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Quantitative Analysis of Acetylsalicylic Acid by q-NMR (Quantitative-Nuclear Magnetic Resonance Spectroscopy)

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QUANTITATIVE ANALYSIS OF ACETYLSALICYLIC ACID
BY Q-NMR
(QUANTITATIVE-NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY)

By

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TABLE OF CONTENTS

ACKNOWLEDGMENTS	1
LIST OF TABLES	4
LIST OF FIGURES	5
ABSTRACT.....	6
INTRODUCTION	7
MATERIALS AND INSTRUMENTATION.....	9
Materials.....	9
Instrumentation.....	9
EXPERIMENTAL PROCEDURES.....	9
NMR Test Solutions.....	9
Solubility of Acetylsalicylic Acid	9
Preparation of Acetylsalicylic Acid Solvent System.....	9
Preparation of High and Low Concentration Acetylsalicylic Acid Stock Solutions.....	9
Preparation of High and Low Concentration Acetylsalicylic Acid Diluted Solutions.....	10
HPLC Test Solutions.....	10
Solubility of Acetylsalicylic Acid	10
Preparation of Acetylsalicylic Acid Solvent System.....	10
Preparation of Low Concentration Acetylsalicylic Acid Stock Solutions	10
Preparation of Low Concentration Acetylsalicylic Acid Diluted Solutions.....	10
Preparation of Mobile Phase.....	10
General Procedure for Operation of the Bruker NMR Spectrometer.....	11
General Procedure for Operation of the Agilent HPLC	12
RESULTS	13
NMR Spectra.....	13
NMR Analysis of High Concentration Acetylsalicylic Acid Solutions	17
NMR Analysis of Low Concentration Acetylsalicylic Acid Solutions.....	29
HPLC Analysis of Low Concentration Acetylsalicylic Acid Solutions	42
DISCUSSION.....	45
Qualitative Analysis	45
NMR of CDCl_3	45
NMR of tert-butanol in CDCl_3	45

TABLE OF CONTENTS (continued)

NMR of Acetylsalicylic Acid in CDCl_3	45
NMR of Acetylsalicylic Acid in Solvent System (tert-butanol / CDCl_3).....	45
Quantitative Analysis	46
NMR - High Concentration Acetylsalicylic Acid Methyl Peaks	46
NMR - High Concentration Acetylsalicylic Acid Aromatic Ring Proton Peaks	46
NMR - Low Concentration Acetylsalicylic Acid Methyl Peaks	47
NMR - Low Concentration Acetylsalicylic Acid Aromatic Ring Proton Peaks	48
HPLC - Low Concentration Acetylsalicylic Acid Peaks.....	49
Comparison of q-NMR vs. HPLC.....	49
Analysis Time Requirements.....	49
Solvent Management	49
Cost of Operation.....	50
Efficacy of Analysis	50
FUTURE WORK.....	50
CONCLUSION.....	51
REFERENCES	52
APPENDIX.....	53

LIST OF TABLES

Table 1. The NMR high concentration acetylsalicylic acid solutions average methyl peak areas	17
Table 2. The NMR high concentration acetylsalicylic acid solutions average aromatic ring H _A proton peak areas	19
Table 3. The NMR high concentration acetylsalicylic acid solutions average aromatic ring H _B proton peak areas	21
Table 4. The NMR high concentration acetylsalicylic acid solutions average aromatic ring H _C proton peak areas	23
Table 5. The NMR high concentration acetylsalicylic acid solutions average aromatic ring H _D proton peak areas	25
Table 6. The NMR high concentration acetylsalicylic acid solutions average aromatic ring H _A - H _D proton peak areas	27
Table 7. The NMR low concentration acetylsalicylic acid solutions average methyl peak areas	29
Table 8. The NMR low concentration acetylsalicylic acid solutions average aromatic ring H _A proton peak areas	31
Table 9. The NMR low concentration acetylsalicylic acid solutions average aromatic ring H _B proton peak areas	33
Table 10. The NMR low concentration acetylsalicylic acid solutions average aromatic ring H _C proton peak areas	35
Table 11. The NMR low concentration acetylsalicylic acid solutions average aromatic ring H _D proton peak areas	37
Table 12. The NMR low concentration acetylsalicylic acid solutions average aromatic ring H _A - H _D proton peak areas	39
Table 13. The NMR low concentration acetylsalicylic acid solutions average acetylsalicylic acid peak areas	42
Table 14. Compare low concentration acetylsalicylic acid q-NMR and HPLC data	44

LIST OF FIGURES

Figure 1. The NMR spectrum of CDCl_3	13
Figure 2. The NMR spectrum of 100.4 mM <i>tert</i> -butanol in CDCl_3	14
Figure 3. The NMR spectrum of 101.1 mM acetylsalicylic acid in CDCl_3	14
Figure 4. The NMR spectrum of 100.1 mM acetylsalicylic acid in solvent system (<i>tert</i> -butanol in CDCl_3).....	15
Figure 5. A close-up view of the NMR spectrum of 100.1 mM acetylsalicylic acid in solvent system (<i>tert</i> -butanol in CDCl_3).	15
Figure 6. The NMR spectrum of 10.1 mM acetylsalicylic acid in solvent system.....	16
Figure 7. A plot of the NMR high concentration acetylsalicylic acid solutions and the average methyl peak areas.....	18
Figure 8. A plot of the NMR high concentration acetylsalicylic acid solutions and the average aromatic ring H_A proton peak areas	20
Figure 9. A plot of the NMR high concentration acetylsalicylic acid solutions and the average aromatic ring H_B proton peak areas	22
Figure 10. A plot of the NMR high concentration acetylsalicylic acid solutions and the average aromatic ring H_C proton peak areas	24
Figure 11. A plot of the NMR high concentration acetylsalicylic acid solutions and the average aromatic ring H_D proton peak areas	26
Figure 12. A plot of the NMR high concentration acetylsalicylic acid solutions and the average aromatic ring $\text{H}_A - \text{H}_D$ proton peak areas	28
Figure 13. A plot of the NMR high concentration acetylsalicylic acid solutions average methyl peak areas and the average aromatic ring $\text{H}_A - \text{H}_D$ proton peak areas	28
Figure 14. A plot of the NMR low concentration acetylsalicylic acid solutions and the average methyl peak areas.....	30
Figure 15. A plot of the NMR low concentration acetylsalicylic acid solutions and the average aromatic ring H_A proton peak areas	32
Figure 16. A plot of the NMR low concentration acetylsalicylic acid solutions and the average aromatic ring H_B proton peak areas	34
Figure 17. A plot of the NMR low concentration acetylsalicylic acid solutions and the average aromatic ring H_C proton peak areas	36
Figure 18. A plot of the NMR low concentration acetylsalicylic acid solutions and the average aromatic ring H_D proton peak areas	38
Figure 19. A plot of the NMR low concentration acetylsalicylic acid solutions and the average aromatic ring $\text{H}_A - \text{H}_D$ proton peak areas	40
Figure 20. A plot of the NMR low concentration acetylsalicylic acid solutions average methyl peak areas and the average aromatic ring $\text{H}_A - \text{H}_D$ proton peak areas	40
Figure 21. The HPLC chromatograph of 10.0 mM acetylsalicylic acid in solvent system	431
Figure 22. A plot of the HPLC low concentration acetylsalicylic acid solutions and the average acetylsalicylic acid peak areas	43

ABSTRACT

Quantitative analysis is an important step during pharmaceutical drug development. Currently, the pharmaceutical industry uses high performance liquid chromatography (HPLC) for quantitative analysis of organic compounds as the preferred technique. The proton nuclear magnetic resonance (NMR) technique has an inherent ability to quantify a given analyte and may be used to quantify organic compounds. Quantitative nuclear magnetic resonance spectroscopy (q-NMR) of acetylsalicylic acid (ASA) was conducted using *tert*-butyl alcohol (*tert*-butanol) as the internal standard and deuterated chloroform (CDCl₃) as the solvent. Preparations of ASA solutions in low and high concentration ranges, 1.0 mM – 10.1 mM and 10.0 mM – 100.1 mM respectively, were analyzed to determine a linear correlation between the concentrations of ASA with the intensities of methyl peaks and aromatic ring proton peaks using Topspin software.

The q-NMR analysis of high concentration ASA solutions showed a strong linear correlation (R^2 values > 0.9994) and high precision (% RSD values < 1%) for both the average methyl peak areas and the average aromatic ring proton peak areas. However, the low concentration ASA solutions showed a weak linear correlation (R^2 values > 0.9914) but, fairly good precision (% RSD values < 5%) for both the average methyl peak areas and the average aromatic ring proton peak areas. Therefore, the q-NMR technique is a viable alternative for quantitative analysis of high concentration ASA methyl peak areas and aromatic ring proton peak areas, but it is not suitable for the low concentration ASA solutions.

The low concentration (1.0 mM – 10.1 mM) ASA solutions were also analyzed by the conventional HPLC method; the results obtained were compared with the results from the q-NMR technique. A comparison of q-NMR data to HPLC data focused on linearity, precision, and acquisition time for the acetylsalicylic acid solutions at low concentrations. The HPLC data showed a strong linear correlation with an R^2 value of 0.9997 and high precision with a % RSD < 0.9% for the average ASA peak areas. A comparison of q-NMR data to HPLC data for the low concentration ASA solutions showed that the q-NMR technique used less solvents and less time for data acquisition.

Keywords: Proton nuclear magnetic resonance, spectroscopy, q-NMR, chromatography, HPLC

INTRODUCTION

The ability to quantify an active pharmaceutical ingredient (API) is a crucial step in drug development. Currently, in the pharmaceutical industry high performance liquid chromatography (HPLC) is the preferred technique for purity determinations due to its selectivity and reproducibility. HPLC implements a combination of mobile phases and stationary phases (analytical column) for quantitatively separating organic compounds. The mobile phase flows through the HPLC system and a volume (microliters) of analyte is injected into the mobile phase (1). The components of the analyte elute from the HPLC column according to their affinity to the stationary phase and mobile phase (1). As the components pass thru the flow cell, the ultraviolet-visible (UV-VIS) detector measures the absorbance of the analyte (1). The area under the peak is directly proportional to the concentration of the compound in solution.

Quantitative nuclear magnetic resonance (q-NMR) is gaining attention as a viable alternative technique for quantification of organic compounds. Proton nuclear magnetic resonance (NMR) spectroscopy is a powerful technique, which is mainly used for providing information for elucidating structures of organic compounds. The NMR spectrometer applies a magnetic field to the organic compound in solution; the protons are excited to a higher energy level after absorbing electromagnetic energy in the form of radio frequencies (2). The NMR spectrum is a plot of the frequencies of the absorption peaks versus the peak intensities (2). Proton (NMR) technique has an inherent ability to quantify organic compounds (3). By means of quantitative nuclear magnetic resonance (q-NMR) spectroscopy technique, various pharmaceutical ingredients have been utilized for quantification of organic compounds using an internal standard (4, 5). The area under the q-NMR signal is directly proportional to the number of protons for a given peak, and hence directly proportional to the concentration of the compound in solution.

The cost of acquiring and operating HPLC instrumentation is increasing as the chromatographic technology advances. The time necessary to development HPLC methods requires many operator hours, multiple columns, and a high volume of solvents (mobile phase) leading to increased waste disposal. However, it costs less to obtain an HPLC than a NMR spectrophotometer. The q-NMR method development does not use columns, requires less operator time, and uses less solvent, therefore decreasing waste disposal. The cost of HPLC and the q-NMR technique for quantitative analysis are becoming comparable, thus making q-NMR a viable option.

Acetylsalicylic acid (ASA) is the active pharmaceutical ingredient in Aspirin, an anti-inflammatory pharmaceutical for the treatment of pain and fever. In a previous study, ASA was analyzed by q-NMR using ethanol as an internal standard and dimethyl sulfoxide-d6 (DMSO-d6) as the solvent (6). In this study, the q-NMR analysis of ASA showed linearity in respects to the concentrations of ASA in solutions with the methyl peak areas and aromatic ring proton peak areas. In another study, two pharmaceuticals were analyzed by q-NMR, acetaminophen the API in Tylenol (treats minor aches, pains) and ASA. In this study, the separate acetaminophen and ASA solutions were prepared with diethyl ether (internal standard) and deuterated chloroform (CDCl₃) as the solvent (7). The methyl peak areas and aromatic ring proton peak areas showed linearity in respects to the concentrations of the acetaminophen and ASA solutions.

In the present study, the q-NMR technique is used to quantify acetylsalicylic acid solutions from 1 mM – 100 mM using *tert*-butanol as the internal standard and deuterated chloroform (CDCl₃) as the solvent. The linear correlation was determined by plotting the acetylsalicylic acid concentrations versus the average methyl peak areas and the average aromatic ring proton peak areas; the precision was determined by calculating the percent relative standard deviation (% RSD). A comparison of q-NMR data to HPLC data focused on linearity, precision, solvent management, operation costs, and acquisition time for the acetylsalicylic acid solutions at low concentrations.

MATERIALS AND INSTRUMENTATION

Materials

Acetylsalicylic acid (Sigma-Aldrich, CAS: 50-78-2, $\geq 99.0\%$), deuterated chloroform (Sigma-Aldrich, CAS: 865-49-6, for NMR 99.8 atom D), and acetic acid (Sigma-Aldrich, CAS: 64-19-7, $\geq 99.0\%$), were obtained from Sigma-Aldrich. Methanol (Honeywell, CAS: 67-56-1, HPLC), water (Honeywell, CAS: 7732-18-5, HPLC), and *tert*-butanol (Fisher, CAS: 75-65-0), was obtained. The HPLC analytical column was a Keystone BDS or Hypersil, C-18, (15 cm x 4.6 mm x 5 μ m). The glassware used included glass pipets, 1 mL graduated syringe, NMR tubes (Kimax), HPLC vials (Agilent Technologies), volumetric flasks (Kimax) 1 mL, 10 mL, 25 mL and a 100 mL graduated cylinder (Kimax).

Instrumentation

Analytical balances, a 300MHz Bruker Nuclear Magnetic Resonance Spectrometer (NMR), and an Agilent 1260 High Performance Liquid Chromatograph (HPLC) were used for this study.

EXPERIMENTAL PROCEDURES

NMR Test Solutions

Solubility of Acetylsalicylic Acid

A solubility test of acetylsalicylic acid (ASA) was successfully performed using about five mg ASA and 1.0 mL of deuterated chloroform (CDCl_3).

Preparation of Acetylsalicylic Acid Solvent System

A 100.4 mM *tert*-butanol (internal standard) solvent system was prepared by adding 186.0 mg of *tert*-butanol to CDCl_3 in a 25 mL volumetric flask for the high concentration ASA solutions. A 10.3 mM *tert*-butanol solvent system was prepared by adding 18.53 mg of *tert*-butanol to CDCl_3 in a 25 mL volumetric flask for the low concentration ASA solutions.

Preparation of High and Low Concentration Acetylsalicylic Acid Stock Solutions

A 100.1 mM stock solution of ASA was prepared by adding 180.3 mg of ASA to the solvent system in a 10 mL volumetric flask. A 10.1 mM stock solution of ASA was prepared by adding 18.2 mg of ASA to the solvent system in a 10 mL volumetric flask.

Preparation of High and Low Concentration Acetylsalicylic Acid Diluted Solutions

Preparation of the high concentration acetylsalicylic acid solutions 80.1 mM, 60.1 mM, 40.0 mM, 20.0 mM and 10.0 mM was accomplished by adding aliquots of the ASA stock solution (0.8 mL, 0.6 mL, 0.4 mL, 0.2 mL, 0.1 mL) to the solvent system in a 1 mL volumetric flask. Preparation of the 8.1 mM, 6.1 mM, 4.0 mM, 2.0 mM, and 1.0 mM low concentration ASA solutions was accomplished by adding aliquots of the ASA stock solution (0.8 mL, 0.6 mL, 0.4 mL, 0.2 mL, 0.1 mL) to the solvent system in a 1 mL volumetric flask.

HPLC Test Solutions

Solubility of Acetylsalicylic Acid

A solubility test of acetylsalicylic acid (ASA) was successfully performed using about five mg ASA and 1.0 mL of 95:5 methanol / glacial acetic acid.

Preparation of Acetylsalicylic Acid Solvent System

A 95:5 methanol / glacial acetic acid solvent system was prepared by adding 2.5 mL glacial acetic acid to methanol in a 50 mL volumetric flask for the low concentration ASA solutions.

Preparation of Low Concentration Acetylsalicylic Acid Stock Solutions

A 10.0 mM stock solution of ASA was prepared by adding 18.0 mg of ASA to the solvent system in a 10 mL volumetric flask.

Preparation of Low Concentration Acetylsalicylic Acid Diluted Solutions

Preparation of the 8.0 mM, 6.0 mM, 4.0 mM, 2.0 mM, and 1.0 mM low concentration ASA solutions was accomplished by adding aliquots of the ASA stock solution (0.8 mL, 0.6 mL, 0.4 mL, 0.2 mL, 0.1 mL) to the solvent system in a 1 mL volumetric flask.

Preparation of Mobile Phase

The mobile phase was composed of water, methanol, and glacial acetic acid at this ratio 69:28:3, respectively (8). A liter of mobile phase was prepared by combining 690 mL water, 280 mL methanol, and 30 mL glacial acetic acid.

