Whole cell catalyzed synthesis of flavored esters

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in
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Submitted by
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JULY, 2016

Certificate

*This to certified that the thesis entitled "**Whole cell catalyzed synthesis of flavored esters**" being submitted in the partial fulfillment of requirements for the award of degree of **Master of Science in Chemistry** submitted in **School of Chemistry and Biochemistry, Thapar University, Patiala** is a bonafide work carried under the supervision of **Dr. Ranjana Prakash and Dr. Satyendra Kumar Pandey**, School of Chemistry and Biochemistry, Thapar University, Patiala and that no part of this project has been submitted for the award of any other degree.*

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Declaration

*I hereby declared that the work presented in this thesis entitled **"Whole cell catalyzed synthesis of flavored esters"** submitted in the partial fulfillment of requirements for the award of degree of **Master of Science in Chemistry** submitted in **School of Chemistry and Biochemistry, Thapar University, Patiala** is an authentic record of my own work carried out under is a bonafide work carried out under our guidance and supervision and that no part of this project has been submitted for the award of any other degree.*

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Needless to say errors and omissions are solely mine.

Gurpinder Kaur

Summary

The work presents the findings on the whole cell catalyzed esterification of aliphatic acids and ethanol with dry biomass of *Aspergillus* species. The extent of esterification was examined using aliphatic acids and ethanol for generation of variety of aroma esters. The results indicated that increase in chain-length significantly favours the reaction towards enhanced yield of alkyl esters especially with decanoic acid. The study also demonstrate the effect of chain length of alcohol with hexanoic acid. The results obtained through the esterification of hexanoic acid with long chain alcohol, the yield of esters increases when chain length of alcohol increases.

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1.1 Introduction

Fragrance chemistry is a fascinating blend of natural product, synthetic, analytical, and physical chemistry with a certain amount of creative fantasy for odors, and molecular structures. Besides the rigor and logic of isolation, analysis and synthesis, it should be carried away by serendipitous, artistic, intuitive moods. In the broadest sense all compounds with a smell belong to the category *of fragrance chemistry.*[1] The term *aroma chemicals* are used for both fragrance and flavor compounds, although there is no strict separation in practice. Thus, compounds with a sufficiently high vapor pressure will have an odor and this is normally the case up to a molecular weight of around 300, a relatively low polarity granted. It is often incorrectly assumed that only compounds with a pleasant smell belong to the realm of fragrance chemistry. Not only is pleasant odour a very subjective notion, but fragrance compositions often include single compounds which in higher concentrations or in pure form have an animal, faecal, sulfurous or sweaty obnoxious odor. Fragrance chemistry also deals with malodor, in order to counteract it, therefore, the analysis and chemistry of e.g. sweat is also a topic of concern. Fragrance chemistry[2,3] emerged from the very old tradition in virtually all civilizations of making fragrances from naturally occurring materials, mostly of plants but also of animal origin, as there are essential oils, exudates, balsams, and resins. The preparation of flavors and fragrances by isolating them from natural resources began in ancient times. Concurrently, the production of fermented foods (beer, wine and others) allowed the generation of new aromas and formed the roots of modern biotechnology. For many centuries these were the only methods for obtaining this type of compound, albeit in complex mixtures. Rapid progress began with the development of synthetic organic chemistry. From the very beginnings of chemistry the improvement of analytical and synthetic knowledge allowed the isolation and preparation of an impressive number of aromas at the industrial scale.[4] Moreover, the non-natural fragrances were created both by emulation of natural structures – by systematic studies on the relationship between odor and chemical structure and by serendipity.[5] Although the majority of these products were prepared by chemical synthesis or by extraction from plants, the employment of new biotechnological processes has increased considerably in the past decades.[6-10] Chiral flavors often occur in nature as single enantiomers. Because different enantiomers or regioisomers could show different sensorial properties, their specific synthesis is beneficial.[11] Bio catalysis represents a useful tool in this field catalyzing a large number of stereo- and regioselective chemical manipulations that are not easily achieved by the less selective classical synthetic procedures. Furthermore, the increasing sensitivity of the ecological systems supports the choice of environmentally friendly processes and consumers have developed a preference for 'natural' or 'organic' products, thus developing a market for flavors of biotechnological origin.[12] During the past years bio catalytic production of fine chemicals has been expanding rapidly. Flavors and fragrances belong to many different structural classes and therefore represent a challenging target for academic and industrial research. Lipases are one of the most important industrial biocatalyst which catalyzes the hydrolysis of lipids. It can also reverse the reaction at minimum water activity. Because of this pliable nature, it is widely exploited to catalyze the diverse bioconversion reactions, such as hydrolysis, esterification, interesterification, alcoholysis, acidolysis and aminolysis. Lipases, which have proved to be effective catalysts for catalyzing the esterification reactions in non-aqueous phase, have been commonly applied in preparing various flavor and fragrance esters, such as methyl caproate, ethyl caproate, butyl caproate, allyl caproate, ethyl butyrate, butyl butyrate, isovaleryl butyrate, geranyl butyrate, geranyl acetates, ethyl valerate, hexyl acetate and butyl acetate.[13-19] The application of whole cell biocatalysts is another cost-saving approach in place of chemical and enzymatic approaches to potentially enhance the productivity of the reactions associated with generation of flavored esters.[20]

Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be robust organisms for industrial applications. In the present study, we demonstrate that the use of fungal whole cell catalyst for generation of flavored esters from aliphatic acid by transesterification reaction.

1.2 Literature review

1.2.1 Flavor

Flavor is a complex sense noticed when food or beverage with flavor character is placed in the mouth. The British Standard Institution (1975) previously defined flavor as "the combination of taste and odor". However, flavor can be influenced by sensations of pain, heat and cold, and by tactile sensations. Therefore, the flavor research community has defined flavor as "the sum of those characteristics of any material taken in the mouth, perceived principally by senses of taste and smell and also by the general pain and tactile receptors in the mouth, as received and interpreted by the brain."[21] In 1991 it explained as “the combined impression perceived via the chemical senses from a product in the mouth.”[22] .Some foods like chili peppers or other seasonings where taste and aroma senses are considered to be the two most important factors of flavor. Non-volatile as well as volatile compounds in foods are responsible for flavors.

1.2.2 Fruit Aromas

The word “aroma” derives from Latin *arōma,* and was coined in the 13 century to define “sweet odors”. Fresh fruit aromas are described by a particular set of natural volatile compounds. Various chemical structure that are esters, alcohols, aldehydes, ketones, lactones, acids, acetals, phenols, hydrocarbons and heterocyclic compounds are found in fruits . Nursten and co-workers23-24 did the very first journals on volatile flavor profiles of various fruits. In both of these journals structure in fruits such as apple, banana, orange and strawberry were studied. Also, volatiles in fruit are generally present in really small amount.

