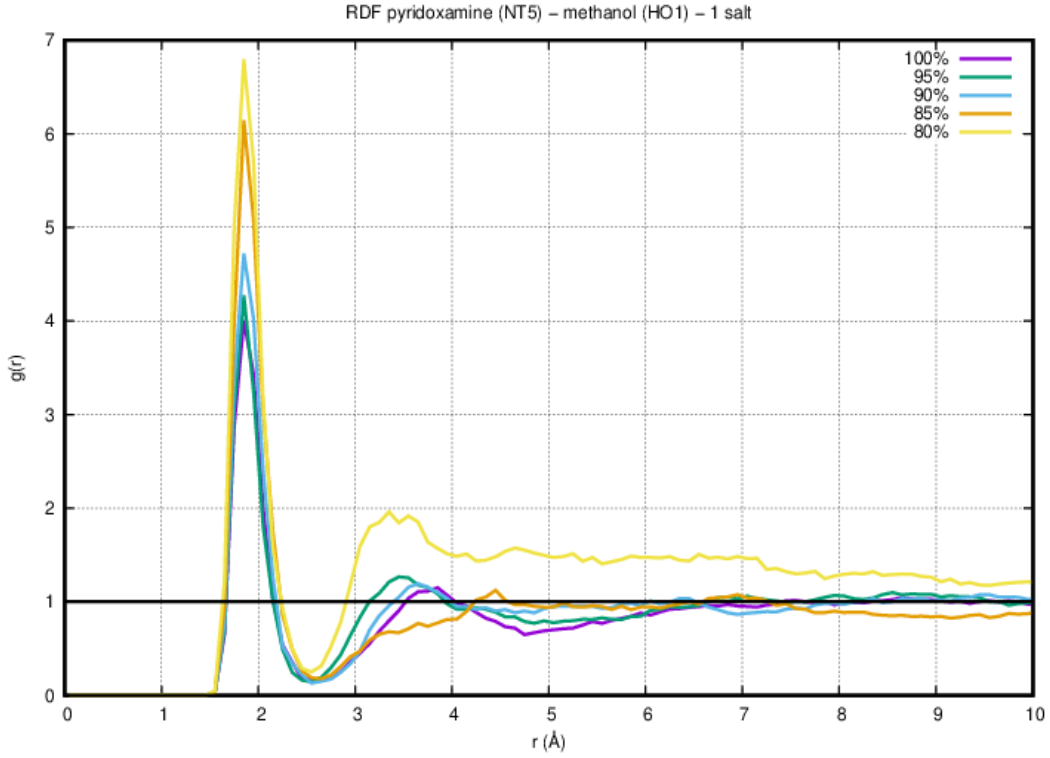


Fig 6a. The RDF of pyridoxamine's NT5 atom to Methanol's HO1 atoms when 1 salt molecule of NaCl is present.

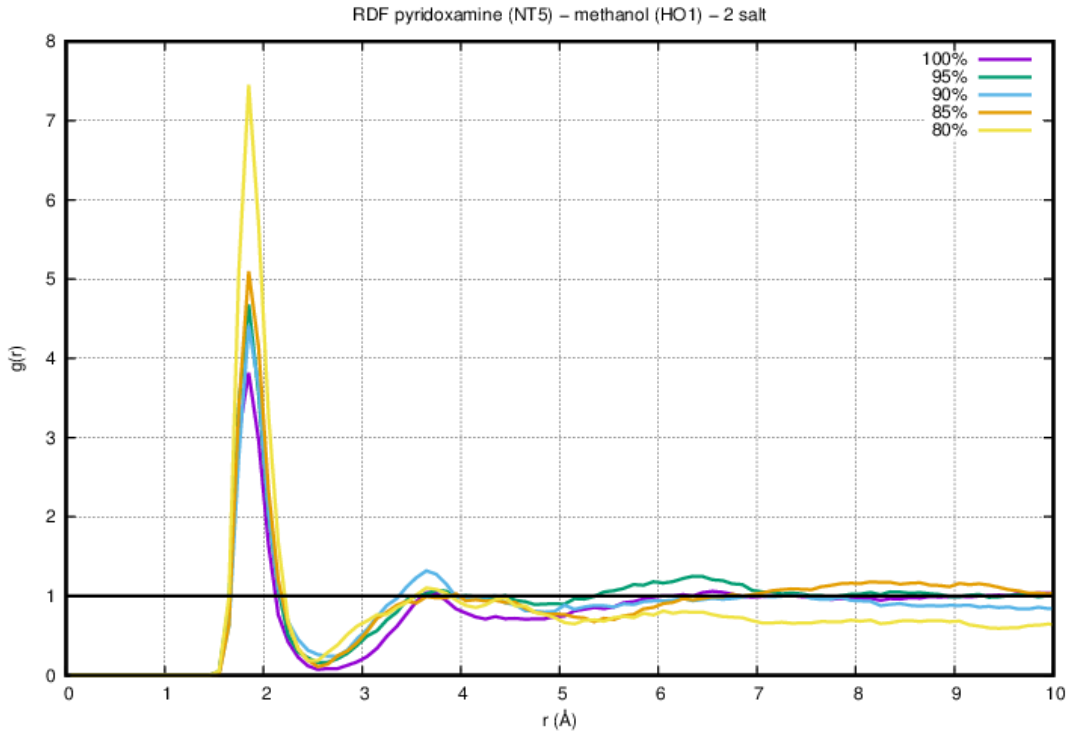


Fig 6b. The RDF of pyridoxamine's NT5 atom to Methanol's HO1 atoms when 2 salt molecules of NaCl are present.

3.3. Interaction between Pyr(N1) and moh(HO1)

3.3.1. Effects of Composition

The RDF from Fig 7. below shows the effects of change in composition of the mobile phase on the specific N1-HO1 interaction.

It can be observed that the RDF for this N1-HO1 interaction has a broad and weak peak at 2 angstroms for all compositions. This indicates that this interaction is a very weak interaction and its contribution to the solubility of our analyte in methanol is not significant. This also suggests that the N1 atom is obstructed by other parts of the pyridoxamine molecule (steric hindrance). Enhancing this interaction may result in higher solubility of our analyte in methanol as it will strengthen one of the weakest interactions between

methanol and our analyte. This initial peak represents the first solvation shell and the second peak indicates the second solvation shell for the analyte. The solvation shells show a different pattern in strength of the interaction as composition is changed.

The composition of our mobile phase is represented by the amount of methanol molecules present in the phase by percentage that is from 100% to 80% and color coded for better presentation. The interaction is observed to a maxima at 90% composition and a minima at 80% in the first peak or first solvation shell while in the second solvation shell it is vice versa.

3.3.2. Effects of Ions

The RDFs from Fig 8a. and Fig 8b. below shows the effects of ions on the specific N1-HO1 interaction.

Addition of 1 molecule of NaCl salt has a positive effect on the first peak of the RDF increasing its sharpness thus the salt facilitates the N1-HO1 interaction and enhances it even though the peak height isn't affected. Also the difference in strength due to composition is reduced keeping the peaks at a close range.

