NMR Metabolomics of Lotus

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Certificate of Examination

This is to certify that the dissertation titled "**NMR metabolomics of Lotus**" submitted by **Mr. Guru Gaurav (Reg. No. MS13027)** for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. S. Kumar Goyal Dr. S. Kumar Biswas Dr. Kavita Dorai

(Supervisor)

Declaration

The work presented in this dissertation has been carried out by me with Dr. Kavita Dorai at Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

GURU GAURAV

(Candidate)

Dated: 1 April, 2018

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr.Kavita Dorai

(Supervisor)

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I would like to express my sincere gratitude to Dr.Kavita Dorai, for her great confidence and support throughout this project. I also thank Sumit Mishra for reading drafts of this dissertation and providing valuable comments. I am grateful to Mukesh Kumar and sandeep Singh for their constant support and many valuable discussions we had. I would like to acknowledge Ankit Singh , Chandan Kumar Sharma for various helpful discussions.

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For/Dedicated to/To my Papa . . .

Chapter 1

INTRODUCTION

1.1 NMR spectroscopy

Historically NMR (Nuclear magnetic resonance) was invented in 1945 with two simultaneous incidents. In first incident weak radio frequency signals were detected from paraffin wax by Purcell , Torry and Pound . In the same time radio signals from nuclei in water were detected by Bloch , Packard and Hanson [1] . NMR spectroscopy is a analytic technique and used for identification of compounds. NMR is used in quantum computation , biophysics , Medicine and many other important fields of physics , chemistry and biology .

1.1.1 Energy levels

All nuclei have internal spin which is intrinsic property of nuclei. Nuclear spin are characterize by Spin quantum number I .The interaction between nuclear spin and external magnetic field give rise to different energy levels from $-I$ to I in integer steps . These values are m quantum numbers . In single spin nuclei only only two energy levels can be generated in presence of magnetic field . We can denote them by E_α for $m = 1/2$ and E_β for $m = -1/2$ .The energy$E$ of these quantum levels is related to Larmor frequency v and

m quantum number .

$$E = mv$$

where v is

$$v = \frac{-\gamma(1-\theta)B_0}{2\pi}$$

here γ is gyromagnetic ratio , θ is chemical shift and B_0 is applied magnetic field .

spectrum

The transition of energy follow selection rule where m can only change by ± 1 . for one spin case we can say up spin change to down.

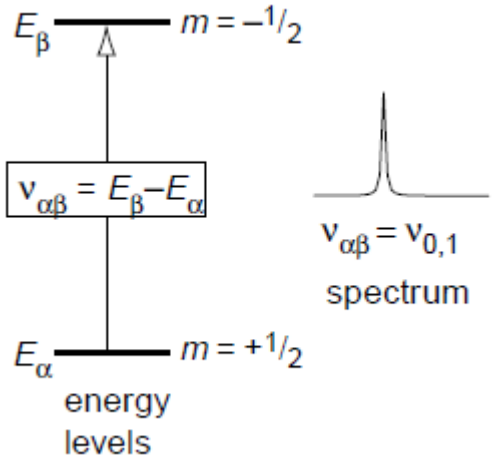


FIGURE 1.1: transition between two energy level of one spin[16]

for spin half system the allowed transition Larmor frequency $v_(\alpha\beta)$ is difference between energy levels E_α - E_β as shown in figure 1.1 . In NMR spectrum we will see the transition from upper to lower energy level so we will observe $-v$.

1.1.2 Vector Model

Energy level model and selection rules can work in spectroscopy but for NMR Vector Model is better approach . For example we can not explain pulse sequence NMR by energy level model . According to quantum mechanics all nuclei have magnetic moment which can be in any direction . In the presence of applied magnetic field B_0 all nuclei in sample align themselves in the direction of B_0 and create a magnetization vector as shown in figure 1.2 .

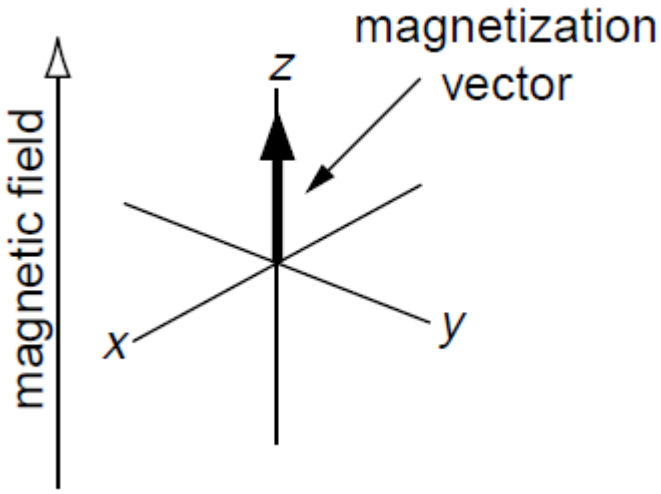


FIGURE 1.2: magnetization vector along B_0 direction[17]

A frequency pulse applied to magnetization vector tilt it from the direction of B_0 and magnetization vector start precessing in direction of B_0 in a cone. This is called Larmor precession . The frequency of precession is Larmor frequency ω_0.

If a coil is placed in XY plane perpendicular to B_0 the precessing vector create a electric pulse in coil when it pass through coil. The electric pulse is called free induced signal. This pulse is amplified and detected in NMR pulse experiment .

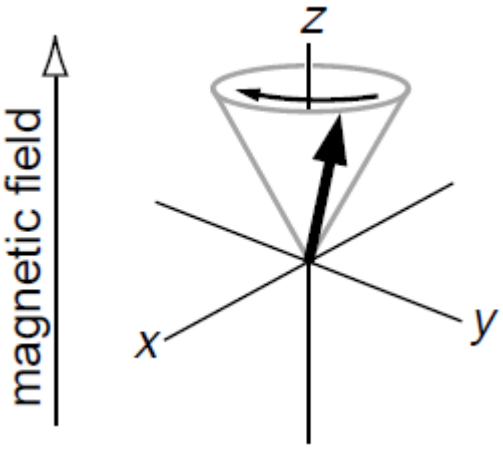


FIGURE 1.3: magnetization vector spinning around B_0[18]

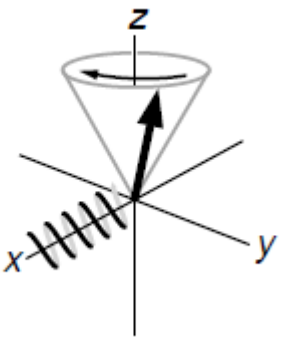


FIGURE 1.4: coil placed in XY plane[19]

1.1.3 1D NMR spectroscopy

$1D$ NMR refer to the ^1H proton NMR , $^(13)C$ carbon NMR and NMR of other nuclei. In NMR spectroscopy there are two kind of $1D$ experiment.

Regular $1D$ NMR experiment

In regular $1D$ NMR experiment an RF pulse of few watt is projected on magnetization vector for few microsecond . This RF pulse move magnetization vector in XY plane or toward XY plane[9].

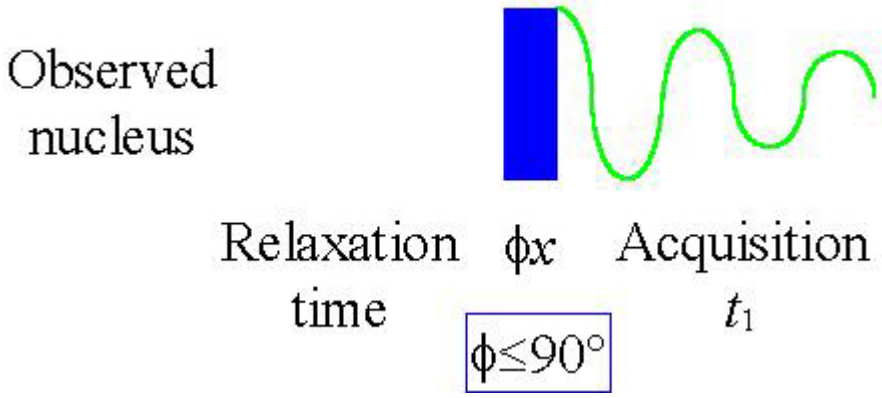


FIGURE 1.5: free induction decay signal[20]

The magnetization vector precess and relax to it's previous state after the RF pulse . The time require to relaxation after pulse is called relaxation time . All this process is called free induction decay of magnetization vector . The signal is observed during precession of magnetization vector in time domain which is converted to frequency domain by Fourier transformation .