In addition, among the large number of volatiles generated by fruit, typically only a small fraction of those compounds are aroma active or odor-active and even fewer are aroma impactors.

1.2.3 Natural and Artificial Flavors

In nature, esters are normally found in fruits in low concentrations. According to Montero-Calderon[25] in different fruits containing different esters, for example, in pineapple there are ethyl butanoate, methyl butanoate, methyl hexanoate and a small amount of ethyl octanoate and esters of propanoic acid. In Citrus fruits there are not only aliphatic esters but also the monoterpenic esters such as geranyl acetate are present.[26] In these fruits, the esters are generally produced by alcohol acyltransferases which have been investigated in a variety of fruits such as papaya,[27] apple,[28] banana,[29] strawberry.[30] Besides fruits, esters are also found in rose.[31]

Artificial flavors are very different from natural flavors. Artificial flavors are made of many compounds, which enhance or help to create flavor. These compounds are not similar to natural flavor but because of similar character they are named after their flavor. In the previous years, natural flavors were difficult to replace, but as technology became more superior, the differences between natural and artificial flavors became less. Flavor analysis and identification of flavor esters are difficult tasks. Earlier only 1500 flavor compounds were known in the 1970's but now there are over 7000 flavor compounds that have been recognized.

Aroma oils first synthesize in between 1845 and 1850. In these lower molecular mass fatty acid esters of several alcohols and were produced by the chemical industry for their fruity flavor. Haarmann and co-workers founded industrial synthesis of flavor esters of vanillin[3] and coumarin.[1] Traditionally, the flavor esters are extracted from natural materials, and these compounds could be labeled as natural.[31]

1.3 Methods of flavored ester preparation

1.3.1 Esterification

Esterification is the chemical process for making esters, which are compounds of the chemical structure R-COOR', where R and R' are either alkyl or aryl groups. The most common method or preparing esters is to heat a carboxylic acid with an alcohol while removing the water that is formed. Esters can also be formed by various other reactions. Other reactions include the reaction of an alcohol with an acid chloride or an anhydride. Simple alcohols such as methanol and ethanol react very fast because they are relatively small and contain no carbon atom side chains that would hinder their reaction. These different rates of reaction were first reported by Nikolay and co-workers in 1879-83. The esterification process has a broad spectrum of uses from the preparation of highly specialized esters in the chemical laboratory to produce several commercial ester products. Commercial esters are manufactured by either a batch or a continuous synthetic process. The continuous process for making esters was first reported in 1921 and has been used widely in the synthesis of large quantities of esters.

$$\mathrm{ROH + R'\text{-}COOH \rightarrow R'COOR}$$

Fischer esterification is used to produce all acetate esters and is the most widely used commercial method. The process involves the reaction of acetic acid with an alcohol in the presence of an acid catalyst, usually sulphuric or sulphonic acids. Most processes involve the addition of the alcohol to a reactor containing acetic acid. The reaction is equilibrium limited, and in most cases the water of reaction is removed by distillation in order to drive the equilibrium to completion.

1.3.2 Acid and ester

In many essential oils and foods straight chain and aliphatic acids are found. Acids are not important as fragrance substances but they contribute in aroma. Aliphatic acids up to C_3- C_{10} are used in production of many essential oils and foods, certain aroma characteristics (C_3–C_8 for fruity smell, C_4, C_6–C_{12} for blossom fragrances). The aroma of nearly all fruits and many foods is due to aliphatic esters. They are responsible for a particular fruit aroma, smell of particular flower. In nature, most esters are obtained from alcohols and acids. Aroma of aliphatic esters with a small number of carbon atoms is strictly fruity but it changes as the number of carbon atoms increases. Aliphatic esters are preferred for artificial fruit aromas in aroma compositions.

1.3.3 Alcohols

Primary alcohols occur widely in nature mostly in fruits. Their smell is weak but they are used as components in aroma compositions. Their use in fruit flavors is very important. Many alcohols are used as natural ingredients in flavor compositions. In most rich aroma compounds higher alcohols are important part.[32] Strongly smelling aliphatic alcohols, such as 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and the aromatic alcohol phenethyl alcohol may be formed from sugars by the pathways of amino acids synthesis. The homologous series of primary alcohols from methanol to decanol are present in rum.[33]

1.3.4 Catalysts

The choice of the proper catalyst for an esterification reaction is dependent on several factors. The most common catalysts used are strong mineral acids such as sulfuric and hydrochloric acids. Lewis acids such as boron trifluoride, tin and zinc salts, aluminum halides, organo titanates have been used. Cation exchange resins and zeolites are often used. Sulfuric acid and hydrochloric acid have classically been used as esterification catalysts, in laboratory preparation. Sulfonic acids, such as benzene sulfonic acid, or methanesulfonic acid, are extensively used in plant processes because of their less acidic nature. Phosphoric acid is sometimes leads to rather slow reactions. Metal salts minimize side reactions but usually require higher temperatures than strong acids.

1.3.5 Chemical catalysis

Due to the low content in natural resources the flavor compounds obtained by extraction methods are quite costly. By chemical synthesis flavor esters can be obtained in large quantity. Nowadays, most of the flavor compounds used commercially are produced by chemical synthesis.[34]The chemical structure of the alcohol, the acid, and the acid catalyst used in the esterification reaction all effect its rate of reaction. Although relatively high yields could be obtained by applying cheap chemical catalysts but high energy will be essential and environmental hazards are mostly produced during the chemical synthetic reactions. Furthermore, the formation of racemic mixtures and chemical wastes makes this method environmentally unfriendly.

1.3.6 Enzymatic catalysis

To overcome drawbacks in chemical catalysis and meet the requirement of food consumers for natural flavor compounds, various biotechnological methods for the production of flavor compounds have been developed.[34-35] The available biotechnological procedures include plant and plant cell culture, fermentation and enzymatic bio catalysis.[36-37]

1.3.7 Microbial Fermentation

Fermentation is a more favorable method for the bio production of flavor esters. Several fungi are now famous for their ability to synthesize aroma esters. *Ceratocystis moniliformis* was reported to be able to synthesize ethyl, propyl, isobutyl , and isoamyl acetates through liquid fermentation in synthetic media.[38] Soares *el al.* found that *Ceratocystis fimbriata* was able to develop banana and pineapple flavor esters.[39] Besides fungi, yeasts are another group of main microorganisms that are well known for synthesizing aroma esters. *Hansenula mrakii* was reported to be able to synthesize is amyl acetate and ethyl hexanoate during sake brewing, which is mainly due to the esterase in this yeast.[40]