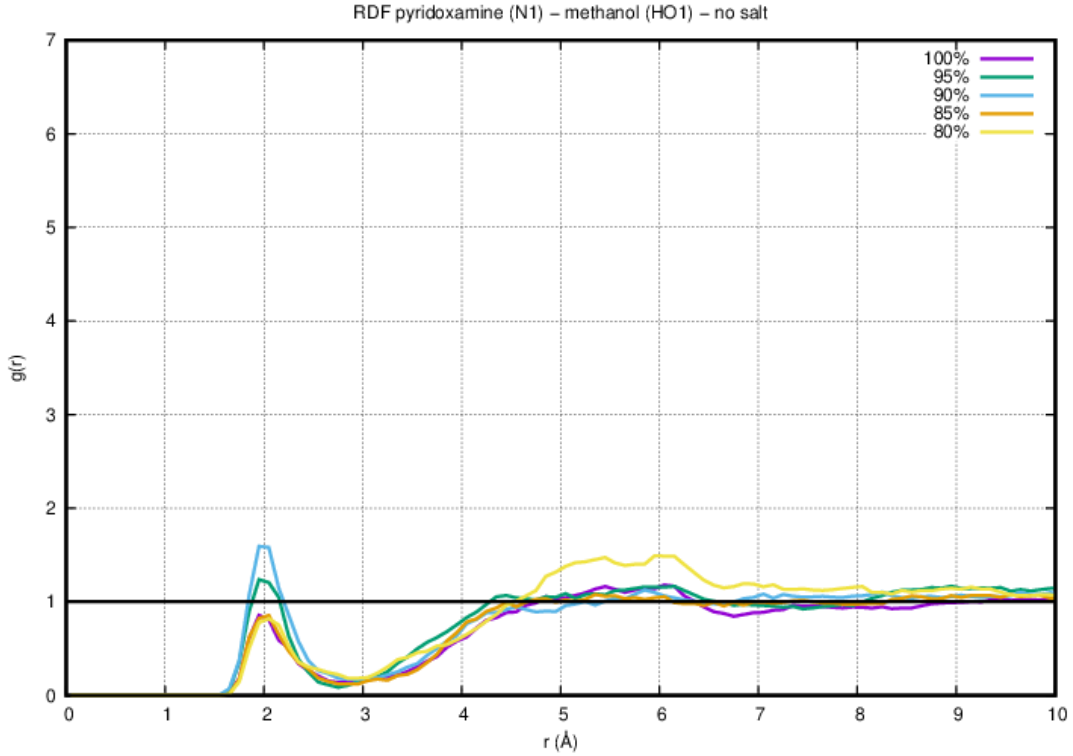


Fig 7. The RDF of pyridoxamine's N1 atom to Methanol's HO1 atoms.

Addition of 2 molecules of NaCl to the solution not only increases the sharpness of the first peak but an increase in peak height is also observed meaning the interaction is a bit stronger with the addition of these ions. Also a change in the maxima and minima is observed where the maxima is at 85% and minima at 80% composition of methanol.

From Fig 3. the second solvation shell doesn't exactly have peaks as the observed RDF shows very broad peaks converging and normalizing to the value 1. This indicates that ions have a sizable effect on both the first and second solvation shell as there is an emergence of a less broad second peak at all compositions of methanol. This effect is observed better at higher

concentrations of the salt in the solution.

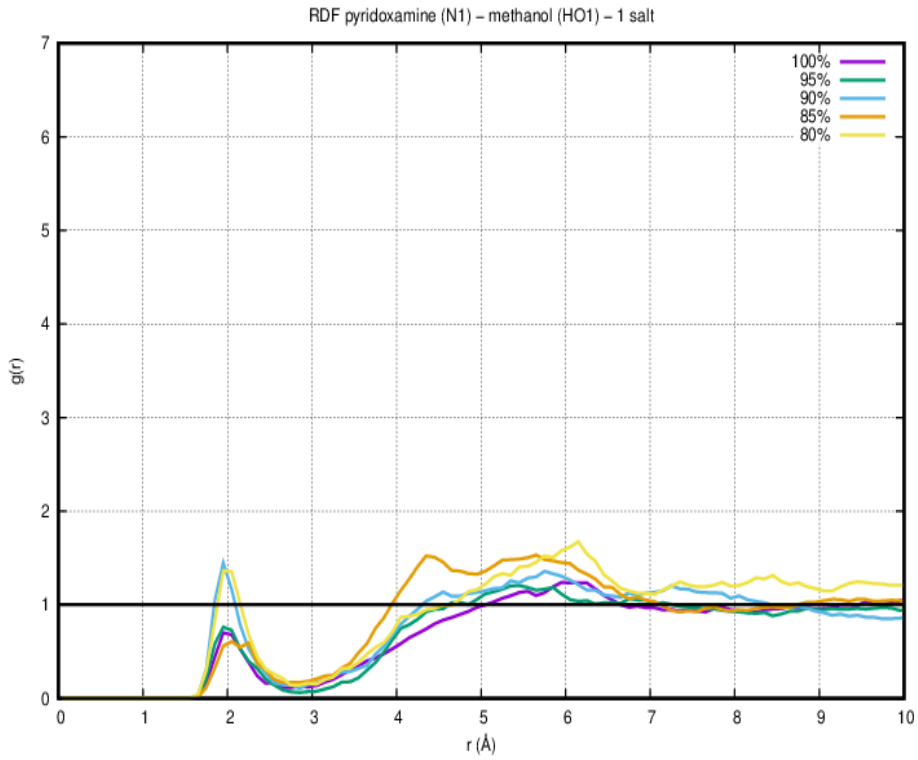


Fig 8a. The RDF of pyridoxamine's N1 atom to Methanol's HO1 atoms when 1 salt molecule of NaCl is present.

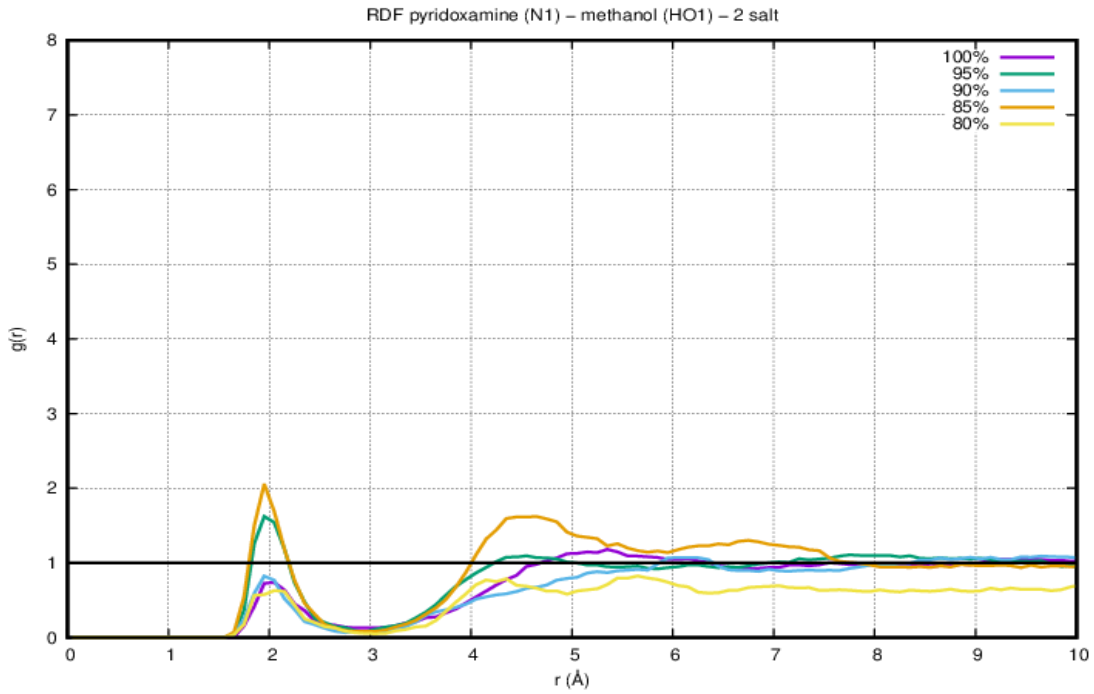


Fig 8b. The RDF of pyridoxamine's N1 atom to Methanol's HO1 atoms when 2 salt molecules of NaCl are present.

3.4. Interaction between Pyr(O3) and moh(HO1)

3.4.1. Effects of Composition

The RDF from Fig 9. below shows the effects of change in composition of the mobile phase on the specific O3-HO1 interaction.

It can be observed that the RDF for this O3-HO1 interaction has a completely weak almost no significant first peak. This shows that the interaction in interest is not happening at shorter distances below 3 angstroms. Although the second and third peaks show a much stronger peak compared to the first they are still very broad and weak peaks indicating a very weak interaction in general. Just below the distance of 2 angstroms regardless of the composition. The oxygen

atom is thus not accessible for plausible interactions to take place and does not affect the solubility of the analyte in the methanol.