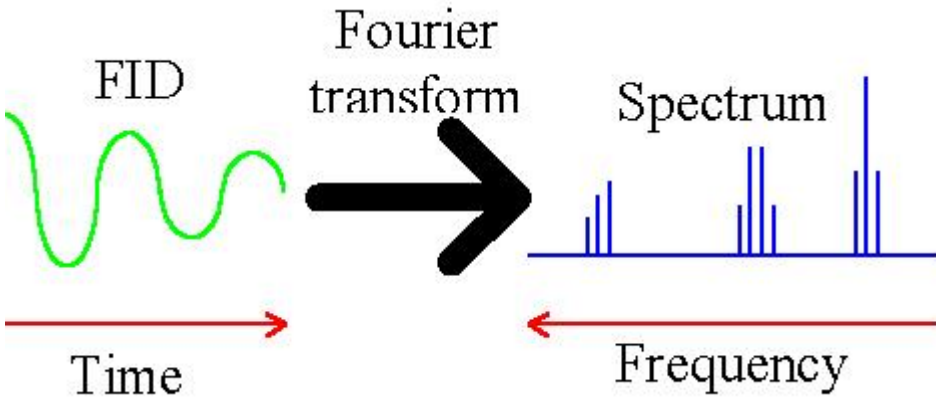


FIGURE 1.6: Fourier transformation of observed signal[21]

Decoupled $1D$ **NMR experiment**

In many cases the coupling between different nuclei cause heteronuclear coupling . This coupling reduce the sensitivity of signal and split the signal . For decoupling a Rf pulse window of few kilohertz is applied to other nuclei who

are creating coupling in observable nuclei . This nullify coupling effect and the spectrum appear more stronger .

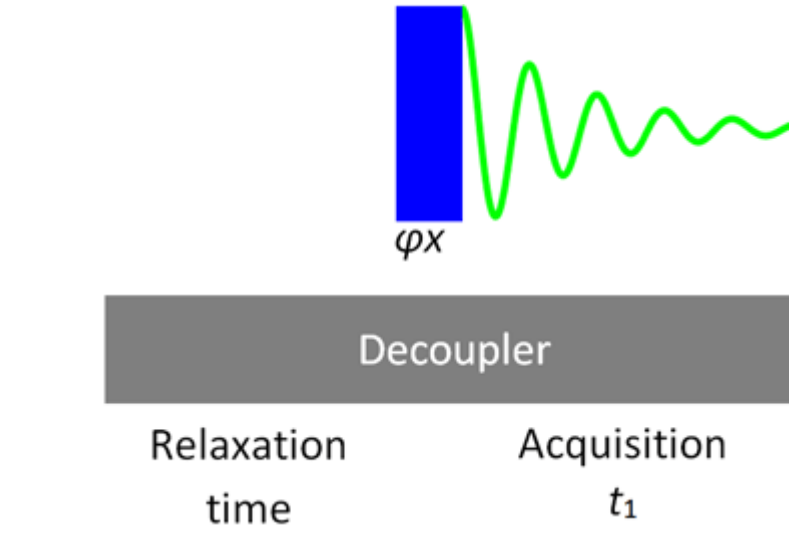


FIGURE 1.7: pulse sequence and decoupling[22]

This nullify coupling effect and the spectrum appear more stronger .

1.1.4 2D NMR spectroscopy

In 2D NMR intermediate stages evolution and mixing is performed before signal acquisition . In evolution process t_1 time is set to 0 and slowly increased by Δt . At the same time large data set is collected from t_1 evolution . In $2D$ graph the F_1 axis represent t_1 and F_2 by t_2 . The F_2 axis represent the acquisition after evolution and mixing similar like $1D$.

t_1 is called evolution time and t_2 is called acquisition time . The Fourier transformation is applied in both dimension and a $2D$. This shows a contour diagram .Here F_1 represent evolution frequency and F_2 represent acquisition frequency .

This shows a contour diagram .Here F_1 represent evolution frequency and F_2 represent acquisition

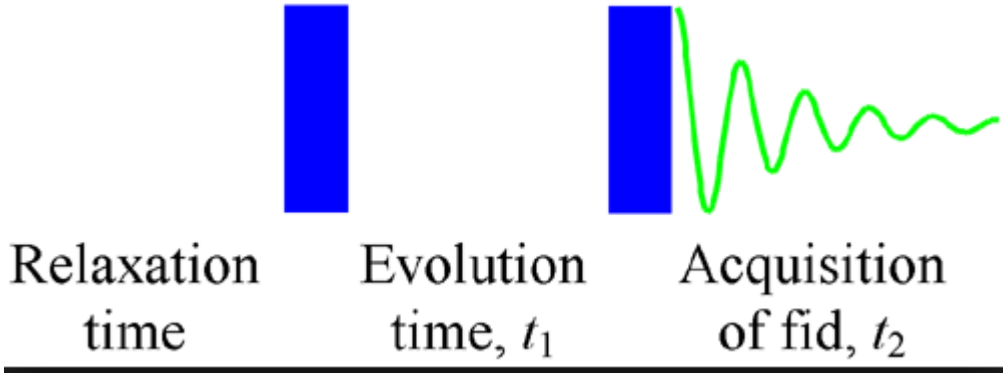


FIGURE 1.8: pulse sequence in 2*D* NMR[23]

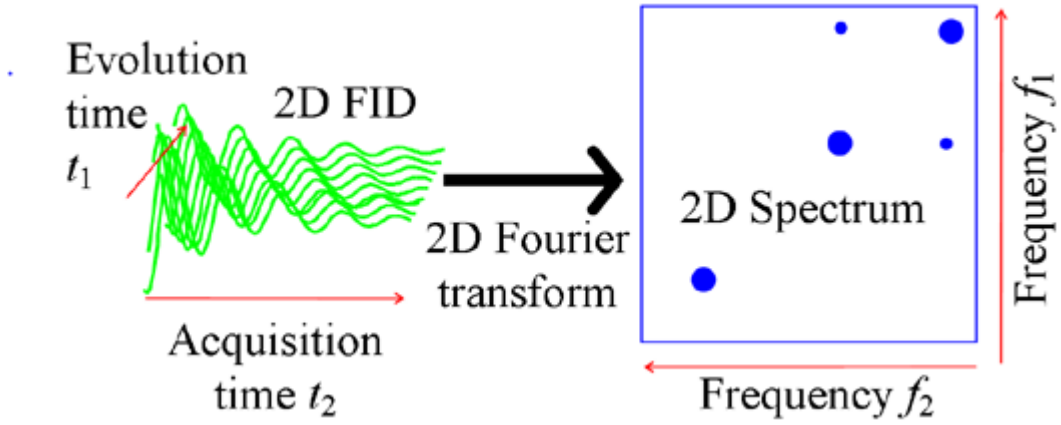


FIGURE 1.9: 2*D* Fourier transformation and contour digram[24]

1.2 NMR metabolomics

NMR metabolomics is a branch of NMR where endogenous and exogenous metabolites are identified and quantified in biological samples. with the help of NMR metabolomics we identifies different form of molecules and there catabolic and anabolic pathways. After the knowledge of all metabolites in any organism it provides a complete bio-molecular picture under certain conditions . For complete bio-molecular picture of an organism it is very hard to find all metabolites , for example 3000 metabolites are recorded in tobacco leaves[2] . In metabolic experiments not only the number of metabolites but also there polarity , concentration , chemical behavior and stability make it

hard to analysis all metabolites in one experiment . NMR provides an batter approach for metabolic experiments . NMR detect secondary metabolites among many primary metabolites and also detect there molar concentration .

Besides all benefits of NMR there are few limitation . Because NMR spectroscopy is low sensitive a large number of samples are required than any other spectroscopic methods .The overlap of signals in $1H$ NMR is another limitation which causes error in peak investigation but $2D$ NMR reduce this limitation with better resolution .

1.3 Multivariate analysis of Metabolites

In metabolomics we find a large amount of dataset ,which is hard to interpret without special analysis methods . Metabolites are mostly outproducts of cell reaction and provide a global overview of all outcome metabolite .Like in many NMR metabolomic experiment only few significant metabolite explain the behaviour of problem who change there conecntration in different sample of experiment . In many multivariate analysis like principal component analysis (PCA) , Partial least square(PLS) Metabolites responsible for variation among diffent classes are selected for future analysis [3]. Metabolic fingerprinting is used to identify metabolic difference among samples and explain biological relationship. A data matrix X with N observation row vector and K observable is used as input matrix for multivariate analysis . Linear transformation on input matrix X is performed and transformed into p dimensional T matrix .

$$T = XA$$

here A is K by p matrix and A^T is liner transformation weight matrix . This is dimensional reduction of large data set if k is larger then p. The main goal of

analysis is to reduce Data set by A^T , here A^T depends on chosen algorithm i.e. PCA , PLS etc .

1.3.1 Principal component analysis

Principal component analysis is used for variance preservation in reduced data set from original data set. This method is mainly used for understanding metabolites variance among different sample group . The transformation matrix used for PCA is constructed by making column of eigenvectors of covariance matrix S.

$$S = \frac{X^T H X}{N-1}$$

where H is centering matrix .

$$S = Q \Lambda Q^{-} 1$$

here S is eigendecomposed in Q matrix of eigenvector of S and Λ is diagonal matrix . The variance in new transformed data is given by eigenvalue of Λ.

1.3.2 Partial least square

PCA is mainly used for expressing groups and variance among groups but this method is only applicable when within a group variance is small comparable to variance among groups . The class membership withing group is also used in this analysis . transformation is given by help of covariance between X and Y

$$S = \frac{X^T H Y Y^T H X}{(N-1)^2}$$

1.3.3 T-test

T-test is used to compare samples of population .For two sample from same population t value is given by

$$S = \frac{X_1 - X_2}{\sqrt{\frac{S_2}{N_2} + \frac{S_1}{N_1}}}$$

where S is variance of sample and N is number of points in sample . With the help of degree of freedom between samples $N_1 + N_2 - 2$ and t and Z table we calculate p value . p value ≤ 0.05 indicate corresponding metabolite or point is a noise among samples . In metabolomics these kind of signal are called significant metabolites responsible for difference between samples of same population or plant.