General Procedure for Operation of the Bruker NMR Spectrometer

The procedure for acquiring NMR spectra for the Bruker 300 MHz NMR spectrophotometer with Topspin 3.2 software was as follows:

- Create a new data set using start > create data set.
- Use “rpar” command to choose experiment parameters for the proton-NMR spectrum. Select proton-all.
- Select “lift” to eject previous sample.
- Place 0.7 mL of the solution in the NMR tube and insert it in the NMR probe through a turbine.
- Use “ij” to inject present sample.
- Use “lock” command to lock the signal of the solvent.
- Use “tune” command to tune instrument to solvent.
- Use “prosol single pulse calibration” to acquire the 90° pulse value at -8.2 dB
- Use “rga” command to check the receiver gain of the amplifier.
- Use “zg” command to begin the experiment and acquire data in the free induction decay (FID) format.
- Use “efp” command to convert the FID format to a frequency domain spectrum.
- Use “apk” to correct the baseline.
- Use “Integrate” to obtain peak areas. TE Topspin 3.2 software assigns an area of 1.000 for the first peak and the other peaks are assigned numbers proportional to their areas.
- Use “xwinplot” command to display the spectrum with legends.

General Procedure for Operation of the Agilent HPLC

The procedure for acquiring HPLC spectra for the Agilent 1260 High Performance Liquid Chromatograph with ChemStation software was as follows:

- Turn on the column heater (column temperature 4°C).
- Turn on the ultraviolet or visible light detector (wavelength 275 nm).
- Connect the HPLC analytical column.
- Condition the column with the mobile phase (flow rate 1.5 ml/min).
- Create a new method set.
- Create a new sample set.
- Perform the sample analysis

RESULTS

In this study, various solutions were analyzed; their proton-NMR spectra, chromatograms, tables and plots are below. The proton-NMR spectrum of CDCl_3 shows the chemical shifts of the residual protons of the solvent (CDCl_3) peak and unknown impurity peak that was observed, but could not be reproduced. The internal standard (*tert*-butanol) peak is the most intense peak of the solvent system (CDCl_3 / *tert*-butanol). The acetylsalicylic acid (ASA) methyl peaks and aromatic ring peaks are distinct and separate from the peaks created by the solvent system.

The ASA high and low concentration solutions were analyzed in septuplicate, tables were created using the ASA methyl peak areas and aromatic ring peak areas, the average methyl peak areas and average aromatic ring peak areas were used to calculate % RSD values as seen in the tables. Separate plots of the ASA concentrations vs. average methyl peak areas and the average aromatic ring peak areas are used to show the linear correlation between the ASA concentrations and the peak areas. Using the plots, R^2 values were also calculated.

NMR Spectra

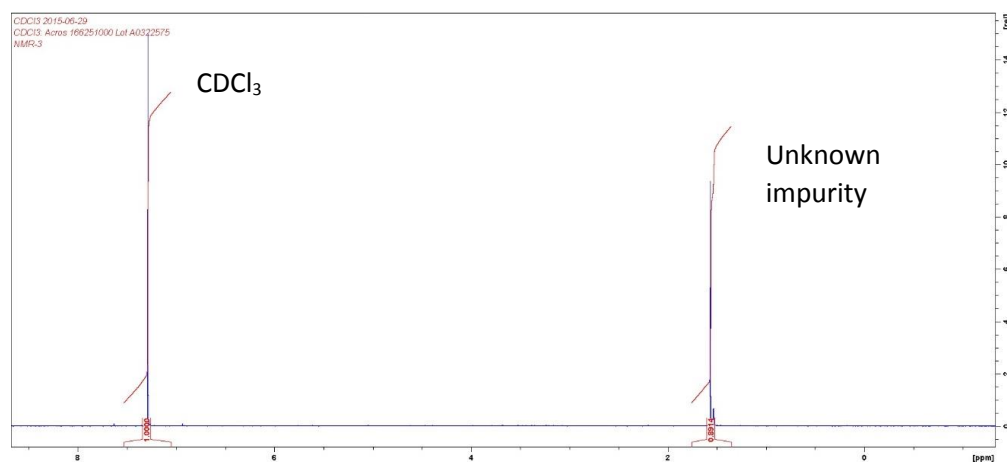


Figure 1. The NMR spectrum of CDCl_3 , shows a residual solvent proton peak at 7.3 ppm and an impurity peak at 1.5 ppm that was not reproducible.

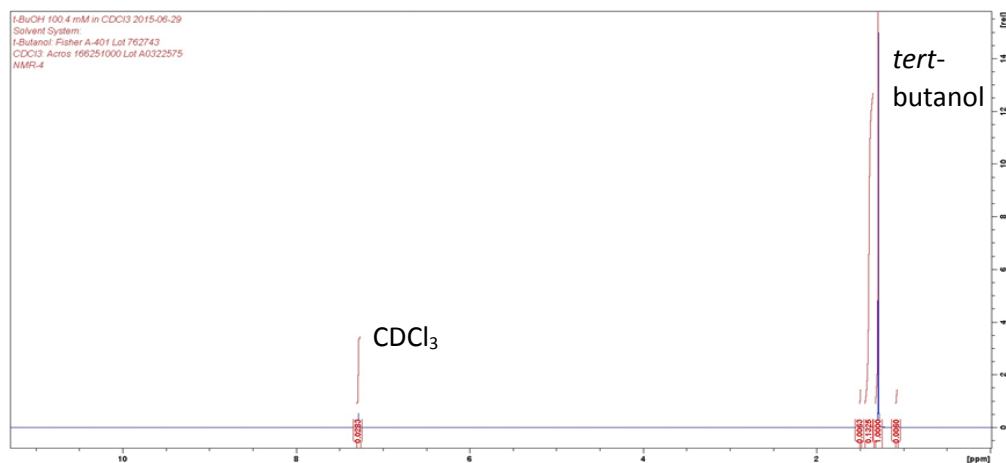


Figure 2. The NMR spectrum of 100.4 mM *tert*-butanol in CDCl_3 , shown the *tert*-butanol methyl peak is at 1.4 ppm and the CDCl_3 residual solvent proton peak is at 7.3.

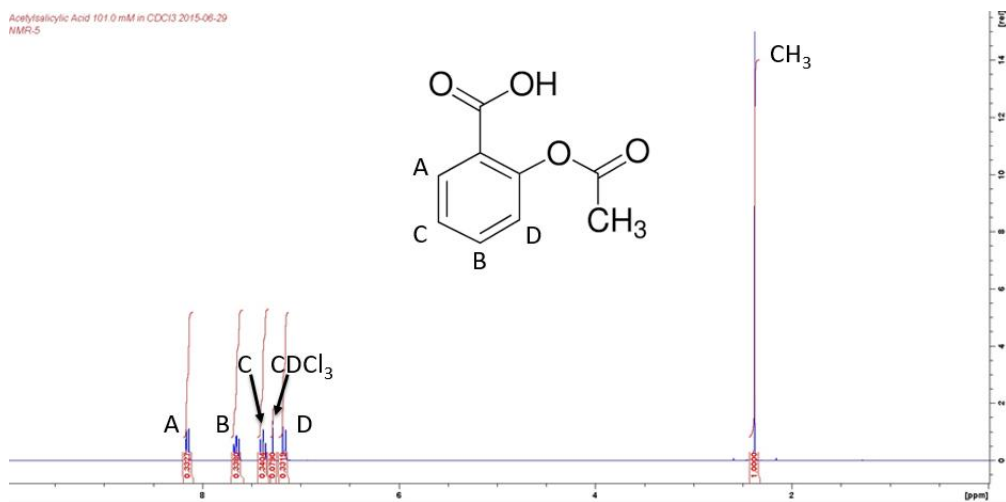


Figure 3. The NMR spectrum of 101.1 mM acetylsalicylic acid in CDCl_3 , shows an ASA methyl peak at 2.4 ppm. The ASA aromatic ring group proton peaks appear at 7.1 ppm, 7.4 ppm, 7.7 ppm, and 8.2 ppm. The CDCl_3 displays a residual solvent proton peak at 7.3 ppm.

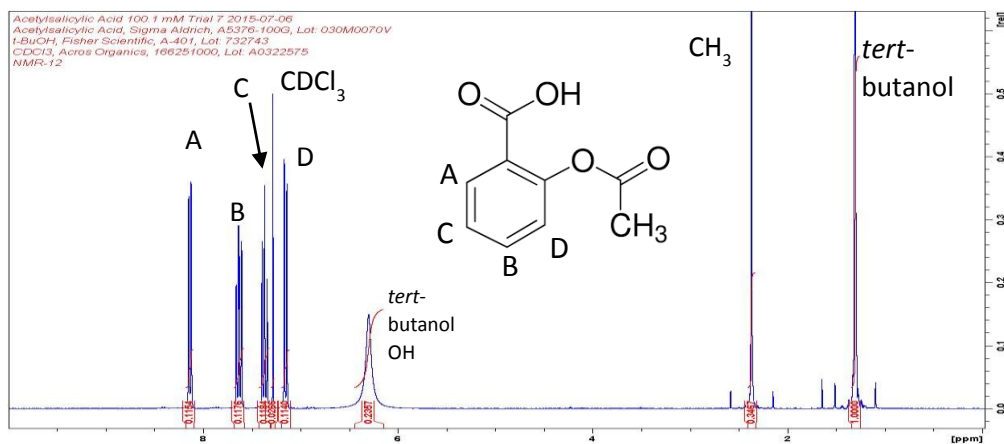


Figure 4. The NMR spectrum of 100.1 mM acetylsalicylic acid in solvent system (*tert*-butanol in CDCl₃) displays an ASA methyl peak at 2.4 ppm. The ASA aromatic ring group proton peaks appear at 7.1 ppm, 7.4 ppm, 7.7 ppm, and 8.2 ppm. The CDCl₃ displays residual solvent proton a peak at 7.3 ppm. The internal standard *tert*-butanol displays a methyl peak 1.4 and a hydroxyl peak at 6.3 ppm.

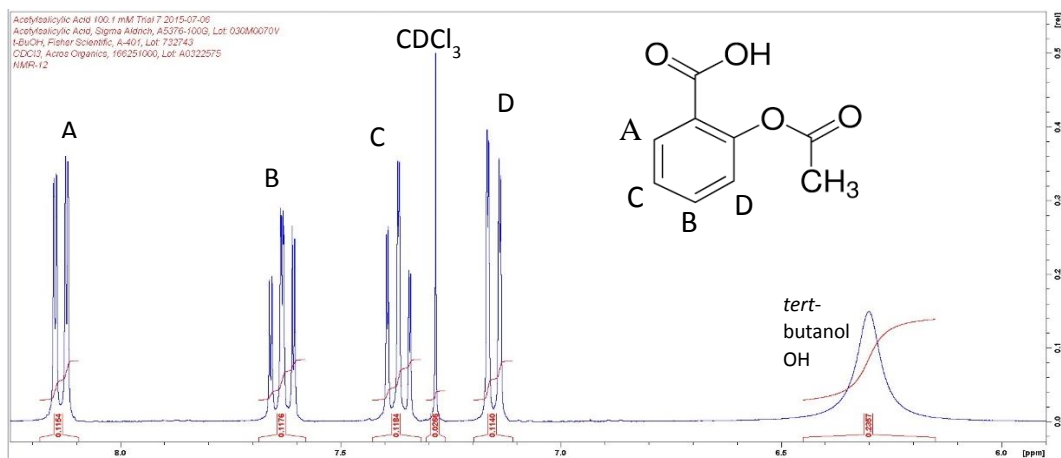


Figure 5. A close-up view of the NMR spectrum of 100.1 mM acetylsalicylic acid in solvent system (*tert*-butanol in CDCl₃), showing aromatic ring group peaks at 7.1 ppm, 7.4 ppm, 7.7 ppm, and 8.2 ppm. The CDCl₃ displays a residual solvent proton peak at 7.3 ppm. The internal standard *tert*-butanol displays a hydroxyl peak at 6.3 ppm.

NMR Analysis of High Concentration Acetylsalicylic Acid Solutions

Table 1. The NMR high concentration acetylsalicylic acid solutions: Average methyl peak areas, the % RSD ranged from 0.21275% (100.1 mM) – 0.70522% (10.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	Methyl Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	10.0 mM	1	0.03570	0.03609	0.00025	0.70522
		2	0.03630			
		3	0.03590			
		4	0.03620			
		5	0.03590			
		6	0.03620			
		7	0.03640			
2	20.0 mM	1	0.07300	0.07294	0.00038	0.52040
		2	0.07265			
		3	0.07250			
		4	0.07252			
		5	0.07322			
		6	0.07322			
		7	0.07344			
3	40.0 mM	1	0.13571	0.13644	0.00071	0.52326
		2	0.13604			
		3	0.13783			
		4	0.13641			
		5	0.13619			
		6	0.13601			
		7	0.13687			
4	60.1 mM	1	0.20414	0.20471	0.00074	0.36191
		2	0.20517			
		3	0.20357			
		4	0.20447			
		5	0.20584			
		6	0.20506			
		7	0.20470			
5	80.1 mM	1	0.27935	0.27890	0.00094	0.33848
		2	0.28014			
		3	0.27937			
		4	0.27885			
		5	0.27729			
		6	0.27807			
		7	0.27922			
6	100.1 mM	1	*	0.34655	0.00074	0.21275
		2	0.34583			
		3	0.34624			
		4	0.34784			
		5	0.34670			
		6	0.34593			
		7	0.34673			

* Represents data that could not be obtained due to a software error during the saving process.

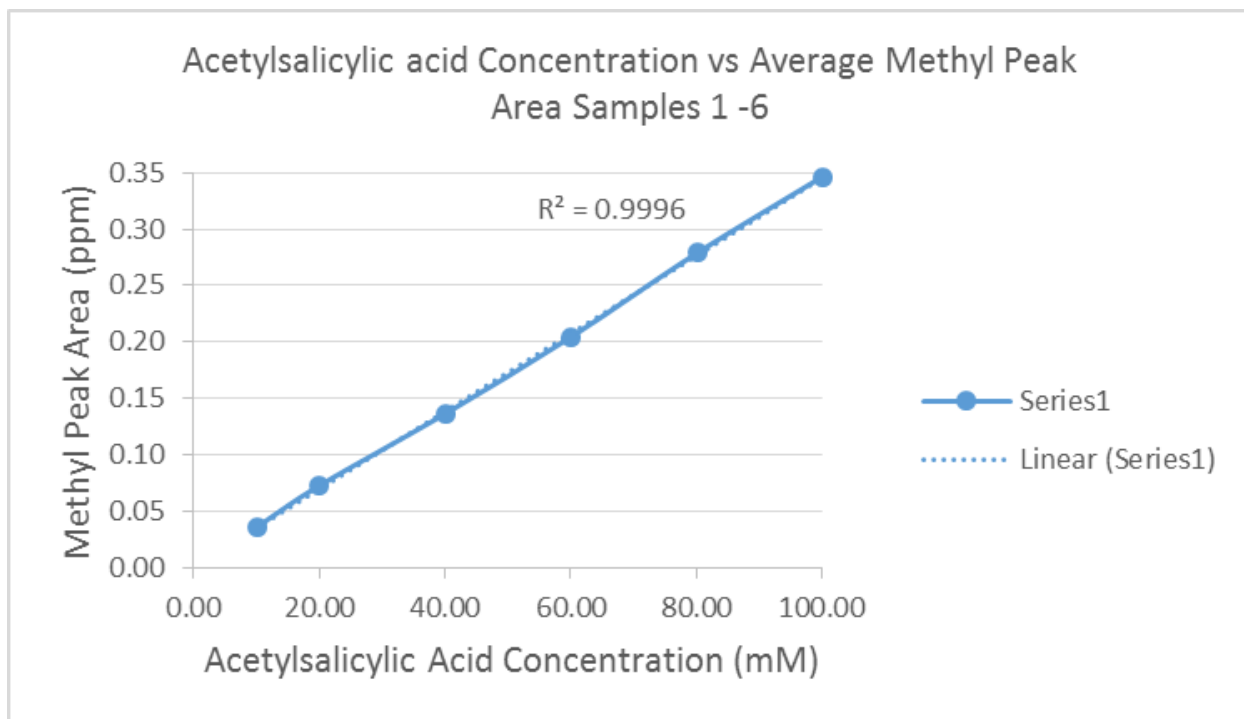


Figure 7. A plot of the NMR high concentration acetylsalicylic acid solutions (10.0 mM - 100.1 mM) and the average methyl peak areas shows a R^2 value of 0.9996.

Table 2. The NMR high concentration acetylsalicylic acid solutions: Average aromatic ring H_A proton peak areas, the % RSD ranged from 0.19161% (10.0 mM) – 0.73369% (100.1 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	H _A of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	10.0 mM	1	0.01166	0.01167	0.00002	0.19161
		2	0.01171			
		3	0.01168			
		4	0.01166			
		5	0.01166			
		6	0.01164			
		7	0.01168			
2	20.0 mM	1	0.02363	0.02392	0.00018	0.73369
		2	0.02376			
		3	0.02388			
		4	0.02398			
		5	0.02399			
		6	0.02411			
		7	0.02409			
3	40.0 mM	1	0.04526	0.04534	0.00022	0.47685
		2	0.04519			
		3	0.04569			
		4	0.04513			
		5	0.04559			
		6	0.04534			
		7	0.04519			
4	60.1 mM	1	0.06783	0.06801	0.00025	0.36306
		2	0.06797			
		3	0.06818			
		4	0.06778			
		5	0.06848			
		6	0.06795			
		7	0.06785			
5	80.1 mM	1	0.09350	0.09308	0.00063	0.67998
		2	0.09310			
		3	0.09322			
		4	0.09228			
		5	0.09275			
		6	0.09417			
		7	0.09256			
6	100.1 mM	1	*	0.11537	0.00027	0.23137
		2	0.11531			
		3	0.11530			
		4	0.11584			
		5	0.11503			
		6	0.11530			
		7	0.11545			

* Represents data that could not be obtained due to a software error during the saving process.

