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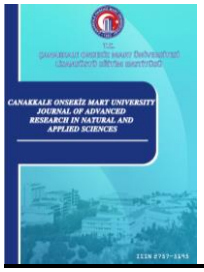

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Novel Benchtop ^{1}H NMR and High Field ^{1}H NMR spectroscopy as practical tools for characterization of chemically exchanging systems in Ascorbic acid

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Research Article

Abstract – Nuclear magnetic resonance (NMR) spectra and its action on characterization of pH dependent systems are among the most useful tools in spectroscopic techniques. Nevertheless, practical considerations often restrict the application of High field NMR owing to its huge dimensions, expense and time-consuming property. On the other hand, benchtop devices could be another option with transportable permanent magnet systems (1–2 T) but with several disadvantages such as reduced sensitivity and low resolution. In this study, proton exchange rates depending on pH of a selected molecule, were monitored by both 800 MHz High-Field and 60 MHz Benchtop H^{1} NMR to be compared. L-Ascorbic acid (AA) which is an important substrate for body and biological reactions was chosen as a target model molecule. This study supported the use of NMR for pH dependent chemical changes due to protonation. According to spectrum results and previous literature studies, shifting behaviour of Peak 1 (assigned to H13) towards lower frequencies upon pH increase, gave us its feasibility for pKa determination. While High-field NMR spectroscopy demonstrated the compositional analysis of AA and proton exchanges showing couplings between protons, Benchtop NMR was displayed as a limited tool owing to broadened lines of splitting peaks. Even so, shifting tendency of Peak 1 was still obtained quantitatively and promoted the feasibility of Benchtop NMR for characterization of a model molecule which was L-ascorbic acid. In brief, this study supported the effect of magnetic field strength on characterization of pH dependent chemical exchange regimes for vitamins.

Keywords – *Ascorbic acid, Benchtop NMR, chemically exchanging system, pH dependency, proton transfer*

1. Introduction

Nuclear magnetic resonance (NMR) is among the most powerful spectroscopic techniques for analysis of molecular structure and detection of chemical species (M. P. Ledbetter et al., 2009). In polymer chemistry, the peak intensities and chemical shifts in NMR spectra enable some structural quantities such as polymer composition, branching, sequence distribution and crosslinking to be measured or characterized (Park, Kim, Seok, Kim, & Kim, 2015; Shapiro, 2011). On the other hand, spectral sensing of local pH changes in vivo has been of long-standing interest for characterization of metabolites, pathological conditions, substrates etc. (Shchepin et al., 2016). Depending on exchange regime and reaction rate constant, spectral lineshapes show unique pattern in NMR spectroscopy. Labelled pyruvic acid and ascorbic acid are among the substrates that are widely used as biomarker probes for differentiation of healthy and cancer cells by analyzing their chemical transformation into their other forms (pyruvate, dehydroascorbic acid, etc.).

Ascorbic acid also called as Vitamin C, is one of the most outstanding members of antioxidants (reducing agent) by donating electrons to many enzymatic / nonenzymatic reactions. It is converted into oxidized state

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mainly as dehydroascorbic acid (DHA) and DHA could be used as a probe for redox chemistry in biological systems (Keshari et al., 2019). Injection of hyperpolarized form of DHA and its rapid conversion to Vitamin C in tissues having high oxidative stress including kidney, liver highlights its biochemical role. These reactions like reduction-oxidation are also affected by pH variations. Chemical exchange like proton exchange due to such kind of changes in media is important phenomena to be considered in so many areas from biochemistry to medical applications. These concepts could be characterized and studied by several techniques. One of them, NMR, allows to differentiate chemical species through their resonance frequencies (chemical shifts and *J* couplings) (Barskiy et al., 2019). In addition to other features, one of the strongest aspect of NMR is its ability for detection of chemical exchange phenomena even in case where system is in equilibrium (Levitt, 2000). It is a non-invasive and non-destructive method probing the chemical composition of samples. Although there are past studies related to ascorbic acid spectra (Paukstelis J.V., Mueller D.D., Seib P.A., 1982; Reid, 1989; Singh, Mohanty, & Saini, 2016), the use of recent, new-generation instruments with different settings were proposed in this study. In this work, the utility of NMR spectra was presented to show pH-dependency of metabolic substrates. For this aim, L-Ascorbic acid (AA) was chosen as a model substrate.

Especially, traditional high field NMR has a tremendous utility with high resolution, high signal to noise ratio and high sensitivity. Nevertheless, the use of high magnetic fields requires high cost, immobile and large superconducting magnets with constant cryogenic temperature setup making sometimes conventional NMR as a limited tool (Micah P. Ledbetter & Budker, 2013). Nowadays, Benchtop NMR instruments (frequencies varying up to 100 MHz) with permanent magnets have opened the ways to access several applications such as pharmaceutical and biotechnology. They are built in a way that is cheaper and highly portable compared to high field equipment but limited devices due to low resolution, low signal to noise ratio, etc. (Heerah, Waclawek, Konzuk, & Longstaffe, 2020). To achieve better results such as better signal to noise ratio (SNR) in Benchtop NMR, it is required to manipulate several parameters like number of scans (Dopona, 2015). In this study, 60 MHz Benchtop NMR spectra was demonstrated to analyze its efficiency in chemical exchange analysis and its use in pH- pK_a relationship. The objective of the study was to show the application of Benchtop NMR spectroscopy as a practical tool for analysis of pH dependent exchange system in a biological molecule.

2. Materials and Methods

2.1. Sample preparation

A stock solution of L-Ascorbic acid (AA) (99% Sigma Aldrich, St. Louis, Missouri, USA) was prepared in a 200 mM concentration by dissolving AA in deuterium oxide (D_2O) for signal locking. Some samples were prepared in DMSO_d6 (Sigma-Aldrich, USA), as well. Sodium hydroxide (Fisher Chemical, USA), hydrochloric acid (Macron Fine Chemicals, Avantor, USA) or acetic acid (Macron Fine Chemicals, Avantor, USA) at different molarities were used to adjust pH. Sample pH values were measured at room temperature using benchtop pH/ORP meter (HI 2211, Hanna Instruments, Rhode Island, USA) with micro pH combination electrode, glass body (Sigma Aldrich, St Louis, MO). Then, NMR measurements were performed immediately.