1.4 Quantitative NMR experiment

NMR spectroscopy used for structural elucidation of metabolites present in sample is called quantitative NMR spectroscopy . The study of chemical shift and and coupling constant provide better explanation of inter molecular resonance in proton ^1H NMR.[4,7 ,6]

Quantitative^1H NMR spectroscopy is used to quantitative estimation of molecules and recent development in technology like use of high field magnets and CryoProbe has open gates for low sensitive molecular $^(13)P$ and $^(13)C$;based qNMR . Qualitative NMR provide qualitative description of molecules present in complex solution without isolation of molecules .

Inaccuracy in qNMR method is less than 2% which is very low in quantitative experiments . The precision , accuracy and robustness make NMR a

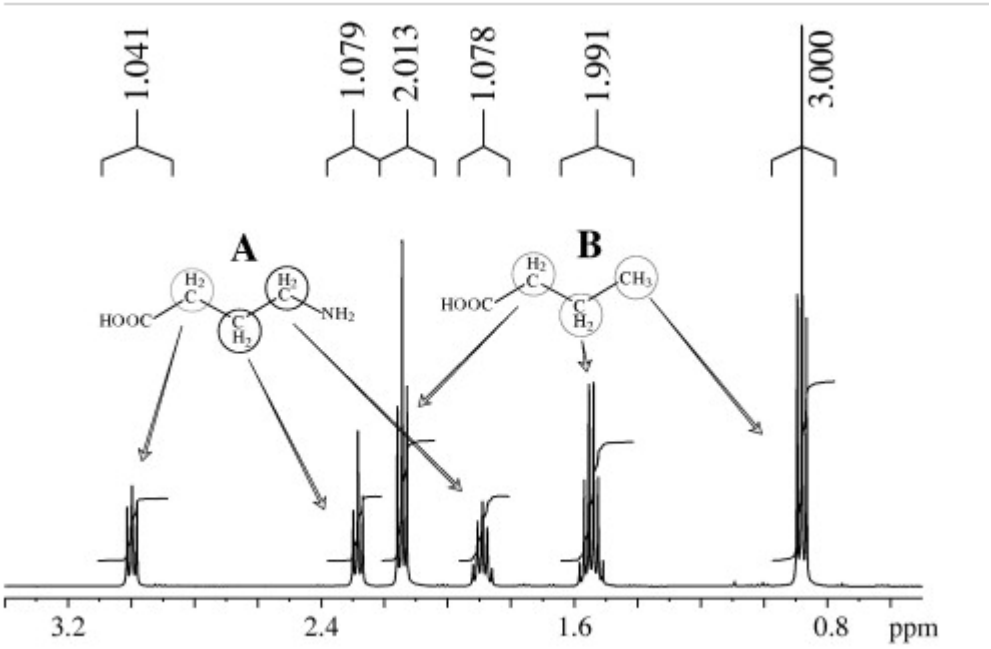


FIGURE 1.10: The relative integration value of A and B molecules peaks shows inter molecular resonance[4]

good analytic technique . The error in qNMR is introduced because of improper acquisition and wrong use of processing parameter . Because NMR is sensitive technique many error are introduced during sample preparation who effect the accuracy of result .

1*D* NMR experiments are generally used for analysis of low weight molecules or simple molecules . Peak overlap of noise to signal is a major limitation while 2*D* NMR experiment are used for resolving peak overlapping problem .

1.4.1 2*D* Quantitative NMR spectroscopy

2*D* NMR spectroscopy spread resonance frequency in two orthogonal direction and reduce peak overlapping . 2*D* NMR based metabolic profiling have advantage that it provide clear picture of metabolites while 1*D* NMR based metabolic profiling is biased toward abundant molecules . BY the help of Multivariate analysis and 2*D* NMR low abundant molecules can be achieved

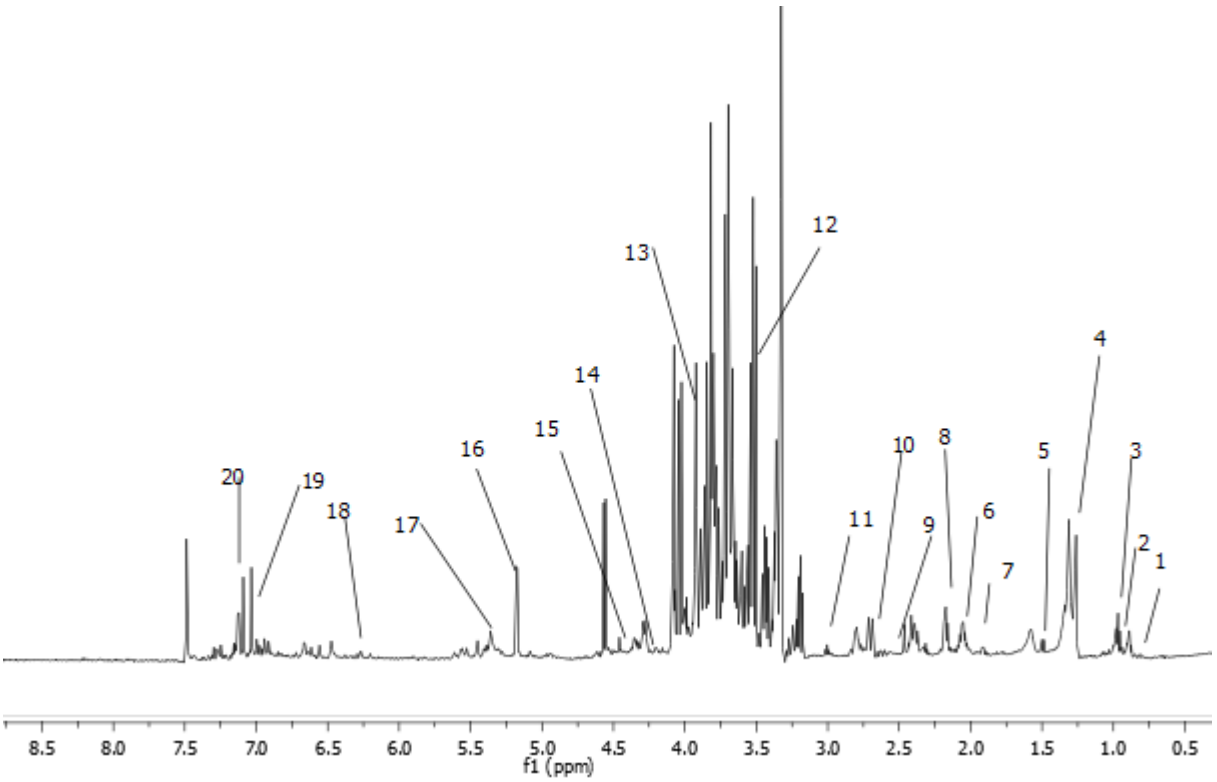


FIGURE 1.11: The Single pulse ^1H NMR spectrum shows metabolite peaks of lotus leaf and their intensity is proportional to their concentration

in metabolic profiling .

1H- 13C HSQC (hetronuclear single quantum correlation) is an example of 2D NMR experiment . 2D HSQC provide better dispersion in the $^(13)C$ direction . It have few limitation like it is low sensitive and take long acquisition time [8].

COSY(correlation spectroscopy) is another example of 2D NMR experiment . In COSY a single RF pulse (p_1) is followed by specific evolution time (t_1) followed by second pulse (p_2) and second pulse is followed by measurement period (t_2)[11].

Only one type of isotope is used in COSY for both direction like (^1H) . In COSY NMR spectrum there are two types of peaks diagonal and cross peaks . Diagonal peaks show metabolite peak of 1D NMR spectrum while cross peak express coupling between nuclei pairs .

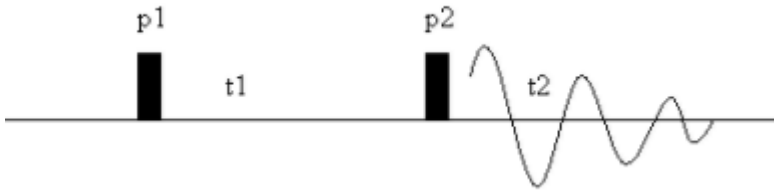


FIGURE 1.12: COSY pulse sequence[25]

TOCSY (total correlation spectroscopy) is same like COSY but the cross peaks show coupling between nuclei directly paired and paired by chain of coupling . It express the larger interconnected spin coupling .

1.4.2 Application of Qualitative NMR Experiment

Quantitative NMR is used in Chemistry , biology and medicine. Qualitative and quantitative analysis of natural product are nowadays carried out by NMR spectroscopy . NMR spectroscopy technique provides chemical structure and relationship between chemicals in natural product . It also provide purity and impurity standard in natural products . Quantitative NMR spectroscopy is used in organic synthesis without any standard compound . It provide concentration of new compound in mixture with clear structural information . In the field of metabolomics, qNMR is used in pharmaceutical research , disease diagnosis and animal study. Quantitative NMR is also used for environmental analysis. NMR spectroscopy is used in reaction monitoring and kinetic analysis[12,13] .

