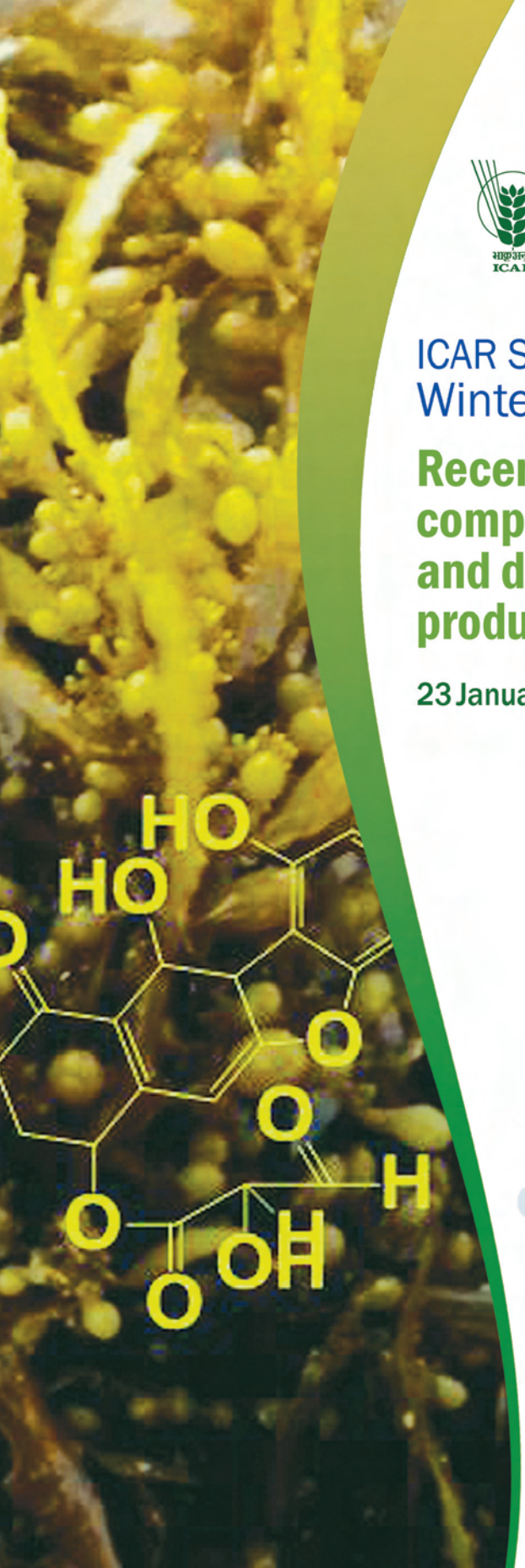
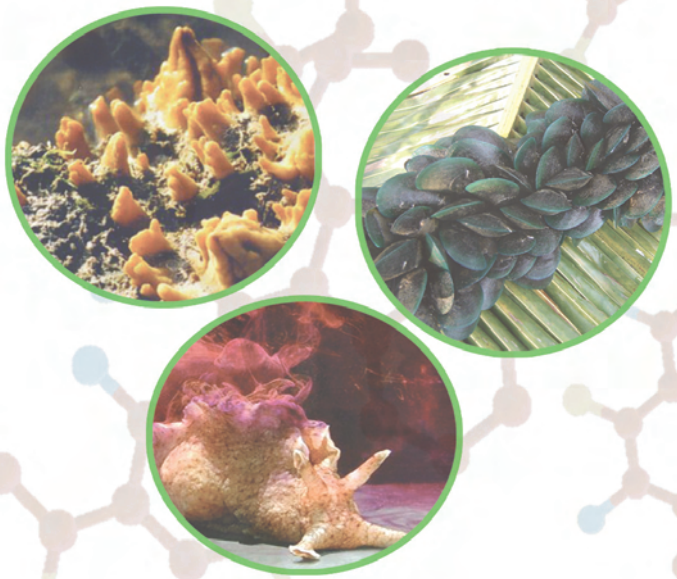
CMFRI Training Manual Series No.13/2018



ICAR Sponsored Winter School on

Recent advances in bioactive compounds from marine organisms and development of high value products for health management

23 January to 12 February 2018



Marine Biotechnology Division
ICAR-Central Marine Fisheries Research Institute
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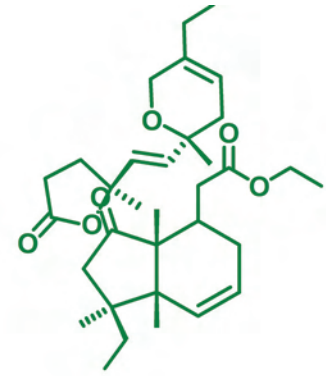

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Course Manual

ICAR-Winter School on

Recent advances in bioactive compounds from marine organisms and development of high-value products for health management

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ICAR-Central Marine Fisheries Research Institute,
23 January - 12 February, 2018

Publisher :

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Ernakulam North P. O., Kochi - 682 018, Kerala

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This manual has been prepared as a reference material for the ICAR funded Winter School on "Recent advances in bioactive compounds from marine organisms and development of high-value products for health management" held at Central Marine Fisheries Research Institute, Kochi during 23 January - 12 February, 2018

Edited by Foxit Reader

FOREWORD

There has been a growing interest in the marine derived bioactive compounds in the recent years, and the functional foods, enriched with natural ingredients have been proved to provide beneficial action for human health. Marine derived bioactive components and the functional food ingredients demonstrated to possess potential health benefits. High value secondary bioactive metabolites from the marine organisms are attracting attention because of the growing demand for new compounds of 'marine natural' origin, having potential applications in pharmaceutical fields, and concerns about the adverse effects by synthetic drugs and their derivatives. The pioneering R & D works at ICAR-Central Marine Fisheries Research Institute on marine bioprospecting envisaged a systematic approach involving chemical profiling of major species of marine organisms for bioactive pharmacophore leads for activity against various diseases, and a library of molecules with bioactive potential. The research work in this institute developed protocols to prepare various pharmaceutical leads, nutraceuticals/ functional food supplements enriched with lead molecules with different properties against various drug targets for use against various life-threatening diseases.