Microbial fermentation yields numerous flavor esters during metabolism. Several metabolic steps are involved during fermentation to synthesize one specific compound. Enzymes involved in flavor compound synthesis are isolated from different sources involving microorganisms, plants and animals. In enzyme as catalyst biosynthesis needs one or more steps. This enzymatic biosynthesis overpowers the weaknesses of chemical synthesis such production of unwanted byproducts (racemic mixture), pollution etc. Furthermore, enzymatic bio catalysis shows high substrate specificity, regio-and enantioselectivity, and the reactions can be performed under mild conditions.[8] Enzymes show different substrate specificities due to their different structures especially in the active site for the synthesis of isoamyl acetate using the immobilised *Rhizomucor miehei* lipase. Response surface methodology was applied in the biosynthesis of isoamyl butyrate using lipases from *Rhizopus sp*. and *Geotrichum sp.* (ester yield 75%).[41] Yet, in some cases, the lipase activity is withdrawn by the substrate or product, which causes the decrease in the yield of flavor esters. For example, the lipase preparation from *Rhizopus oryzae* was reported to synthesize butyl acetate in a solvent- free system, 60% of yield was observed by using the immobilized lipase.[42] The low yield was believed to be because of the denaturation effect of acetic acid on the lipase.[19] Similarly, Ozyilmaz and Gezer[43] reported that using *Candida rugosa* lipase as the biocatalyst and the yields of isoamyl acetate, ethyl valerate and butyl acetate in solvent-free systems were 34%, 19% and 33%. To overcome the inhibitory effect of polar substrates (e.g. acetic acid), organic solvents or other reaction media are used to minimize the deactivation of lipases through diluting the concentration of polar substrates in the reaction mixtures.[44]

1.3.8 Enzymatic Esterification

Lipases are capable to catalyze three different types of reactions to synthesize flavor esters that are esterification, alcoholysis of esters with alcohols, and trans esterification of esters[45]. Among these three reactions, esterification is the most frequently used one by researchers for ester synthesis. One important flavor ester imparting banana flavor is isoamyl acetate, has been well synthesized with esterification of acetic acid with isoamyl alcohol catalyzed by different lipases including lipases from *Rhizomucor miehei*, *Candida antarctica* and *Thermomyces lanuginosus*, *Candida rugosa.*[46-48,43] The lipase-catalyzed synthesis of other flavor esters such as ethyl butyrate, ethyl hexanoate, ethyl valerate, butyl acetate and terpenoid alcohol esters are also reported in literature.[49-52,13] Though high yields of esters have been found by this method, enzyme activity is certainly affected by short-chain fatty acids and alcohols. The polar acids, especially acetic acid, could change the micro aqueous pH of the enzyme and cause dead-end inhibition of the lipase by reacting with the serine residue in the activate site.[53] Instead, water produced during esterification may push the reaction direction towards hydrolysis, which may result in the low yield of esters. Therefore, water needs to be continuously removed from the reaction mixture to ensure a high yield of esters.[54] In the literature, hetero-azeotropic distillation and addition of dehydrating agents, molecular sieves, membranes and zeolites into the reaction media have been used for water removal during esterification reactions.[54-57]

1.3.9 Enzymatic transesterification

Synthesis of esters through lipase-catalyzed trans esterification reaction (alcoholysis) can relieve the inhibitory effect of polar fatty acids on lipase activity, since it uses triglycerides and simple esters as substrates instead of short-chain fatty acids. Furthermore, no water is produced during trans esterification reaction. Paramita Mahapatra reported the synthesis of two esters of acetic acid, *n*-butyl acetate and *n*-propyl acetate through lipase-catalyzed trans esterification of vinyl acetate with *n*-butanol and *n*-propanol in solvent-free systems[58]. However, this method needs the preparation of corresponding esters as feedstock's, which is not cost-effective, and low conversions are achieved. Instead of using simple esters as the source of fatty acids for ester synthesis, applying fats and oils, the natural lipids, as feedstock's, is an attractive alternative approach. Lipids mainly vegetable oils, are low-cost and environmental natural alternatives of simple esters that can be used to synthesize fatty acid esters through transesterification reactions. Few studies described are lead at using vegetable oils as the substrates in production of flavor esters. Thus, it is quite new and challenging to study the application of whole cell studies in the production of flavor esters.

1.4 Applications

Esters have many uses in both the living world and industries. Esters called parabens, are used as food and drug preservatives because they can prevent the growth of microorganisms such as molds and yeast. Furthermore, esters have sweet fruit smells and with low molecular mass. Their properties make them suitable for the preparation of cosmetics and perfumes. Esters with lower the molecular mass carry stronger odors .These esters are used as food additives to improve the flavor and smell of processed foods. In addition, the small esters like ethyl ethanoate are excellent solvents for organic compounds. Sunburn lotions, nail polish removers, plasticizers and glues use esters as solvents. For example, polystyrene cement is a mixture of polystyrene dissolved in ethyl ethanoate. When the ester evaporates, a solid plastic is left behind to bind together the surfaces being joined. Besides, large esters are used in the production of soaps and detergents. Soaps and detergents can remove dirt from surfaces of human skin, cloths and other solids when dissolved in water. Esters which have small molecular weight and very nice odor are responsible for the pleasant odor of jasmine, roses, etc. and for the odors of apples, bananas, strawberries, etc. Esters with higher molecular mass carry weaker odors are used for the fragrance and flavoring in industries. Esters of short-chain fatty acids are aroma-impact compounds found in fermented dairy products. These esters are responsible for fruity flavors that can be regarded either ethyl esters of the longer-chain fatty acids (C_{12} or above) at excessive levels may impart an undesirable soapy odor to the product[59] as a defect or as an attribute by the consumer. Fruit like aroma in esters is important compound in rum .The main esters formed in rums are acetate-esters of alcohols (e.g. ethyl acetate), and ethyl esters of fatty acids (e.g., ethyl decanoate). The ester content of rum varies from 4 to 64% w/v, with ethyl acetate typically being the main ester and fermentation between the yeast cells and medium. There are two main groups of volatile esters in fermented beverages. The first group contains the acetate esters (in which the acid group is acetate and the alcohol group is ethanol), such as ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), and phenyl ethyl acetate (roses, honey). The second group is the ethyl esters (in which the alcohol group is ethanol and the acid group is a medium-chain fatty acid) and includes ethyl hexanoate (anise seed, apple-like aroma), ethyl octanoate (sour apple aroma), and ethyl decanoate (floral aroma).[60]

1.4.1 Lacunae

Different aroma synthesis by lipases is well known, but still all reactions were carried out by commercially known lipases such as, *Mucor miehei, Candida antarctica, Candida rugasa, etc* lipases. In the present study we have tested the capacity of whole cell of *Aspergillus* sp in our laboratory for synthesis of important aroma molecules by esterification.