2.2. NMR methods

Each sample of 600 µL was pipetted into a standard 5 mm NMR tube. High-field 1H NMR measurements were performed at room temperature using an 800 MHz Avance III Bruker NMR spectrometer. High field spectrum of each solution was acquired using 4 scans and 35 s of delay time. Benchtop NMR measurements were conducted at room temperature using a 60 MHz Benchtop NMR (NMReady-60PRO, Calgary, Alberta,

Canada). The optimization of setting parameters of Benchtop NMR was discussed in detail at the next section.

3. Results and Discussion

3.1. pH-dependent chemical exchange in L-Ascorbic acid

Ascorbic acid (AA) (shown as $AscH_2$ in chemical structure), an essential nutrient for human health, plays a significant role in so many pH dependent biological functions acting as enzymatic cofactor, reducing agent, substrate, etc. There exist several spectrum studies of ascorbic acid by different techniques such as Raman spectroscopy , ^{13}C NMR, ^{1}H NMR at different conditions in the literature (Karakurt, Aydoğdu, Çıkrıkcı, Orozco, & Lin, 2020; Paukstelis J.V., Mueller D.D., Seib P.A., 1982; Singh et al., 2016). As given in Figure 1, structurally it is a water-soluble ketolactone with two ionizable hydroxyl groups in which γ-lactone makes it highly reactive (J. Du, J. J. Cullen, 2013). It has two pK_a's, pK_1 is 4.2 and pK_2 is 11.6; so, the ascorbate monoanion, $AscH^-$, is the dominant form at physiological pH (approximately 7.4) (J. Du, J. J. Cullen, 2013). To quantify pH dependent exchange behaviour, we performed 800 MHz High-Field NMR spectra by studying aqueous solutions of L-Ascorbic acid (AA) under varying pH conditions from 0.37 to 12.07. Firstly, the recorded ^{1}H NMR spectra for AA, 200 mM stock solution in D_2O adjusting pH to 2.3 was shown in Figure 2. Since -OH hydroxy protons undergo rapid exchange with solvent, they are not seen as separate resonances. Similar to the study of Reid (1989) (Reid, 1989), the stable system could be defined as "**ABMX**" system as figured out in Figure 1a. **A** and **B**, two methylene protons have an adjacent chiral center causing their environments to become different. Although they are similar, they are not exactly chemically equivalent and do not have exact coupling constants, as well. *J* coupling constants belonging to system at specific pH values were listed on Table 1. Geminal coupling in -10 to -20 Hz range was ignored.

As given in Figure 2, AA gave peaks at around 4.94 ppm assigned to H13 (**X** proton); a triplet peak at around 4.06 ppm (**M** proton) and a doublet peak at around 3.74 ppm (**A** and **B** proton). Peak 1 (**X** proton) at around 4.94 ppm assigned to C4-H13 is expected to be a doublet due to the presence of **M** proton. However, most probably due to low coupling constant value, it was seen as a doublet just in stock solution at pH 2.3. Proton **X** was magnified to see peak splittings in detail in Figure 2a. Peak 2 as a solvent peak was observed at around 4.8 ppm similar to the literature (Reid, 1989; Singh et al., 2016). Figure 3 was obtained at around 4.06 ppm as a triplet peak due to **M** proton assigned to H14 bond coupling with C6-H_2. Peak 4, another splitting pattern as doublet peak, at around 3.74 ppm rose due to two freely rotating protons of C6-H_2 having a three bond coupling with H14 (Reid, 1989).

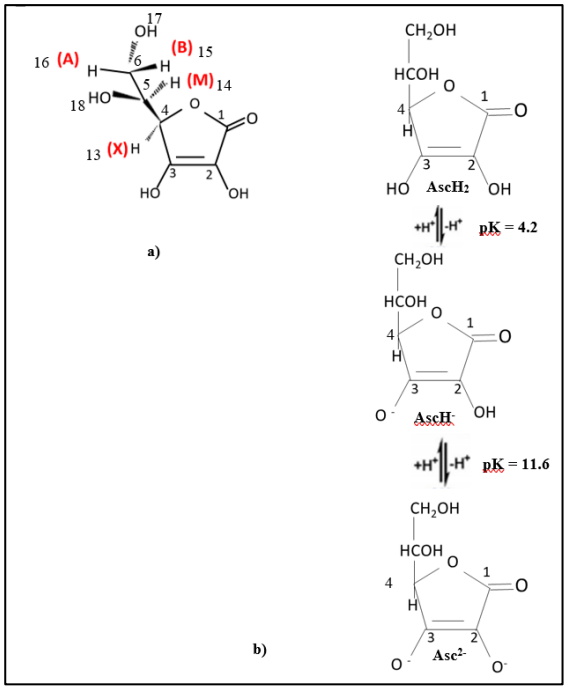
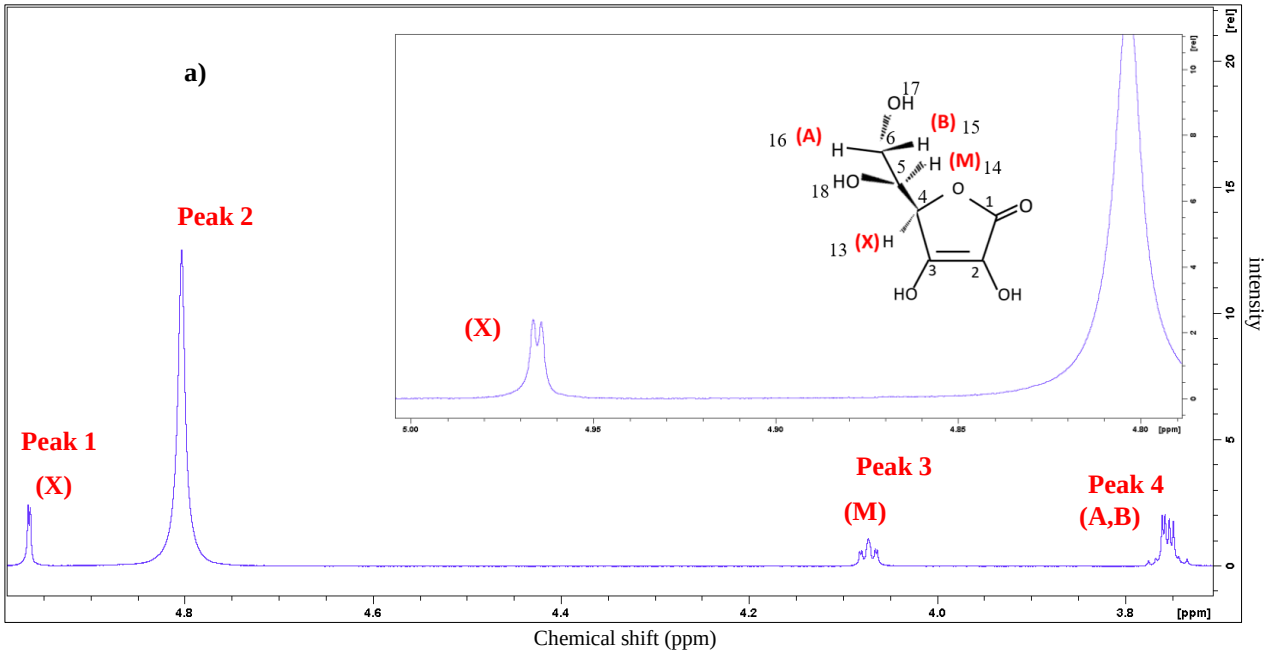