Chapter 2

NMR metabolomics And Lotus

2.1 Metabolomic analysis of plant

NMR metabolomic analysis is applied in many fields . In biophyics plant study based on NMR metabolomics have many advantages . Different species of plants are characterized and classified based on their special and unique behavior in medicinal science and in behavioral science . The study of stress and infection can be studies as change in metabolic profile of plant . The unexpected changes in metabolites in plants mostly in wild is a common phenomena which can be explained by the means of NMR metabolomics[2,6] .

2.2 Lotus

Nelumbonucifera also called Indian Lotus , Chinese water Lilly and sacred Lotus , all are placed in Mono generic Family Nymphaeaceae . In ancient time Lotus was spread over Nile river with his closely related species Blue Lotus . Starting from Egypt Lotus spread in Assyria , Persia , India and China . It is also called by names Padma and Kamala in India . Lotus Flower are spread in all over India and they are used in Decoration, as vegetable , medicinal purposes and as a cash crop . Lotus is a aquatic plant and grows up to 8ft [5] .

2.3 Metabolmic study of lotus

2.3.1 Objective

Lotus has a cyclic behavior related to Sun movements . As we know many phenotype behavior are linked to metabolites and their biochemical pathways . The primary objective is to find responsible metabolites for Lotus Cyclic Behavior . Plants leaf contain phytocromes to sense light signals . Many plant light controlled action other then photosynthesis are directly because of phytocromes . The cyclic behavior of lotus are called type 2 response of phytocromes[10].

2.3.2 practical consideration

The metabolites in plants are closely related to their developmental stages and environment .

The Leaves of Lotus plant were collected from unperturbed environment of IISER Mohali Pond . The samples were taken from many Lotus plants because studying the general behavior of species there is a probability of taking sample from infected or unhealthy plants .

2.3.3 Sample preparation

Lotus leaves were collected from IISER Mohali pond for 10 time points within 27 hours . For each time point 6 replicates were analyzed . The leaf sample was washed by distilled water after harvesting and placed in -80^0C for 2 days . After freezing leaves were lyophilized with help of vacuum freezer machine .Leaves were lyophilized because water can start enzymatic reaction in Biological sample And also show overlapping spectra for Proton NMR

.Leaves were crushed in fine powder and stored in -20^0C in air tight Eppendrof tubes.NMR Samples were prepared by using Methanol$-D4$ 840μl , D_2O 340μl,TMSP 20μl, Sodium hydrogen Phosphate 5.11mg and 0.1g plant leaf.D_2O and Sodium hydrogen phosphate are main extraction solvent . Leaf crushed samples were properly mixed in extraction solvent by centrifuging at 5000rpm for 5min and supernatant was removed from NMR sample[15] .

2.3.4 1D and 2D NMR Spectroscopy of Lotus

1D and 2D NMR spectroscopy was performed in Bruker Biospin 600MHz Spectrometer . Methanol D_4 was used for internal lock . Standard of spectrum is TMSP at 0.00ppm frequency .1D NMR spectra were acquired at 16 scans . For Better understanding of molecular behavior in Lotus 2D NMR were performed .COSY , HSQC ,HMQC were performed at 8 scan .

2.3.5 Database and software

Peaks of NMR spectrum were checked on Human Metabolome Database . $SIMCA$, metrenova , topshim , metabohunter , metaboanlysit software are used of metabolic study .