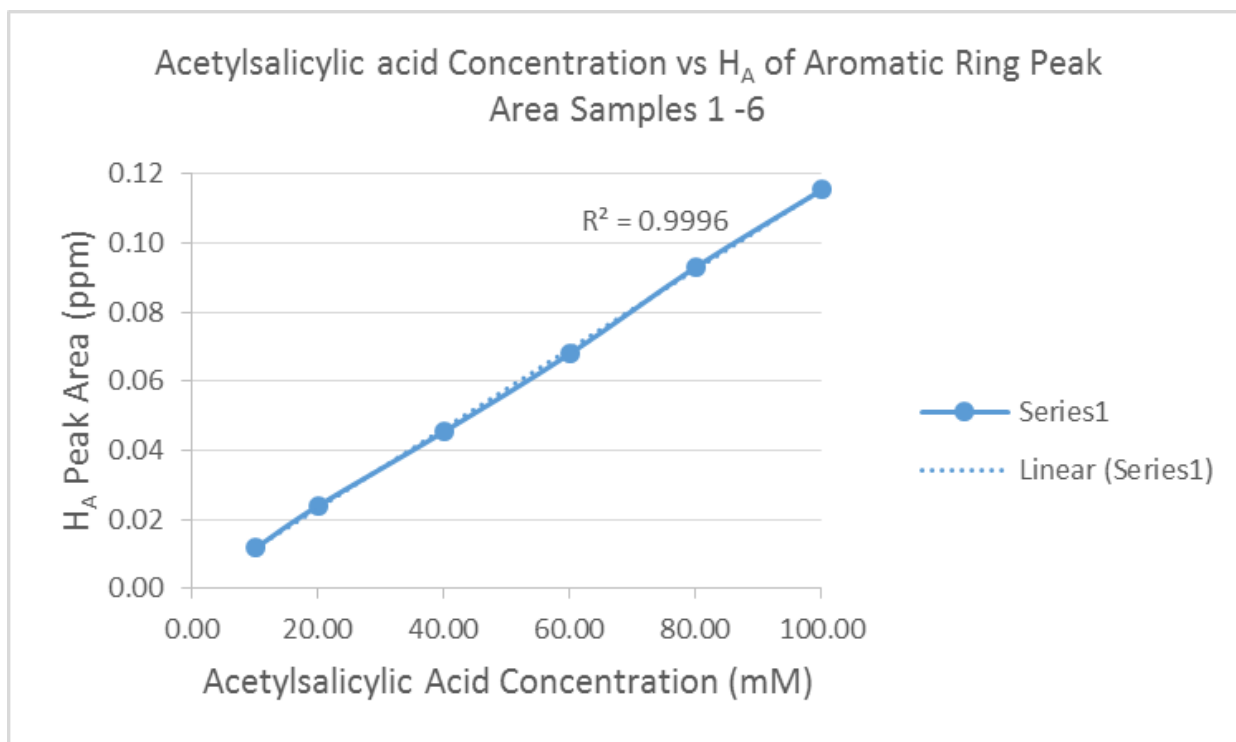


Figure 8. A plot of the NMR high concentration acetylsalicylic acid solutions (10.0 mM - 100.1 mM) and the average aromatic ring H_A proton peak areas shows a R^2 value of 0.9996.

Table 3. The NMR high concentration acetylsalicylic acid solutions: Average aromatic ring H_B proton peak areas, the % RSD ranged from 0.34918% (10.0 mM) – 1.05501% (20.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	H _B of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	10.0 mM	1	0.01217	0.01220	0.00004	0.34918
		2	0.01215			
		3	0.01221			
		4	0.01215			
		5	0.01222			
		6	0.01223			
		7	0.01226			
2	20.0 mM	1	0.02440	0.02453	0.00026	1.05501
		2	0.02422			
		3	0.02423			
		4	0.02465			
		5	0.02463			
		6	0.02468			
		7	0.02492			
3	40.0 mM	1	0.04617	0.04613	0.00025	0.54382
		2	0.04612			
		3	0.04652			
		4	0.04630			
		5	0.04616			
		6	0.04585			
		7	0.04579			
4	60.1 mM	1	0.06916	0.06921	0.00029	0.41438
		2	0.06949			
		3	0.06925			
		4	0.06924			
		5	0.06962			
		6	0.06891			
		7	0.06882			
5	80.1 mM	1	0.09493	0.09503	0.00075	0.78831
		2	0.09531			
		3	0.09562			
		4	0.09407			
		5	0.09460			
		6	0.09624			
		7	0.09443			
6	100.1 mM	1	*	0.11757	0.00050	0.42484
		2	0.11794			
		3	0.11753			
		4	0.11823			
		5	0.11679			
		6	0.11732			
		7	0.11762			

* Represents data that could not be obtained due to a software error during the saving process.

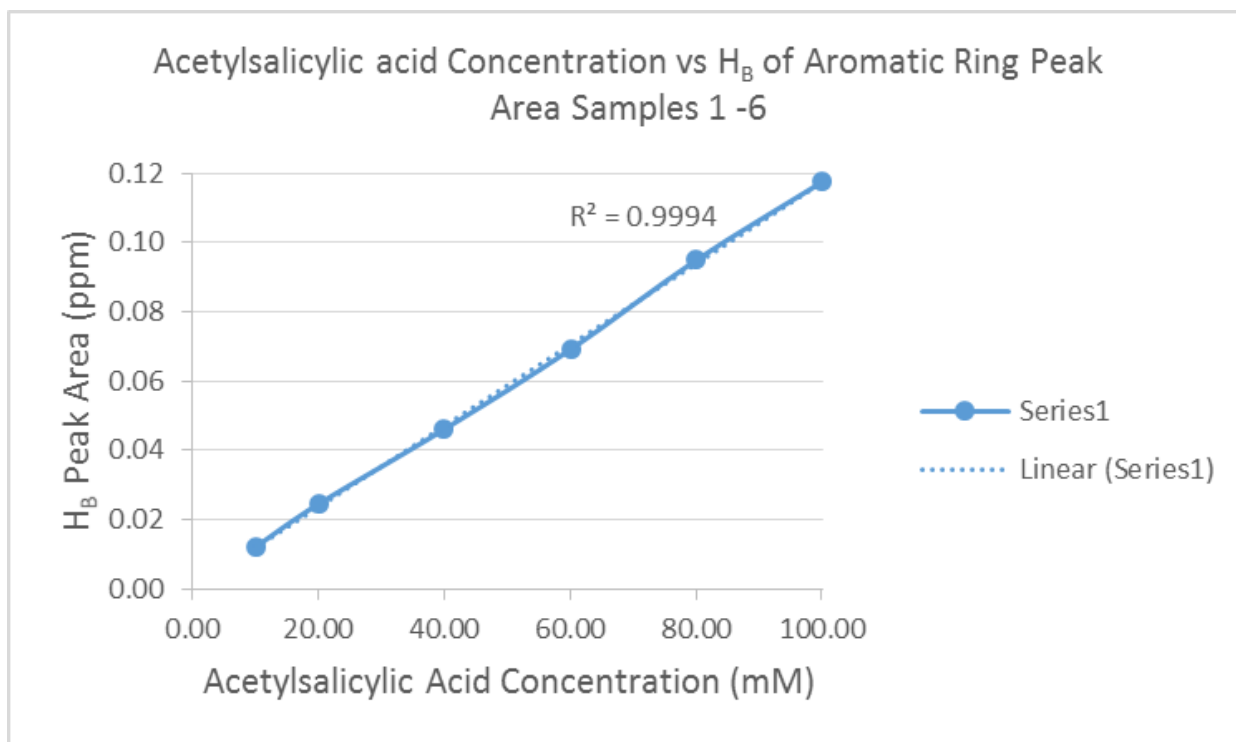


Figure 9. A plot of the NMR high concentration acetylsalicylic acid solutions (10.0 mM - 100.1 mM) and the average aromatic ring H_B proton peak areas shows a R^2 value of 0.9994.

Table 4. The NMR high concentration acetylsalicylic acid solutions: Average aromatic ring H_c proton peak areas, the % RSD ranged from 0.36002% (100.1 mM) – 1.11126% (20.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	H _c of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	10.0 mM	1	0.01208	0.01223	0.00013	1.04071
		2	0.01218			
		3	0.01217			
		4	0.01212			
		5	0.01227			
		6	0.01241			
		7	0.01238			
2	20.0 mM	1	0.02429	0.02456	0.00027	1.11126
		2	0.02421			
		3	0.02441			
		4	0.02474			
		5	0.02485			
		6	0.02450			
		7	0.02489			
3	40.0 mM	1	0.04629	0.04621	0.00032	0.68992
		2	0.04611			
		3	0.04660			
		4	0.04629			
		5	0.04656			
		6	0.04583			
		7	0.04580			
4	60.1 mM	1	0.06936	0.06960	0.00035	0.50075
		2	0.06987			
		3	0.06964			
		4	0.06957			
		5	0.07006			
		6	0.06971			
		7	0.06899			
5	80.1 mM	1	0.09490	0.09522	0.00039	0.40868
		2	0.09534			
		3	0.09547			
		4	0.09474			
		5	0.09508			
		6	0.09590			
		7	0.09509			
6	100.1 mM	1	*	0.11823	0.00043	0.36002
		2	0.11856			
		3	0.11804			
		4	0.11881			
		5	0.11774			
		6	0.11784			
		7	0.11841			

* Represents data that could not be obtained due to a software error during the saving process.

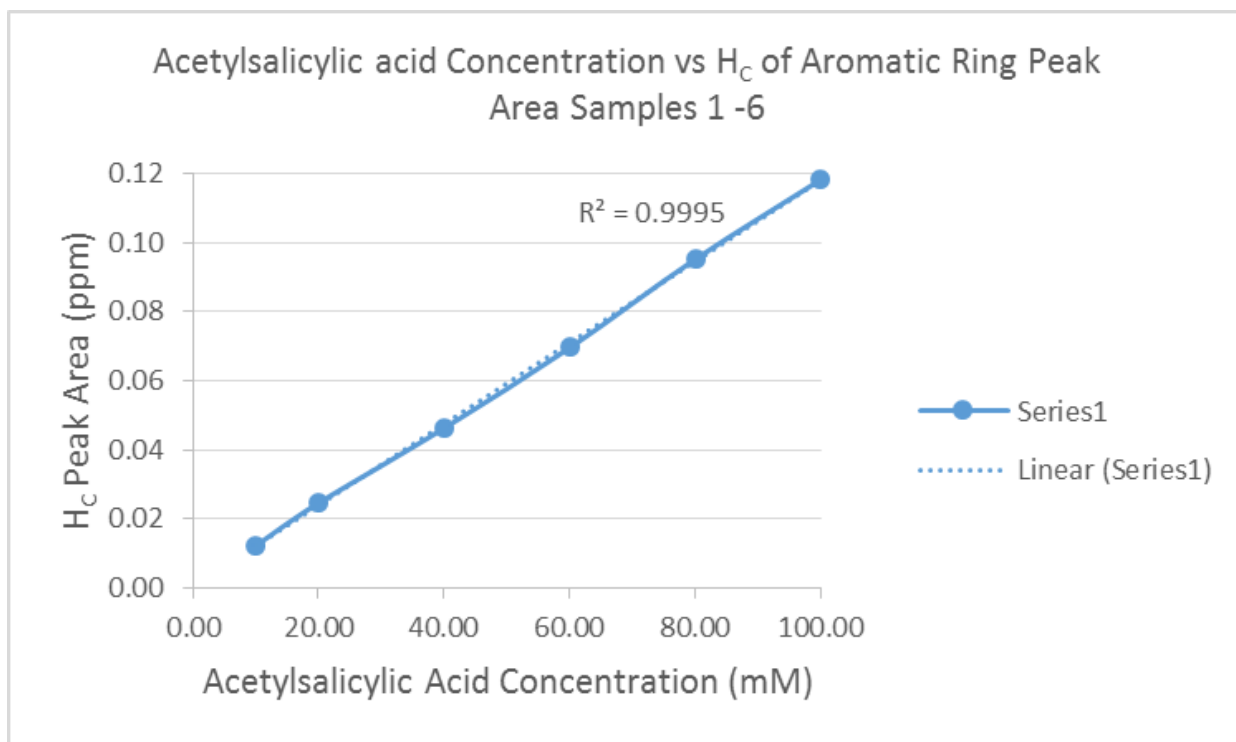


Figure 10. A plot of the NMR high concentration acetylsalicylic acid solutions (10.0 mM - 100.1 mM) and the average aromatic ring H_C proton peak areas shows a R² value of 0.9995.

Table 5. The NMR high concentration acetylsalicylic acid solutions: Average aromatic ring H_D proton peak areas, the % RSD ranged from 0.27292% (100.1 mM) – 0.62974% (20.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	H _D of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	10.0 mM	1	0.01131	0.01145	0.00007	0.62974
		2	0.01151			
		3	0.01151			
		4	0.01147			
		5	0.01142			
		6	0.01141			
		7	0.01149			
2	20.0 mM	1	0.02388	0.02383	0.00008	0.33435
		2	0.02384			
		3	0.02371			
		4	0.02394			
		5	0.02376			
		6	0.02379			
		7	0.02388			
3	40.0 mM	1	0.04527	0.04532	0.00028	0.62127
		2	0.04533			
		3	0.04589			
		4	0.04507			
		5	0.04541			
		6	0.04506			
		7	0.04522			
4	60.1 mM	1	0.06720	0.06742	0.00035	0.51426
		2	0.06764			
		3	0.06748			
		4	0.06717			
		5	0.06798			
		6	0.06754			
		7	0.06694			
5	80.1 mM	1	0.09113	0.09107	0.00025	0.27452
		2	0.09103			
		3	0.09060			
		4	0.09115			
		5	0.09131			
		6	0.09092			
		7	0.09132			
6	100.1 mM	1	*	0.11387	0.00031	0.27292
		2	0.11417			
		3	0.11347			
		4	0.11426			
		5	0.11372			
		6	0.11365			
		7	0.11397			

* Represents data that could not be obtained due to a software error during the saving process.

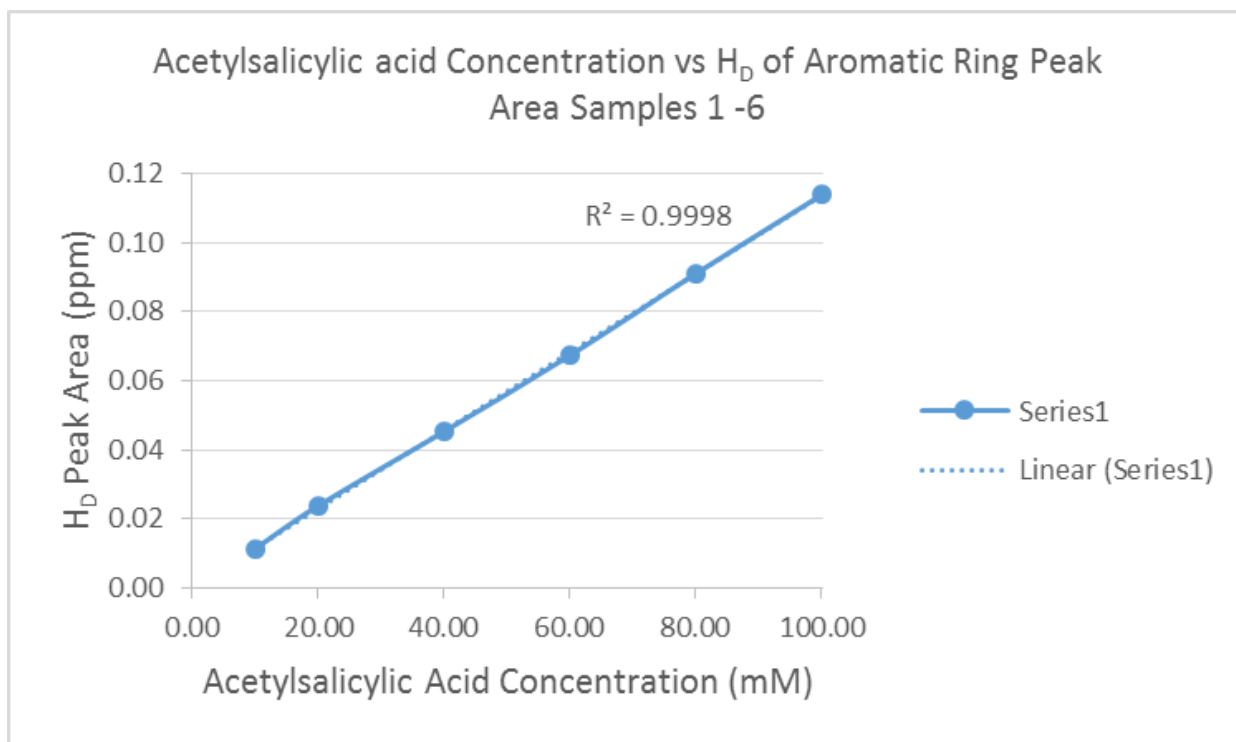


Figure 11. A plot of the NMR high concentration acetylsalicylic acid solutions (10.0 mM - 100.1 mM) and the average aromatic ring H_D proton peak areas shows a R² value of 0.9998.