ICAR-Central Marine Fisheries Research Institute is the pioneering marine research institute in India to work in the frontier area of bioactive molecule discovery from marine organisms as promising therapeutic agents against various diseases, aquatic food product technology, and development of high value products for health management. This prestigious research institute of Indian Council of Agricultural Research is working in the broad national interest of producing high value bioactive leads from the marine organisms, which would provide promising therapeutic agents against various diseases. This institute has developed and commercialized the nutraceutical products Cadalmin™ Green Algal extract (Cadalmin™ GAe) and Antidiabetic extract (Cadalmin™ ADe) as green alternatives to synthetic drugs to combat rheumatic arthritic pains and type-2 diabetes, respectively to a leading biopharmaceutical company in India. The anti-inflammatory nutraceutical Cadalmin™ Green Mussel extract (Cadalmin™ GMe) from Asian green mussel Perna viridis has been commercialized with Amalgam Group of Companies. Cadalmin™ Antihypercholesterolemic extract (Cadalmin™ ACe) has been developed from seaweeds to combat dyslipidemia leading to obesity, and the product was out-licensed to a leading Indian MNC in wellness and obesity management. Antimicrobial therapeutic product from marine bacteria as oral applicant has been developed and the product is in pipeline for commercialization. Seaweed-derived natural template inspired synthetic derivatives as potential pharmacophores were designed and developed. Several nutraceutical and cosmeceutical products from marine organisms are in pipeline, and are being commercialized.

The objective of the National level ICAR Winter School on "Recent advances in bioactive compounds from marine organisms and development of high-value products for health management" is to provide up-to-date information and acquaint the participants with the latest technologies on isolation and characterization of marine natural products of pharmaceutical importance from marine organisms, general and advanced methods of isolation procedures by chromatography, classification of organic compounds and their characterization by advanced spectroscopic experiments. This program further aims to give exposure to the chemical perspectives of marine organisms, primary and secondary bioactive metabolites from fish and marine organisms to develop bioactive compounds and high-value functional food products. Theory and practical classes will be conducted in these areas to provide the participants a hands-on experience.

This ICAR Winter School is organized with the full funding support from ICAR, New Delhi, and the twenty-five participants from various parts of India who are attending this programme were selected after scrutiny of their applications based on their bio-data. They are serving as academicians, such as Professors/Scientists, and in similar posts. The faculties include the knowledgeable scientists and professors from various parts of India and abroad. This training will enable the participants to efficiently carry out their academic programmes, and to plan research on bioactive molecule discovery in their respective laboratories and institutes so that they can formulate the strategies for research.

The Winter School on "Recent advances in bioactive compounds from marine organisms and development of high value products for health management" is very ideal for the current scenario of increasing lifestyle diseases and human health. Understanding the importance of natural products in the health care system of India, ICAR-Central Marine Fisheries Research Institute has reasonably contributed in the various aspects. The Manual released on this occasion covers all aspects of marine natural products prepared by the experts in their respective fields. I congratulate the Course Director of this programme, Dr. Kajal Chakraborty and Head of the Marine Biotechnology Division, Dr. P. Vijayagopal, along with other staff members of Marine Biotechnology Division and Central Marine Fisheries Research Institute for their sincere efforts in bringing out the manual in time, and to arrange the programme in a befitting manner.

A. Gopalakrishnan
Director, ICAR-Central Marine Fisheries Research Institute
Kochi, Kerala

PREFACE

Marine-derived bioactive components and the functional food ingredients with potential health benefits are an emerging area of research. The rich diversity of flora and fauna in the marine and coastal habitats of the Indian subcontinent represent an untapped reservoir of bioactive compounds with valuable pharmaceutical and biomedical use. Considering the underutilization of these groups of marine organisms, exploring bioactive compounds and development of any biologically useful products have benefits as health products. Comprehensive analyses demonstrated that during the last decade the average proportion of bioactive compounds among the new compounds is declining, though there are a large number of marine natural products yet to be explored. This may indicate that the research level of bioactivity is not keeping up with the discovery of new compounds. Thus, the research tools and methods for finding bioactivity need to be improved. The first improvement is about methods of spectral and bioactivity-guided separation and purification of marine-derived secondary metabolites, which combine the discovery of new compounds. These improvements in technology are dependent upon the automation in spectroscopy, which also allows the study of the functions of new compounds extracted from the target marine organisms. Second, for the discovery of new lead compounds and artificial intelligence for drug development evolved to a more mechanistic approach that targets specific molecular lesions. Combined with high-throughput screening through a large number of drug targets, bioactivity research against various life-threatening diseases will be effective in revealing the potentially useful biological properties of marine natural products. Furthermore, the discovery of new bioactive compounds from marine metabolites will form the basis for new drug leads. Thus, the new compounds will absolutely compose an abundant resource for future bioactivity research and drug development. Various medicinal and biomedical products from marine flora and fauna provide a myriad of benefits for human health and multiple life-threatening diseases, and therefore, are the attractive options for the food and pharmaceutical industry. The increasing interest in marine-based functional food ingredients and nutraceutical formulations in the last decade along with increased number of patents filed/granted have appropriately demonstrated the possibilities of bioactive from marine organisms to maintain and improve human health and well-being.

The present ICAR Winter School on "Recent advances in bioactive compounds from marine organisms and development of high-value products for health management" is designed to acquaint the participants with the advances in marine bioactive compounds with emphasis on the latest technologies on isolation and characterization of marine natural products of pharmaceutical importance. The course is planned in such a way that it covers both theoretical and practical aspect of recent advances in bioactive compounds from marine organisms. This programme will strengthen the knowledge of participants with regard to

the general and advanced methods of isolation procedures by chromatography, and their characterization by advanced spectroscopic experiments aspects.

I wish to thank the Education Division of Indian Council of Agricultural Research for giving us an opportunity to organize this ICAR Winter School. We are grateful to Dr. A. Gopalakrishnan, Director, ICAR-Central Marine Fisheries Research Institute, for his guidance, continuous interest in the course and providing all necessary facilities. I am highly obliged to Dr. P. Vijayagopal, Head, Marine Biotechnology Division for his guidance and support for the programme. All the scientists of Marine Biotechnology Division, technical staff, supporting staff and research scholars supported us in organizing the ICAR Winter School. I recall with gratitude the marvellous effort and help in preparing this manual by Minju Joy, Research Scholar of Marine Biotechnology Division. I take this opportunity to thank all the faculty members who have devoted their valuable time and contributed material for the preparation of the manual. I am confident that the Course Manual would aid the participants to enhance their knowledge and competence in the area of marine bioactive compounds and their applications for the development of high-value products for health management.