1.5 Material and Methods

Culture media *viz.* mycological peptone, Bushnall Hass Broth (BHB) and Potato Dextrose Broth (PDB) was purchased from HiMedia, India. Other chemicals such as alcohols (methanol to decanol), acids (propionic to decanoic), hexane, ethyl acetate, silica gel 100-200 mesh for column chromatography, Bi-ammonium hydrogen ortho-phosphate ($(NH_4)_2HPO_4$), were purchased from SD Fine-chem limited, India. All the reagents used were of analytical grade.

1.5.1 Preparation of Biomass

The given spores of *Aspergillus sp*. inoculated aseptically in 500 ml Erlenmeyer flask containing 200 ml of sterile PDB and incubated at 30°C, 120 rpm for 3 days. The active culture obtained from PDB was further used for experimentation. The active culture obtained from PDB was inoculated into 200ml minimal media containing nitrogen and carbon source. The minimal media BHB containing $MgSO_4$ (0.2 g/l), $CaCl_2$ (0.02 g/l), KH_2PO_4 (1.0 g/l), K_2HPO_4 (1.0 g/l) and $FeCl_3$ (0.05 g/l) supplemented with mycological peptone (0.5% w/v), $(NH_4)_2HPO_4$ (0.5%w/v) and cotton seed oil (10% v/v). Mycological peptone and $(NH_4)_2HPO_4$ were used to supplement nitrogen and cotton seed oil was used as main carbon source for fungal growth. Culture flask was incubated at 30°C, 120 rpm for 3 days. Fungal biomass was separated by filtering through Whatman filter (No.1) paper, washed with hexane to remove the excess oil and dried with blotting paper. The partially dried biomass was crushed in liquid nitrogen to make homogenous powder using pestle mortar.

1.5.2 Optimization of esterification reaction conditions

To optimize the esterification reaction conditions, various step-wise modifications were carried out to achieve maximum yield of alkyl esters (Table. 1).

Table 1 Optimization of parameters for esterification reaction using dried biomass.

S.No	Constant Parameter	Variable Parameter	Range
Step 1	Molar ratio of acid/alcohol 1:1 + addition of alcohol at 0 hour and 36h reaction time	Amount of biomass	500 mg to 2000 mg
Step 2	1000 mg biomass + addition of alcohol at 0hour and 36 h reaction time	Molar ratio of alcohol	0.5 to 2.0
Step 3	1000 mg biomass + molar ratio of acid/alcohol 1:1.5 and 36h reaction time	Time of addition of alcohol	0 h, Stepwise addition (2 steps) in every 8 h interval, Stepwise addition (3 steps) in every 12 h interval
Step 4	1000 mg biomass + molar ratio of acid/alcohol 1:1.5 addition of alcohol at 0 h	Time of reaction	6 h to 36 h

1.5.3 Esterification reaction

Optimized conditions as described above, esterification reactions of aliphatic acids (propionic to decanoic) except nonanoic acid were carried out with 1.0 g of dried biomass, taken in round bottom flask containing 1:1.5 molar ratio of acid and alcohol (ethanol) and the mixture was stirred for 24 h on magnetic stirrer at 30°C. After 24 h, product was separated through centrifugation at 10,000 rpm for 10 min. The progress of the reaction was checked regularly by thin layer chromatography. Different types of alcohols (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol and decanol) were tried to check the effect of chain length of alcohol and extent of esterification with hexanoic acid.

1.5.4 Identification, purification and quantification of flavored ester

The product (flavored ester) obtained was analysed using thin layer chromatography (TLC) with precoated aluminum TLC plates as stationary phase and hexane: ethyl acetate (49:1) as a mobile phase. The compound was purified by silica gel column chromatography (49:1). The product showed stains in both anisaldehyde and potassium permaganate. Further, product was quantified by using ^{1}H-NMR (JEOL 400 MHz, ^{1}H-NMR) with $CDCl_3$ as solvent and chemical shifts were expressed in parts per million with tetra methyl silane (TMS) as internal standard. ^{1}H NMR spectra were recorded with pulse duration of 2.18 sec with a relaxation delay of 4 sec and 16 scans. The product formed from the esterification reactions were quantified by NMR using the equation proposed by Sharma *el al.*[61]

$$C = 100 \times (AE_{\alpha\text{-}CH2} / A_{\alpha\text{-}CH2})$$

Wherein:

C – Conversion of acid to the corresponding alkyl ester.

$AE_{\alpha\text{-}CH2}$ -Integration value of the protons of the alkyl esters.

$A\alpha - CH_2$ – Integration value of the methylene protons

1.6 Results and discussion

1.6.1 Effect of amount of biomass

In present study, varying the amount of biomass is used to achieve the maximum conversion of esters because the amount of biomass influences the esterification reaction. In figure 1, Amount of biomass was increased from 500 mg to 2000 mg, the maximum conversion (yield=48%) was observed when 1000 mg biomass was used in the reaction. In case of 500 mg and 700 mg the conversion of ester was observed in only 2% and 7% yields respectively. Further increase of biomass leads to decrease in conversion of esters i.e. 31% conversion when 2000 mg biomass was used. Further increase in biomass decreased the product formation and reduced the yield, presumably due to increased viscosity of the reaction mixture in which homogeneous stirring could not take place. The observations on the excess biocatalyst not contributing to increase in percentage conversion are also further supported by observations of Torres and Otero.[62]

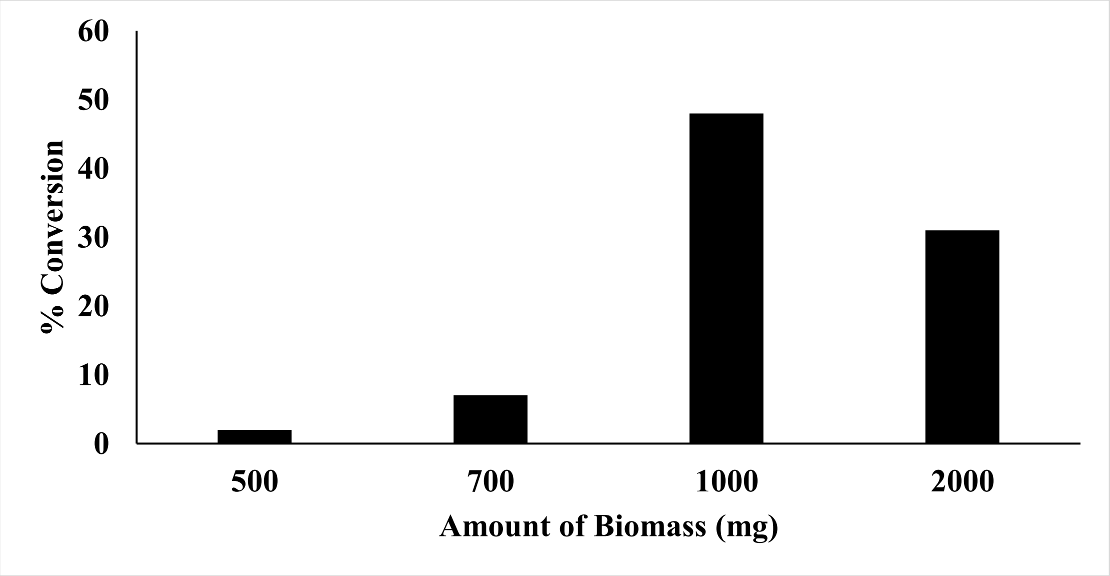

Figure 1 Effect of biomass on esterification reaction.