Table 6. The NMR high concentration acetylsalicylic acid solutions: Average aromatic ring H_A - H_D proton peak areas, the % RSD ranged from 0.30278% (100.1 mM) – 0.69936% (20.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	Average H _A - H _D of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	10.0 mM	1	0.04722	0.04754	0.00019	0.40199
		2	0.04755			
		3	0.04757			
		4	0.04740			
		5	0.04757			
		6	0.04769			
		7	0.04781			
2	20.0 mM	1	0.09620	0.09684	0.00068	0.69936
		2	0.09603			
		3	0.09623			
		4	0.09731			
		5	0.09723			
		6	0.09708			
		7	0.09778			
3	40.0 mM	1	0.18299	0.18300	0.00095	0.51654
		2	0.18275			
		3	0.18470			
		4	0.18279			
		5	0.18372			
		6	0.18208			
		7	0.18200			
4	60.1 mM	1	0.27355	0.27424	0.00113	0.41179
		2	0.27497			
		3	0.27455			
		4	0.27376			
		5	0.27614			
		6	0.27411			
		7	0.27260			
5	80.1 mM	1	0.37446	0.37439	0.00156	0.41548
		2	0.37478			
		3	0.37491			
		4	0.37224			
		5	0.37374			
		6	0.37723			
		7	0.37340			
6	100.1 mM	1	*	0.46505	0.00141	0.30278
		2	0.46598			
		3	0.46434			
		4	0.46714			
		5	0.46328			
		6	0.46411			
		7	0.46545			

* Represents data that could not be obtained due to a software error during the saving process.

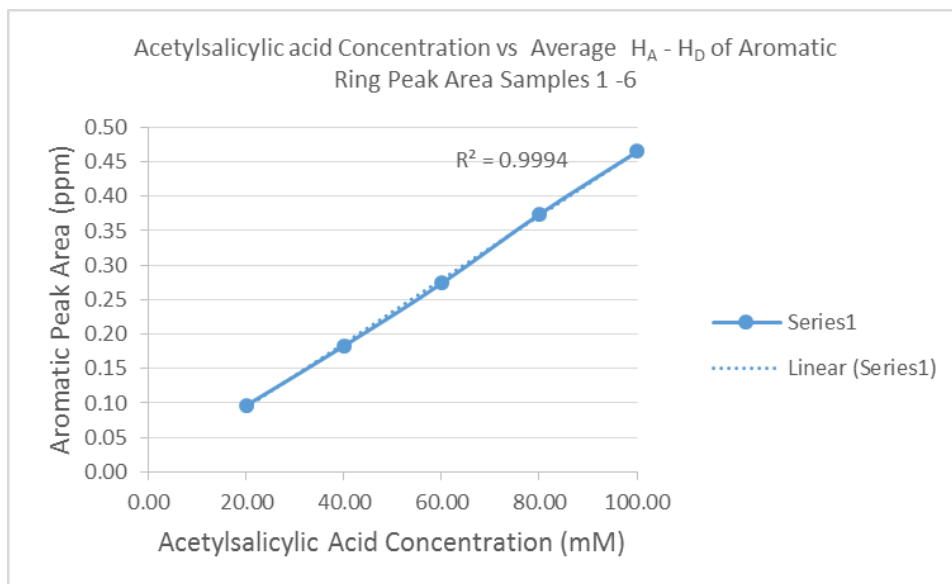


Figure 12. A plot of the NMR high concentration acetylsalicylic acid solutions (10.0 mM - 100.1 mM) and the average aromatic ring $H_A - H_D$ proton peak areas shows a R^2 value of 0.9994.

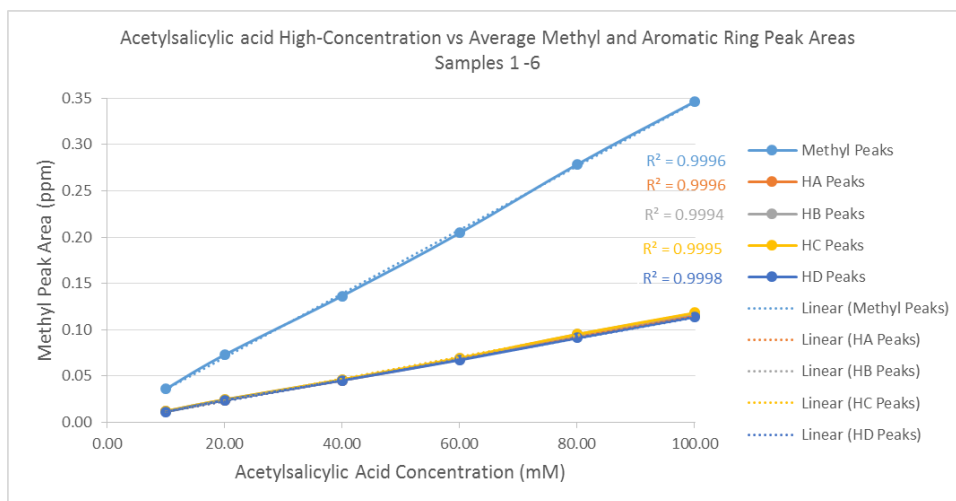


Figure 13. A plot of the NMR high concentration acetylsalicylic acid solutions (10.0 mM - 100.1 mM) average methyl peak areas and the average aromatic ring $H_A - H_D$ proton peak areas shows the overall linearity.

NMR Analysis of Low Concentration Acetylsalicylic Acid Solutions

Table 7. The NMR low concentration acetylsalicylic acid solutions: Average methyl peak areas, the % RSD ranged from 0.94809% (2.0 mM) – 2.22860% (1.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	Methyl Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	1.0 mM	1	0.03486	0.03590	0.00080	2.22860
		2	*			
		3	0.03568			
		4	0.03568			
		5	0.03590			
		6	0.03598			
		7	0.03732			
2	2.0 mM	1	0.08043	0.07999	0.00076	0.94809
		2	0.07994			
		3	0.08053			
		4	0.07893			
		5	0.07893			
		6	0.08070			
		7	0.08045			
3	4.0 mM	1	0.14600	0.14459	0.00161	1.11634
		2	0.14252			
		3	0.14433			
		4	0.14589			
		5	0.14390			
		6	0.14664			
		7	0.14288			
4	6.1 mM	1	0.24709	0.24725	0.00480	1.94265
		2	0.24531			
		3	0.24280			
		4	0.25318			
		5	0.24873			
		6	0.25304			
		7	0.24062			
5	8.1 mM	1	0.27110	0.26705	0.00457	1.70966
		2	0.27181			
		3	0.27181			
		4	0.26660			
		5	0.26455			
		6	0.26119			
		7	0.26230			
6	10.1 mM	1	0.30508	0.30383	0.00305	1.00238
		2	0.30185			
		3	0.30356			
		4	0.30732			
		5	0.30003			
		6	0.30114			
		7	0.30786			

* Represents data that could not be obtained due to a software error during the saving process.

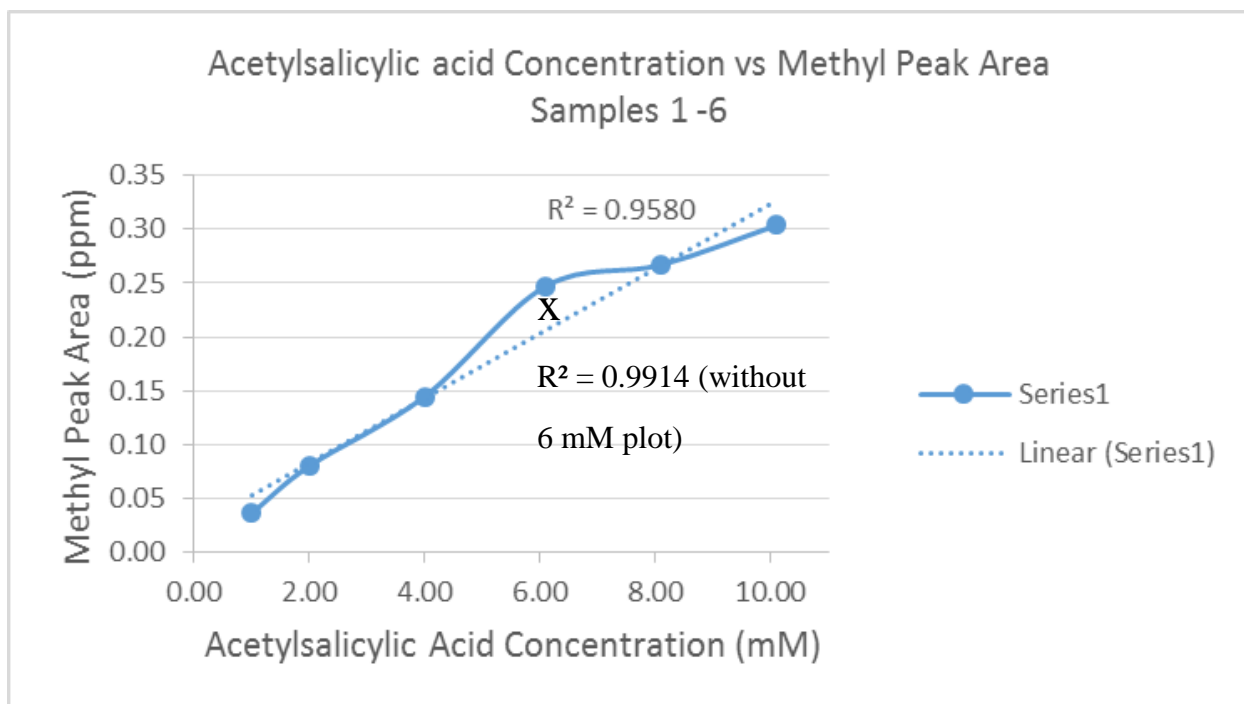


Figure 14. A plot of the NMR low concentration acetylsalicylic acid solutions (1.0 mM - 10.1 mM) and the average methyl peak areas shows a R^2 value of 0.9580. Excluding the 6 mM acetylsalicylic acid solution displays a R^2 value of 0.9914.

Table 8. The NMR low concentration acetylsalicylic acid solutions: Average aromatic ring H_A proton peak areas, the % RSD ranged from 0.83424% (6.1 mM) – 3.42632% (1.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	H _a of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	1.0 mM	1	0.01439	0.01463	0.00050	3.42632
		2	*			
		3	0.01417			
		4	0.01440			
		5	0.01432			
		6	0.01541			
		7	0.01511			
2	2.0 mM	1	0.02691	0.02662	0.00058	2.16298
		2	0.02724			
		3	0.02744			
		4	0.02605			
		5	0.02607			
		6	0.02620			
		7	0.02641			
3	4.0 mM	1	0.03879	0.03788	0.00090	2.37168
		2	0.03769			
		3	0.03764			
		4	0.03782			
		5	0.03821			
		6	0.03882			
		7	0.03617			
4	6.1 mM	1	0.06224	0.06214	0.00052	0.83424
		2	0.06269			
		3	0.06262			
		4	0.06221			
		5	0.06155			
		6	0.06235			
		7	0.06132			
5	8.1 mM	1	0.08045	0.07902	0.00164	2.07927
		2	0.08061			
		3	0.08061			
		4	0.07901			
		5	0.07819			
		6	0.07635			
		7	0.07789			
6	10.1 mM	1	0.08465	0.08553	0.00111	1.30231
		2	0.08677			
		3	0.08666			
		4	0.08657			
		5	0.08450			
		6	0.08531			
		7	0.08424			

* Represents data that could not be obtained due to a software error during the saving process.

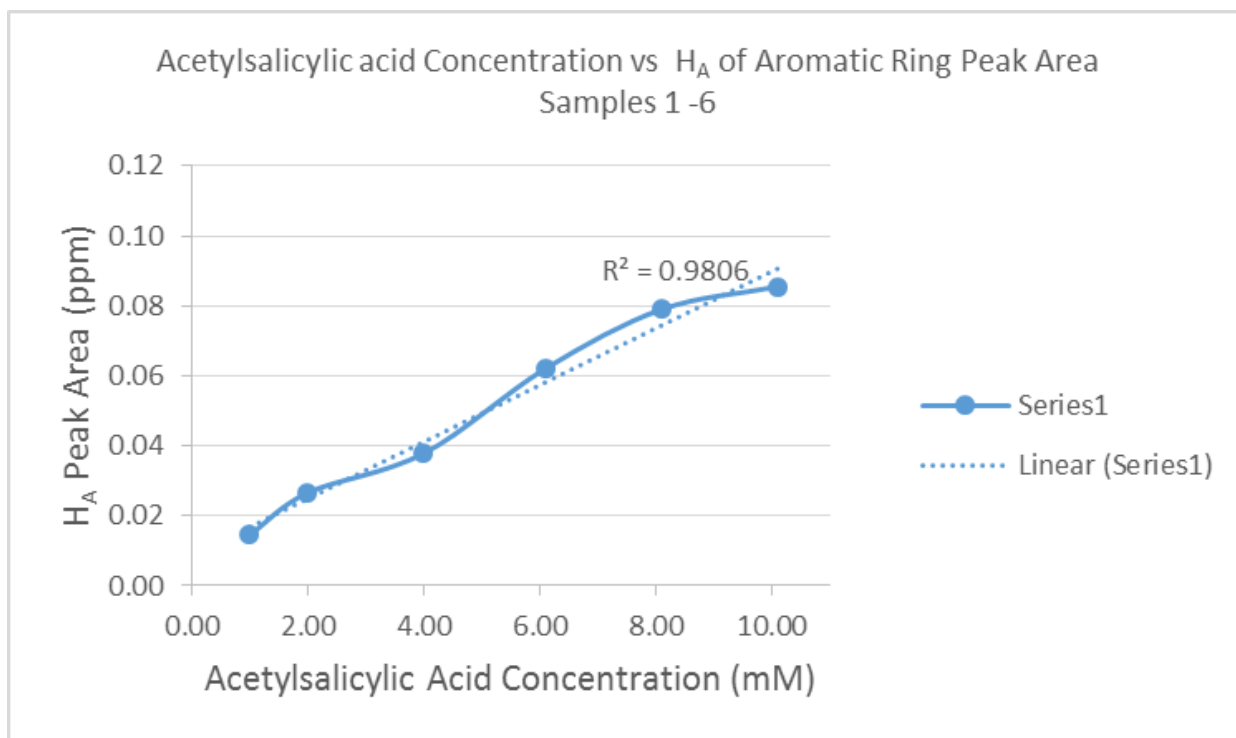


Figure 15. A plot of the NMR low concentration acetylsalicylic acid solutions (1.0 mM - 10.1 mM) and the average aromatic ring H_A proton peak areas shows a R^2 value of 0.9806.

Table 9. The NMR low concentration acetylsalicylic acid solutions: Average aromatic ring H_B proton peak areas, the % RSD ranged from 0.80307% (6.1 mM) – 2.95824% (1.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	H _B of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	1.0 mM	1	0.01714	0.01772	0.00052	2.95824
		2	*			
		3	0.01792			
		4	0.01730			
		5	0.01816			
		6	0.01842			
		7	0.01735			
2	2.0 mM	1	0.03105	0.03046	0.00061	1.99682
		2	0.03071			
		3	0.03141			
		4	0.02980			
		5	0.02990			
		6	0.03016			
		7	0.03020			
3	4.0 mM	1	0.04192	0.04138	0.00051	1.22259
		2	0.04203			
		3	0.04139			
		4	0.04155			
		5	0.04117			
		6	0.04058			
		7	0.04105			
4	6.1 mM	1	0.07009	0.07052	0.00057	0.80307
		2	0.07096			
		3	0.07072			
		4	0.07143			
		5	0.07018			
		6	0.06977			
		7	0.07052			
5	8.1 mM	1	0.09095	0.08905	0.00168	1.88939
		2	0.09052			
		3	0.09052			
		4	0.08875			
		5	0.08662			
		6	0.08735			
		7	0.08864			
6	10.1 mM	1	0.09214	0.09441	0.00125	1.32274
		2	0.09519			
		3	0.09525			
		4	0.09555			
		5	0.09474			
		6	0.09478			
		7	0.09325			

* Represents data that could not be obtained due to a software error during the saving process.

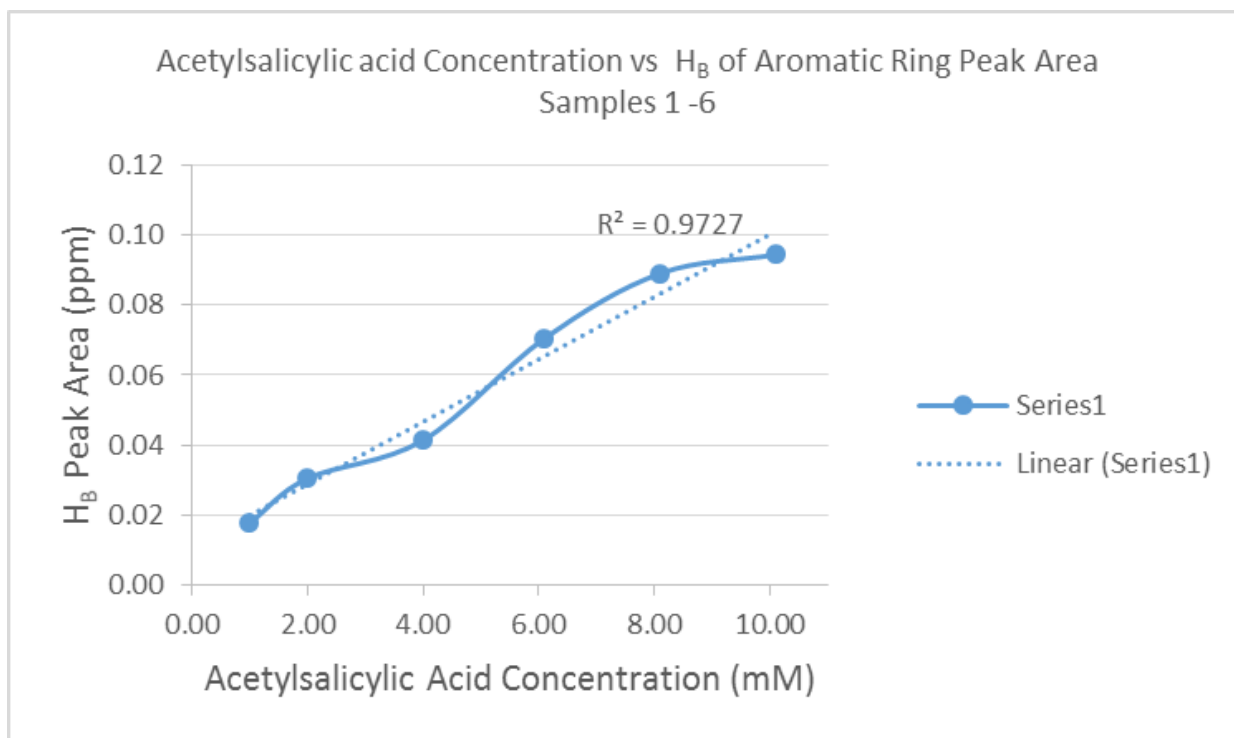


Figure 16. A plot of the NMR low concentration acetylsalicylic acid solutions (1.0 mM - 10.1 mM) and the average aromatic ring H_B proton peak areas shows a R^2 value of 0.9727.