2.4 1*D* And 2*D* NMR spectrum of Lotus leaves

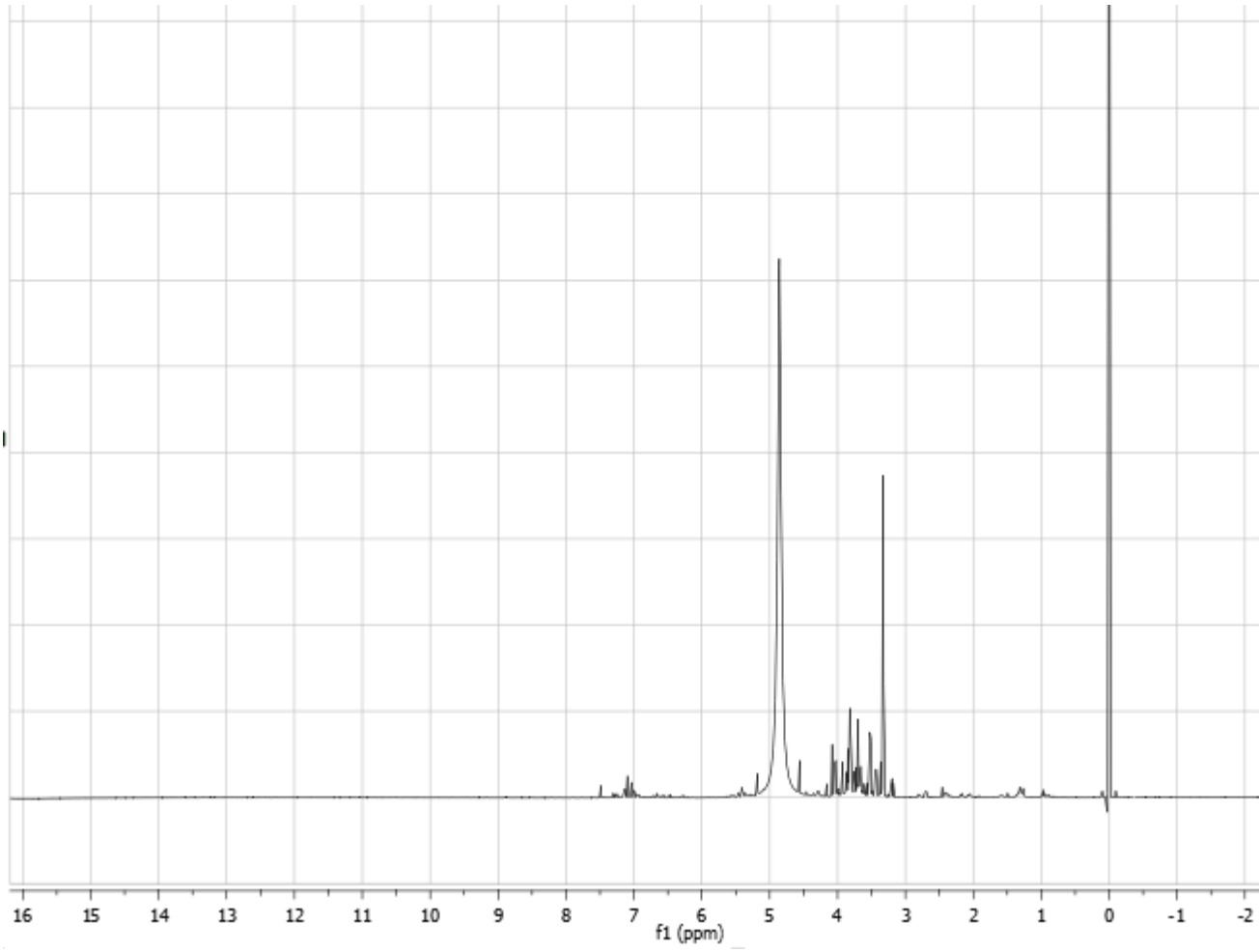


FIGURE 2.1: 1D NMR spectrum of Lotus

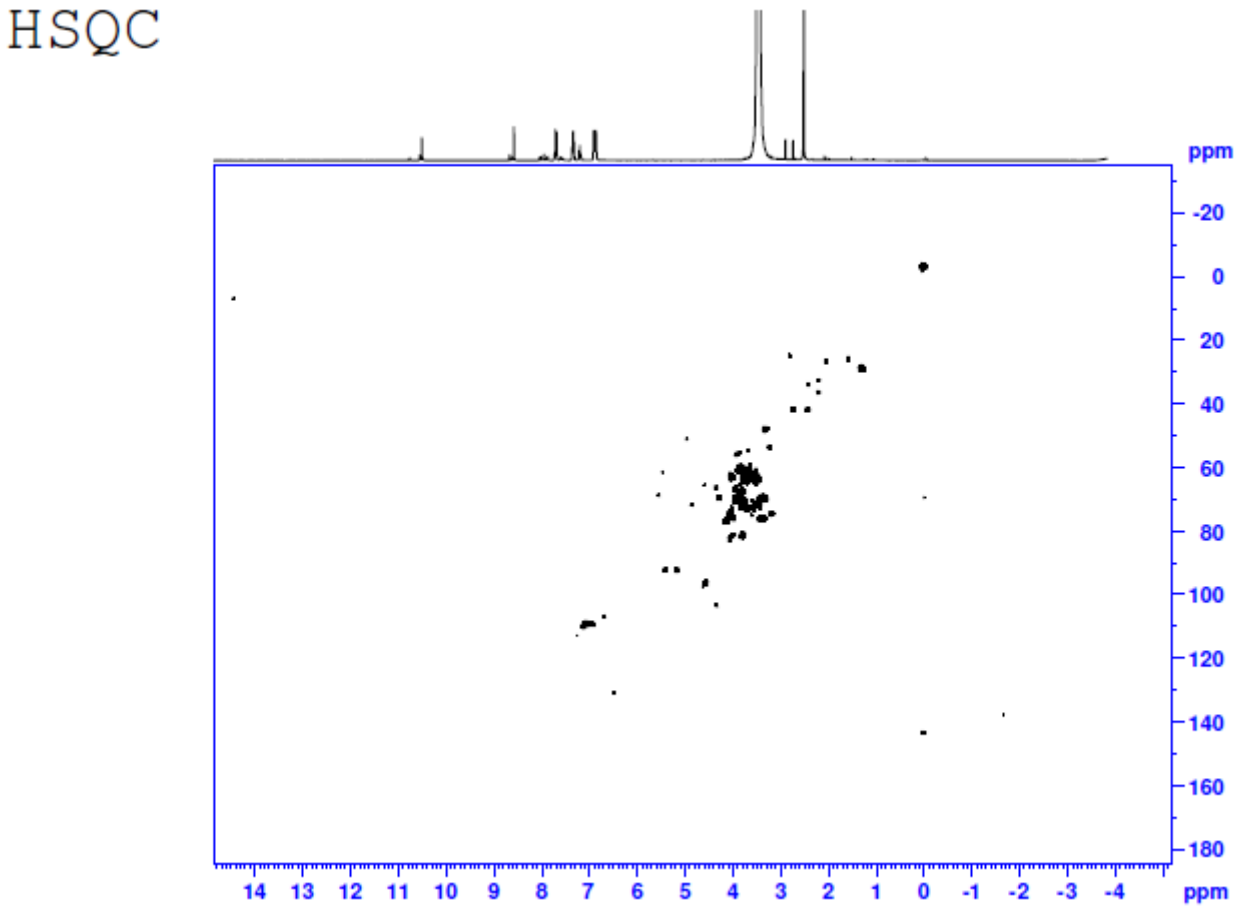


FIGURE 2.2: Heteronuclear single-quantum correlation spectroscopy of lotus leaves