Figure 1. **a)** AA structure in acidic solution showing "ABMX" system (J. Du, J. J. Cullen, 2013) and **b)** AA structures representing pK values associated with AA.

In addition to determined hydrogens, there are four in hydroxyl groups, as well. However, they can not be observed in aqueous solution owing to D_2O which could exchange OH with O^2H (Singh et al., 2016). Therefore, NMR spectrum of AA in DMSO_d6 solution was also figured out to show peaks belonging to H17 and H18 protons at around 5 ppm (Figure 3).



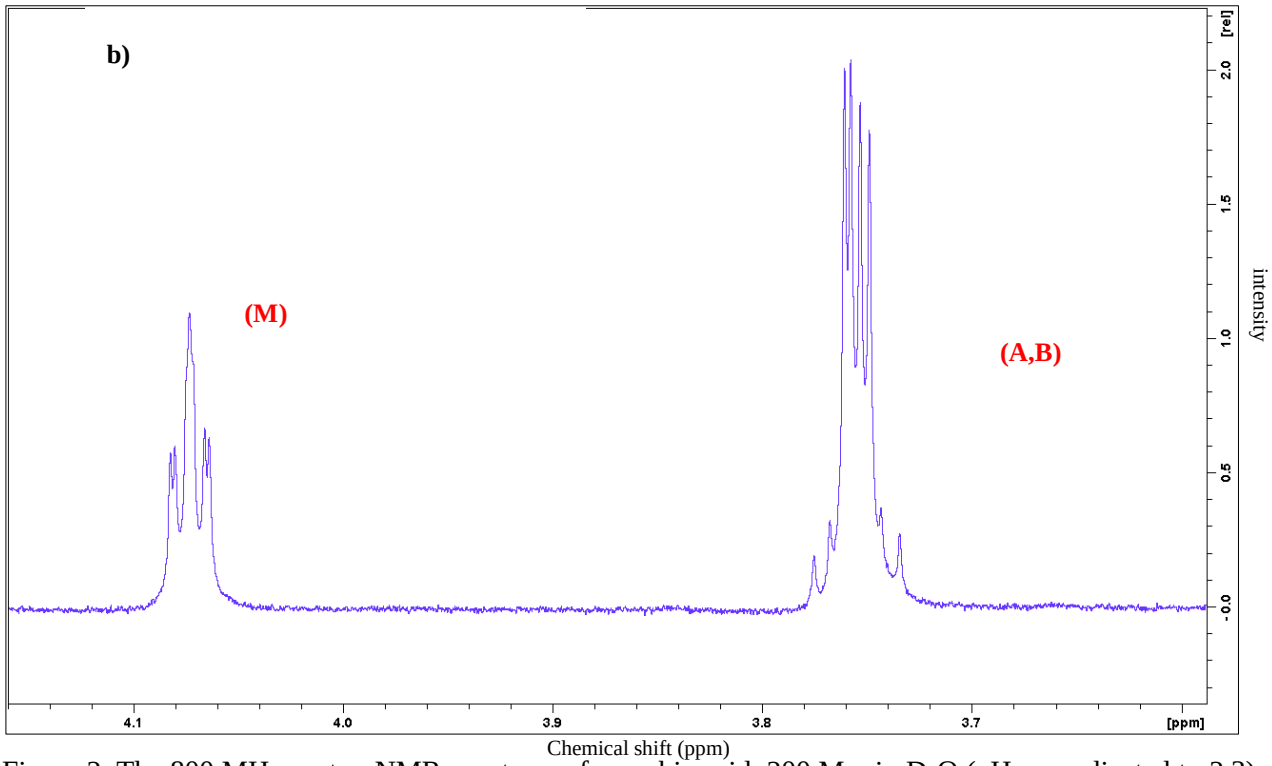


Figure 2. The 800 MHz proton NMR spectrum of ascorbic acid, 200 Mm in D_2O (pH was adjusted to 2.3) **a)** as overview **b)** between 3 and 4 ppm.

Table 1. Coupling constants for AA at certain pH values

Compound	pH	J_{MX}	J_{AM}	J_{BM}	J_{AB}	$\Delta\delta_{A,B}$
L-ascorbic acid	~ 2	1.8	5.8	7.3	-11.5	6.48
	~ 7	1.9	5.6	7.6	-11.6	14.77

Note: The data was taken from (Paukstelis J.V., Mueller D.D., Seib P.A., 1982) measured at 600.2 MHz NMR in D_2O.

pH dependence of chemical shifts was evaluated to see the ability of High Field ^1H NMR in chemical exchange phenomena. Owing to complex nature of AA, it is not easy to interpret its spectra. The long-range coupling patterns of those resonances could be complex and probably unclear. However, in this study it was aimed to refresh the potential of NMR spectroscopy for vitamins to investigate exchange mechanism.