Table 10. The NMR low concentration acetylsalicylic acid solutions: Average aromatic ring H_C proton peak areas, the % RSD ranged from 1.23863% (6.1 mM) – 4.69852% (2.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	H _C of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	1.0 mM	1	0.01656	0.01684	0.00066	3.94244
		2	*			
		3	0.01592			
		4	0.01759			
		5	0.01709			
		6	0.01749			
		7	0.01636			
2	2.0 mM	1	0.03203	0.03027	0.00142	4.69852
		2	0.03075			
		3	0.03212			
		4	0.02967			
		5	0.02918			
		6	0.02837			
		7	0.02979			
3	4.0 mM	1	0.04711	0.04344	0.00171	3.93313
		2	0.04305			
		3	0.04345			
		4	0.04338			
		5	0.04188			
		6	0.04241			
		7	0.04281			
4	6.1 mM	1	0.07290	0.07181	0.00089	1.23863
		2	0.07299			
		3	0.07166			
		4	0.07189			
		5	0.07164			
		6	0.07095			
		7	0.07064			
5	8.1 mM	1	0.09172	0.09109	0.00121	1.32755
		2	0.09196			
		3	0.09196			
		4	0.09166			
		5	0.09085			
		6	0.08855			
		7	0.09092			
6	10.1 mM	1	0.09419	0.09606	0.00165	1.71984
		2	0.09536			
		3	0.09801			
		4	0.09832			
		5	0.09519			
		6	0.09679			
		7	0.09458			

* Represents data that could not be obtained due to a software error during the saving process.

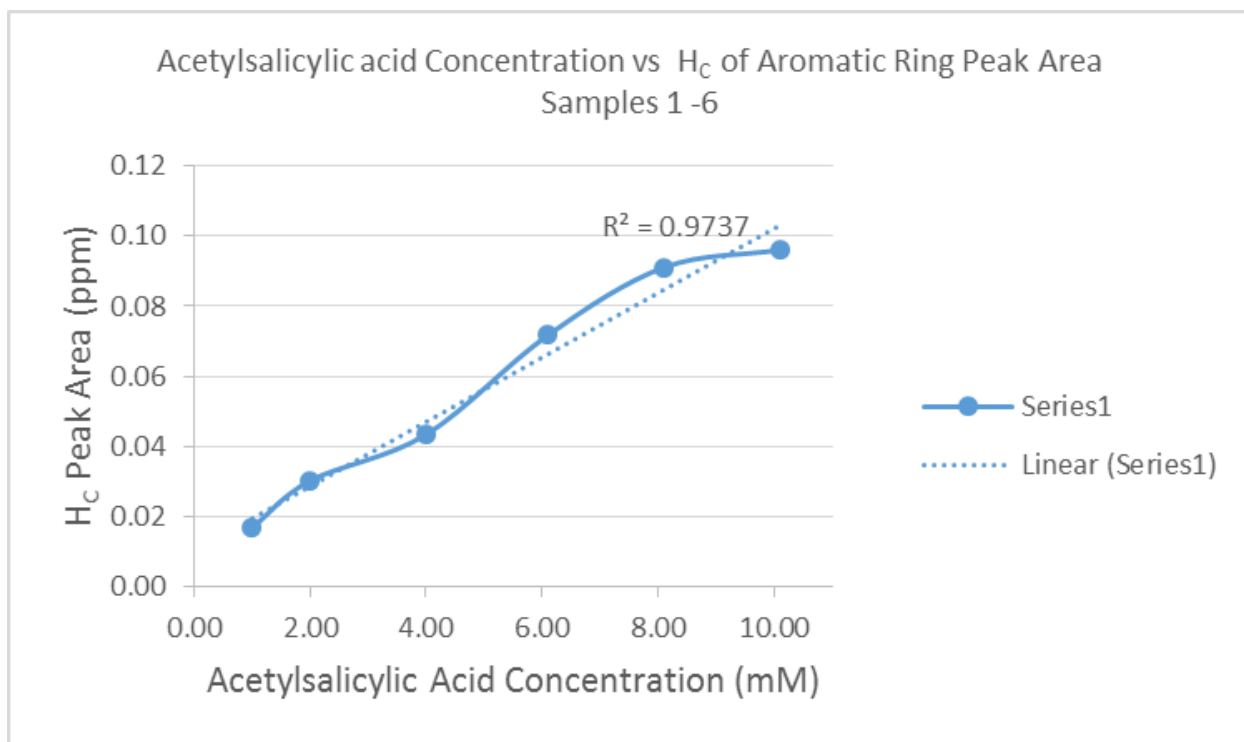


Figure 17. A plot of the NMR low concentration acetylsalicylic acid solutions (1.0 mM - 10.1 mM) and the average aromatic ring H_c proton peak areas shows a R^2 value of 0.9737.

Table 11. The NMR low concentration acetylsalicylic acid solutions: Average aromatic ring H_D proton peak areas, the % RSD ranged from 1.40636% (10.1 mM) – 4.01002% (2.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	H _D of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	1.0 mM	1	0.01529	0.01533	0.00034	2.21081
		2	*			
		3	0.01481			
		4	0.01528			
		5	0.01517			
		6	0.01569			
		7	0.01571			
2	2.0 mM	1	0.02727	0.02735	0.00110	4.01002
		2	0.02611			
		3	0.02643			
		4	0.02646			
		5	0.02866			
		6	0.02771			
		7	0.02883			
3	4.0 mM	1	0.03957	0.04005	0.00071	1.76312
		2	0.03985			
		3	0.04095			
		4	0.03977			
		5	0.03987			
		6	0.04112			
		7	0.03924			
4	6.1 mM	1	0.06586	0.06489	0.00111	1.71367
		2	0.06609			
		3	0.06393			
		4	0.06458			
		5	0.06334			
		6	0.06606			
		7	0.06439			
5	8.1 mM	1	0.08368	0.08214	0.00191	2.32713
		2	0.08362			
		3	0.08362			
		4	0.08252			
		5	0.08194			
		6	0.07839			
		7	0.08118			
6	10.1 mM	1	0.08653	0.08727	0.00123	1.40636
		2	0.08531			
		3	0.08934			
		4	0.08785			
		5	0.08742			
		6	0.08722			
		7	0.08722			

* Represents data that could not be obtained due to a software error during the saving process.

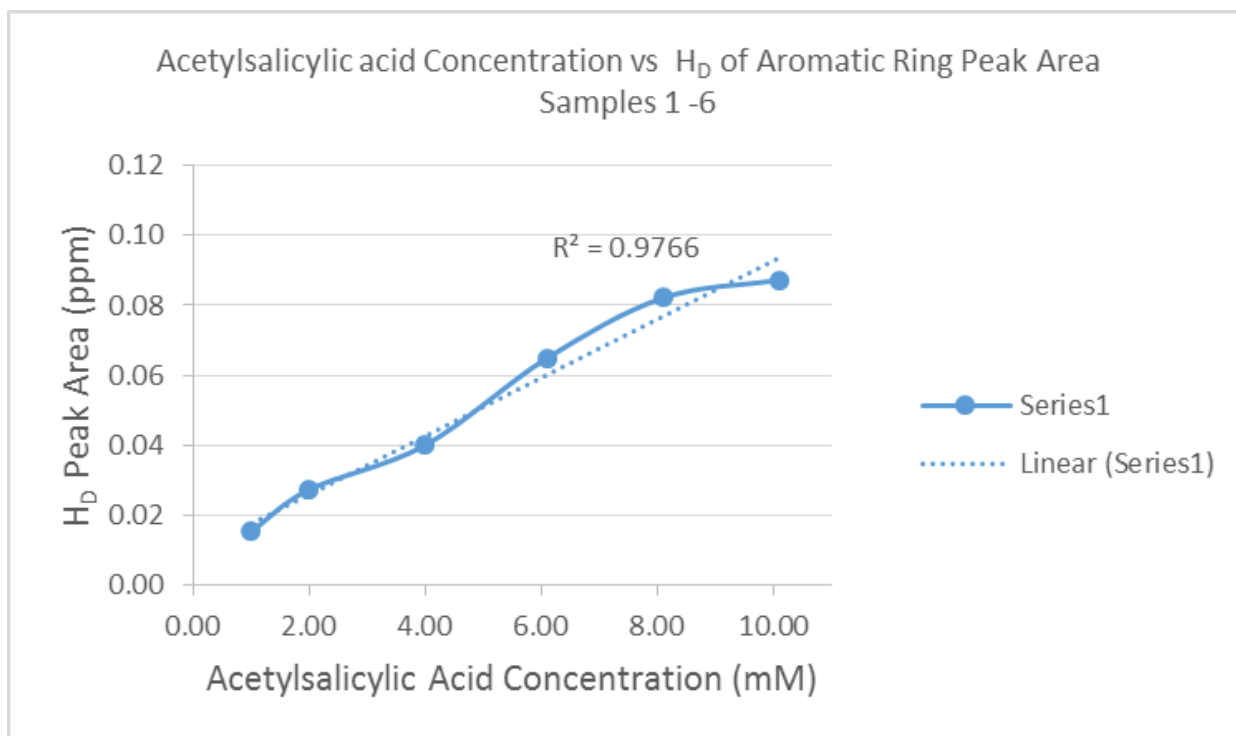


Figure 18. A plot of the NMR low concentration acetylsalicylic acid solutions (1.0 mM - 10.1 mM) and the average aromatic ring H_D proton peak areas shows a R² value of 0.9766.

Table 12. The NMR low concentration acetylsalicylic acid solutions: Average aromatic ring H_A - H_D proton peak areas, the % RSD ranged from 0.80729% (6.1 mM) – 2.23999% (1.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	Average H _A - H _D of Aromatic Ring Peak Area (rel)	Peak Area Mean (rel)	RSD	%RSD
1	1.0 mM	1	0.06338	0.06451	0.00144	2.23999
		2	*			
		3	0.06282			
		4	0.06457			
		5	0.06474			
		6	0.06701			
		7	0.06453			
2	2.0 mM	1	0.11726	0.11470	0.00214	1.86421
		2	0.11481			
		3	0.11740			
		4	0.11198			
		5	0.11381			
		6	0.11244			
		7	0.11523			
3	4.0 mM	1	0.16739	0.16276	0.00248	1.52224
		2	0.16262			
		3	0.16343			
		4	0.16252			
		5	0.16113			
		6	0.16293			
		7	0.15927			
4	6.1 mM	1	0.27109	0.26937	0.00217	0.80729
		2	0.27273			
		3	0.26893			
		4	0.27011			
		5	0.26671			
		6	0.26913			
		7	0.26687			
5	8.1 mM	1	0.34680	0.34129	0.00611	1.78890
		2	0.34671			
		3	0.34671			
		4	0.34194			
		5	0.33760			
		6	0.33064			
		7	0.33863			
6	10.1 mM	1	0.35751	0.36328	0.00434	1.19574
		2	0.36263			
		3	0.36926			
		4	0.36829			
		5	0.36185			
		6	0.36410			
		7	0.35929			

* Represents data that could not be obtained due to a software error during the saving process.

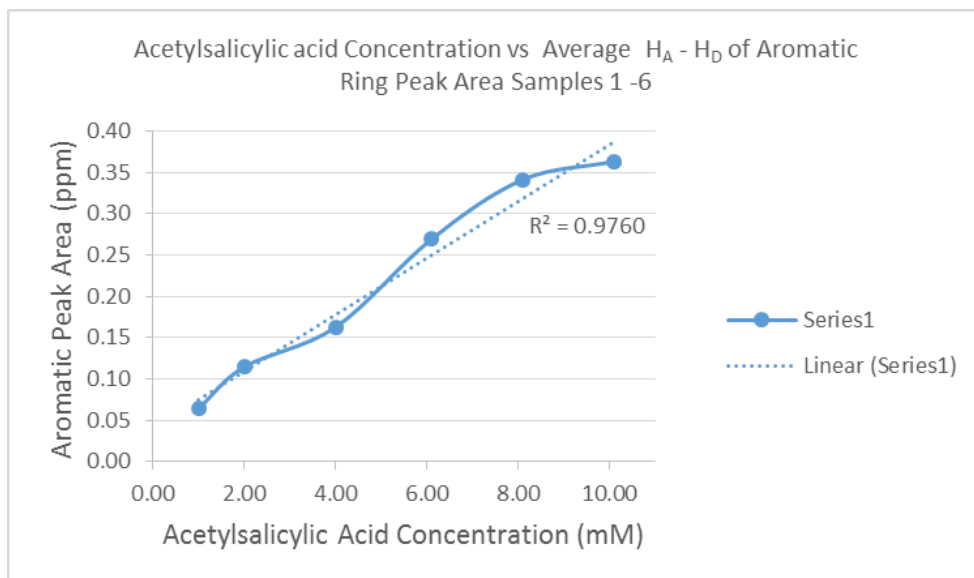


Figure 19. A plot of the NMR low concentration acetylsalicylic acid solutions (1.0 mM - 10.1 mM) and the average aromatic ring $H_A - H_D$ proton peak areas shows a R^2 value of 0.9760.

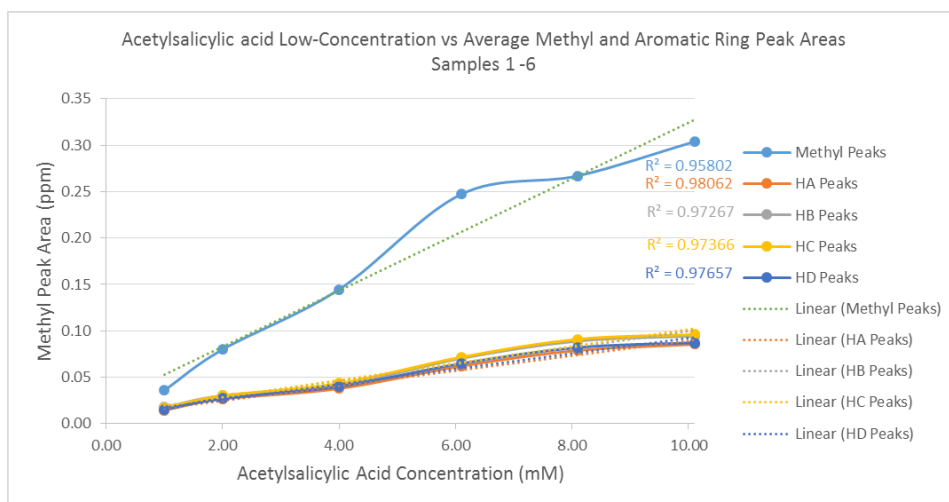


Figure 20. A plot of the NMR low concentration acetylsalicylic acid solutions (1.0 mM - 10.1 mM) average methyl peak areas and the average aromatic ring $H_A - H_D$ proton peak areas shows the overall linearity.