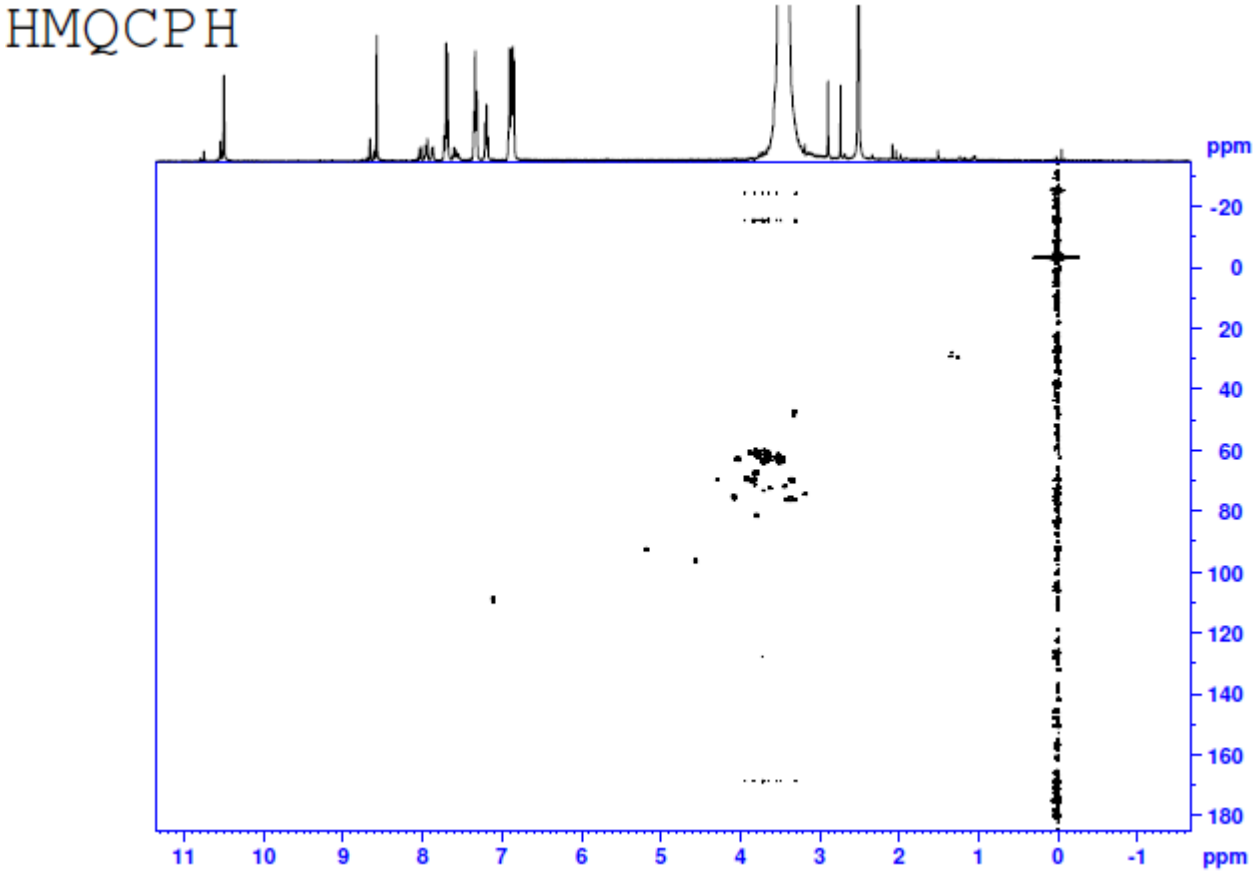


FIGURE 2.3: Heteronuclear single-quantum correlation spectroscopy

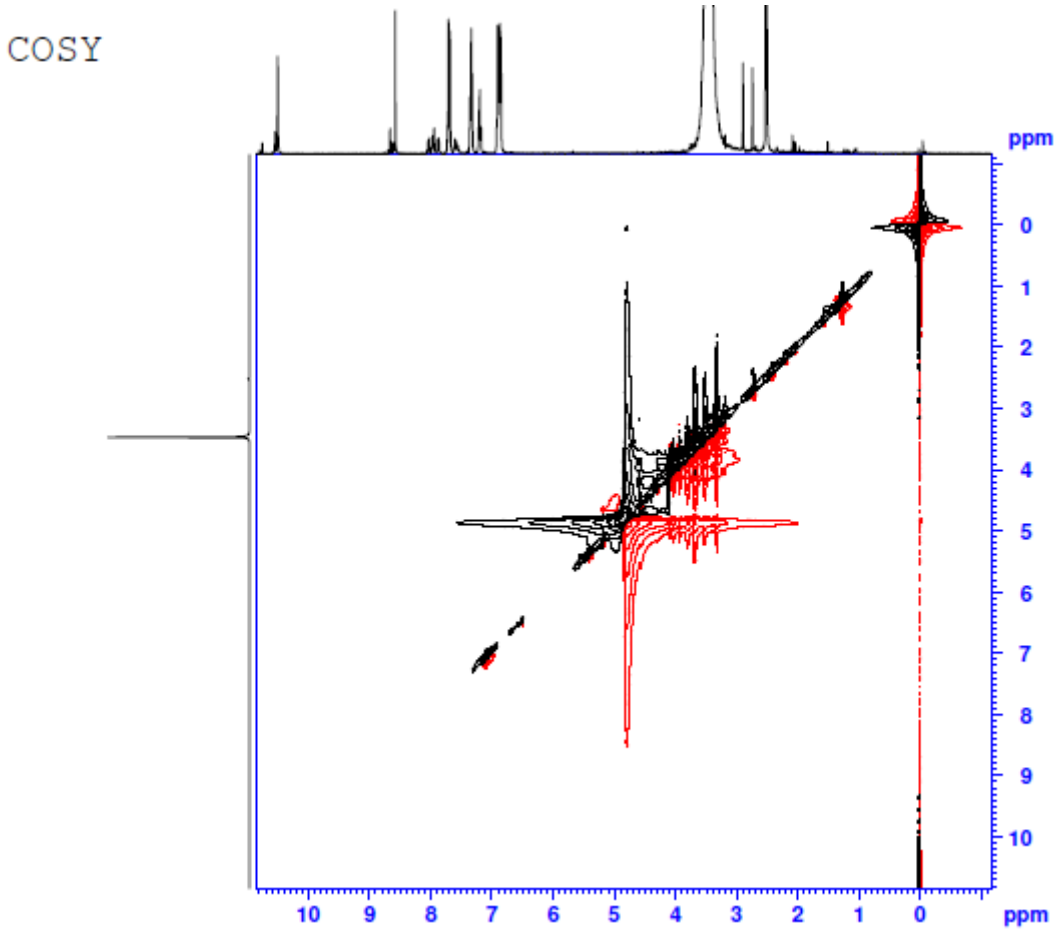


FIGURE 2.4: correlation spectroscopy of lotus leaves

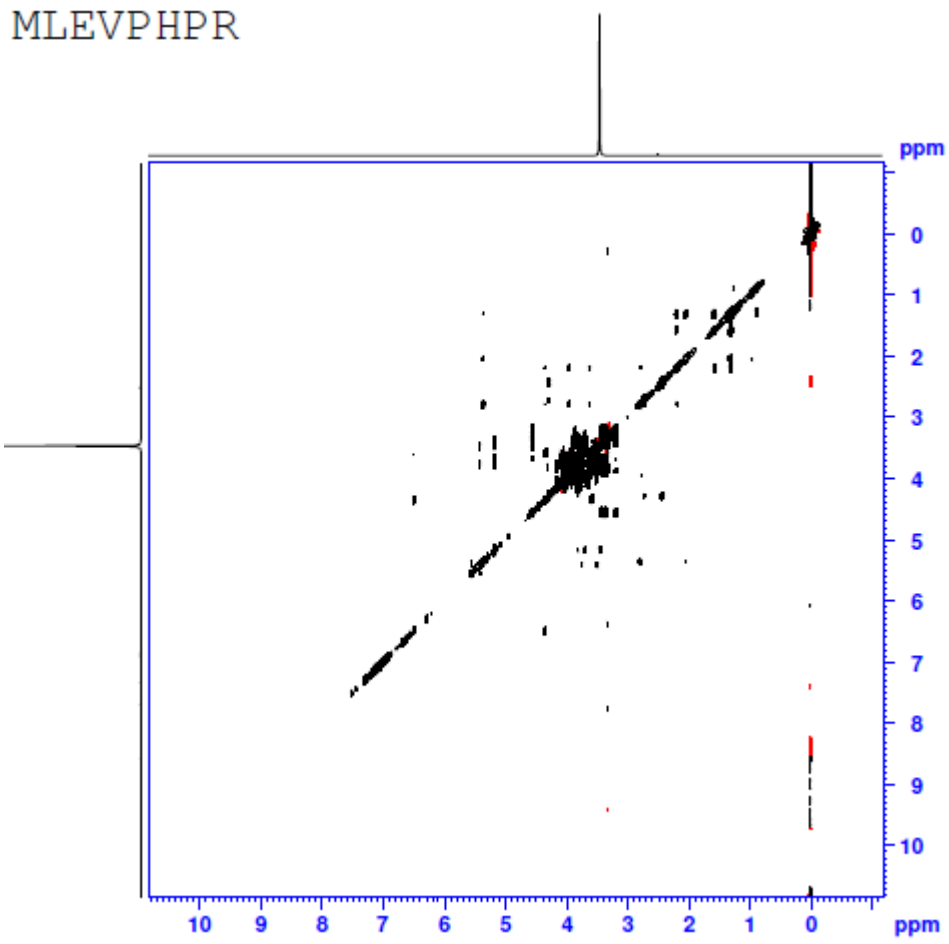


FIGURE 2.5: total correlation spectroscopy of lotus leaves

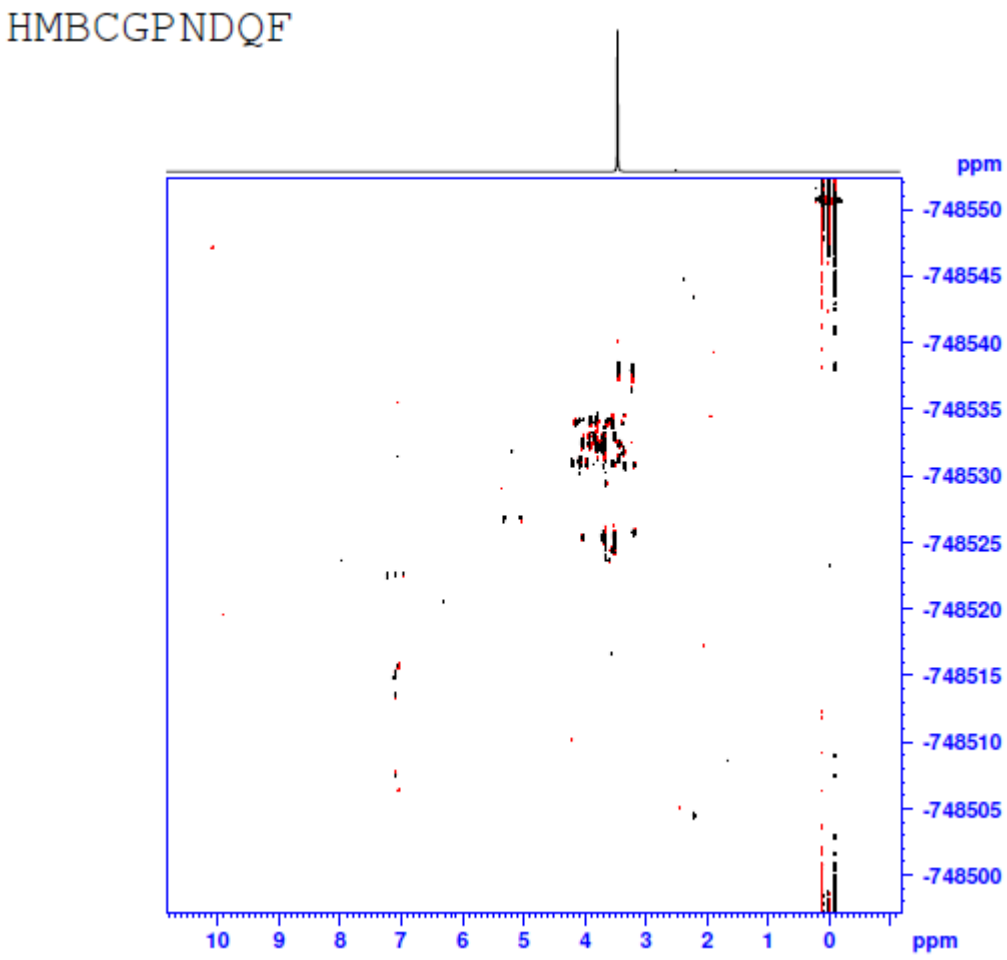
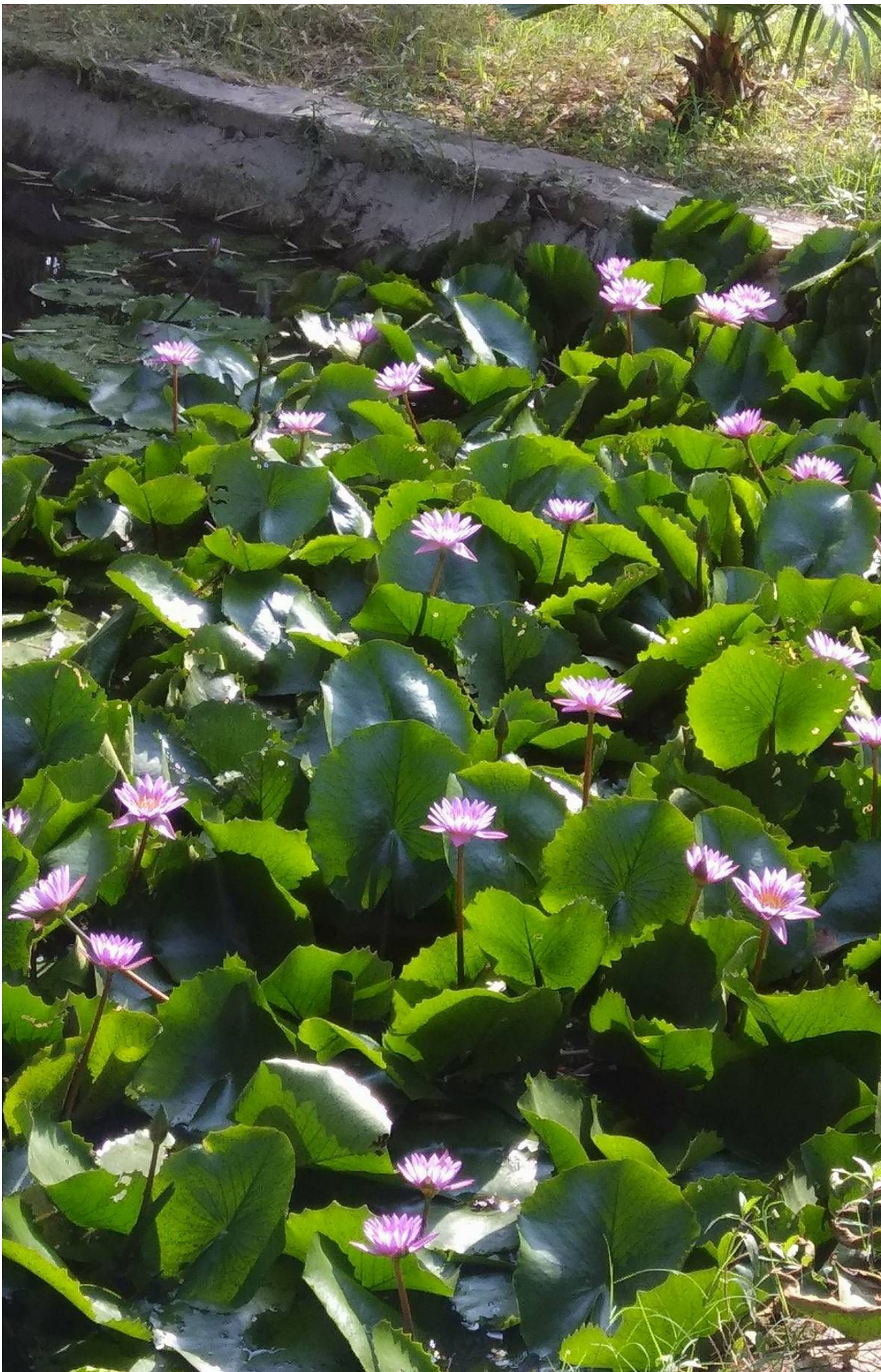


FIGURE 2.6: ^{1}H - $^{3}1P$ HMQC of lotus leaves



Chapter 3

Result and Conclusion

3.1 result

3.1.1 Metabolites in Lotus Leaf

Metabolites are identified based on $1D$ and $2D$ NMR Spectra of Lotus Leaves.