The composition of our mobile phase is represented by the amount of methanol molecules present in the phase by percentage that is from 100% to 80% and color coded for better presentation. The interaction is observed to have maxima 80% composition and minima at 100% composition methanol by looking at the amplitude of the RDF peaks at different compositions. But this is observed clearly from the second peak of the RDF.

3.4.2. Effects of Ions

The RDFs from Fig 10a. and Fig 10b. below shows the effects of ions on the specific O3-HO1 interaction.

The addition of just a single salt into the solution alters the intensity of the O3-HO1 interaction to such an extent that the previously insignificant peaks are now sharp and stronger. For the composition of 80% methanol which was the maxima when no salt was present, the increase in peak size, height and sharpness is most observable for all solvation shells. The maxima for the first peak is at 80% and minima at 100% composition of methanol similar to the data obtained when no salt was introduced.

Addition of 2 NaCl molecules also increases the intensity of the first peak but the effect is readily observed for the 85% composition in methanol. The increase is prevalent at all peaks even though the second and third peaks at this composition are not clearly observed. While addition of 1 NaCl enhanced the first peak at 80% addition of one more completely diminished the peak. This goes on to show the importance of concentrations of ions to the specific interaction.

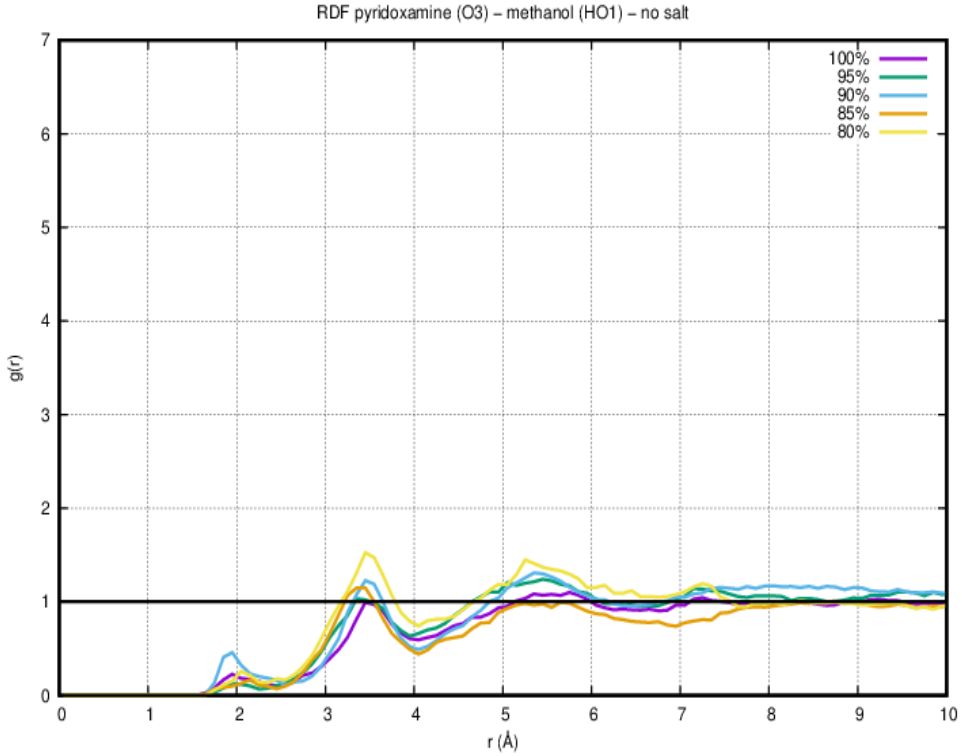


Fig 9. The RDF of pyridoxamine's O3 atom to Methanol's HO1 atoms

3.5. Interaction between Pyr(O4) and moh(HO1)

3.5.1. Effects of Composition

The RDF from Fig 11. shows the effects of change in composition of the the mobile phase on the specific O4-HO1 interaction.

It can be observed that the RDF for this O4-HO1 interaction has a medium peak just below 2 angstroms which is contrary to the almost nonexistent peaks observed on the O3 oxygen of the analyte. This indicates that O4 is more accessible to interaction than O3 and thus is crucial in considering the solubility of the analyte in the methanol. Also the peaks are two distinct solvation shells as expected rather than the multiple solvation peaks observed on the RDF of

O3. The composition of our mobile phase is represented-

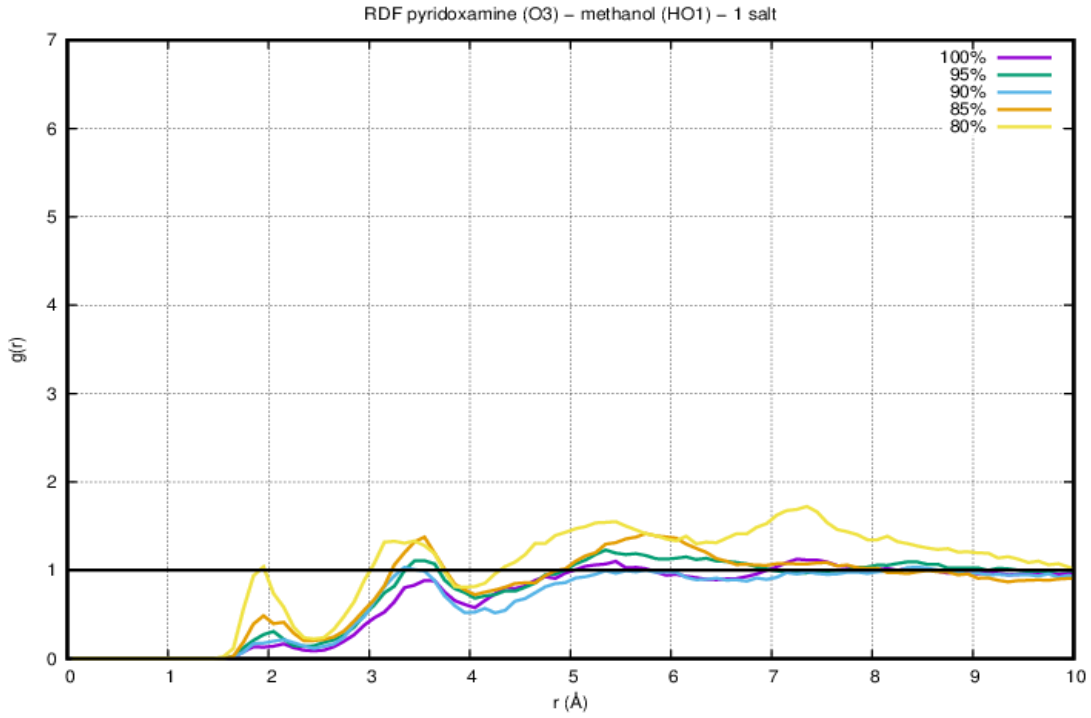


Fig 10a. The RDF of pyridoxamine's O3 atom to Methanol's HO1 atoms when 1 salt molecule of NaCl is present.