1.6.2 Effect of molar ratio of alcohol to acid on the esterification

In present study, alcohol molar ratio plays very important role in esterification. In figure 2, the maximum conversion (52%) was observed when 1:1.5 acid/alcohol molar ratio was used. The conversion of esters was observed 27%, 30% in case of 1:0.5 and 1:1 acid/alcohol molar ratio and 7% in case of 1:2 acid/alcohol molar ratio. It shows the higher amount of alcohol leads to negative effect on enzyme activity. The decrease in conversion is also presumed to be due to inactivation of enzyme by excess of alcohol.[63] It was reported that short-chain alcohols, when added in excess, can modify the hydrophilic end of the enzyme resulting in denaturation.[64]

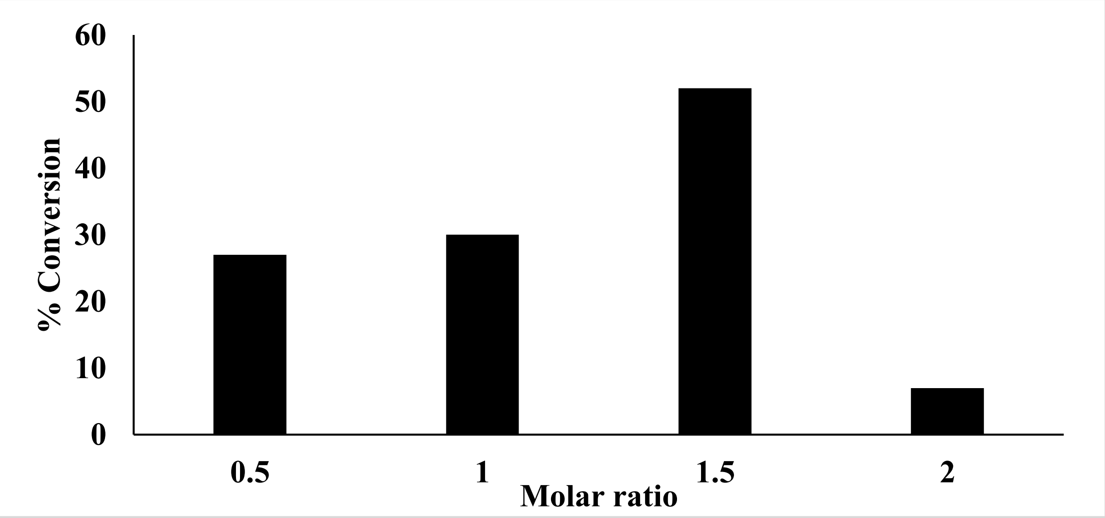

Figure 2 Effect of molar ratio of acid/alcohol.

1.6.3 Effect of time of addition of alcohol

The time of alcohol addition also significantly influence the esterification reaction. Alcohol was added in 0 hour and stepwise (2 step) addition in 8 hour and (3 step) in 12 hour. When alcohol was added in 0 hr, reaction showed very promising results (yield=52%). However, further increase in the time interval from 8 h to 12 h between the successive alcohol additions did not enhance the ethyl ester yield. Comparatively, low yields (3%) were obtained when the time interval of alcohol addition was 12 h, due to reversible nature of reaction. When the time interval of the addition of ethanol is greater than 0 h, the alcohol added initially gets completely utilized in the reaction resulting in a reverse reaction and subsequent decrease in yield of products.[20,63] (Figure 3)

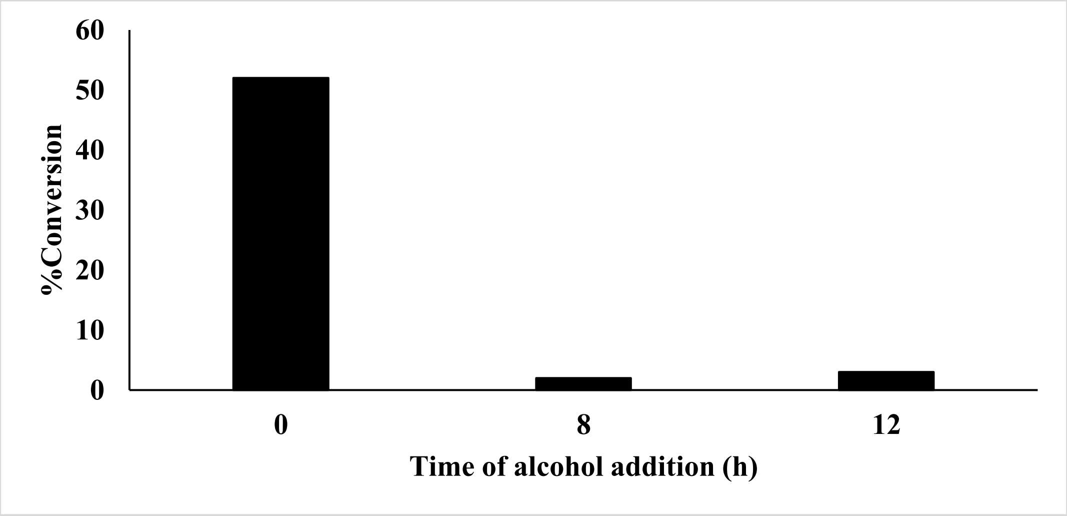

Figure 3 Effect of time of addition of alcohol.

1.6.4 Rate of reaction

The observations indicated that with increase the time of reaction, the extent of esterification increased upto 24 h beyond which it decreased. Figure 4 shows that percent conversion of ester increases from 6 h to 12 h i.e. 2% to 30% yields followed by increase to 52% yield in 24 h.