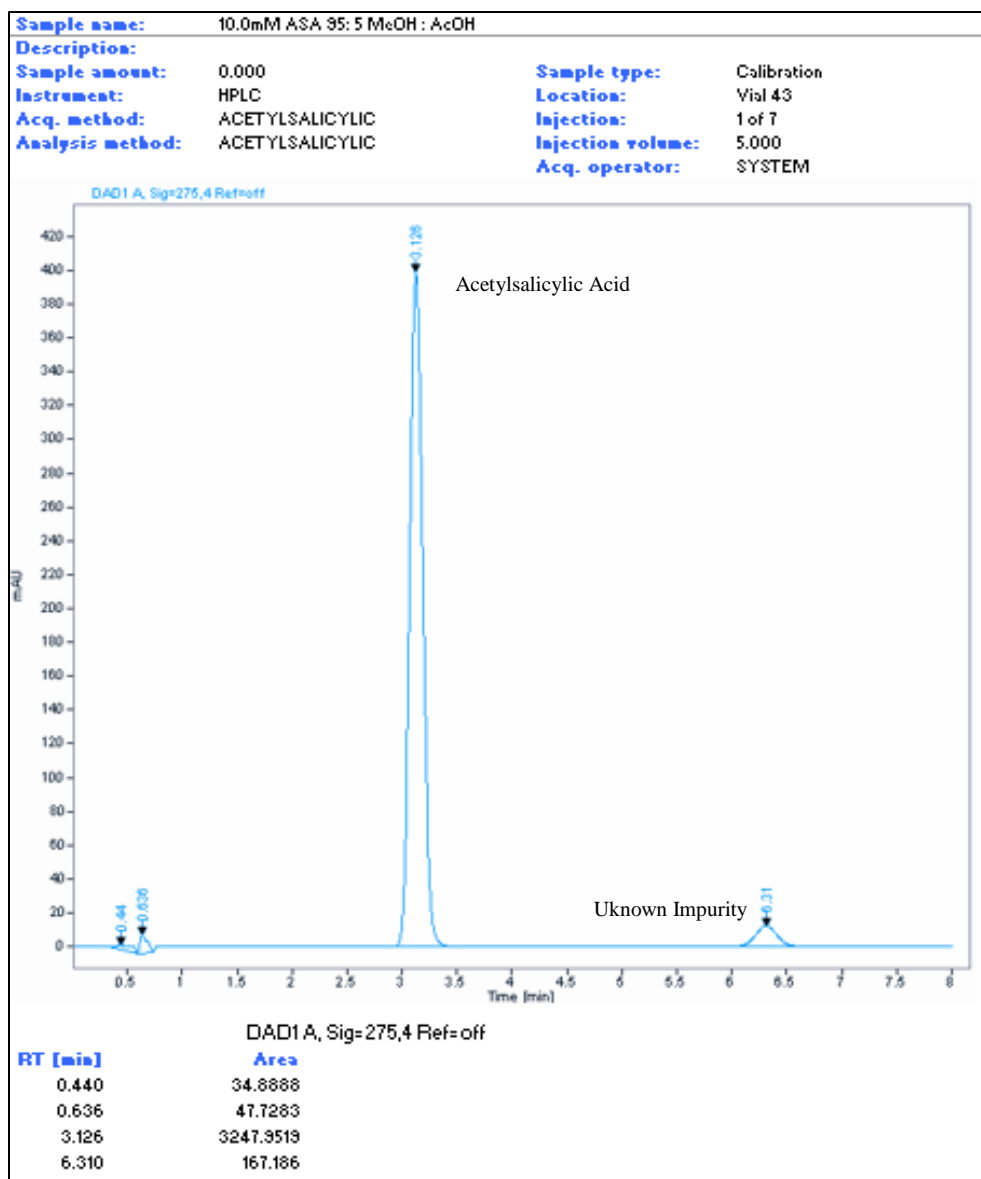


Figure 21. The HPLC chromatograph of 10.0 mM acetylsalicylic acid in solvent system shows an ASA peak at 3.126 minutes and an unknown impurity peak at 6.310 minutes.

HPLC Analysis of Low Concentration Acetylsalicylic Acid Solutions

Table 13. The NMR low concentration acetylsalicylic acid solutions: Average acetylsalicylic acid peak areas, the % RSD ranged from 0.45243% (10.0 mM) – 0.93044% (8.0 mM).

Sample No.	Acetylsalicylic Acid Concentration	NMR Trial No.	ASA Peak Area (mAU)	Peak Area Mean (mAU)	RSD	%RSD
1	1.0mM	1	332.0613	333.4809	1.9282	0.5782
		2	333.8247			
		3	335.3283			
		4	334.9912			
		5	333.8119			
		6	334.5150			
		7	329.8338			
2	2.0mM	1	665.5047	670.2156	5.1599	0.7699
		2	670.2914			
		3	661.8572			
		4	670.8779			
		5	676.0087			
		6	675.9660			
		7	671.0036			
3	4.0mM	1	1325.2648	1331.8140	6.1623	0.4627
		2	1331.7726			
		3	1341.1753			
		4	1336.0219			
		5	1333.7629			
		6	1331.5908			
		7	1323.1099			
4	6.0 mM	1	1943.1215	1962.2265	9.9447	0.5068
		2	1954.1570			
		3	1968.8270			
		4	1965.7344			
		5	1970.1403			
		6	1965.6021			
		7	1968.0034			
5	8.0 mM	1	2577.7690	2576.8584	23.9761	0.9304
		2	2587.4380			
		3	2569.8408			
		4	2529.5024			
		5	2574.2402			
		6	2600.7236			
		7	2598.4949			
6	10.0 mM	1	3247.9519	3273.2387	14.8092	0.4524
		2	3279.8347			
		3	3283.3296			
		4	3274.5098			
		5	3257.2136			
		6	3284.9334			
		7	3284.8982			

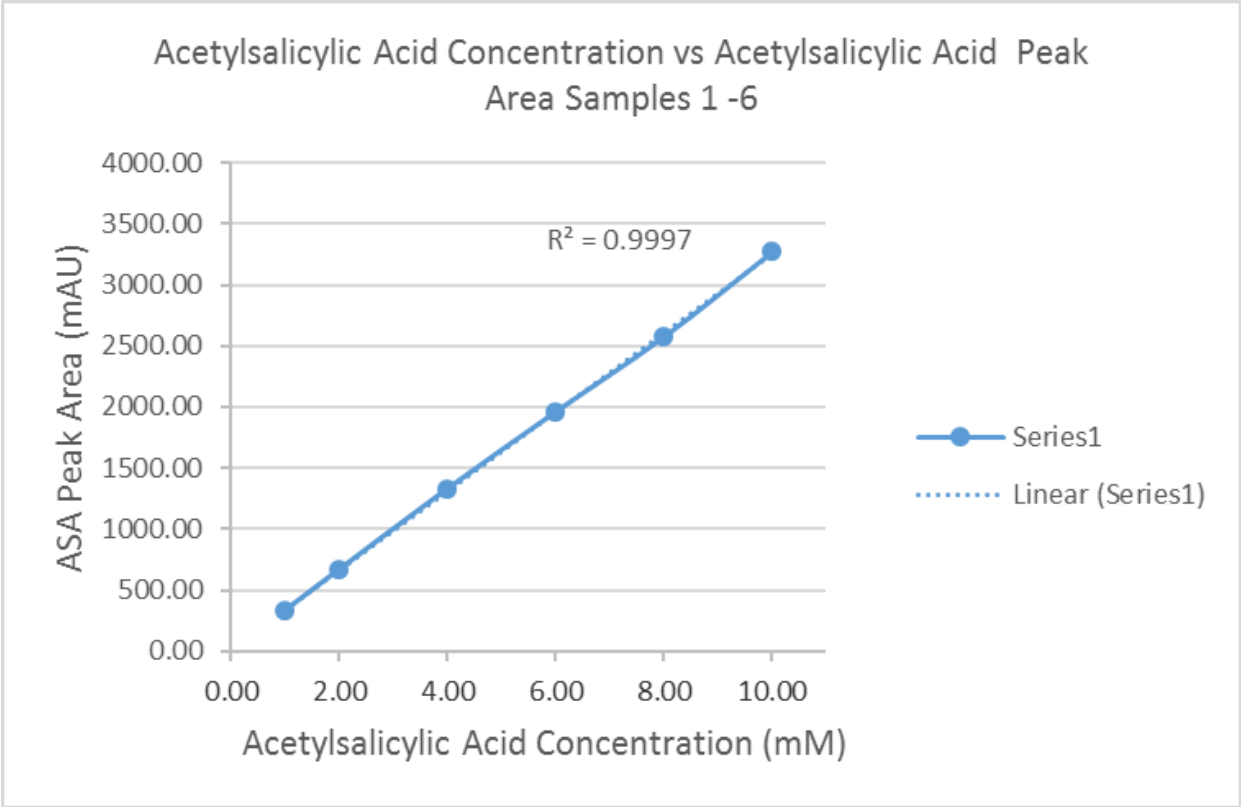


Figure 22. A plot of the HPLC low concentration acetylsalicylic acid solutions (1.0 mM - 10.1 mM) and the average acetylsalicylic acid peak areas shows a R^2 value of 0.9997.

Table 14. Compare low concentration acetylsalicylic acid q-NMR and HPLC: R² values and % RSD.

q-NMR Peak Area R ²				HPLC Peak Area R ²		
Methyl Group		Aromatic Ring		ASA Peak		
0.9995		HB	0.9806	0.9997		
		HC	0.9727			
		HD	0.9737			
		HE	0.9766			
q-NMR % RSD				HPLC % RSD		
Sample No.	ASA Concentration (mM)	Methyl Group (%)	Aromatic Ring (%)	Sample No.	ASA Concentration (mM)	ASA Peak (%)
1	1.0 mM	2.22860	2.23999	1	1.0 mM	0.57820
2	2.0 mM	0.94809	1.86421	2	2.0 mM	0.76989
3	4.0 mM	1.11634	1.52224	3	4.0 mM	0.46270
4	6.1 mM	1.94265	0.80729	4	6.0 mM	0.50681
5	8.1 mM	1.70966	1.78890	5	8.0 mM	0.93044
6	10.1 mM	1.00238	1.19574	6	10.0 mM	0.45243

DISCUSSION

Qualitative Analysis

NMR of CDCl₃

The proton NMR spectrum of CDCl₃ (Figure 1) shows a residual solvent proton peak at 7.3 ppm and an impurity peak at 1.5 ppm. Acetylsalicylic acid is readily soluble in CDCl₃.

NMR of tert-butanol in CDCl₃

The proton NMR spectrum of 100.4 mM *tert*-butanol in CDCl₃ shows a *tert*-butanol methyl peak at 1.4 ppm (Figure 2). The CDCl₃ produces a residual solvent proton peak at 7.3 ppm.

NMR of Acetylsalicylic Acid in CDCl₃

Figure 3 shows a proton NMR spectrum of 101.1 mM acetylsalicylic acid in CDCl₃. The acetylsalicylic acid methyl peak is at 2.4 ppm (singlet). The acetylsalicylic acid aromatic ring proton peaks H_A and H_D, are both doublet of doublets at 8.2 ppm and 7.1 ppm respectively. The remaining aromatic ring proton peaks H_B and H_C are both triplet of triplets at 7.7 ppm and 7.4 ppm respectively. The CDCl₃ produces a residual solvent proton peak at 7.3 ppm.

NMR of Acetylsalicylic Acid in Solvent System (tert-butanol / CDCl₃)

In Figure 4 and Figure 5, the proton NMR spectrum of 100.1 mM acetylsalicylic acid in the solvent system shows an acetylsalicylic acid singlet methyl peak at 2.4 ppm. The acetylsalicylic acid aromatic ring proton peaks are displayed at 8.2 ppm (H_A), 7.7 ppm (H_B), 7.4 ppm (H_C), and 7.1 ppm (H_D). The CDCl₃ residual solvent proton peak is at 7.3 ppm. The *tert*-butanol methyl peak is at 2.4 ppm and hydroxyl peak (broad singlet) has chemical shift of 6.3 ppm. The *tert*-butanol hydroxyl peak does not interfere with the acetylsalicylic acid methyl peaks or the aromatic ring proton peaks and will be used as the internal standard.

Figure 6 shows the proton NMR spectrum of 10.1 mM acetylsalicylic acid in the solvent system. The acetylsalicylic acid methyl peak is at 2.4 ppm and aromatic ring proton peaks are located at 8.2 ppm (H_A), 7.7 ppm (H_B), 7.4 ppm (H_C), and 7.1 ppm (H_D). The CDCl₃ residual solvent proton peak is at 7.3 ppm. The *tert*-butanol peaks do not interfere with the acetylsalicylic acid peaks. However, the acetylsalicylic acid concentrations lower than 10.1 mM shows *tert*-butanol hydroxyl peaks interfering with the methyl peaks (see appendix).

Quantitative Analysis

NMR - High Concentration Acetylsalicylic Acid Methyl Peaks

The high concentration acetylsalicylic acid solutions ranging from 10.0 mM – 100.1 mM are analyzed in septuplicate by q-NMR (Table 1). The acetylsalicylic acid methyl peak (singlet) appears at 2.4 ppm (Figure 4). A plot of concentration of acetylsalicylic acid solutions vs. average methyl peak areas shows a strong linear correlation with a R^2 value of 0.9996 (Figure 7). The % RSD for the analysis ranged from 0.2% to 0.7% showing high precision and robustness (Table 1).

As the acetylsalicylic acid solution concentration decreases, the chemical shift of the *tert*-butanol hydroxyl peak ranges from 2.4 ppm to 6.4 ppm (see appendix). It is likely that acetylsalicylic acid is forming a hydrogen bond with *tert*-butanol. However, the *tert*-butanol hydroxyl peak does not interfere with any methyl peaks of acetylsalicylic acid and, therefore is suitable for quantitative analysis.

NMR - High Concentration Acetylsalicylic Acid Aromatic Ring Proton Peaks

The proton NMR of the acetylsalicylic acid shows aromatic ring proton peaks H_A , H_B , H_C , H_D at chemical shifts 8.2 ppm, 7.7 ppm, 7.4 ppm, and 7.1 ppm, respectively (Figure 4 & Figure 5). The plot of concentration of acetylsalicylic acid solutions vs. average H_A aromatic ring proton peak areas shows a strong linear correlation, with a R^2 value of 0.9996 (Figure 8). The % RSD for the H_A proton peak ranges from 0.2% to 0.7% for the high concentration acetylsalicylic acid solutions, showing high precision (Table 2).

The plot of concentration of acetylsalicylic acid solutions vs. average H_B aromatic ring proton peak areas shows a strong linear correlation, with a R^2 value of 0.9996 (Figure 9). The % RSD for the H_B proton peaks ranges from 0.3% to 1% for the high concentration acetylsalicylic acid solutions showing high precision (Table 3).

The plot of concentration of acetylsalicylic acid solutions vs. average H_C aromatic ring proton peak areas shows a strong linear correlation, with a R^2 value of 0.9995 (Figure 10). The % RSD for the H_C proton peaks ranges from 0.4% to 1% for the high concentration acetylsalicylic acid solutions showing high precision (Table 4).

The plot of concentration of acetylsalicylic acid solutions vs. average H_D aromatic ring proton peak areas shows a strong linear correlation, with a R² value of 0.9998 (Figure 11). The % RSD for the H_D proton peaks ranges from 0.3% to 0.6% for the high concentration acetylsalicylic acid solutions showing high precision (Table 5).

The plot of concentration of acetylsalicylic acid solutions vs. average proton peak areas for H_A - H_D shows a strong linear correlation, with a R² value of 0.9994 (Figure 12). The % RSD for the H_A - H_D proton peaks ranges from 0.3% to 0.7% for the high concentration acetylsalicylic acid solutions showing high precision (Table 6).

The acetylsalicylic acid aromatic ring proton peak areas for the solutions ranging from 10.0 mM to 100.1 mM have R² values with a strong linear correlation and % RSD values <0.8% showing high precision. Therefore, the aromatic ring proton peak areas of acetylsalicylic acid are suitable for quantitative analysis.

The average methyl peak areas and average aromatic ring H_A - H_D proton peak areas for the high concentration acetylsalicylic solution are plotted on the same graph to compare their overall linearity (Figure 13).

NMR - Low Concentration Acetylsalicylic Acid Methyl Peaks

The acetylsalicylic acid solutions in low concentrations ranging from 1.0 mM – 10.1 mM are analyzed in septuplicate by q-NMR (Table 7). The acetylsalicylic acid methyl peak (singlet) appears at 2.4 ppm (Figure 6). A plot of concentration of acetylsalicylic acid solutions vs. average methyl peak areas shows a weak linear correlation with a R² value of 0.9580 (Figure 14).

The % RSD for this analysis ranged from 1% to 2% showing fairly good precision and robustness (Table 7). However, the % RSD for the low concentration solutions is slightly lower compared to the high concentration acetylsalicylic acid solutions.

In addition, as the acetylsalicylic acid solution concentrations decrease, the chemical shift of the *tert*-butanol hydroxyl peak shifts from 3.5 ppm to 6.4 ppm (see appendix). The *tert*-butanol hydroxyl peak does interfere with the acetylsalicylic acid methyl peak for the solutions ranging from 2.0 mM to 10.1 mM. However, if the acetylsalicylic acid solutions in low concentrations are plotted without the 6.0 mM methyl peak areas a strong linear correlation is seen with a R²

value of 0.9914. Therefore, the methyl peak areas of acetylsalicylic acid are not suitable for quantitative analysis at these low concentrations.

NMR - Low Concentration Acetylsalicylic Acid Aromatic Ring Proton Peaks

The proton NMR of the acetylsalicylic acid solutions at low concentrations (1.0 mM – 10.1 mM) shows aromatic ring proton peaks H_A, H_B, H_C, H_D at chemical shifts 8.2 ppm, 7.7 ppm, 7.4 ppm, and 7.1 ppm respectively (Figure 6). The plot of concentration of acetylsalicylic acid solutions vs. average H_A aromatic ring proton peak areas shows a weak linear correlation, with a R² value of 0.9806 (Figure 15). The % RSD for the H_A proton ranged from 1% to 3% for the low concentration acetylsalicylic acid solutions showing fairly good precision (Table 8). However, the % RSD for the H_A proton is not as precise as for the high concentration acetylsalicylic acid solutions.

The plot of concentration of acetylsalicylic acid solutions vs. average H_B proton aromatic ring peak areas shows a weak linear correlation, with a R² value of 0.9727 (Figure 16). The % RSD for the H_B proton ranged from 1% to 3% for the low concentration acetylsalicylic acid solutions showing fairly good precision (Table 9).

The plot of concentration of acetylsalicylic acid solutions vs. average H_C proton aromatic ring peak areas shows a weak linear correlation, with a R² value of 0.9737 (Figure 17). The % RSD for the H_C proton ranged from 1% to 5% for the low concentration acetylsalicylic acid solutions showing fairly good precision (Table 10).