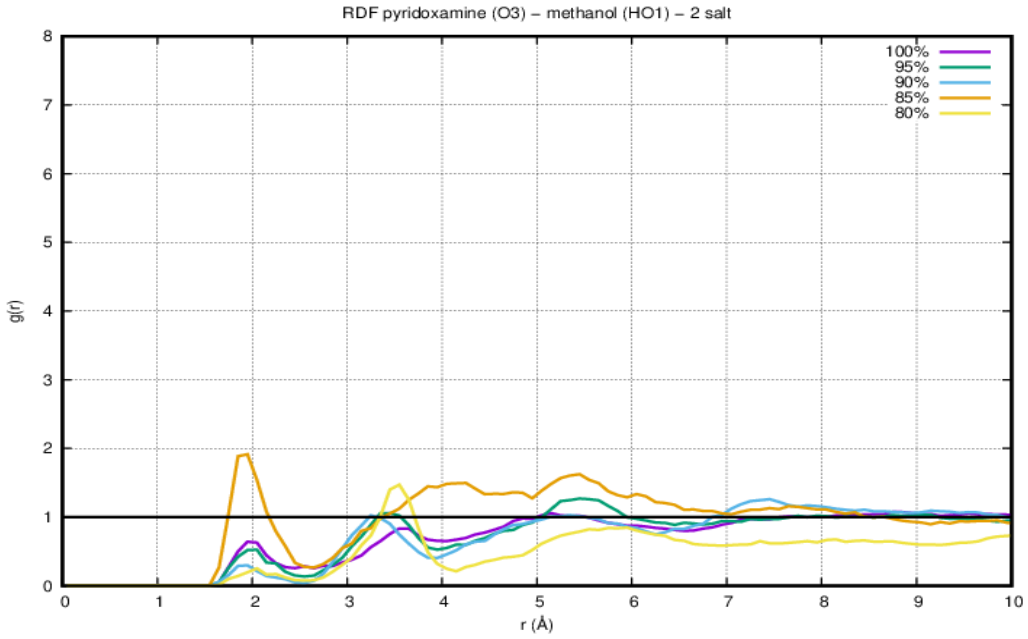


Fig 10b. The RDF of pyridoxamine's O3 atom to Methanol's HO1 atoms when 2 salt molecules of NaCl are present.

by the amount of methanol molecules present in the phase by percentage that is from 100% to 80% and color coded for better presentation. The interaction is observed to have maxima 80% composition and minima at 100% composition methanol by looking at the amplitude of the RDF peaks at different compositions. This goes to show that interaction strength of these two oxygens might be very different but they behave in a similar manner at different compositions of the mobile phase.

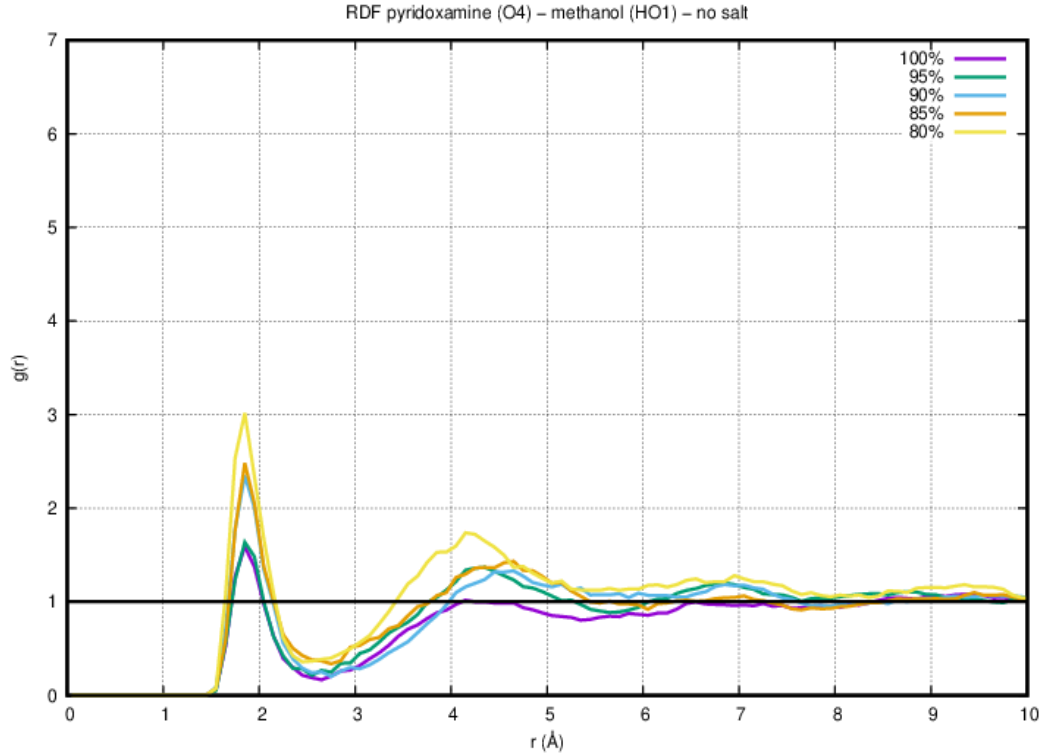


Fig 11. The RDF of pyridoxamine's O4 atom to Methanol's HO1 atoms.

3.5.2. Effects of Ions

The RDFs from Fig 12a. and Fig 12b. below shows the effects of ions on the specific O4-HO1 interaction.

Addition of 1 molecule of salt sharpens all the peaks but there is no increase in their amplitude meaning there is no increase in strength of the interaction; rather unexpectedly there is a decrease in amplitude for peaks at all compositions except for 80% methanol. Thus an addition of 1 NaCl molecule is not favoring this interaction at any composition apart from the 80% methanol where by its second peak has a small increase in amplitude.

Addition of 2 NaCl molecules alters the maxima of the RDFs from a maxima of 80% methanol to that of 90% but the strength of the interactions is decreased

as the peaks have lower amplitudes for both the first and second peaks at all compositions. Thus an addition of ions does not favor

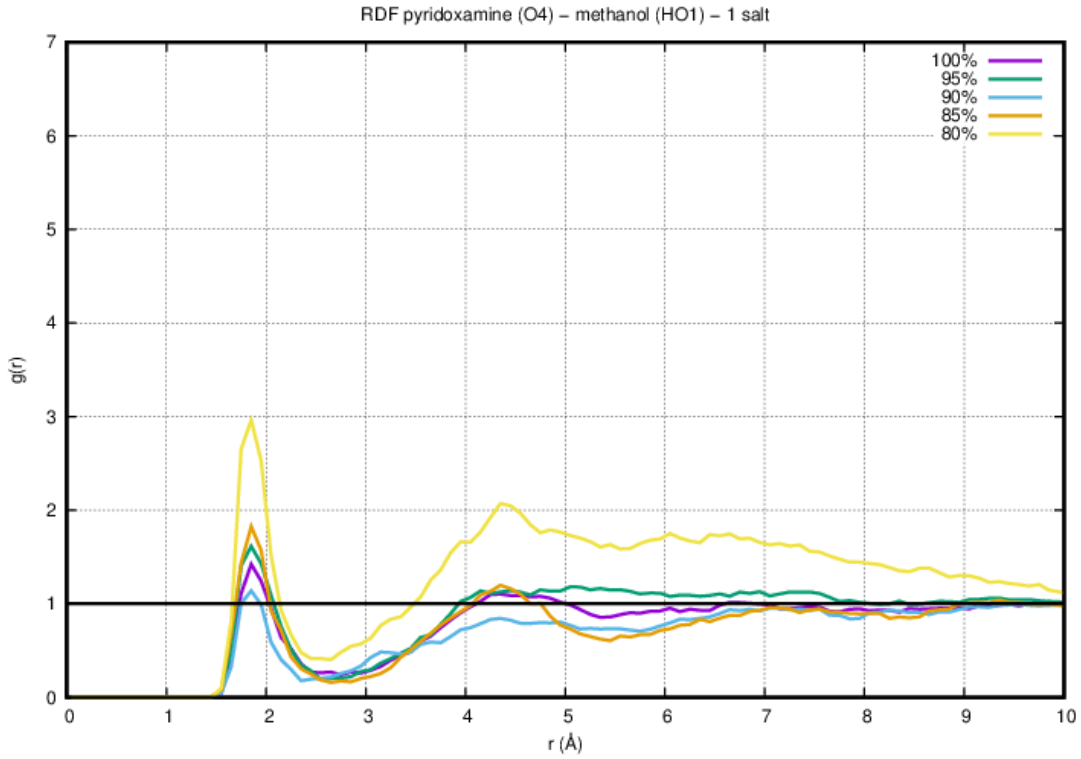


Fig 12a. The RDF of pyridoxamine's O4 atom to Methanol's HO1 atoms when 1 salt molecule of NaCl is present.