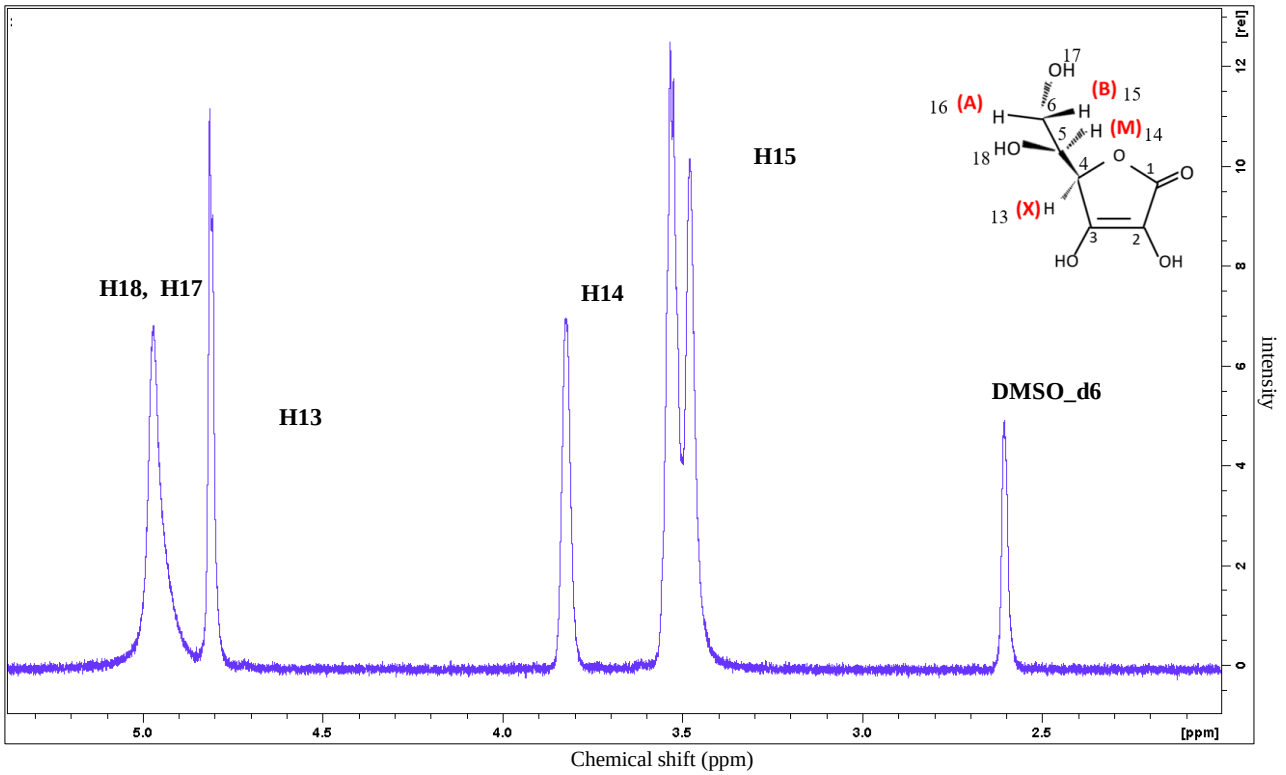
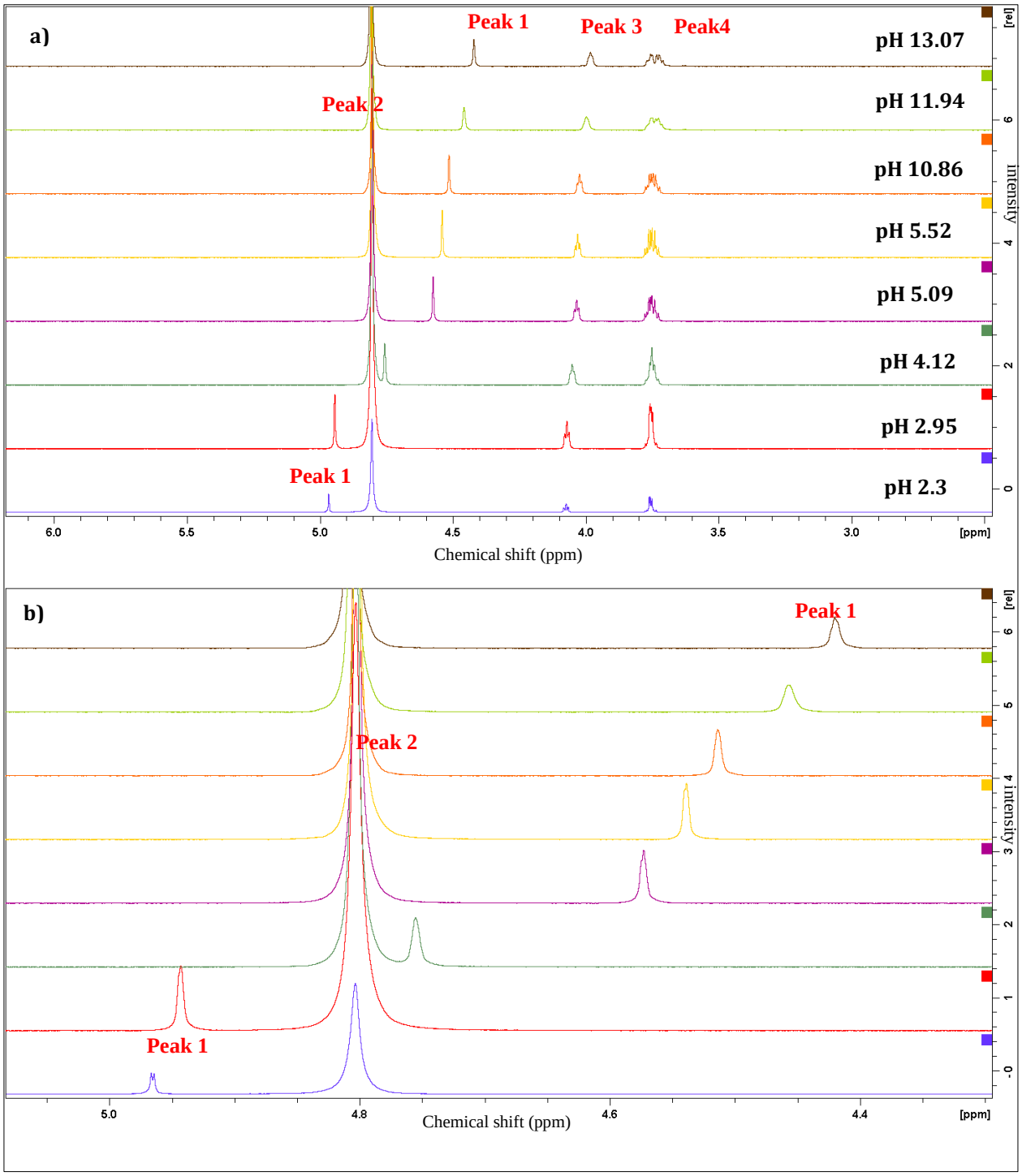