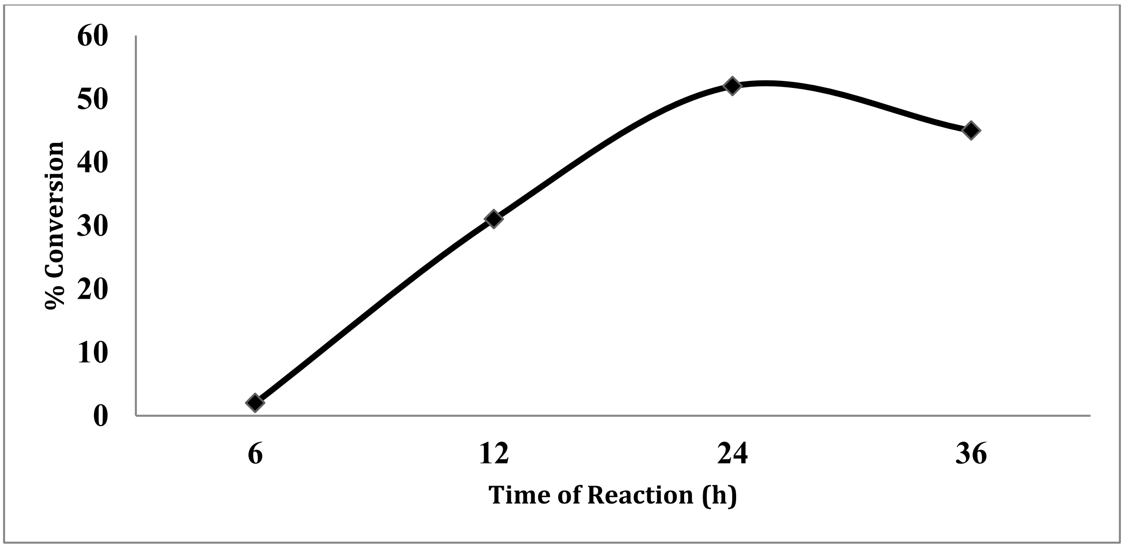

Figure 4 Effect of time of reaction.

1.6.5 Esterification reaction

In the esterification reaction, the whole cell of *Aspergillus sp.* used as catalyst to catalyze the reaction. Ethanol used as an acyl acceptor with all the aliphatic acids (propanoic to octanoic and decanoic). The conversion of acid to esters was very less in case of short chain aliphatic acids. The yield of ethyl propanoate was achieved only 5% yield, ethyl butyrate (2% yield) and ethyl pentanoate (3% yield), due to small carbon chain length these esters are highly volatile. The yields of esters were increases with increasing the chain length of acids. The yield of ethyl hexanoate, ethyl heptanoate and ethyl octanoate was 50%, 54% and 50% yields respectively. The maximum conversion (84% yield) was obtained in case of ethyl decanoate, volatile nature of ester decreases with increasing the carbon chain length in esters(Table 2). Yan Xu reported the biosynthesis of ethyl esters of short chain fatty acids using commercial lipases and whole cell lipase and cell free lipase from *Rhizopus chinesis* used as catalyst for esterification hexanoic acid with ethyl alcohol in solvent phase (heptane) in room temperature (30ºC). The maximum conversion was obtained 96.5% yield in 72h in case of whole cell lipase and 84.9% yield in case of cell free lipase.[13] Abbas and Co-workers in 2003 reported the use of *Mucor sp* lipase immobilized on Amberlite IRC 50 in organic solvent at 35ºC to synthesis aroma of propionic acid, butyric acid, carproic acid as well as methanol, ethanol, allyl butanol, isoamyl, geraniol, citronellol and farnesol alcohol used as substrate[17].

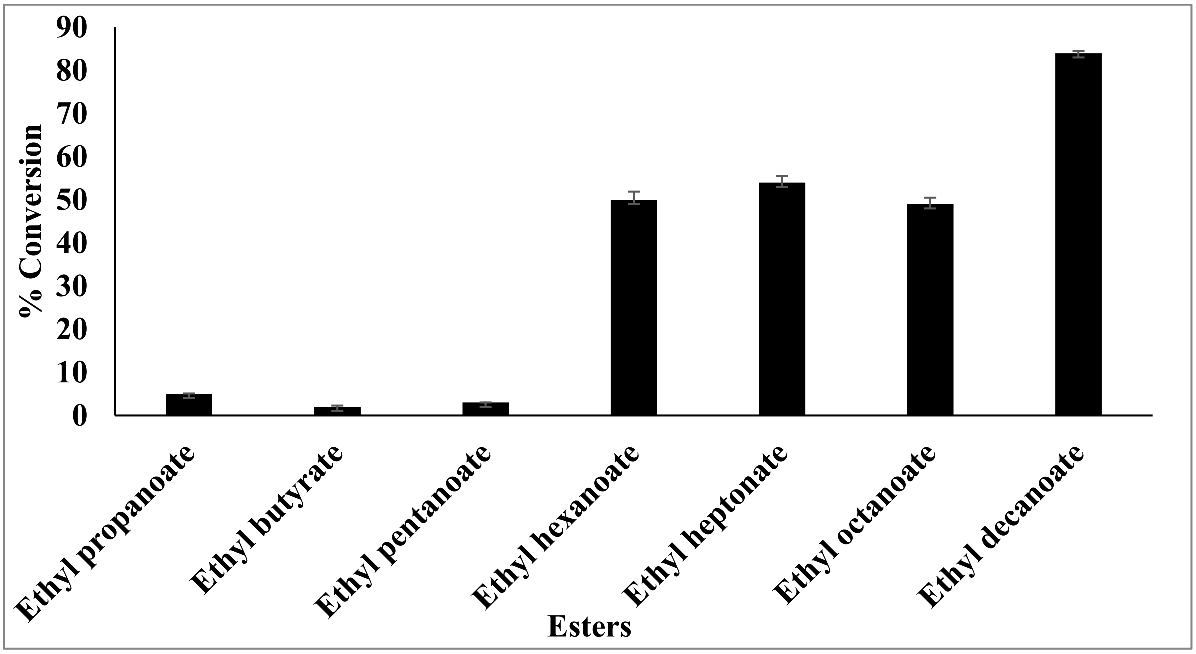

Figure 5 shows the comparison in ester yield from different acid with ethanol.

Table 2 Yield of ethyl esters from different acids

S. no.	Alkyl Esters	Mean Esters (%)
1	Ethyl propanoate	5±0.11
2	Ethyl butyrate	2±0.30
3	Ethyl pentanoate	3±0.025
4	Ethyl hexanoate	50±1.97
5	Ethyl heptonate	54±1.54
6	Ethyl octanoate	50±1.52
7	Ethyl decanoate	84 ±0.57

Table 3 Fragrances of ethyl esters from different acids.