Kajal Chakraborty
Course Director

January, 2018

CONTENTS

CHAPTER

08

SPECTROSCOPIC METHODS TO CHARACTERIZE BIOACTIVE COMPOUNDS: NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Dr. Meledath Govindan[1], Kajal Chakraborty[2], Minju Joy[2]

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[2]ICAR-Central Marine Fisheries Research Institute, Kochi

NMR SPECTROSCOPY

It is a spectroscopic technique that gives us information about the number and types of atoms in a molecule, for example, about the number and types of

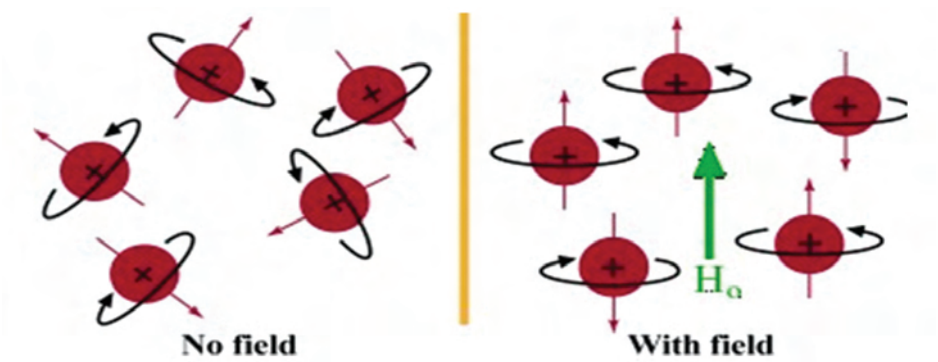
- Hydrogen atoms using ^{1}H NMR spectroscopy
- Carbon atoms using ^{13}C NMR spectroscopy
- Phosphorus atoms using ^{31}P NMR spectroscopy
- Numerous other nuclei - ^{15}N, ^{19}F, etc.

NUCLEAR SPIN STATES

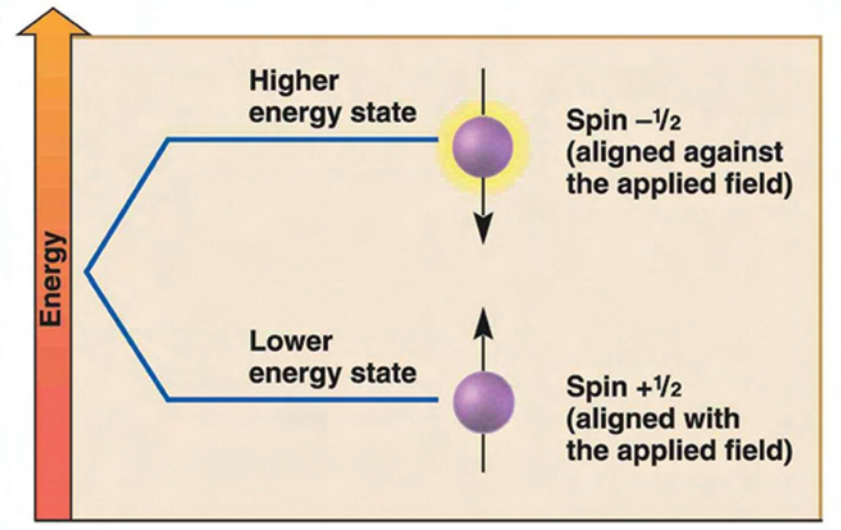
An electron has a spin quantum number of 1/2 with allowed values of +½ and -½. This spinning charge creates an associated magnetic field. In effect, an electron behaves as if it is a tiny bar magnet and has what is called a magnetic moment. The same effect holds for certain atomic nuclei. Any atomic nucleus that has an odd mass number, an odd atomic number, or both also has a spin and a resulting nuclear magnetic moment.

NUCLEAR SPINS IN B_0

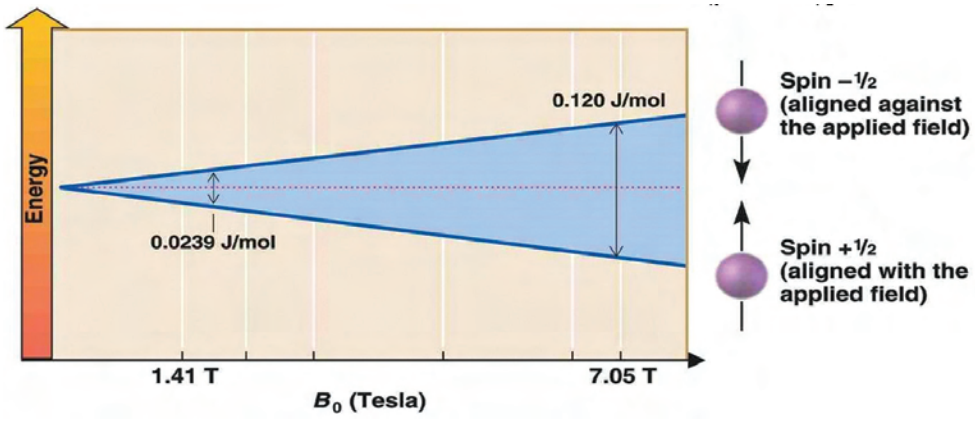
Nuclear spins are completely random in orientation within a collection of ^{1}H and ^{13}C atoms. Only certain orientations of nuclear magnetic moments are allowed only when the interaction between nuclear spins and the applied magnetic field is quantized in a strong external magnetic field of strength B_0.