The plot of concentration of acetylsalicylic acid solutions vs. average H_D proton aromatic ring peak areas shows a weak linear correlation, with a R² value of 0.9776 (Figure 18). The % RSD for the H_D proton ranged from 1% to 4% for the low concentration acetylsalicylic acid solutions showing fairly good precision (Table 11).

The plot of concentration of acetylsalicylic acid solutions vs. average peak areas for H_A - H_D protons shows a weak linear correlation, with a R² value of 0.9760 (Figure 19). The % RSD for the H_A - H_D protons ranged from 1% to 2% for the low concentration acetylsalicylic acid solutions showing fairly good precision (Table 12). Therefore, the aromatic ring proton peak areas of low concentration acetylsalicylic acid solutions are more desirable for quantitative analysis than the methyl peak areas.

The average methyl peak areas and average aromatic ring H_A - H_D proton peak areas for the low concentration acetylsalicylic solution are plotted on the same graph to compare their overall linearity (Figure 20).

HPLC - Low Concentration Acetylsalicylic Acid Peaks

Figure 21 shows the 10.0 mM acetylsalicylic acid in the solvent system. The acetylsalicylic acid peak is at 3.126 minutes and the unknown impurity peak is located at 6.310 minutes. The low concentration acetylsalicylic acid solutions ranging from 1.0 mM – 10.0 mM are analyzed in septuplicate by HPLC (Table 13). The plot of concentration of acetylsalicylic acid solutions vs. the average acetylsalicylic acid peak areas shows a strong linear correlation with a R² value of 0.9997 (Figure 22). The % RSD for the acetylsalicylic acid peak ranges from 0.5% to 0.9% for the low concentration acetylsalicylic acid solutions showing high precision and robustness (Table 13).

Comparison of q-NMR vs. HPLC

Analysis Time Requirements

The q-NMR analysis takes about five minutes per trial for each acetylsalicylic acid (ASA) solution and approximately 35 minutes for septuplicate analysis. The q-NMR analysis requires manually placing the samples into the q-NMR instrument one at a time. Method development for the q-NMR analysis is minimal and only requires general settings to be applied, but requires about 3 days to determine the appropriate standard and solvent system. The HPLC analysis takes approximately 8 minutes per trial for each acetylsalicylic acid solution and approximately 56 minutes for septuplicate analysis. The HPLC contains an auto-sampler tray that holds up to 100 samples; therefore, all of the samples can be added at one time. Method development for the HPLC requires about two weeks to determine the appropriate column, flow-rate, temperatures, and mobile phases.

Solvent Management

The ASA solution does not degrade and is not lost during the q-NMR analysis. Septuplicate analysis of an ASA solution only requires a small aliquot using about 0.7 ml. The septuplicate analysis of all the high and low concentration ASA solutions requires in total about 8 ml of solution. The low volume of solvents decreases the waste disposal and the environmental impact. However, for the HPLC septuplicate analysis of all the low concentration acetylsalicylic acid

solutions requires approximately 504 mL of mobile phase (about 12 mL per injection). The volume of solvent waste produced is significantly greater for HPLC.

Cost of Operation

In this study, the labor costs are not applicable. However, in the literature, the average cost for the q-NMR labor per sample is about \$440 (9) and reagents cost close to \$120 for a total of nearly \$560. The q-NMR analysis of an additional sample is about \$320. The labor cost associated with the analysis of one sample for HPLC is about \$2,940 (9) and reagents cost around \$50 for a total of \$2,990. The analysis of additional samples cost close to \$300 for HPLC.

Efficacy of Analysis

The q-NMR high concentration ASA solutions shows high precision with the low average % RSD values for the methyl peak areas and the average aromatic ring proton peak areas. The R^2 values for the average methyl peak areas and the average aromatic ring proton peak areas show a strong linear correlation. However, the q-NMR low concentration acetylsalicylic acid solutions show fairly good precision for the average % RSD values for the methyl peak areas and the average aromatic ring proton peak areas. The R^2 values for the average methyl peak areas and the average aromatic ring proton peak areas show a weak linear correlation. Comparing the time requirements, solvent management, cost of analysis, and efficacy q-NMR is a viable alternative for quantitative analysis for the high concentration ASA, but not for the low concentration ASA.

For the HPLC analysis, the low concentration acetylsalicylic acid solutions vs. average acetylsalicylic acid peak areas show a R^2 value having a strong linear correlation. The % RSD for the acetylsalicylic acid peak shows high precision for the low concentration acetylsalicylic acid solutions. Based on these results, implementing HPLC analysis of these low concentration acetylsalicylic acid solutions is preferred over q-NMR.

FUTURE WORK

Future q-NMR research will implement the Electronic REference To access In vivo Concentrations method (ERETIC), which uses an electronic device to synthesize a reference signal for determining the absolute concentrations. Further q-NMR studies will include using an internal standard other than *tert*-butanol, which does not interfere with the low concentration acetylsalicylic acid methyl group peaks.

CONCLUSION

The q-NMR analysis of high concentration acetylsalicylic acid solutions showed a strong linear correlation with high R^2 values and high precision with % RSD for both the average methyl peak areas and the average aromatic ring proton peak areas. However, the low concentration acetylsalicylic acid solutions showed a weak linear correlation as indicated by the R^2 values and fairly good precision for the % RSD for both the average methyl peak areas and the average aromatic ring proton peak areas. These results show that q-NMR is a viable alternative for quantitative analysis of high concentration acetylsalicylic acid methyl peak areas and aromatic ring proton peak areas, but it is not suitable for the low concentration acetylsalicylic acid solutions.

The HPLC data showed low concentration acetylsalicylic acid solutions with a strong linear correlation for the R^2 values and high precision for the % RSD for the average acetylsalicylic acid peak areas. The HPLC technique is suitable for the quantitative analysis of low concentration acetylsalicylic acid peak areas.

REFERENCES

1. Siddiqui, M.R. et al., Analytical techniques in pharmaceutical analysis: A review. *Arabian Journal of Chemistry* **2013**, <http://dx.doi.org/10.1016/j.arabjc.2013.04.016>
2. Silverstein, R. M.; Bassler, C. G.; Morrill, T. C. *Spectrometric Identification of Organic Compounds*, 4th ed.; John Wiley & Sons: New York, **1981**; 181-212.
3. Holzgrabe, U. Quantitative NMR spectroscopy in Pharmaceutical Applications. *Prog. Nucl. Magn. Reson. Spectr.* **2010**, *57*, 229-240.
4. Kumar, S., Flemming, C., Villanueva, M., Zuklic, J., “Quantitative Determination of Acetaminophen, Acetylsalicylic Acid, Cetirizine Dihydrochloride, and Fexofenidine Hydrochloride by Quantitative-NMR Technique,” *The International Chemical Congress of Pacific Basin Societies 2015 (Pacifichem 2015), Honolulu, HI, December 15-20, 2015*.
5. Kumar, S., Flemming, C., “Quantitative Determination of Acetylsalicylic Acid by Quantitative Nuclear Magnetic Resonance (q-NMR) technique,” *1st Annual Research Day, Governors State University, University Park, IL, April 1, 2016*.
6. Kumar, S., Kumar, S. M., Mannem, S. K. R., and Gouthami, K., “Quantitative Analysis of Acetaminophen, Acetylsalicylic Acid, and Cetirizine Dihydrochloride by q-NMR (Quantitative Nuclear Magnetic Resonance) Technique”. *247th American Chemical Society National Meeting, Dallas, TX, March 16-20, 2014*.
7. Kumar, S., Kumar, S. M., Gouthami, K., “Quantitative Analysis of Acetylsalicylic Acid and Acetaminophen by q-NMR (Quantitative Nuclear Magnetic Resonance) Technique”, *American Chemical Society Southwest Regional Meeting, Waco, TX, November 16-19, 2013*.
8. Sawyer, M.; Kumar, V. A Rapid High-Performance Liquid Chromatographic Method for the Simultaneous Quantitation of Aspirin, Salicylic Acid, and Caffeine in Effervescent Tablets. *J. Chrom. Sci.* **2003**, *41*, 393-397.
9. Webster, G. K., Kumar, S. (2014). Expanding the Analytical Toolbox: Pharmaceutical Application of Quantitative NMR. *Analytical Chemistry*, **2014**, *86*, 11474-11480.

APPENDIX

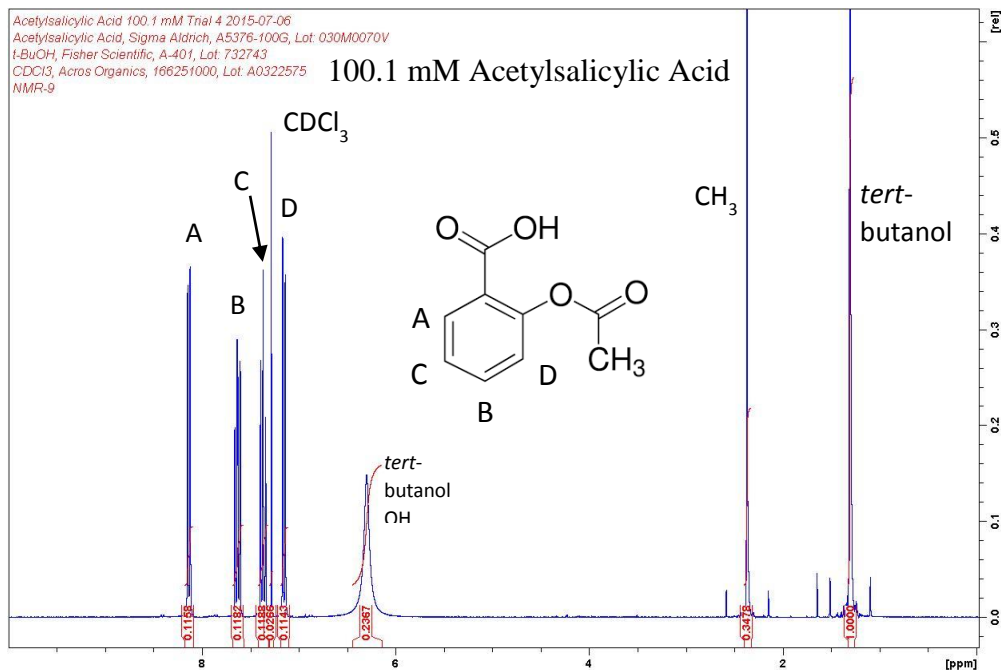


Figure 1. NMR Spectrum of 100.1 mM acetylsalicylic acid in solvent system..

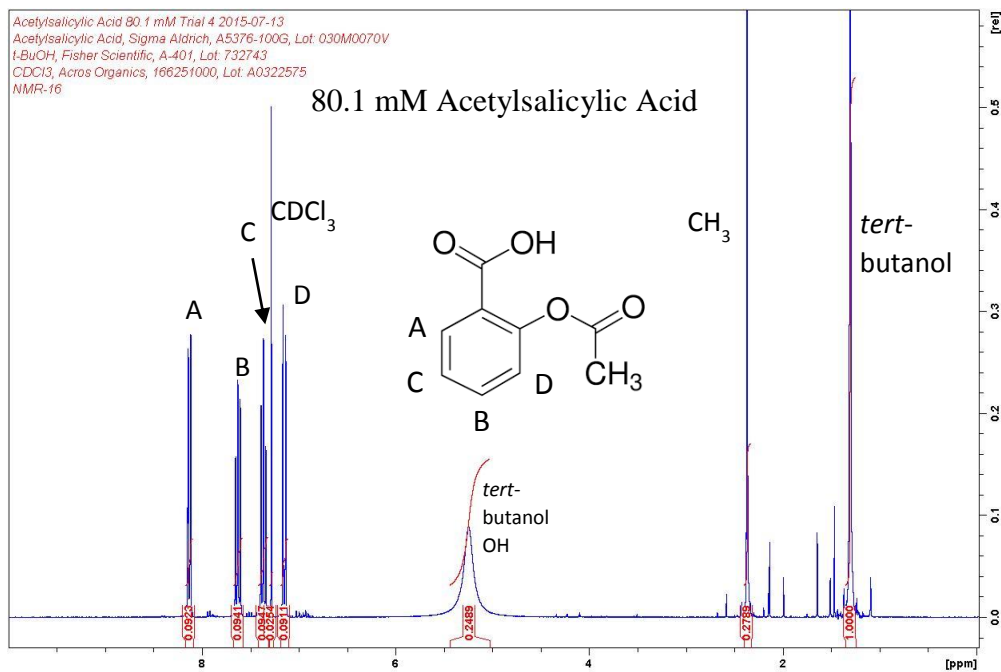


Figure 2. NMR Spectrum of 80.1 mM acetylsalicylic acid in solvent system.

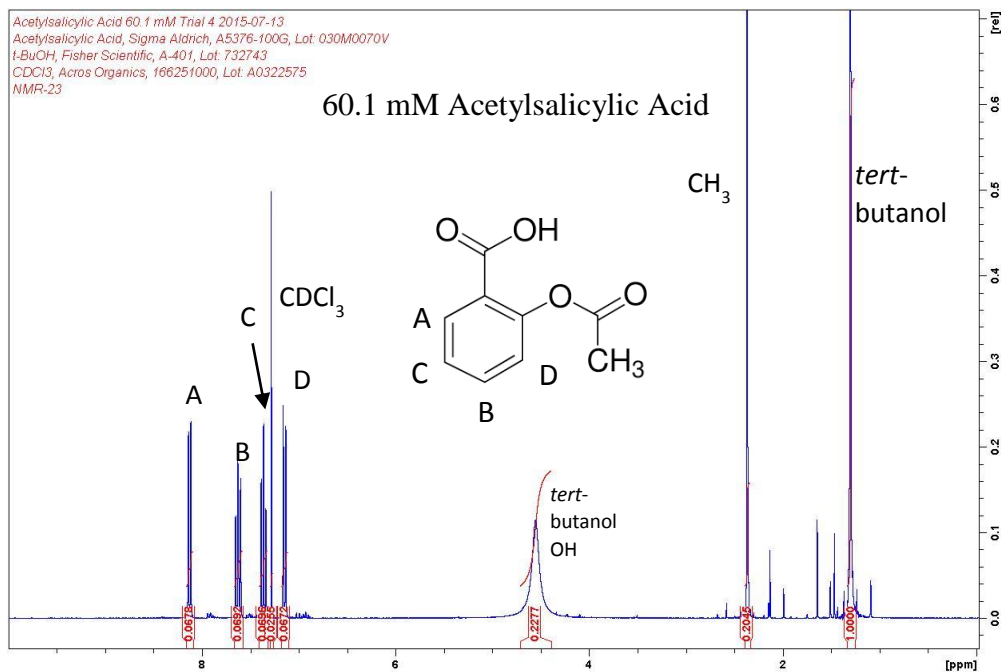


Figure 3. NMR Spectrum of 60.1 mM acetylsalicylic acid in solvent system.

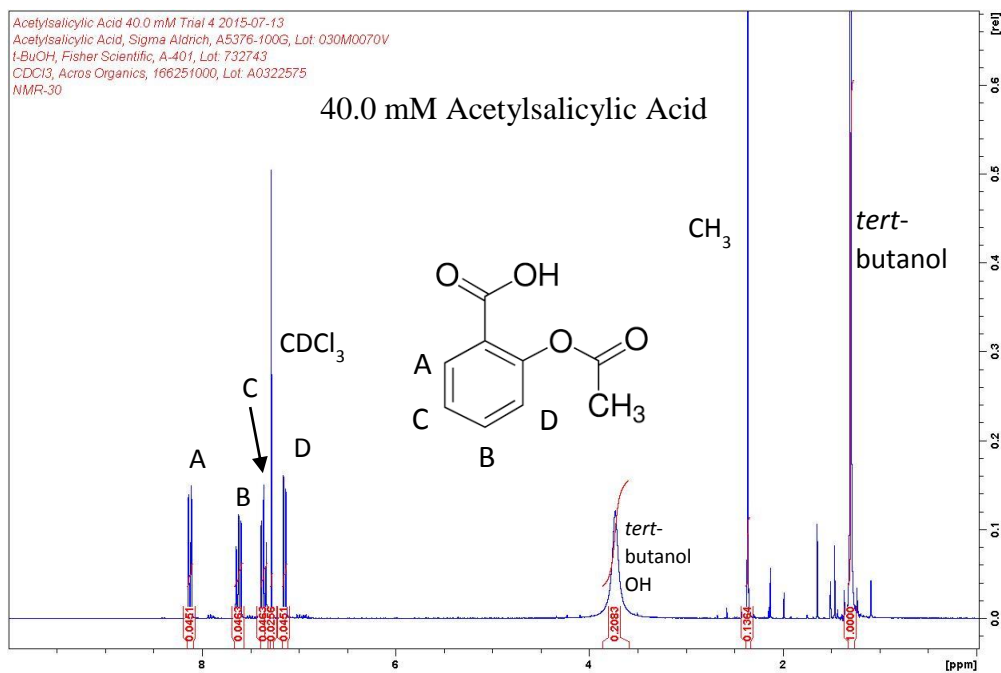


Figure 4. NMR Spectrum of 40.0 mM acetylsalicylic acid in solvent system.