S. no.	Alkyl Esters	Fragrance
1	Ethyl propanoate	Strong ripe apple
2	Ethyl butyrate	Fresh red apple
3	Ethyl pentanoate	Aniseed
4	Ethyl hexanoate	Ripe fruit berry
5	Ethyl heptonate	Ripe grapes
6	Ethyl octanoate	Coconuty
7	Ethyl decanoate	Oily

From above results, it was observed that the percentage conversion of alkyl esters increases from short chain to long chain aliphatic acids and fragrance changed from fruity to waxy flavor. The esterification reaction was carried out of hexanoic acid with all primary alcohols (methanol to decanol), because the ethyl hexanoate gives high fragrance of fruity flavor. The fragrance of ester produced changes with change of alcohol. The conversion of ester increases from methanol to nonanol except hexanol and decanol.

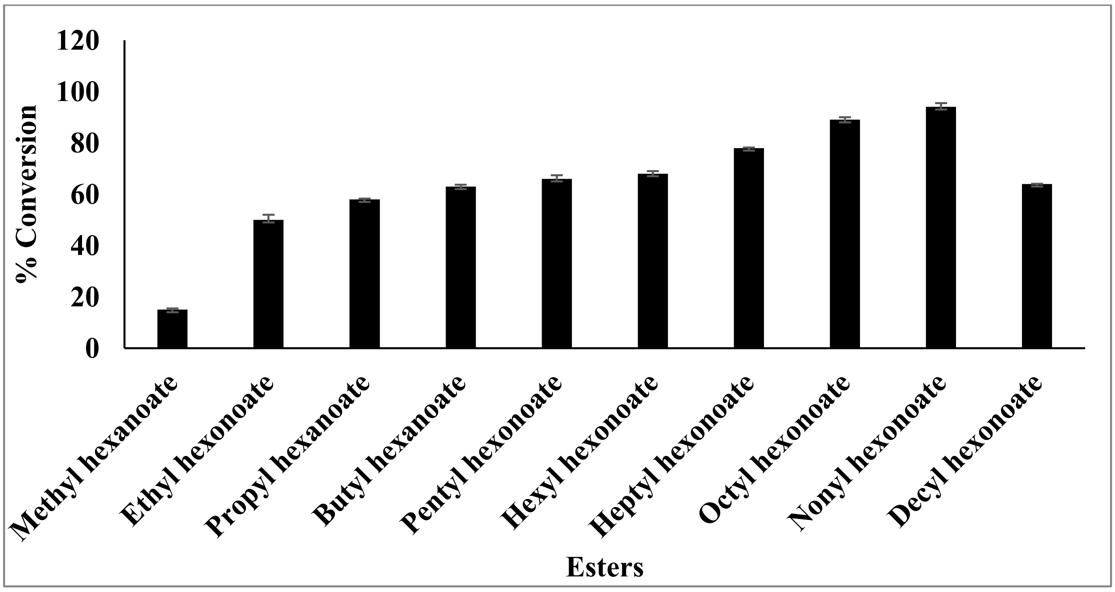

Figure 6 Effect of chain length of alcohols on esterification of hexanoic acid.

Table 4 Yield of alkyl esters of different alcohols with hexanoic acid.

S.no	Alkyl Esters	Mean Ester (%)
1	Methyl Hexanoate	15±0.54
2	Ethyl Hexanoate	50±1.97
3	Propayl Hexanoate	58±0.32
4	Butayl Hexanoate	62±0.68
5	Pentayl Hexanoate	66±1.46
6	Hexayl Hexanoate	68±1.02
7	Heptayl Hexanoate	78±0.27
8	Octayl Hexanoate	89±0.97
9	Nonayl Hexanoate	94±1.57
10	Decayl Hexanoate	64±0.06

The maximum conversion was observed when nonanol (94% yield) use as acyl acceptor (Table 3). *Shuang-Yan Han*[66] *el al*. (2009) reported transesterification of hexanoic acid and ethanol for generation of ethyl hexanoate, a fragrance compound of liquor by recombinant *Saccharomyces cerevisiae* displaying *Candida antarctica* lipase constructed on cell surface and used as a whole-cell biocatalyst and obtained maximum yield 98.2% in 12 h. The ester synthesis was carried out by the *Mucor miehei* lipase for various alcohol substrates: n-propanol, n-butanol, isoamyl alcohol, n-hexanol, n-octanol, 2-ethylhexanol, n-decanol, and lauryl alcohol. The effects of temperature, the nature of the acid, and immobilization of the lipase on its substrate specificity have been elucidated by carrying out esterification's at 29ºC and 50ºC with lauric and oleic acids and by using both the soluble and immobilized (resin-adsorbed) forms of the lipase as catalysts. Higher synthesis rates were obtained with oleic acid than with lauric acid.[67]

Table 5 Fragrances of alkyl esters of different alcohols with hexanoic acid.

S.no	Alkyl Esters	Fragrance
1	Methyl Hexanoate	Strong pineapple
2	Ethyl Hexanoate	Sweet pineapple
3	Propayl Hexanoate	Sweet tropical pineapple
4	Butayl Hexanoate	Fruity berry
5	Pentayl Hexanoate	Fruity green apple
6	Hexayl Hexanoate	Waxy, berry
s7	Heptayl Hexanoate	Green sapy
8	Octayl Hexanoate	Coconut oily
9	Nonayl Hexanoate	Flowery
10	Decayl Hexanoate	Oily

1.7 Conclusion

In conclusion, we have developed a new method in which whole cell of *Aspergillus* sp. is able to catalyzed coupling of aliphatic acids with ethanol to get a variety of important aroma esters. In this study, we explored the effects of chain length of alcohol on biocatalyzed esterification of hexanoic acid. Excellent yields were obtained almost with all the acids, but decanoic acid furnished maximum conversion with ethanol, and also the yield of esters increases when the chain length of alcohol increases.