Two orientations are allowed for ^{1}H and ^{13}C

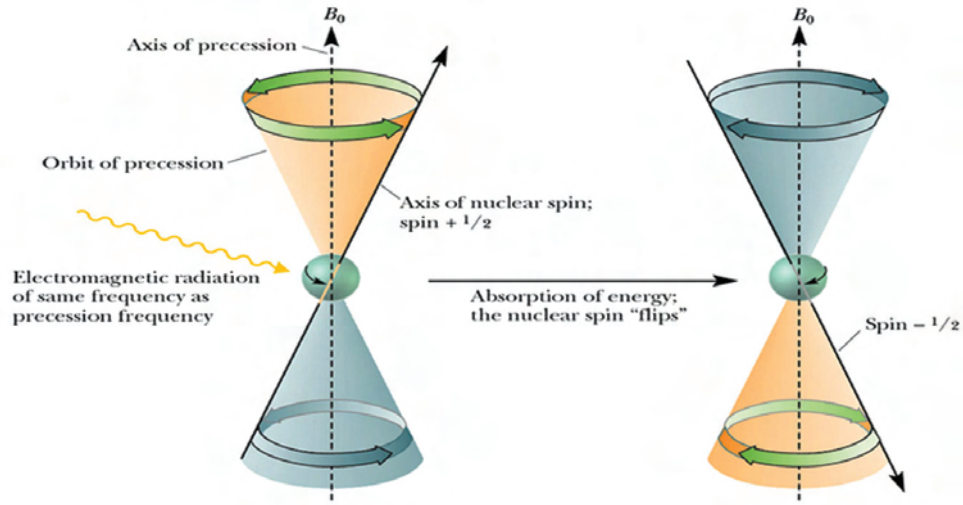


The energy difference between allowed spin states increases linearly with applied field strength [values shown here are for ^{1}H nuclei (protons)].



When nuclei with a spin quantum number of ½ are placed in an applied field, a majority of nuclear spins are aligned with the applied field in the lower energy state (a-state). The nucleus begins to precess and traces out a cone-shaped surface, in much the same way a spinning top or gyroscope traces out a coneshaped surface as it precesses in the earth's gravitational field. The rate of precession represented as a frequency in hertz (Hz). If the precessing nucleus is irradiated with electromagnetic radiation of the same frequency as the rate of precession, (1) the two frequencies couple, (2) energy is absorbed, and (3) the

nuclear spin is flipped from spin state +½ (with the applied field) to -½ (against the applied field).

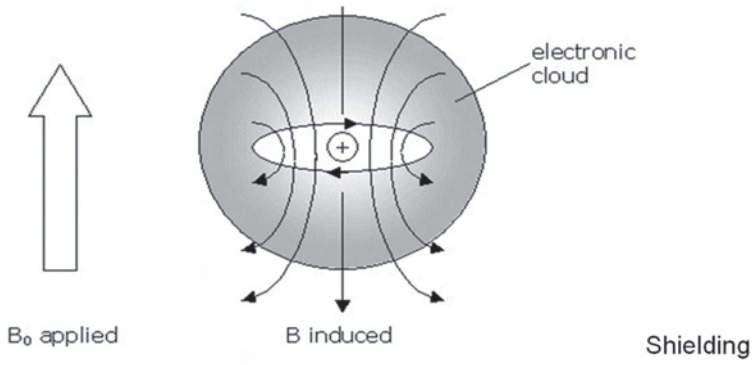


RESONANCE

In NMR spectroscopy, resonance is the absorption of electromagnetic radiation by a precessing nucleus and the resulting "flip" of its nuclear spin from a lower energy state to a higher energy state.

CHEMICAL SHIFT

Hydrogen in organic molecules is not isolated from all other atoms; they are surrounded by electrons, which create another magnetic field. These electrons magnetic fields act as a "blanket" and shield the protons from the external field. Result of this electron shielding is that ^1H nuclei in different electronic environment absorb at different frequencies or they are said to have different chemical shift.



CHEMICAL SHIFT - Ä

Signals are measured relative to the signal of the reference compound tetramethylsilane {$(CH_3)_3Si$}.

For a ^{1}H-NMR and ^{3}C-NMR spectra, signals are reported by their shift from the signal of TMS that is set as 0 ppm.

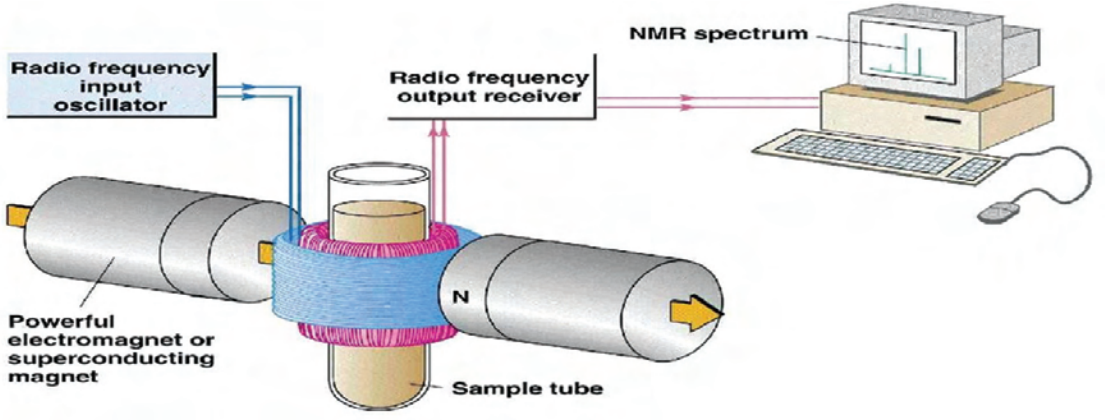
Chemical shift (ä): the shift in **ppm** of an NMR signal from the signal of TMS set as 0 ppm.

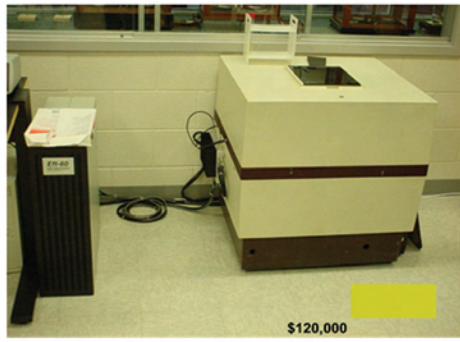
NMR SPECTROMETER

Spectrometer is the instrument used to detect the frequency of electromagnetic radiation and record it as a signal.

Signal: A recording in an NMR spectrum of a nuclear magnetic resonance.