1. Amino acid :L-Alanine,N-Acetyl L-aspartic acid ,L-Cysteine , D-Cysteine , L-tryptophan .

2. Carbohydrate : Alpha Glucose

3 Organic acid : Citric Acid , hesperdine

4 Lipids : terminal methyl , methyl group , $-COOH$ group

5 Large Alkaloids : Ananine , N-nornuciferine , 2-benzylisoquinoline Co-clourine , norcoclaurine

6 Non phenolic base : Iiriodenine

7 Phenolic base : N-methyl-coclaurine

8 other : hyperoside , rutin , astragalin , O-nornuciferine

3.1.2 t test analysis

t - test analysis of lotus leaves at 10 time points with significant metabolites is given in table 3.1.

The concentration of significant metabolites is calculated by

$$m_A = \frac{m_s \times I_A \times MW_A \times N_S}{I_S \times MW_s \times N_A}$$

where m_s is mass of $TMSP$, I_A is peak integral of metabolite under observation , MW_A is molecular weight of metabolite , N_S is number of protons in $TMSP$, I_S is peak integration of metabolite , MW_s is molecular weight of TMSP,146.26$gmol^-1$ And N_A is number of proton in metabolite under observation .

It is shown in table that significant metabolites like $\alpha Glucose$ have different concentration at different time points .

TABLE 3.1: t- test analysis of lotus plant , concentration is given in w/w in percents

significant metabolites	3pm	6pm	9pm	12am	3am	6am	9am	12pm
hesperdine	0.103	0.103	0.1036	0.005	0.103	0.103	0.127	0.103
leucine	0.002	0.002	0.007	0.0002	0.005	0.0001	0.05	0.007
phenylalanine	0.01	0.025	0.007	0.001	0.093	0.0338	0.026	0.012
Alpha glucose	0.02	1.7789	0.0208	0.005	0.033	0.926	0.2397	0.125
kaempherol	0.048	0.48	0.048	0.0004	0.0009	0.16756	0.061	0.06
chlorogenic acid	0.001	0.002	0.004	0.0006	0.0006	0.1002	0.082	0.09
tryptophan	0.02	0.02	0.178	0.001	0.003	0.069	0.099	0.076

3.1.3 Multivariate analysis of Lotus leaves

The main aim of data analysis is to define the difference between group of data and explain in meaningful way . PCA and OPLS-DA of 10 classes of Lotus leaf sample is shown in figure 3.1 and 3.2 .

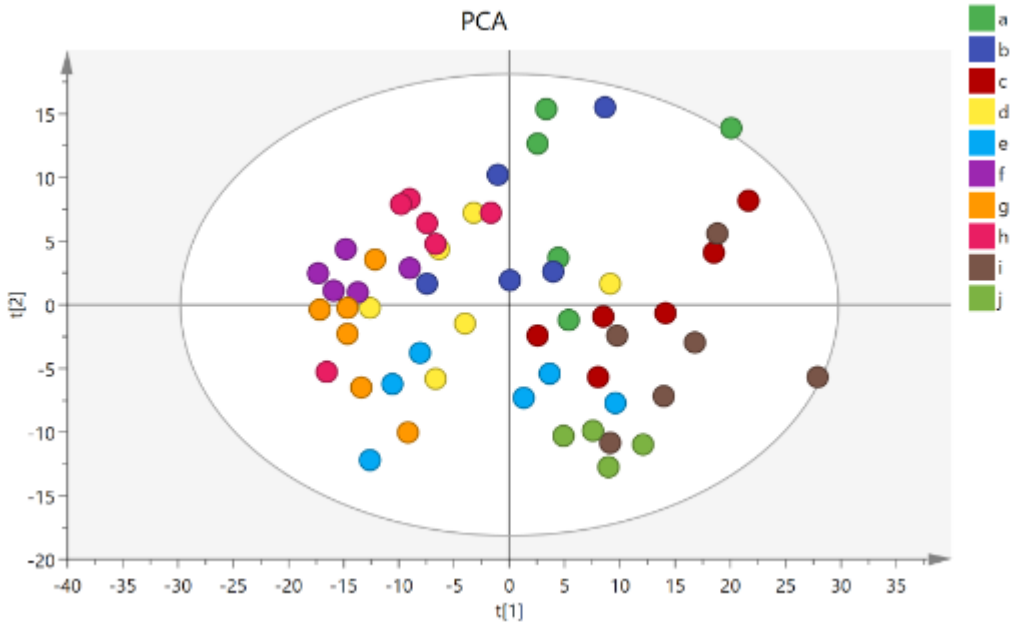


FIGURE 3.1: principal component analysis of lotus leafs (SIMCA)

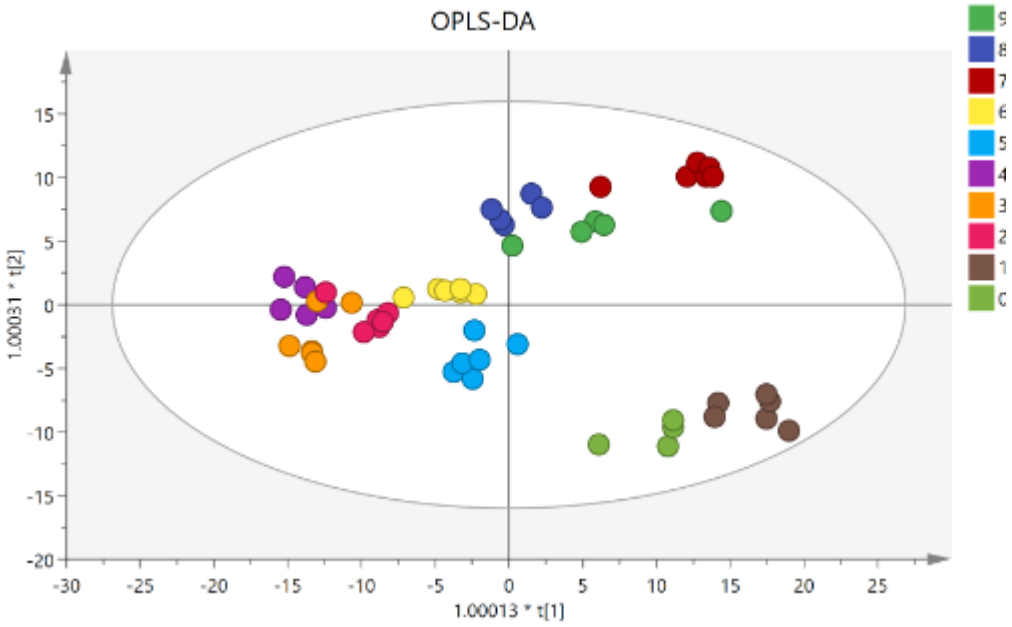


FIGURE 3.2: oplsda analysis of lotus leaves (SIMCA)

Classification of different metabolites in Lotus leafs is presented by multivariate PCA and OPLS-DA methods . Principal component 1 shows 46.9% variance and Principal component 2 shows 18.3% variance among different class of lotus leaf sample figure 3.3. We see a low variance in score plot and it imply less separation among groups figure 3.4. it shows a large similar metabolic profile of leaf sample of 10 different time points .

Loading plot of data is shown with two independent loading independent and orthogonal to each other in figure 3.6. The positive region of loading 1 has a high metabolic level in 3*Pm* and 6*pm* lotus sample . 6*am* lotus leaf sample have maximum variance between group and 6*pm* lotus leaf sample have maximum variance within group. The numbering sequence of timing of leaf collection start from 3*pm*(A) and have 3 hour gap .