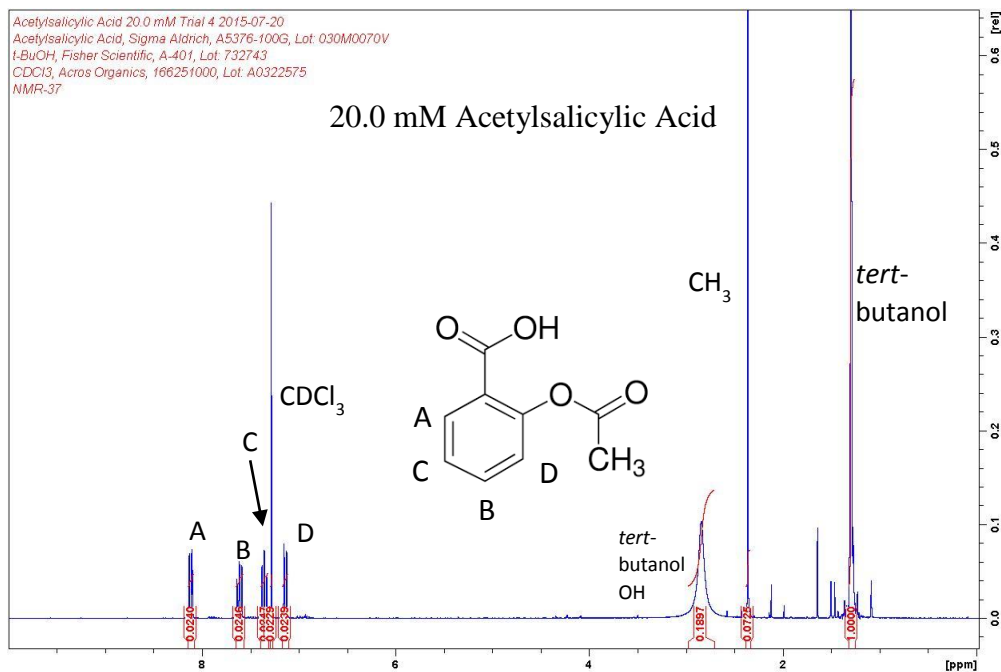


Figure 5. NMR Spectrum of 20.0 mM acetylsalicylic acid in solvent system.

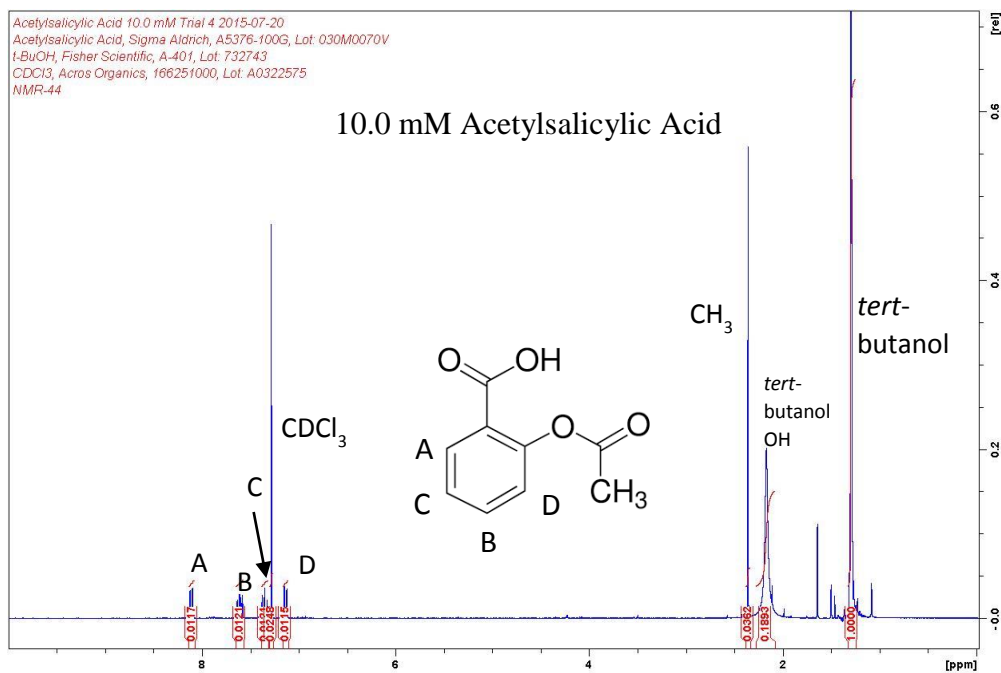


Figure 6. NMR Spectrum of 10.0 mM acetylsalicylic acid in solvent system.

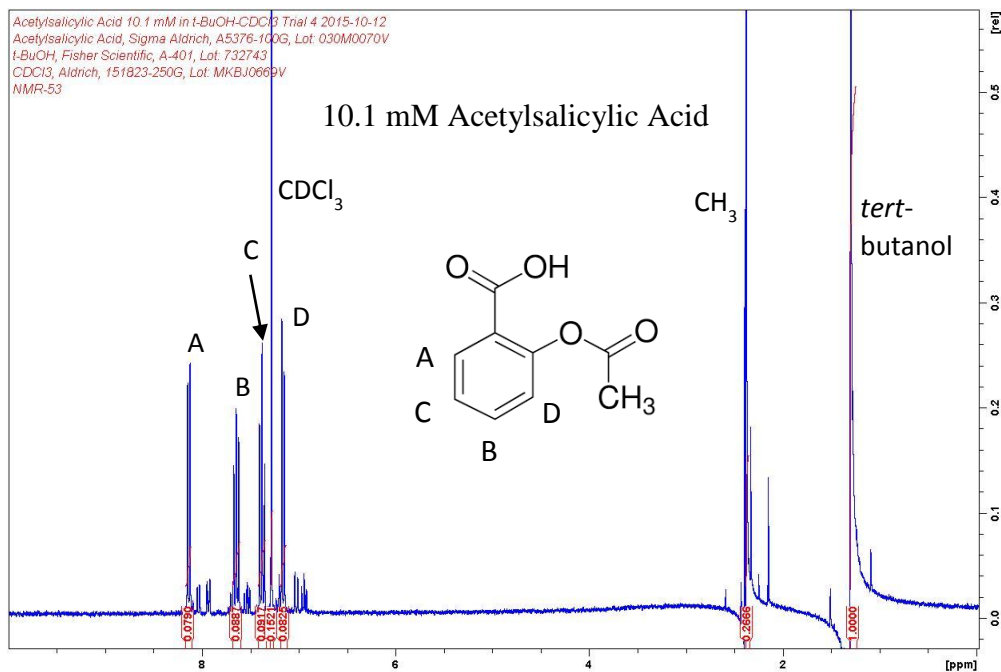


Figure 7. NMR Spectrum of 10.1 mM acetylsalicylic acid in solvent system.

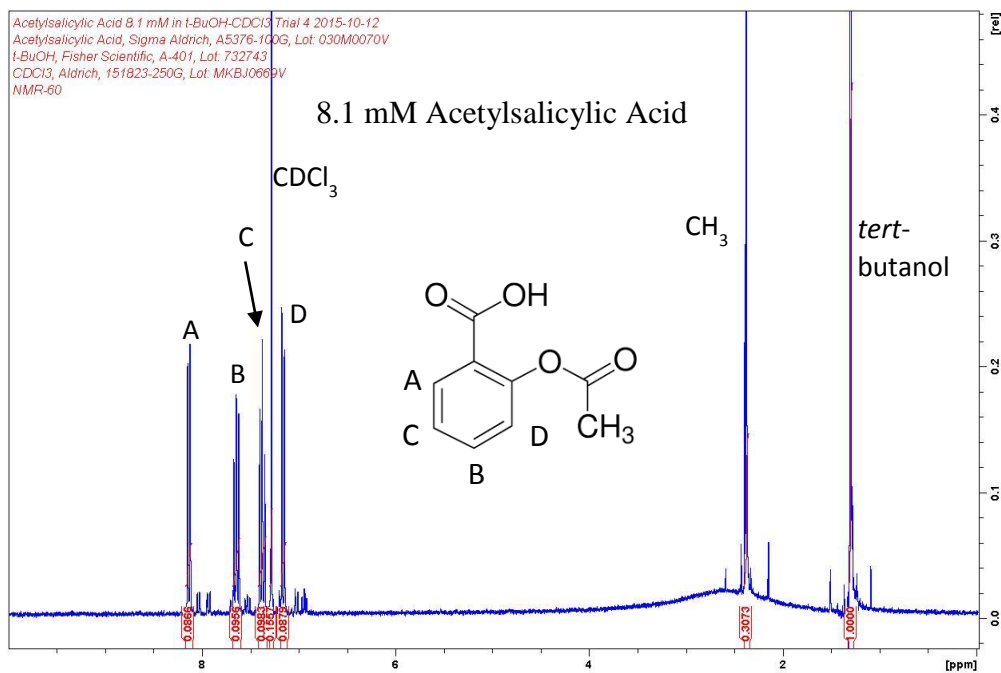


Figure 8. NMR Spectrum of 8.1 mM acetylsalicylic acid in solvent system.

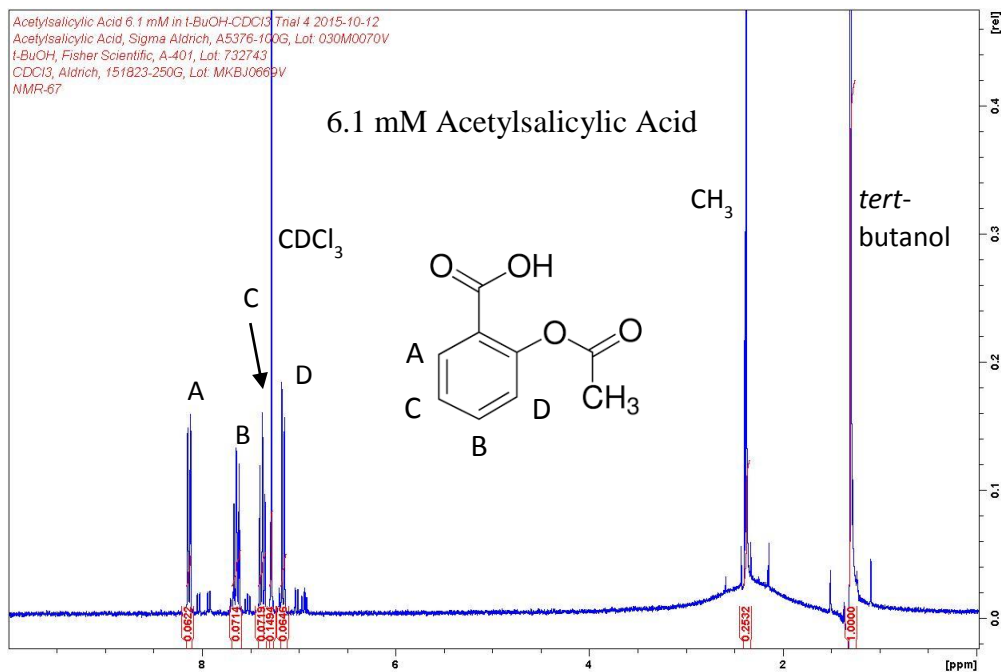


Figure 9. NMR Spectrum of 6.1 mM acetylsalicylic acid in solvent system.

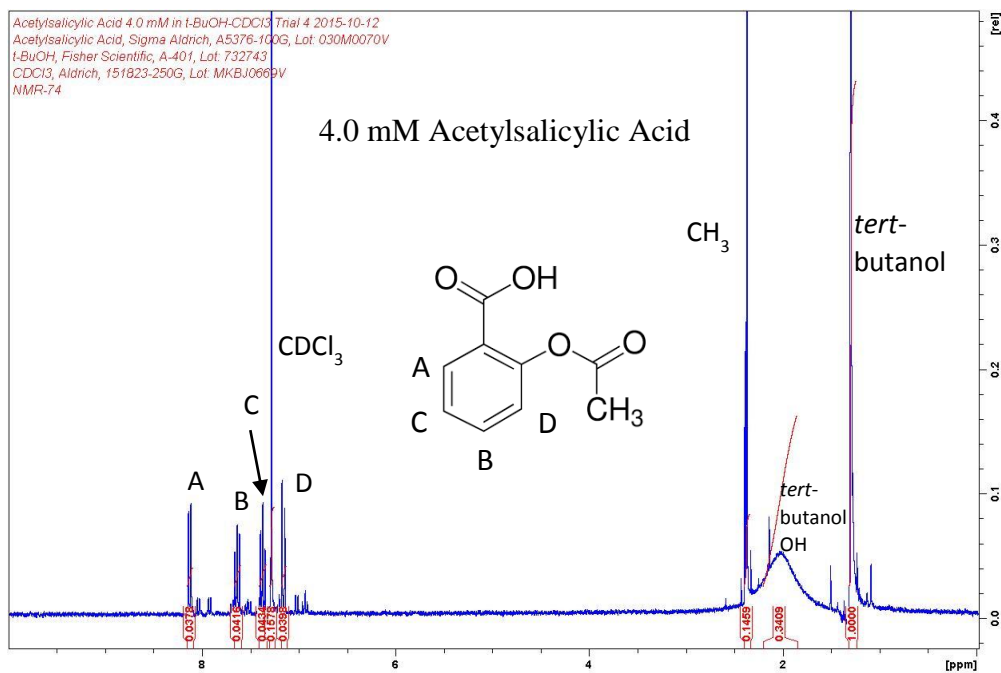


Figure 10. NMR Spectrum of 4.0 mM acetylsalicylic acid in solvent system.

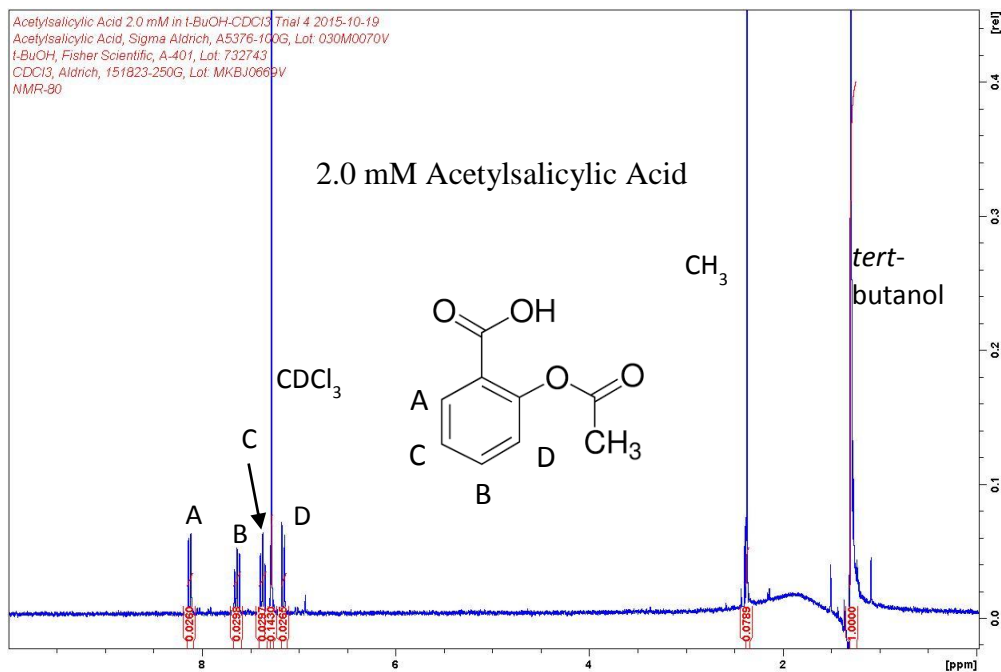


Figure 11. NMR Spectrum of 2.0 mM acetylsalicylic acid in solvent system.

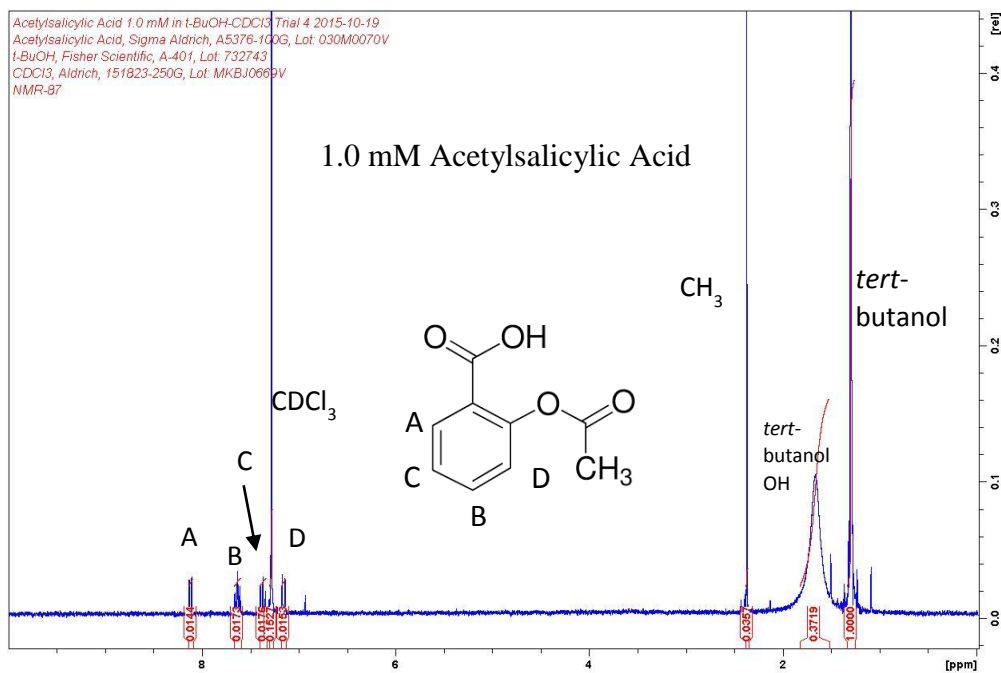


Figure 12. NMR Spectrum of 1.0 mM acetylsalicylic acid in solvent system.