Figure 3. 200 mM AA in DMSO_d6 without TSP in 800 MHz High Field NMR

Figure 4, produced an observable chemical shift change between the substrate and its proton exchanged forms, to allow spectroscopic imaging of both metabolites (Bohndiek et al., 2011). According to the results, three main findings were reached upon increasing pH level. Firstly, Peak 1 shifted to lower frequency at higher pH values. Secondly, splittings at Peak 3 merged into one peak. As a final result, Peak 4 started to split into two main peaks as pH increased.


a)
Peak 1
Peak 3
Peak4
Peak 2
pH 13.07
pH 11.94
pH 10.86
pH 5.52
pH 5.09
pH 4.12
pH 2.95
Peak 1
pH 2.3
[rel]
intensity
6.0
5.5
5.0
4.5
4.0
3.5
3.0
[ppm]
Chemical shift (ppm)
b)
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intensity
5.0
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4.4
[ppm]
Chemical shift (ppm)

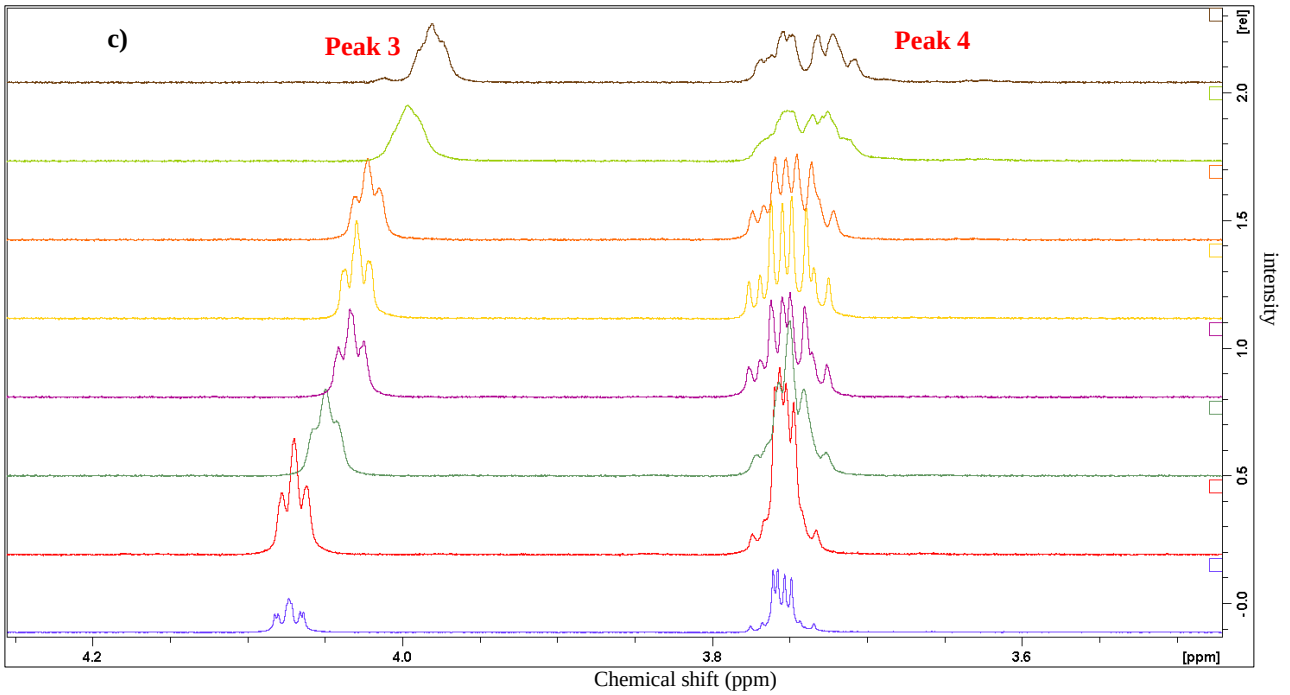


Figure 4. The 800 MHz proton NMR spectrum of AA 200 Mm in D_2O at varying pH values **a)** as overview **b)** belonging to only peak 1 and peak 2 **c)** belonging to only peak 3 and peak 4.

There might be several senarios for these findings. One of them might be related to shielding effect of oxygen especially on **X** proton. As given in Figure 1, the first donated hydrogen is the closest one (belonging to -OH group) to **X** proton. Electronegativity effect of adjacent oxygen might have caused **X** proton to shield and to show its signal at upfield. Clearly, at basic conditions higher than pK_2 of AA, the splitting in proton **M** vanished with the increase of pH dependent proton dissociation rates. Electronegative substituents modify the magnitude of vicinal coupling constants. Moreover, different orientations of a coupled proton with respect to adjacent OH group in cyclic compounds is attributed to difference in coupling constants (Paukstelis J.V., Mueller D.D., Seib P.A., 1982). Upon ionization of AA, conformational changes in AA structure such as different rotamers might also have led changes in J constants and hereby variation in NMR spectral lines.