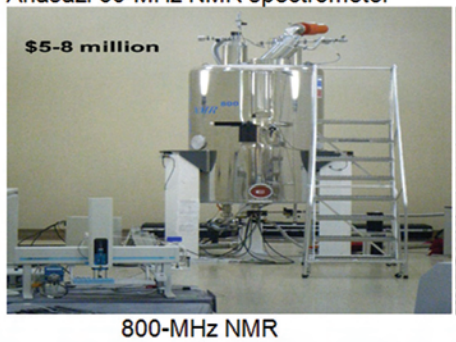
- Essential parts of an NMR spectrometer are a powerful magnet, a radio-frequency generator, and a radio-frequency detector.
- The sample is dissolved in a solvent, most commonly $CDCl_3$ (chloroform-D), CD_3OD (methanol–D_4), or acetone-D_6 and placed in a sample tube which is then suspended in the magnetic field.
- Using a Fourier transform NMR (FT-NMR) spectrometer, a spectrum can be recorded in about 2 seconds.





Anasazi 60-MHz NMR spectrometer

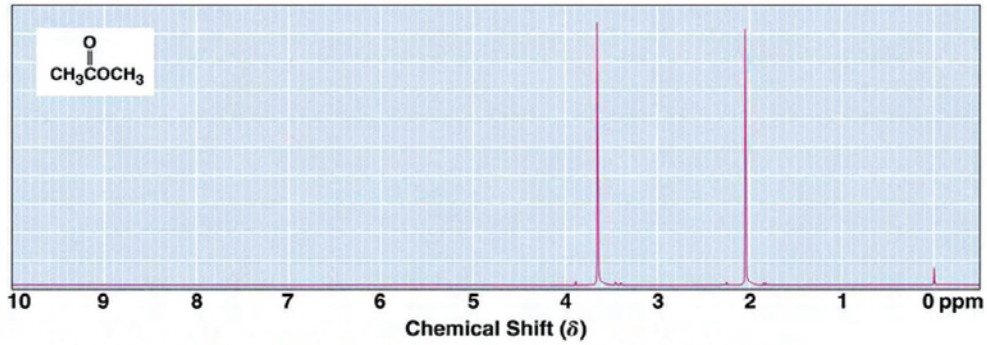
400-MHz NMR



800-MHz NMR

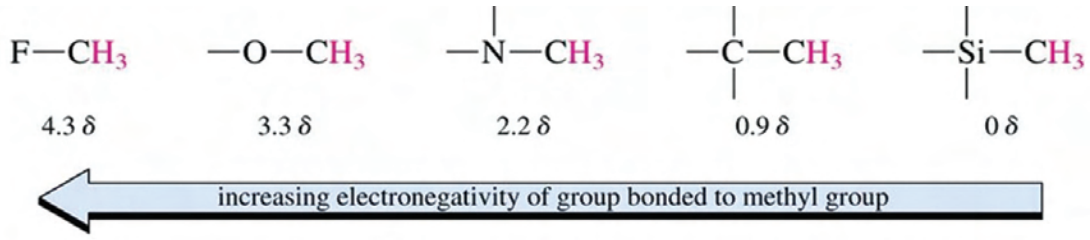
900-MHz NMR

TYPICAL NMR SPECTRUM



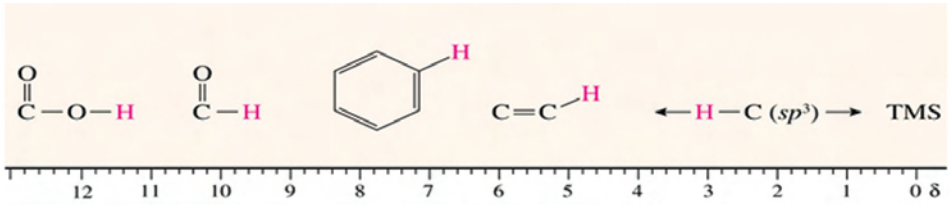
Downfield: the shift of an NMR signal to the left on the chart paper.
Upfield: the shift of an NMR signal to the right on the chart paper.
Equivalent hydrogens: They have the same chemical environment. A molecule with 1 set of equivalent hydrogens gives 1 NMR signal.

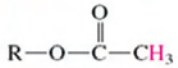
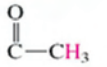
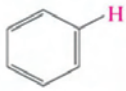
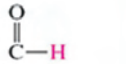
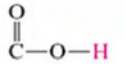
CHEMICAL SHIFT AND ELECTRONEGATIVITY OF ATTACHED ATOMS/GROUPS



Electronegative atoms deshield nearby hydrogens, resulting in a downfield shift.

GENERAL CHEMICAL SHIFT REGIONS

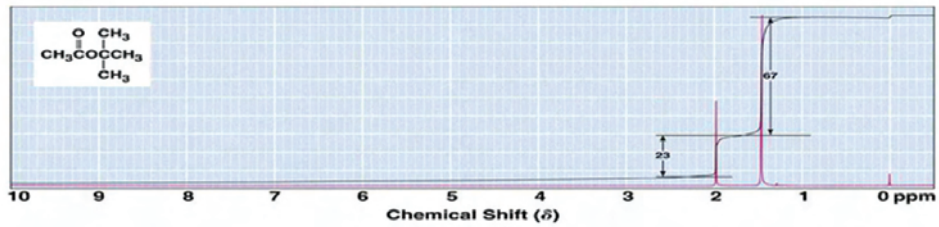


Type of Hydrogen	Chemical Shift (δ)	Type of Hydrogen	Chemical Shift (δ)
—C—CH_3	0.9	Cl—CH_3	3.0
C=C—CH_3	1.6	O—CH_3	3.3
C≡C—H	1.8		3.7
N—H	1–3	O_2N—CH_3	4.1
O—H	2–5	F—CH_3	4.2
	2.0		5.5–6.5
	2.2		7–8
N—CH_3	2.2		10
I—CH_3	2.2		12
N≡C—CH_3	2.2		
Ph—CH_3	2.3		
Br—CH_3	2.7		

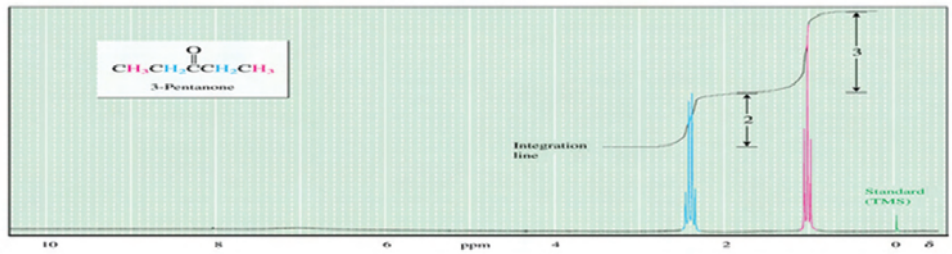
Note that these positions are only approximate. Furthermore, most of these positions are given for CH_3 groups.
CH_2 groups appear farther downfield by about 0.3 ppm and CH groups by about 0.7 ppm.