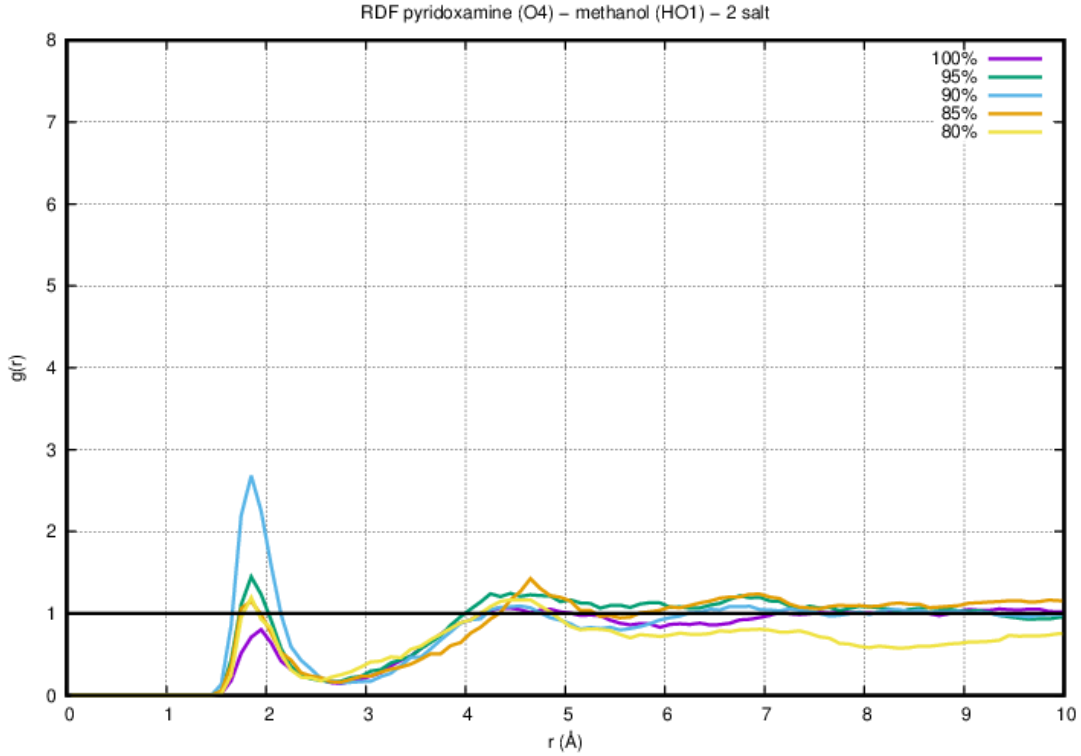


Fig 12b. The RDF of pyridoxamine's O4 atom to Methanol's HO1 atoms when 2 salt molecules of NaCl are present.

this interaction at any composition apart from the 90% methanol composition.

3.6. Interaction between Pyr(HN*) and moh(O1)

3.6.1. Effects of Composition

The RDF from Fig 13. below shows the effects of change in composition of the mobile phase on the specific HN*-O1 interaction.

It can be observed that the RDF for this HN*-O1 interaction has one medium peak and remaining weak peaks just above 2 angstroms. This indicates that HN atoms are fairly accessible to interaction with the oxygen of the methanol. The interesting factor is the presence of two different peaks with almost the same

height for all compositions except 85% methanol. That is for this specific interaction the change in distance between the atoms has a very small effect on their overall positioning in the solution between the range in which they can interact.

The composition of our mobile phase is represented by the amount of methanol molecules present in the phase by percentage that is from 100% to 80% and color coded for better presentation. The interaction is observed to have maxima 80% composition and minima at 100% composition methanol by looking at the amplitude of the RDF peaks at different compositions. But for the second solvation shell the maxima is observed at 85% methanol.

3.6.2. Effects of Ions

The RDFs from Fig 14a. and Fig 14b. show the effects of ions on the specific HN*-O1 interaction.

Upon addition of a NaCl molecule the first and second peaks at all compositions are improved and no longer at the same height. The second solvation shell peak becomes more intense than the first. The minima is changed from 80% methanol to 85% methanol for the first peak. Overall the sharpness and strength of the interactions are also increased even if it is at a very small amount.

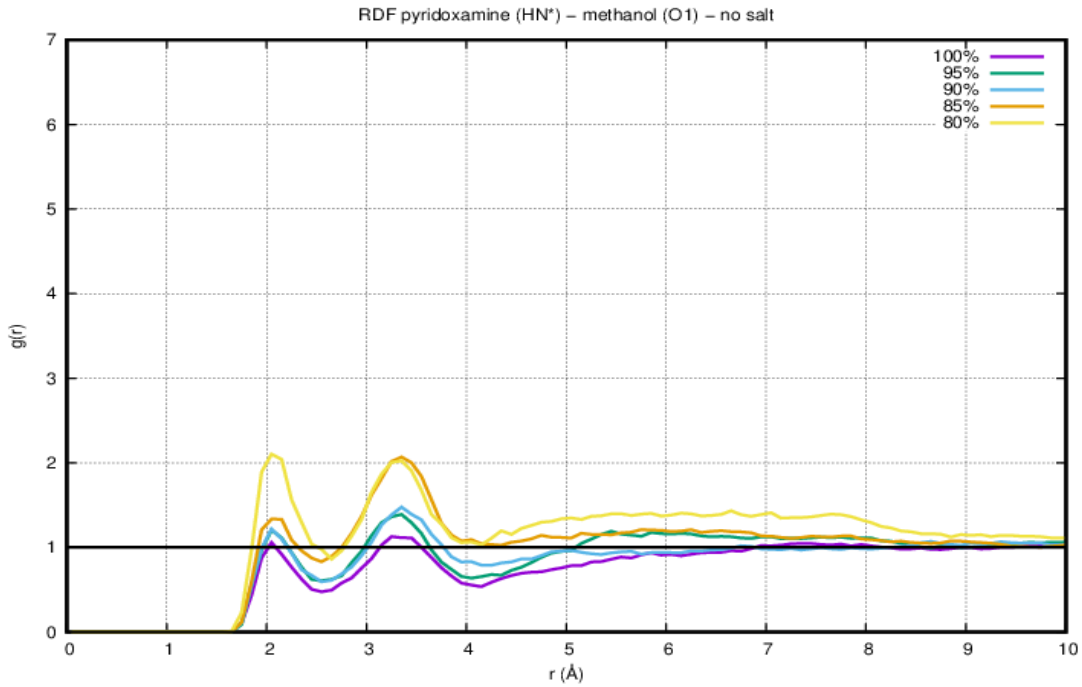


Fig 13. The RDF of pyridoxamine's HN* atom to Methanol's O1 atoms.

Addition of 2 salt molecules has an opposite effect at all compositions as it decreases the height of the peaks showing a decrease in strength of the specific interaction. Also the maxima shifts to 100% methanol for the first peak and minima is 85% methanol.