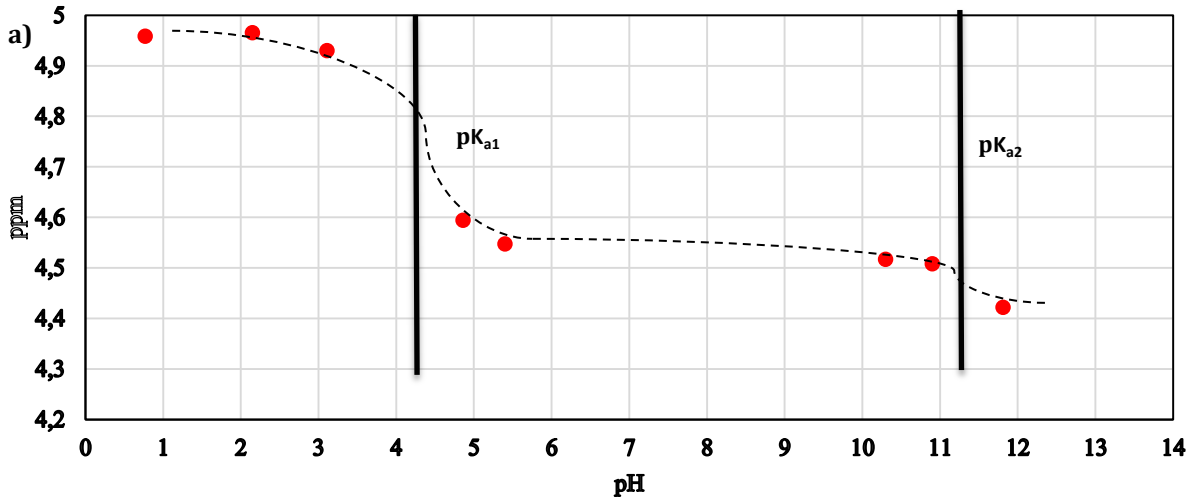


Figure 5. ppm-pH relationship for peak 1 at High Field NMR.

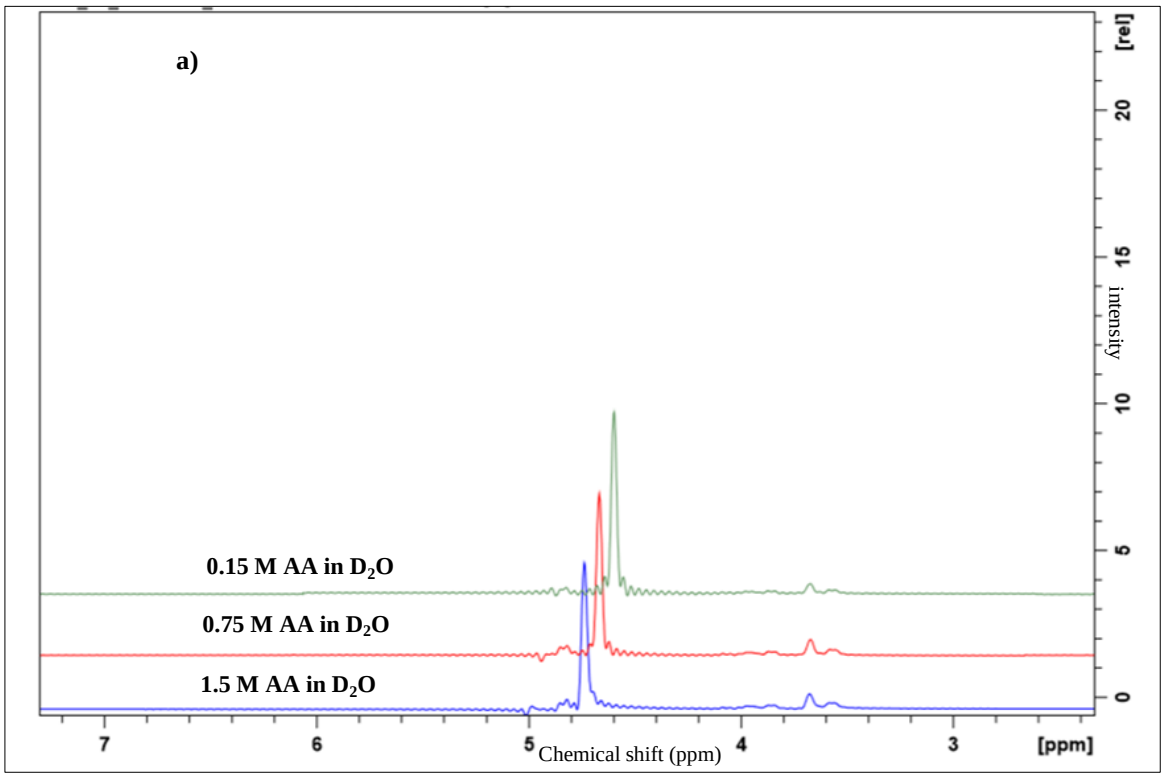
Another reason could be explained by dissociation / association mechanism with corresponding conjugates. As pH value showed an increase, it might have been resulted in higher k_d (dissociation constant) values indicating decrease in mean life time of protons. Thus, NMR peaks could lose some splitting patterns and give much more wider line shapes at high pH conditions. Here, relationship between pH and pK_a is an important event. Another relationship between pK_a and shifting of Peak 1 was found as given in Figure 5. Two sharp decreases in chemical shift points of Peak 1 were observed around pH values (~4 and ~11.5) corresponding to two pK_a values of AA. It was expected from our basic chemistry knowledge, the populations of acids and base change fastest around the pK_a in a titration.

Finally, Peak 4 gave different patterns depending on pH. Since Proton **A** and **B** are close enough, they can affect each other hereby coupled together. Coupled protons moved to each other and this geminal coupling led to a root effect in the presence of strong couplings. Upon increase in pH, this root effect began to change by overlapping weak coupled protons and eventually showing a separation between two.

In the light of findings, it has been proposed that proton exchange, in other words, pH dependency of NMR spectra could be featured for a variety of molecules such as ascorbic acid which is valuable substrate for biomedical applications.

3.2. Benchtop 60 MHz NMR detection of L-Ascorbic acid

In order to investigate the performance of NMR system working with permanent magnet, similar analyses were also conducted at 60 MHz Benchtop ^1H NMR which has a user friendly, cost effective and portable design. In order to increase sensitivity and signal to noise ratio, we conducted preliminary experiments to optimize parameters. For this purpose, firstly relaxation time constants (T_1) of AA molecule were considered and found as 3.61 s (for Peak 1), 3.11 s (for Peak 3) and 1.09 s (for Peak 4), to reach equilibrium and to determine scan delays. Then, different sample concentrations and different number of scans were performed as given in Figure 6. While Figure 6a represented the effect of sample concentration, Figure 6b displayed the effect of number of scans on device efficiency.