SIGNAL AREAS

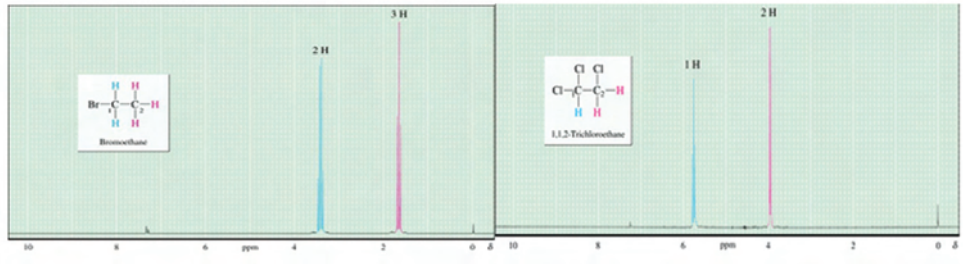
- Relative areas of signals are proportional to the number of H's giving each signal.
- Modern NMR spectrometers electronically integrate and provide integration lines.



INTEGRATION OF PEAKS



SPIN-SPIN COUPLING



SIGNAL SPLITTING: THE (n + 1) RULE

Peak: The units into which an NMR signal is split; doublet, triplet, quartet, etc.

Signal splitting: Splitting of an NMR signal into a set of peaks by the influence of neighboring nonequivalent hydrogens – usually this means H's on neighboring carbons.

(*n* + 1) rule: If a hydrogen has *n* hydrogens nonequivalent next to it but equivalent among themselves on the same or adjacent atom(s), its ^{1}H-NMR signal is split into (*n* + 1) peaks

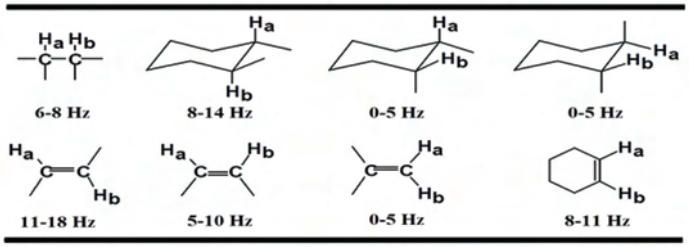
ORIGINS OF SIGNAL SPLITTING

Signal coupling: It is the interaction in which the nuclear spins of adjacent atoms influence each other and lead to the splitting of NMR signals. In the simplest case we expect to see a single peak for each type of proton in a molecule. But consider what happens if a proton that we are looking at (H_A) is near another nonequivalent proton (H_B). In half of the molecules the H_A proton will be adjacent to an H_B aligned with the field and in the other half the H_A proton will be adjacent to an H_B aligned against the field. Thus, half the H_A's in the sample will feel a slightly larger magnetic field than they would in the absence of H_B and half will feel a slightly smaller magnetic field. Thus, we will observe two absorptions for the H_A proton. Of course we would also observe the same thing for H_B. This is called scalar or J-coupling. The spin-spin coupling is transmitted through the electrons in the bonds and so depends on the bonding relationship between the two hydrogens.

Coupling constant (J): The separation on an NMR spectrum (in hertz) between adjacent peaks in a multiplet. It is a quantitative measure of the influence of the spin-spin coupling with adjacent nuclei and can give us further structural information.

COUPLING CONSTANTS

Coupling constant (J): The distance between peaks in a split signal, expressed in hertz. It is the value is a quantitative measure of the magnetic interaction of nuclei whose spins are coupled.



CARBON-13 NMR:

The principles of carbo-13 NMR are similar to that of proton NMR with the following important exceptions.

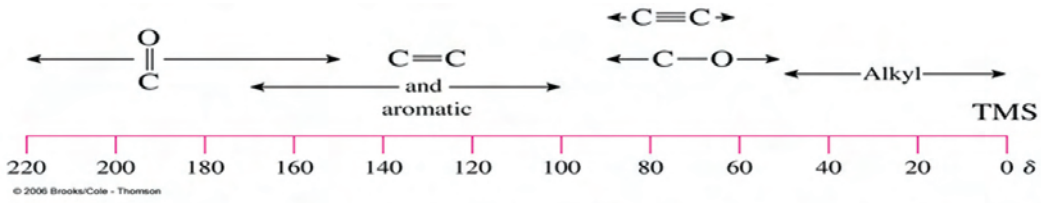
1. Since the natural abundance of ^{13}C isotope is only 1.1% the signal strength is inherently low. Before the advent of Fourier-transform it used to take hours or even days to acquire the spectrum. With the new FT-NMR spectrometers we can generate spectra in minutes.
2. The chemical shifts range from 0 to 220 ppm.
3. Since there is little chance that two ^{13}C atoms are going to be next to each other there is no splitting between carbons. However, carbons can be split by the H's attached to them. The same (n+1) rule applies. Such spectra are going to be very messy and difficult to interpret. Therefore, we usually generate spectra without

the H's being able to split the carbons and all carbons appear as singlets.