3.7. Interaction between Pyr(HO3) and moh(O1)

3.7.1. Effects of Composition

The RDF from Fig 15. show the effects of change in composition of the mobile phase on the specific HO3-O1 interaction. It can be observed that the RDF for-

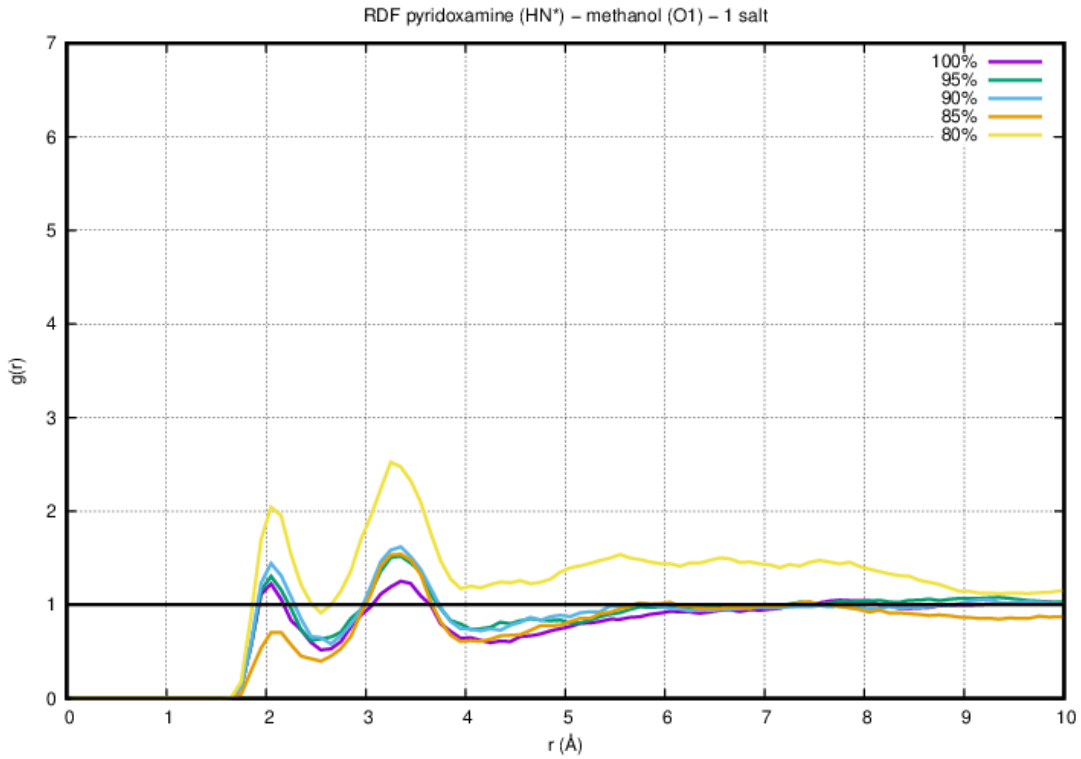


Fig 14a. The RDF of pyridoxamine's HN* atom to Methanol's O1 atoms when 1 salt molecule of NaCl is present.

HO3-O1 interactions have very strong and sharp peaks at all compositions of the mobile phases. This shows that the interaction between HO3 and O1 are strong and major determiners of the solubility of our analyte in methanol and the mobile phase. Hindering this interaction will have an observable effect on the solubility of the analyte.

The composition of our mobile phase is represented by the amount of methanol molecules present in the phase by percentage that is from 100% to 80% and color coded for better presentation. The interaction is observed to have maxima 80% composition and minima at 100% composition methanol by looking at the amplitude of the RDF peaks at different compositions. The RDF shows only the first peak and the second peak is not displayed meaning that the interaction is only clearly observable just below 2 angstroms

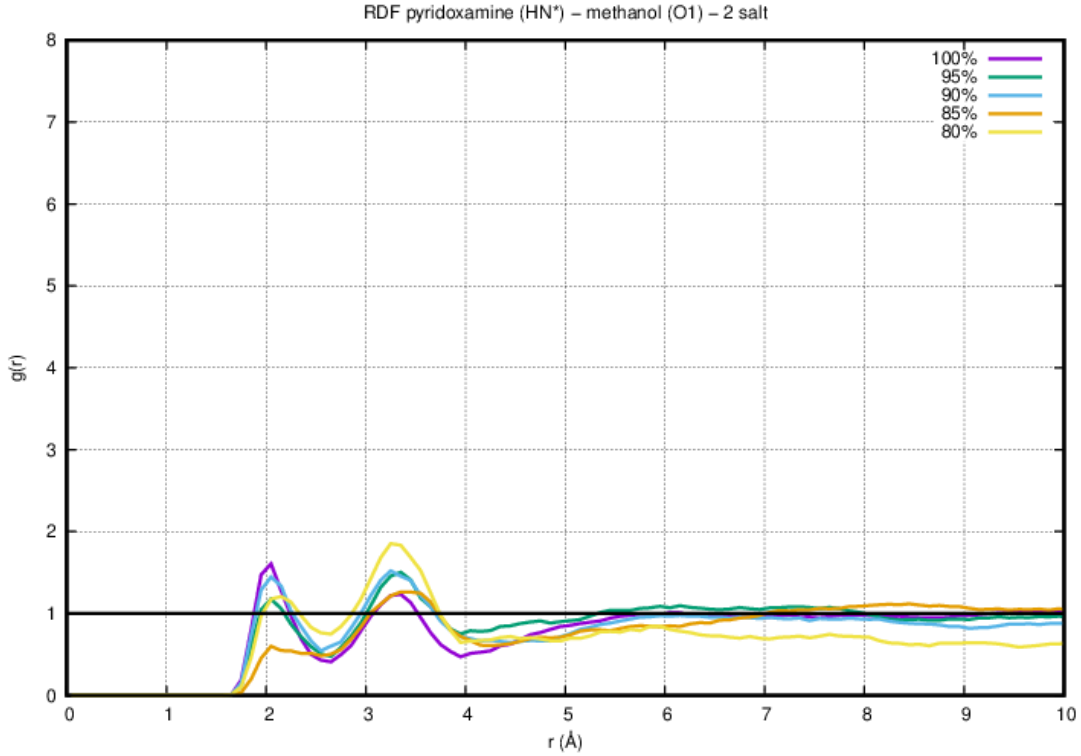


Fig 14b. The RDF of pyridoxamine's HN* atom to Methanol's O1 atoms when 2 salt molecules of NaCl are present.

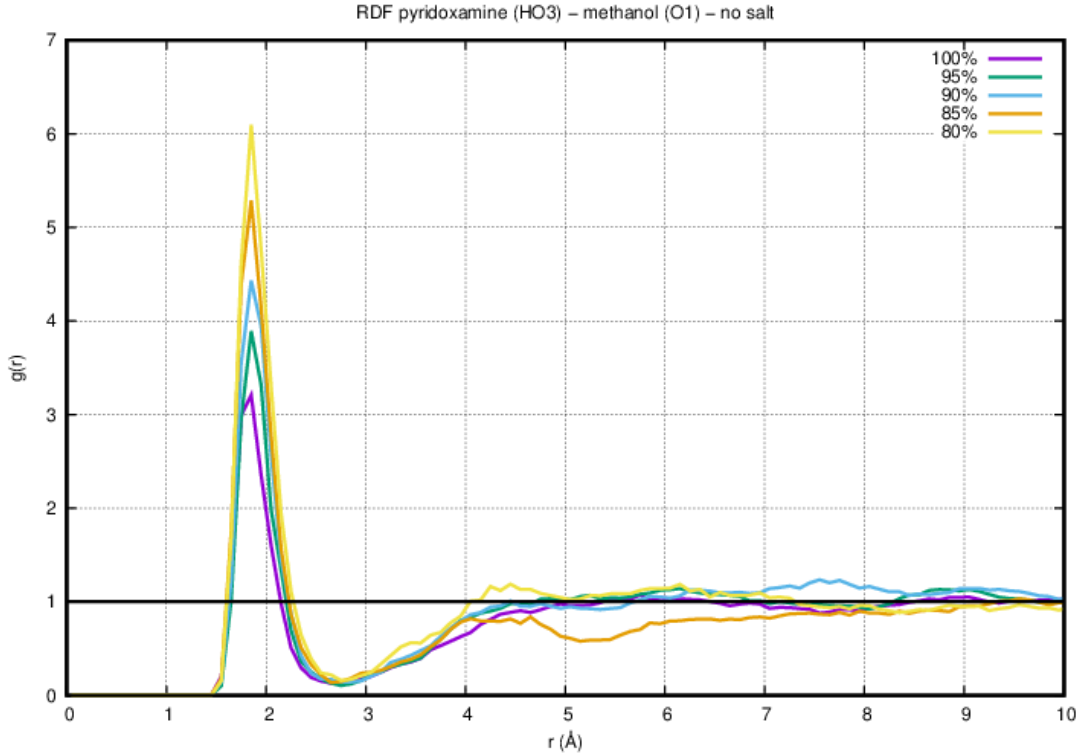


Fig 15. The RDF of pyridoxamine's HO3 atom to Methanol's O1 atoms.