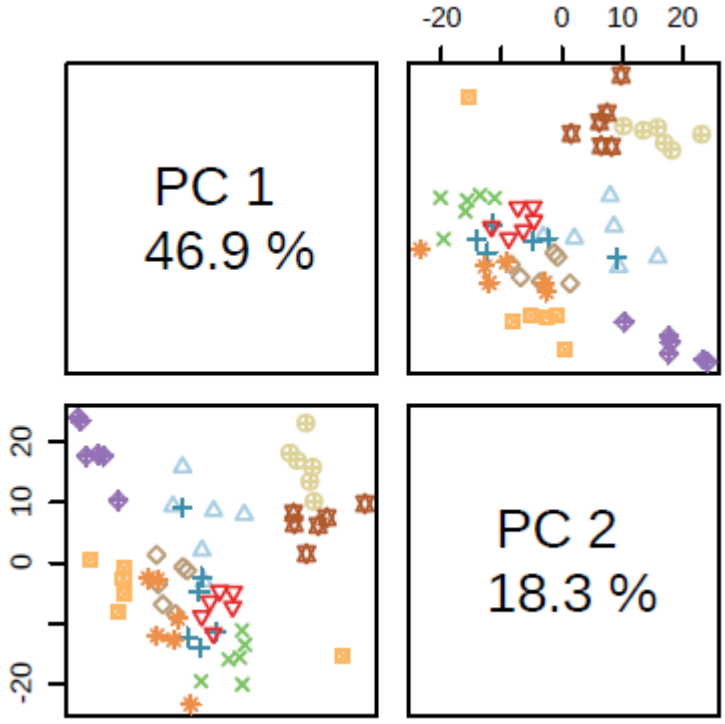


FIGURE 3.3: oplsda analysis of lotus leaves

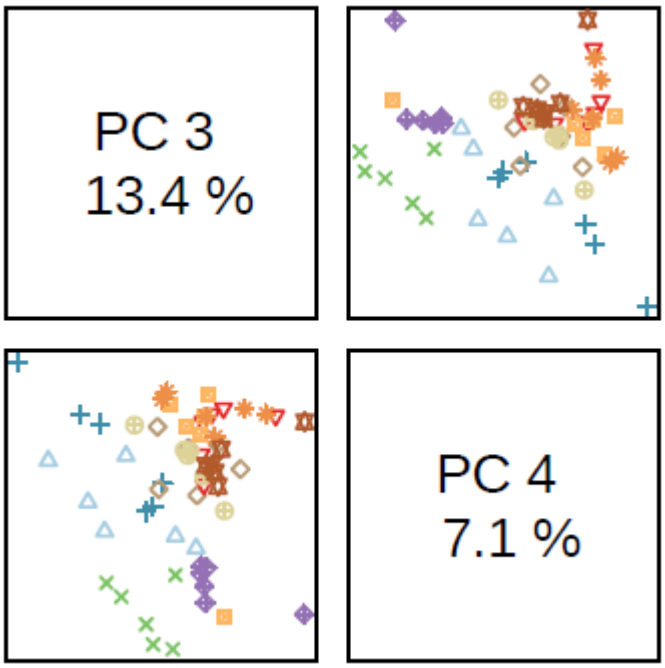


FIGURE 3.4: oplsda analysis of lotus leaves

3.2 Conclusion

L-Alanine,N-Acetyl L-aspartic acid ,L-Cysteine , D-Cysteine , L-tryptophan, Alpha Glucose,Citric Acid , hesperdine, terminal methyl , methyl group , $-COOH$ group ,Ananine , N-nornuciferine , 2-benzylisoquinoline Coclourine , norcoclaurine, Iiriodenine , N-methyl-coclaurine, hyperoside , rutin , astragalin , O-nornuciferine metabolites are identified in lotus plat leaf. Multivariate analysis of lotus leaf samples at 10 different time point show less variance among data . The concentration of α-glucose change from $6pm$ evening to $6am$ morning by a large amount relative to other molecules .

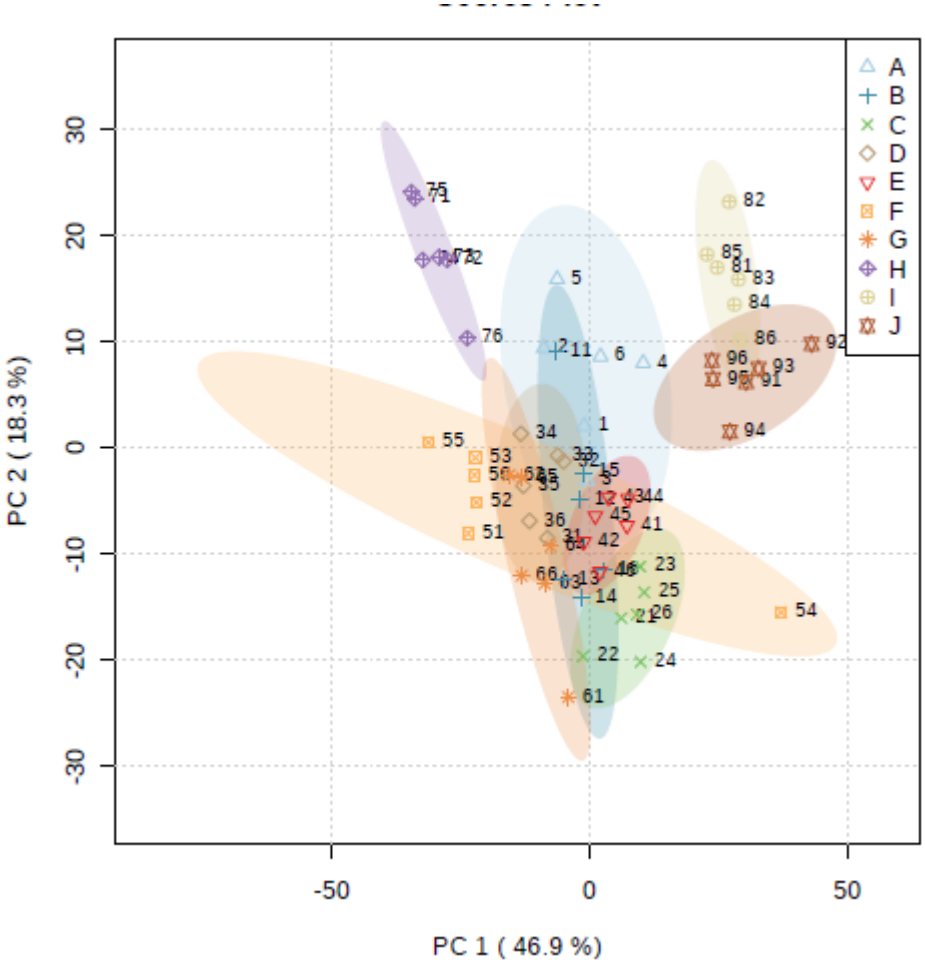


FIGURE 3.5: score plot of different class of lotus leaf samples

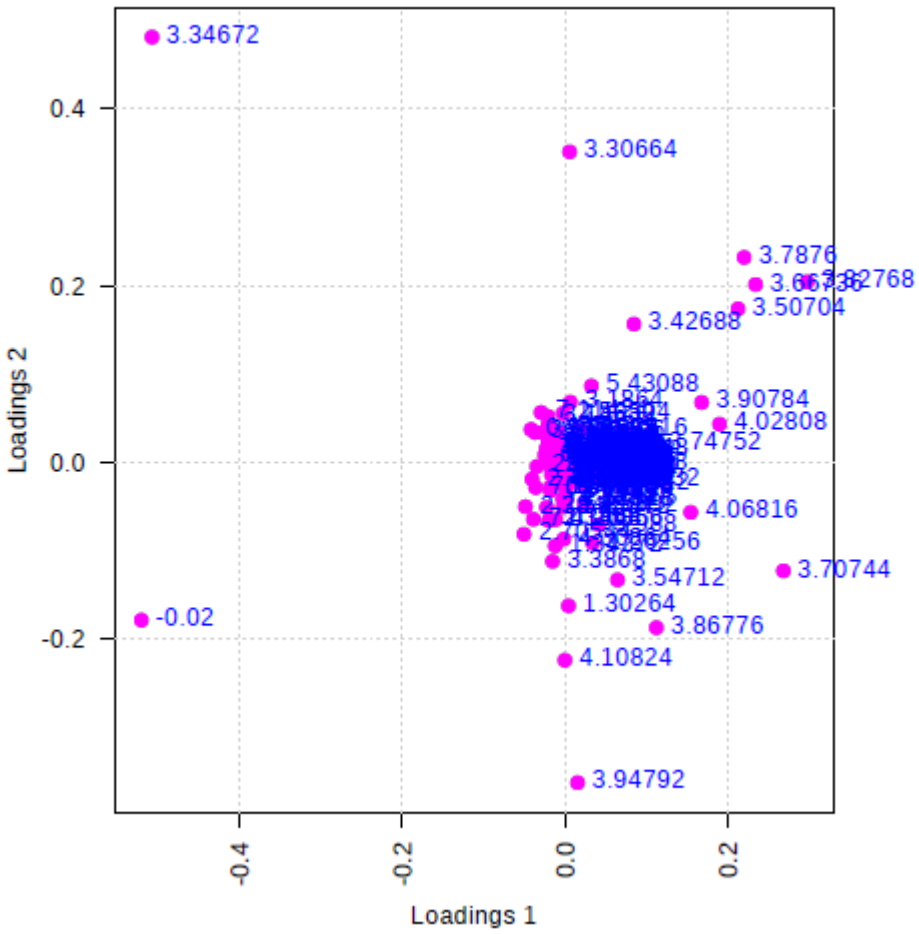


FIGURE 3.6: loading plot of different class of lotus leaf samples

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