4. Size of the peak is not proportional to the number of carbons as is the case in proton NMR.

5. We can get information on the attached H's on carbons by the use of additional techniques – APT or DEPT.

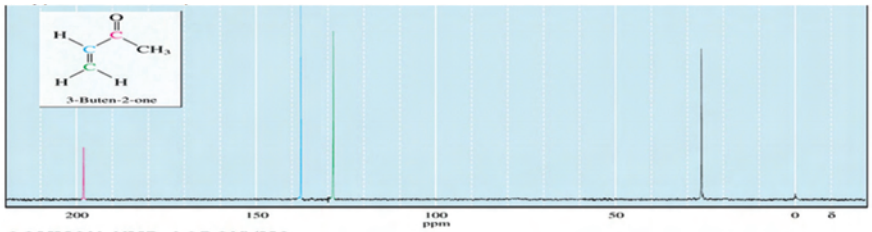
Carbon Chemical Shifts – General Range



More specific chemical shifts

Type of Carbon	Chemical Shift (δ)
1° Alkyl, RCH_3	0–40
2° Alkyl, RCH_2R	10–50
3° Alkyl, $RCHR_2$	15–50
Alkyl halide or amine, $-\overset{\vert}{\underset{\vert}{C}}-X$ (X = Cl, Br, or $\overset{\vert}{N}-$)	10–65
Alcohol or ether, $-\overset{\vert}{\underset{\vert}{C}}-O$	50–90
Alkyne, $-C\equiv$	60–90
Alkene, $C=$	100–170
Aryl, 	100–170
Nitriles, $-C\equiv N$	120–130
Amides, $-\overset{O}{\overset{\Vert}{C}}-\overset{\vert}{N}-$	150–180
Carboxylic acids, esters, $-\overset{O}{\overset{\Vert}{C}}-O$	160–185
Aldehydes, ketones, $-\overset{O}{\overset{\Vert}{C}}-$	180–215

A typical Carbon spectrum



COMMON NMR ACRONYMS

DEPT: Distortionless Enhancement by Polarization Transfer. It provides multiplicity, whether a C is CH_3, CH_2 or CH.

COSY: Correlated Spectroscopy - H-H connectivity.

HMQC: Heteronuclear Multiple Quantum Coherence - C-H correlation.

HMBC: Heteronuclear Multiple-Bond Coherence - 2 and 3 bond C-H connectivity.

NOE: Nuclear Overhauser Enhancement - stereo chemical information.

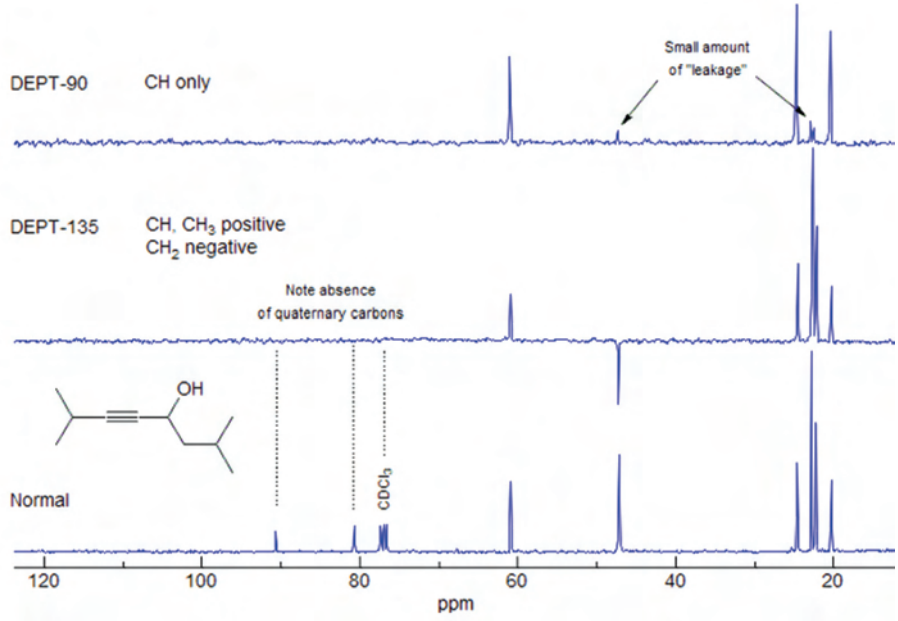
TOCSY: TOtal Correlation Spectroscopy - longer range H-H connectivity.

HSQC: Heteronuclear single quantum coherence - one-bond correlation between H and attached C or a heteroatom like N. HSQC is very useful in protein NMR (http://www.nmr2.buffalo.edu/resources/edu/matr/nmr2_2004.pdf)

ADVANCED NMR

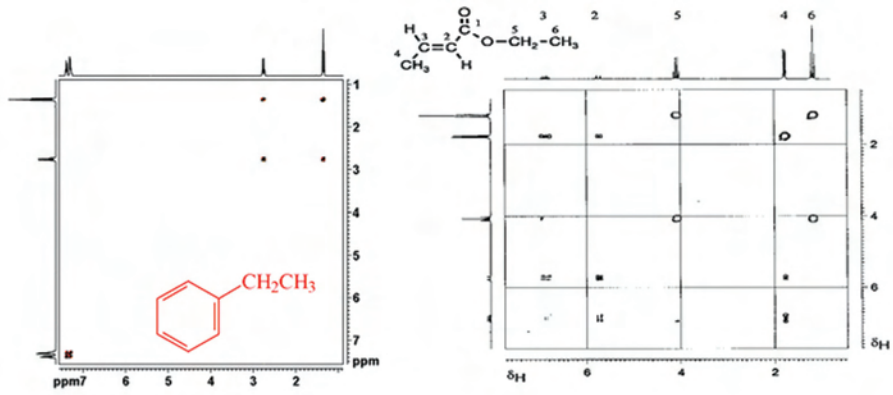
DEPT: Distortionless Enhancement by Polarization Transfer

It is a preferred procedure for determining # protons attached to carbons. The variable proton pulse angle è is set at 90° and 135°. DEPT-90 showed only CH. In DEPT-135, CH_2's are phased down, CH and CH_3 are phased up. A sample DEPT spectrum is shown below:

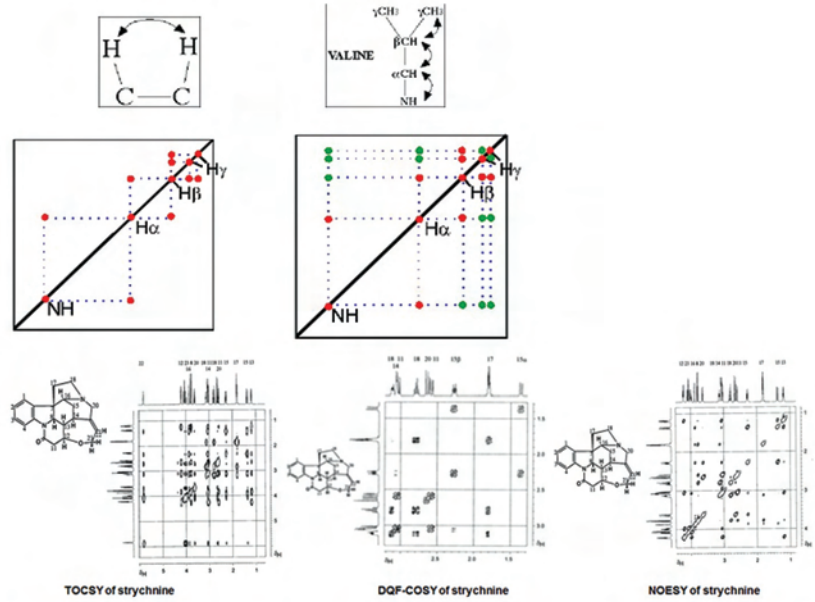


2-DIMENSIONAL NMR

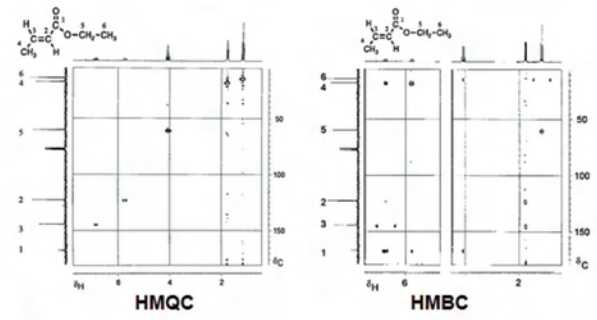
COSY SPECTRUM

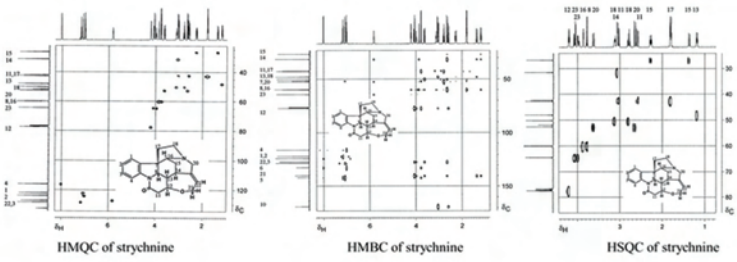


COSY & TOCSY



HMQC: The HMQC experiment provides correlation between protons and their attached heteronuclei through the heteronuclear scalar coupling.
HMBC: The HMBC experiment suppresses correlations via $^{1}J_{C,H}$ while ^{2}J, ^{3}J can be conserved.
HSQC: The HSQC experiment gives the same results as HMQC but the signal-to-noise is better. It correlates protons with their directly attached heteronuclei and especially useful in obtaining N-H correlations in protein NMR.





CONCLUSIONS

With the advent of micro and nanoprobes, we are able to generate spectra with minute quantities of the isolated compound. Further, the coupling of NMR to HPLC has made it significantly better to obtain spectra without having to go through tedious separation processes. Bruker has a system that couples an HPLC more or less directly to the NMR spectrometer. The sample is transferred into the NMR "as is", that is, it arrives with the concentration and the solvent as supplied by the chromatography system. The sample can be transferred during the chromatographic separation and the NMR spectra are then acquired either in on-flow mode (continuously, while the chromatography is running) or in stop-flow mode (while a selected peak is parked in the NMR probe and the chromatography is paused). In a more sophisticated procedure the selected samples are intermediately parked in sample loops and transferred to the NMR after the end of the chromatographic separation. In this procedure the sample handling is minimized. With these kinds of developments NMR is expected to remain the primary tool for structure determination.

SUGGESTED READINGS

Sanders, J., Hunter B.K. 1993. Modern NMR spectroscopy: A guide for chemists, Oxford University Press, 2nd Edition.

Understanding NMR Spectroscopy by James Keeler, 2nd Edition, Wiley, 2005.

NMR Spectroscopy: Basic principles, concepts and applications in chemistry by Harold Gunther, Wiley, 2013.

Inauguration of winter school 2018 by Padma Bhushan Dr. Manju Sharma

Photo with Dr. K. Gopakumar, Formerly DDG ICAR (Fy)

Field visit to India Sea Foods

Field visit to BOS Naturals

Field visit to Accelerated Freeze Drying Co. Ltd

Photo with Dr. Meledath Govindan

Lectures and Interactive Sessions

Practical Sessions


ICAR-CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
Ernakulam North P.O., Kochi-682018, Kerala
ICAR Sponsored Winter School on Recent Advances in Bioactive Compounds from Marine Organisms and Development of High Value Products for Health Management
January 23 to February 12, 2018
CMFRI
केन्द्रीय समुद्री मात्स्यिकी अनुसंधान संस्थान
Central Marine Fisheries Research Institute
കേന്ദ്ര സമുദ്ര മത്സ്യ ഗവേഷണ സ്ഥാപനം
WELCOME
Sitting (L to R) Minimol K.C., Grace Thomas, Kajal Chakraborty (Course Director), P. Vijayagopal (Head, Marine Biotechnology Division), A. Gopalakrishnan (Director), Paulson Mathew, Sathu T., Radhakrishnan E.K.
Standing (L to R) Aswathy Elizabeth Mani, Sreemol C.K., Prima Francis, Soumya Krishnan, Minju Joy, V. Rani, Seeja Thomachan Panjikkaran, Sheneya Festus, Drishya K., Anie Y., Suja Rani S., Sindhu Issac, Teena P. Varghese, Magna Thomas, Santwana Palai, Norma Xavier Chelat, Naheef K., Satya Narayan Sahoo, Jaimin Hareshbhai Bhatt, Ajoy Saha, Senthil Kuppusamy, Kedar Shashikant Damle, Shubhajit Dhara, Midhun Dominic C.D., Manukuttan K.S., Suji Chandru, Tima Antony, Soumya Salas

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ISBN-978-93-82263-21-0
9 78-93-82263-21-0