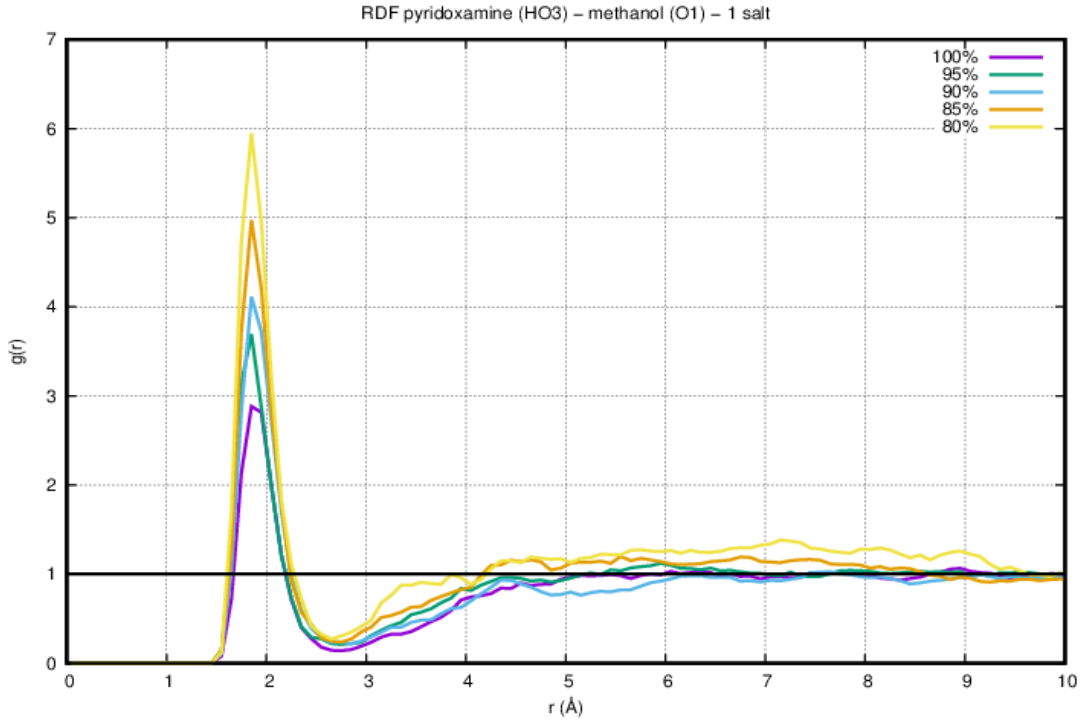


Fig 16a. The RDF of pyridoxamine's HO3 atom to Methanol's O1 atoms when 1 salt molecule of NaCl is present.

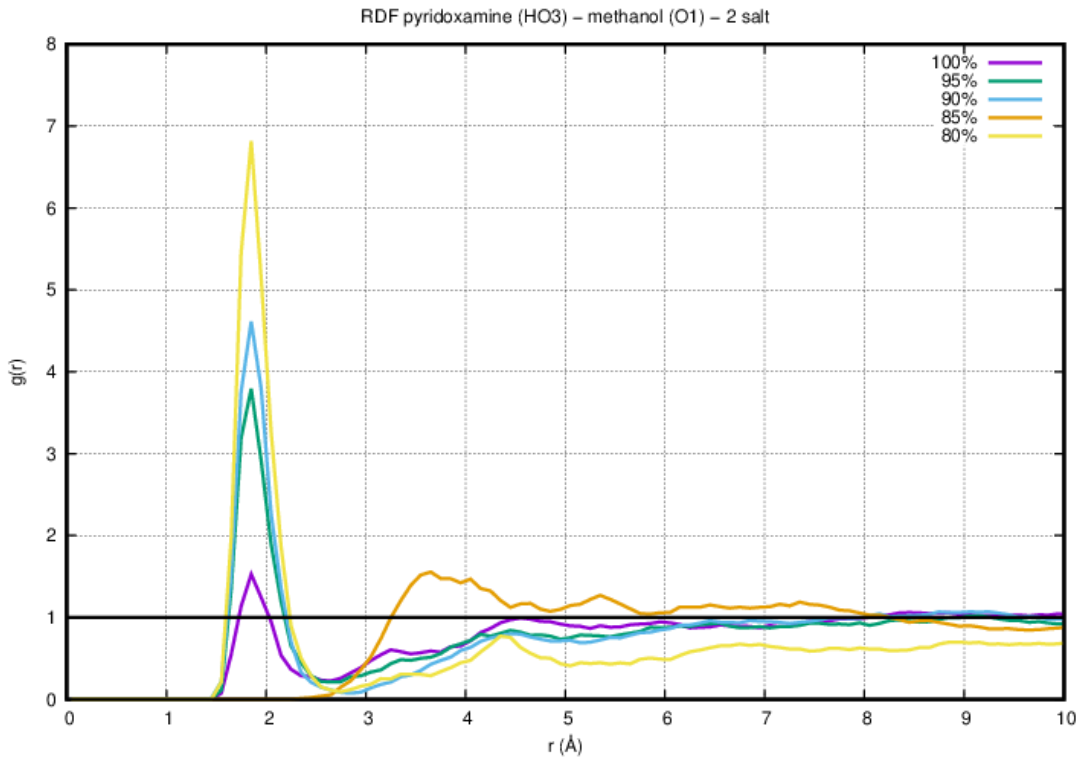


Fig 16b. The RDF of pyridoxamine's HO3 atom to Methanol's O1 atoms when 2 salt molecules of NaCl are present.

3.7.2. Effects of Ions

The RDFs from Fig 16a. and Fig 16b. show the effects of ions on the specific HO3-O1 interaction.

Upon addition of 1 NaCl molecule there is no observable change on the RDF for all compositions. Also the maxima and the minima are maintained.

Upon addition of 2 NaCl molecules the amplitude of the peaks is affected dramatically and in a different pattern. For 80% methanol the peak is sharp and increased in height suggesting stronger interactions at that composition. For 100% methanol the peak is broad and decreased in height significantly so

weaker interactions. For 85% methanol the first peak disappears altogether and for the rest there is a slight increase in peak height thus stronger interaction.

3.8. Diffusion Constants

The diffusion constant of a molecule in a solution is directly proportional to the rate of the molecules movement across the solution. The gives light on to how easy the molecule can move from point A to B in the solution.

The data for diffusion constants of methanol, pyridoxamine and methanol in the presence of pyridoxamine were obtained and tabulated according to the change in composition of the mobile phase and the change in the number of salt molecules.

3.8.1. Diffusion constants of Methanol

Table 1. below shows the diffusion constants of methanol at different compositions including 100% methanol and 0 ions where self-diffusion takes place. The self-diffusion was found to be 2.455 which is close to the experimental value of 2.4[18]. This provides confidence to the results obtained by the simulations although they are not experimental a semi-quantitative analysis could be performed.

As we move from left to right across the table the composition of methanol decreases and as we move from top to bottom down the table the number of NaCl molecules increases. When there are no NaCl molecules present the diffusion constant of methanol decreases with decreasing in methanol composition until when it reaches 80% methanol where it increases abruptly from 2.0006 to 2.0638.

In the presence of ions, the diffusion constant decreases with decrease in composition of methanol until it reaches 85% methanol where it increases and drops back down again at 80% methanol.

	Methanol/Triethylamine ratio	1000/0	950/50	900/100	850/150	800/200
NaCl molecule no.	0	2.4255	2.2245	2.1102	2.0006	2.0638
	1	2.3071	2.2523	2.1562	2.2163	2.026
	2	2.476	2.1218	2.1129	2.1173	2.0029

Table 1. Diffusion constants of methanol at different compositions of the solution.

3.8.2. Diffusion constants of Pyridoxamine

Table 2. Below shows the diffusion constants of the analyte at different compositions of the mobile phase and different concentrations of the ions.