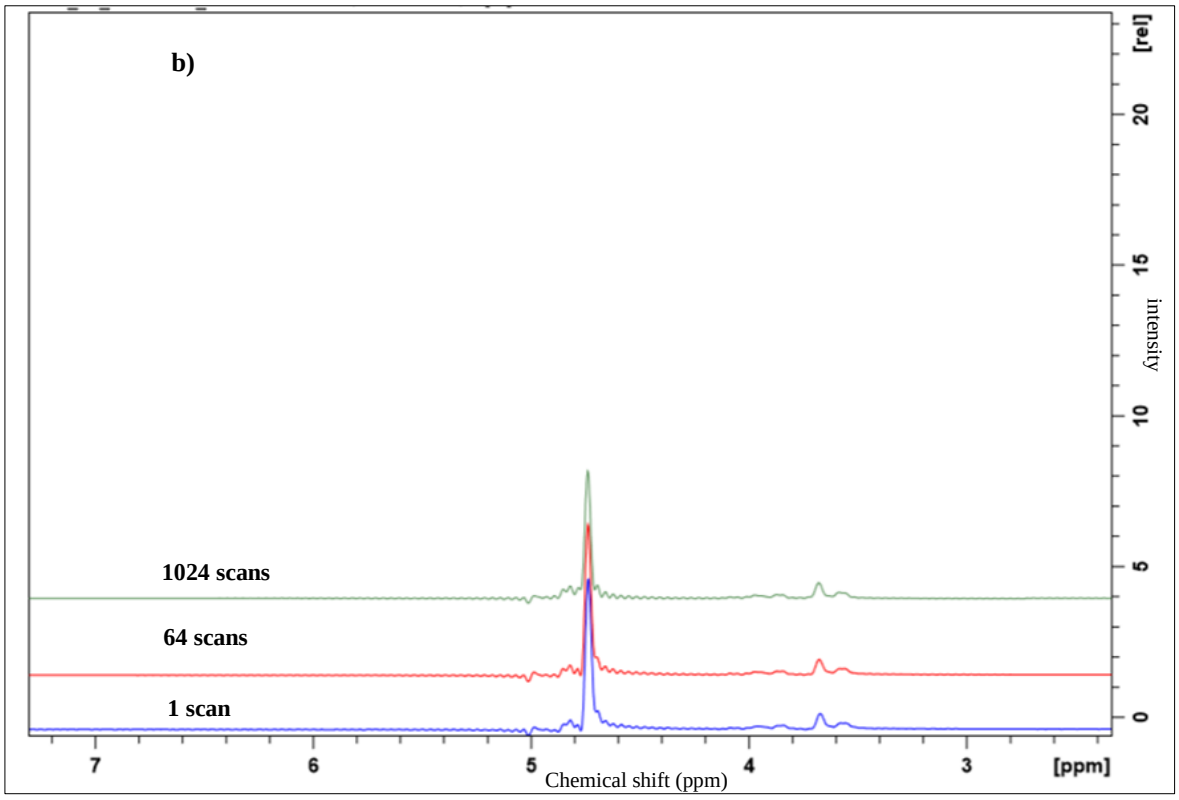
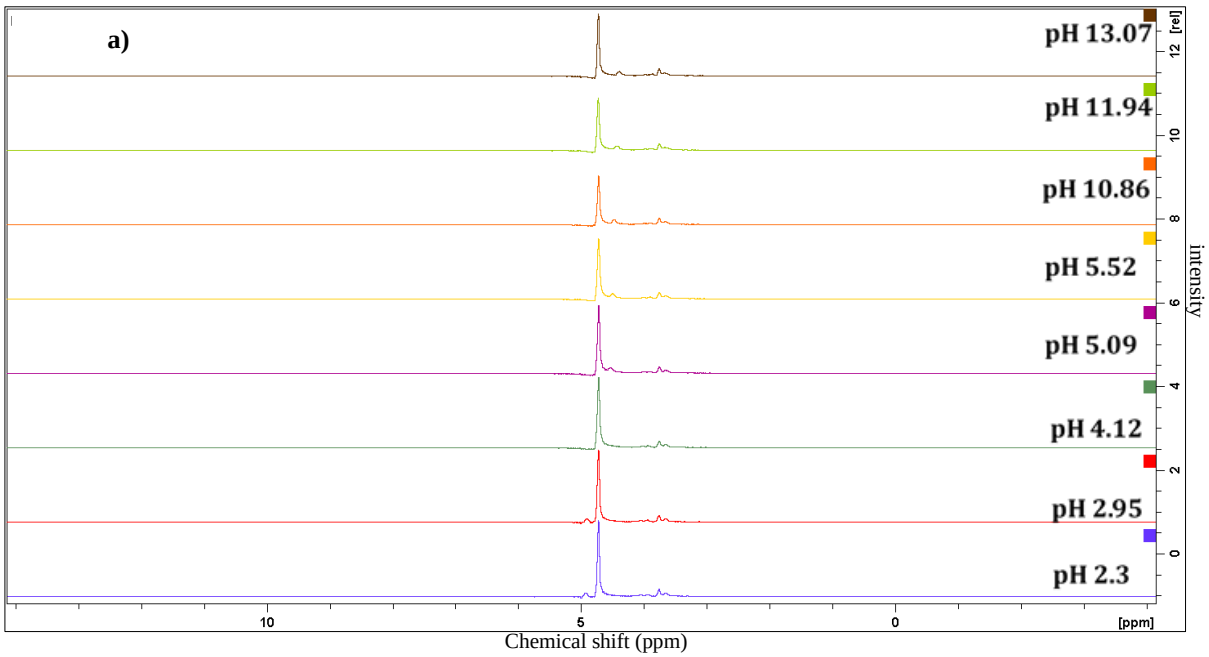


Figure 6. 60 MHz Benchtop NMR measurements **a)** at 32 scans for different AA concentrations in D_2O and **b)** at different number of scans for 1.5 M AA solution.