When the concentration of the ions is zero the diffusion constant of the analyte decreases with the decrease in composition of methanol that is the rate of movement of the analyte is decreased as you change the composition towards lesser amount of methanol until when it reaches 90% methanol where it increases before decreasing again. This is the same when there is one molecule of NaCl but the difference is the increase here happens twice at 905 and at 80% methanol.

In the presence of 2 molecules of NaCl then the diffusion constant decreases with decrease in methanol concentration until when it reaches 85% methanol where it increases before going back down.

This shows that there is no observable trend as the composition changes or as the concentration of the ions changes. But each and every composition of the mobile phase will give a different diffusion constant and these constants can be used to choose the suitable mobile phase.

	Methanol/Triethylamine ratio	1000/0	950/50	900/100	850/150	800/200
NaCl molecule no.	0	1.327	0.0082	1.6857	1.5722	0.6468
	1	1.5378	0.7969	1.3019	0.2934	0.8382
	2	0.926	0.7569	0.5435	1.1631	0.3074

Table 2. Diffusion constants of pyridoxamine at different compositions of the solution.

3.8.3. Diffusion constants of Methanol containing Pyridoxamine

Table 3. below shows the diffusion constants of methanol at different compositions when containing pyridoxamine. The presence of pyridoxamine affects the diffusion constant even at 0 NaCl and 100% methanol solution.

When there are no NaCl molecules present the diffusion constant of methanol decreases with decreasing in methanol composition until when it reaches 90% methanol where it increases before going down again.

In the presence of one NaCl molecule the diffusion constant decreases with decrease in composition of methanol. But when there are 2 NaCl molecules the decreasing stops at 90% methanol where it increases and after that drops.

	Methanol/Triethylamine ratio	1000/0	950/50	900/100	850/150	800/200
NaCl molecule no.	0	2.3482	2.1159	2.1793	2.1289	1.9886
	1	2.5894	2.4324	2.2499	2.1148	1.9243
	2	2.409	2.1513	2.374	2.0725	1.7711

Table 3. Diffusion constants of methanol containing pyridoxamine at different compositions of the solution.

3.9. Retention Estimations

From the obtained data of the calculated RDFs and diffusion constants a few estimations and predictions can be made on the retention of the analyte under study. Retention by definition is the ability of the analyte to bind to the stationary phase but this extent of bondage to the stationary phase is facilitated by strength of interactions between the analyte and the mobile phase. The retention factor is therefore increased as the interactions between analyte and mobile phase are minimized and the vice versa is also true.

In order to understand how the retention factor is changing with respect to composition, a table was made that shows the composition of the mobile phase and the type of interactions that dominate at that specific composition. This was achieved by calculating the integrals of the RDFs for all the interactions at every composition. These integrals represent the strength of an interaction at a given composition. The values of the integrals were normalized to the highest value to facilitate easier comparison and analysis of the obtained data. Below is a

table of the normalized values of the integrals and their sum at each composition.

	NT5-HO1	N1-HO1	O3-HO1	O4-HO1	HN*-O1	HO3-O1	SUM
100%	0.58	0.27	0.07	0.38	0.73	0.63	2.67
1 NaCl - 100%	0.67	0.21	0.06	0.39	0.83	0.63	2.80
2 NaCl - 100%	0.60	0.25	0.19	0.24	0.92	0.38	2.58
95%	0.55	0.31	0.04	0.36	0.78	0.66	2.71
1 NaCl - 95%	0.59	0.20	0.08	0.37	0.82	0.67	2.72
2 NaCl - 95%	0.66	0.38	0.13	0.31	0.74	0.67	2.89
90%	0.51	0.37	0.09	0.43	0.71	0.70	2.81
1 NaCl - 90%	0.63	0.28	0.06	0.23	0.79	0.71	2.71
2 NaCl - 90%	0.59	0.17	0.06	0.47	0.79	0.68	2.76
85%	0.71	0.19	0.04	0.43	0.80	0.73	2.91
1 NaCl - 85%	0.74	0.18	0.11	0.14	0.40	0.72	2.29
2 NaCl - 85%	0.59	0.34	0.35	0.23	0.40	0.02	1.91
80%	0.56	0.20	0.05	0.46	1.00	0.77	3.03
1 NaCl - 80%	0.75	0.26	0.17	0.46	0.97	0.78	3.38
2 NaCl - 80%	0.75	0.14	0.05	0.20	0.67	0.78	2.60

Table 4. Normalized integral sums of g(r) for analyte-mobile phase interactions at different compositions.

Using Table 4. a rationale can be made between the retention factor and the strength of the interactions of an analyte in the mobile phase. It can be stated that 'The retention factor is inversely proportional to the total strength of the interactions between analyte and mobile phase because an increase in strength of interactions causes a decrease in retention.'

$$K \propto 1/\int \quad \sum \quad g(r)$$

The retention factor is represented by K while the total strength of the interaction is represented by $\sum g(r)$. This relation is purely hypothetical since there isn't any experimental proof. Nevertheless, the relation can still be used to estimate the decrease or increase in retention factor as the composition of the mobile phase is changed

If we assume that all other factors affecting the retention factor are the same for all compositions, then by using the relation we can estimate that;

$$K\ (2\ NaCl - 85\%) >> K\ (1\ NaCl - 80\%)$$

This estimation suggests that for the best retention the composition to be used should be of 85% methanol and contain an equivalent of two molecules of NaCl per 64000 angstroms cubic of the mobile phase. But it is known that in HPLC best retention times do not equal best peaks. Therefore, a range of compositions from the table can be selected for optimization purposes.

The data obtained from diffusion constants of pyridoxamine (Table 2. can be used to choose the types of compositions to be used during optimization because the higher the diffusion coefficient the better the retention as shown by the resistance to mass transfer coefficient (Cm) found in the Van Deemter equation.

The table below shows five different compositions in which pyridoxamine has attained high diffusion constants.

D(90%)	**D(1 NaCl - 100%)**	**D(100%)**	**D(1 NaCl - 90%)**	**D(2 NaCl - 100%)**
1.6857	1.5378	1.327	1.3019	0.926

Table 5. Diffusion constants of compositions for optimization.

Combining all the data obtained from the analysis we can now reduce the number of candidates from 15 different compositions to only 6.

4. CONCLUSION

Multiple simulations were performed in order to facilitate an investigation towards the molecular interactions taking place between the analyte and mobile phase. The mobile phase was modeled to contain different kinds of common solvents and ions at different compositions. These models allowed for a wide range of interactions to be studied while at the same time remaining similar to an actual method development approach of altering compositions and ion concentrations to achieve better separations.

By calculating the RDFs and diffusion constants these interactions were quantified and painted a logical picture. The relation between the interactions and the retention of the analyte was successfully established despite the limited amount of data used. The data obtained from the simulations has proven to be useful qualitatively to the selection of a suitable composition or compositions of mobile phases to be used in the RP-HPLC separation. A combination of the results of the simulation and experimental data will go a long way in proper method development. Usually method developments are tedious trial and error procedures performed based on experience and a few guidelines, these procedures usually lead to a waste of a lot of chemicals and time. Molecular simulations of these processes will help to counteract these effects.

In this study there have been a lot of limitations that stopped us from making any quantitative conclusions on the relationship between the interactions of our analyte and its retention factor at a given composition. These limitations include hard drive capacity, total simulation times and the nature of the analyte in the mobile phase. The capacity of our hard drive was not enough to hold the tons of data that would be processed if we increased our output frequency enhancing the diffusion constant results. Total simulation times had to be limited to allow for the finalization of the study within the required

period. Lastly the nature of pyridoxamine allowed for protonation to take place which was not simulated although in practice protonation is minimized by adjustment pH of the mobile phase suggesting that the use of a neutral molecule of our analyte isn't far off.

In conclusion the role of molecular dynamics and its techniques in the method development for RP-HPLC separations is prevalent. The part that molecular dynamics can play involves a lot of theory but combining that information with physical experiments can only improve the predicted results and in turn develop into better models of representation for the actual processes. The steps taken to everyday analysis with all chromatographic procedures could be reimagined.

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