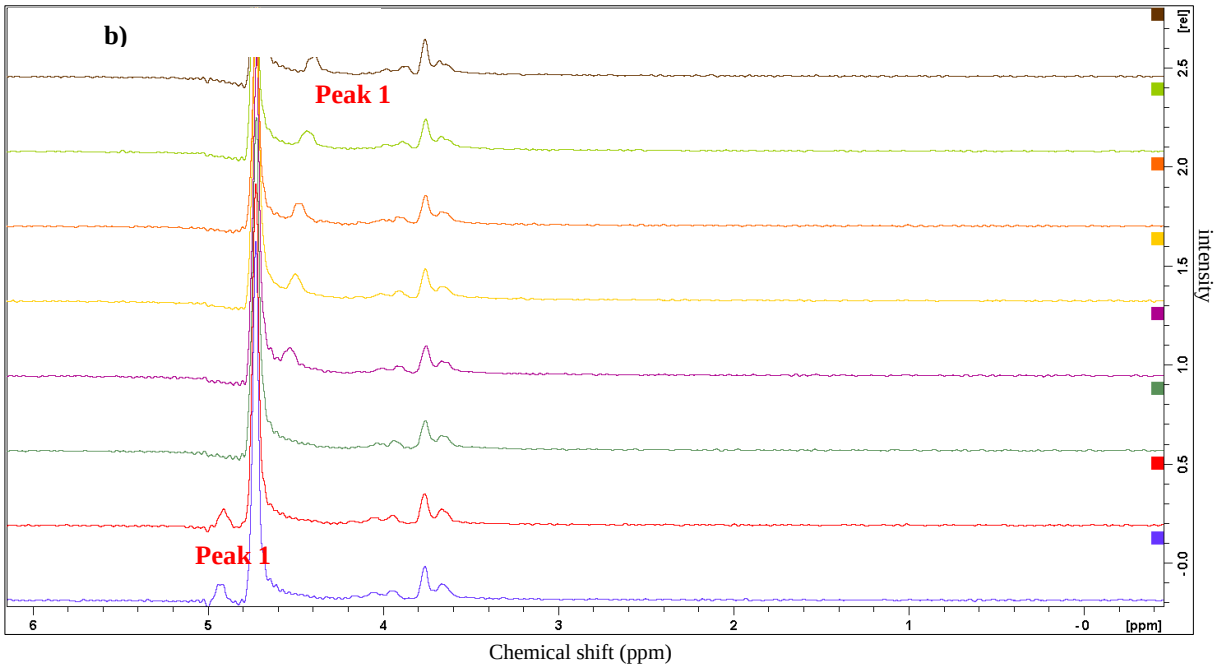


Figure 7. The 60 MHz Benchtop NMR spectrum of AA, 1.5 M in D_2O at varying pH values **a)** as overview **b)** magnified version

Similar to previous part, pH dependency of proton chemical shift of AA in 60 MHz NMR in D_2O was figured out in Figure 7. Similarly, shifting in Peak 1 regarding to pH was observed at Benchtop results, as well. However, couplings of the protons could not have clearly seen at low field NMR. Splitting of NMR peaks was overlapped and disappeared due to low resolution and low sensitivity. Thus, Peak 1 was mainly considered in Benchtop NMR analysis. Another relationship between pK_a and shifting of Peak 1 was also given for benchtop measurement results (Figure 8). These results pointed out that Benchtop NMR spectroscopy could be still challenging for some molecules like vitamins if you aim to identify changes in conformational side chains.

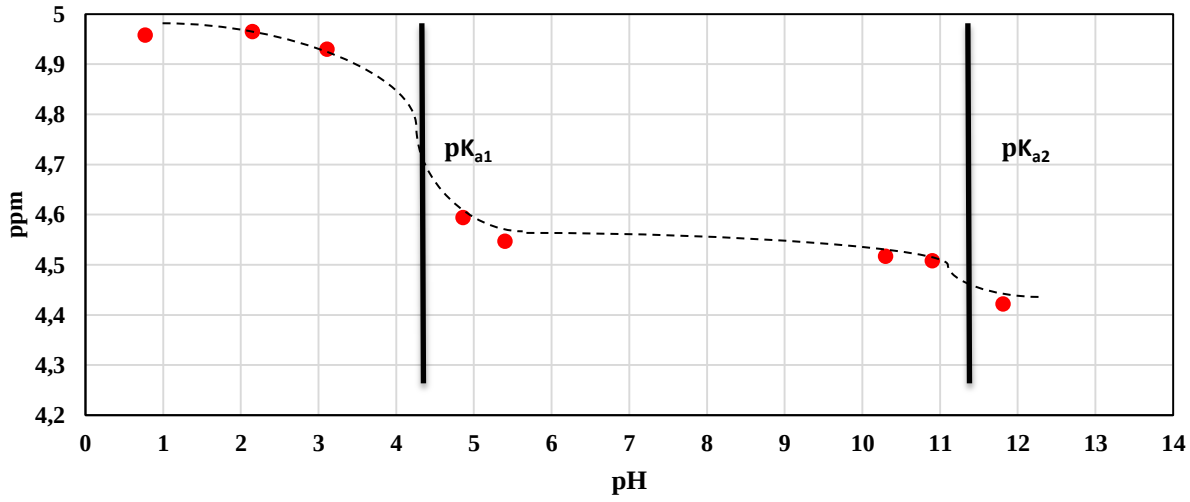


Figure 8. Observed 60 MHz Benchtop ^{1}H NMR chemical shift (ppm) of Peak 1 relative to sample pH

4. Conclusion

In this study, 60 MHz Benchtop NMR and 800 MHz High-Field NMR were performed to verify proton peaks in ascorbic acid as model vitamin, successfully. The obtained figures could also be used in undergraduate student laboratory experiments to show chemical shift dependence on pH. According to the results, the sensitivity of Benchtop NMR limited the detection of peak splitting and multiplets in 60 MHz, but it still showed shifting behaviour of Peak 1 which was assigned to H13 and gave us its feasibility for pK_a determination. Overall, Benchtop NMR could be a promising and a useful tool to test the vitamins exposed to chemical exchange when conventional NMR spectroscopy is used for detailed characterization. The latest higher performance, 100 MHz Benchtop NMR devices could be more promising for future studies, as well. Overall, Benchtop NMR could be of interest as a low-cost alternative method for characterization of pH dependent chemical exchange regimes for complex molecules.

Acknowledgement

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Author Contributions

Author S.Ç: Performed all the study and has currently wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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