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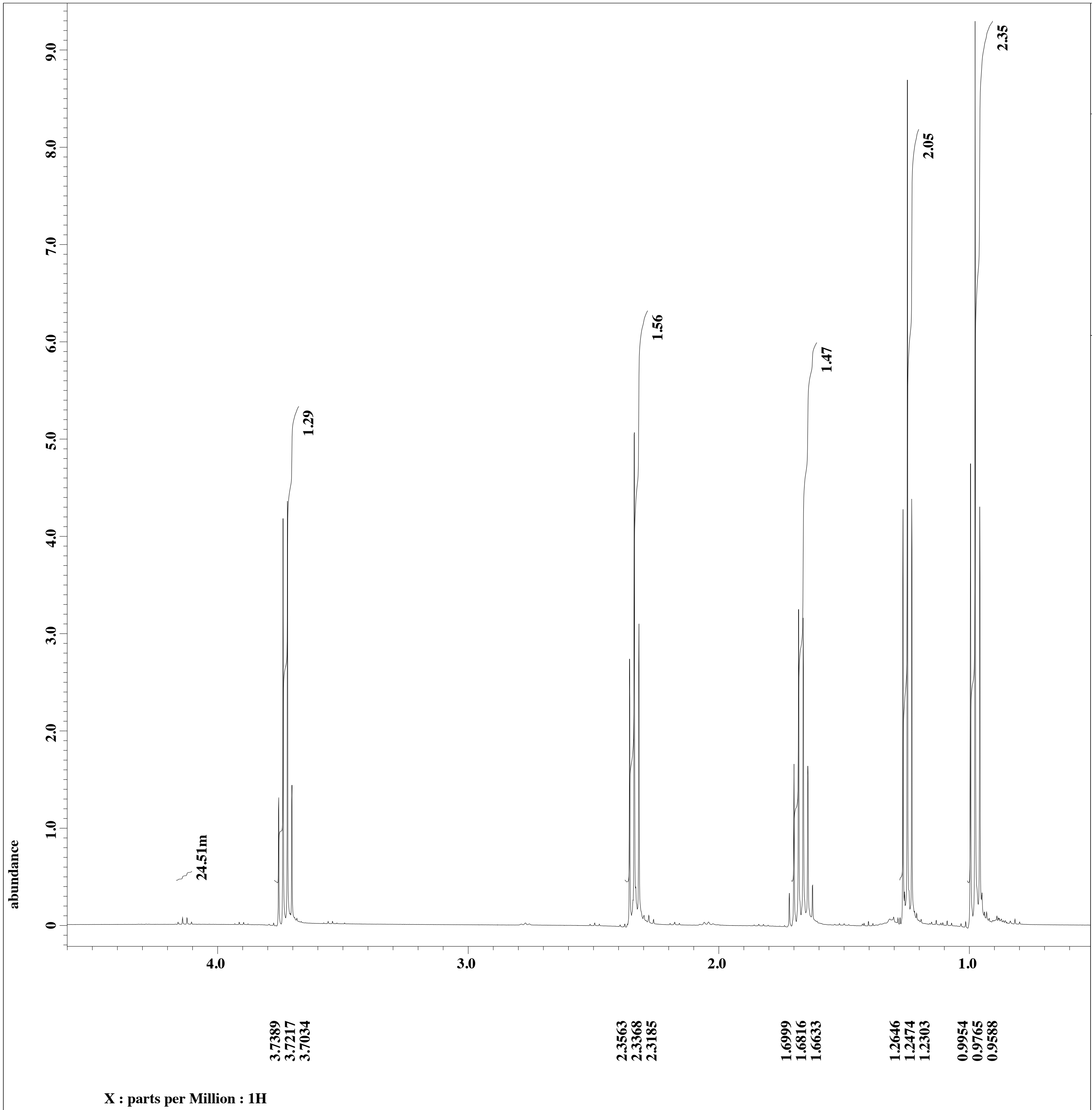

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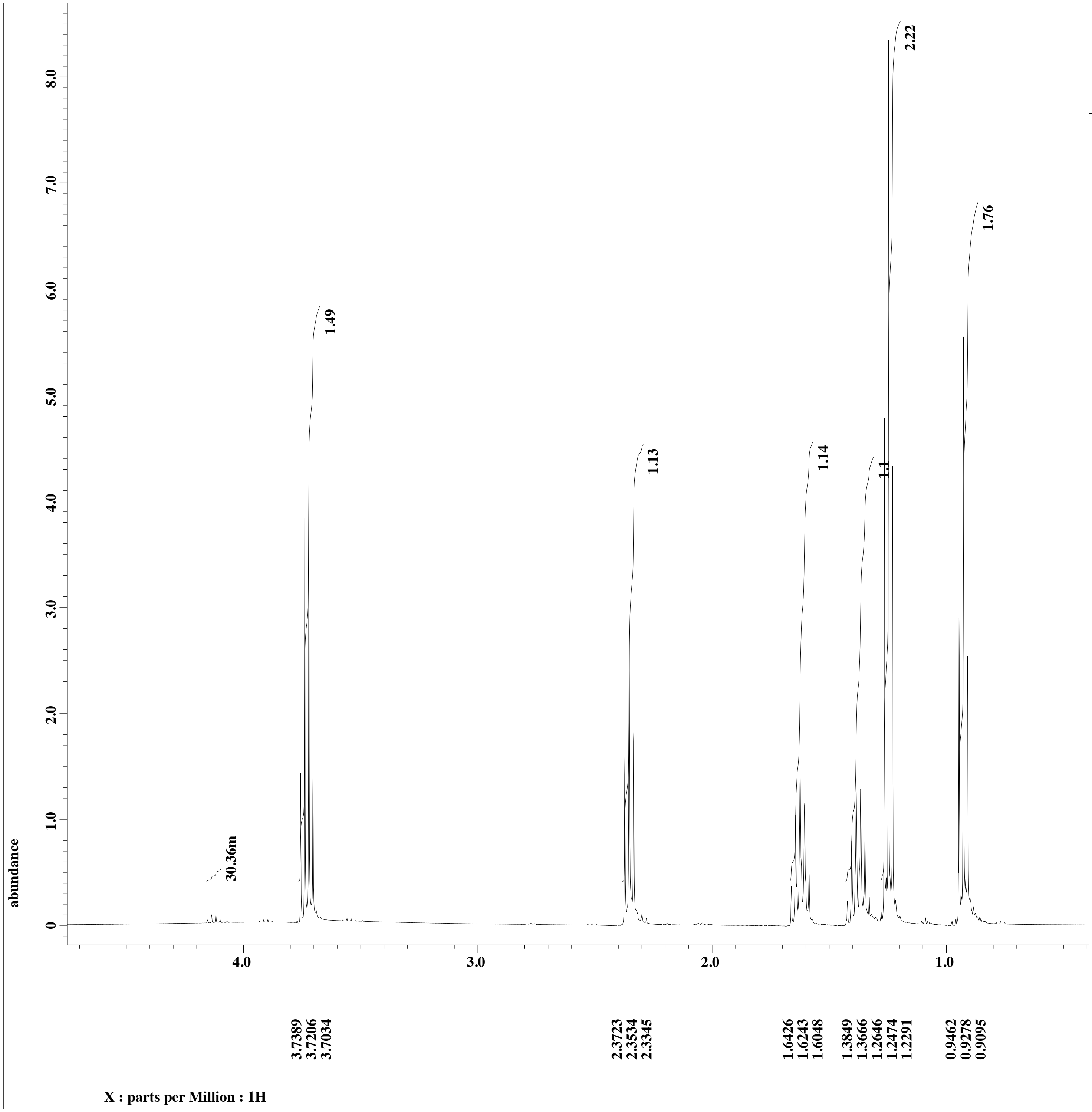

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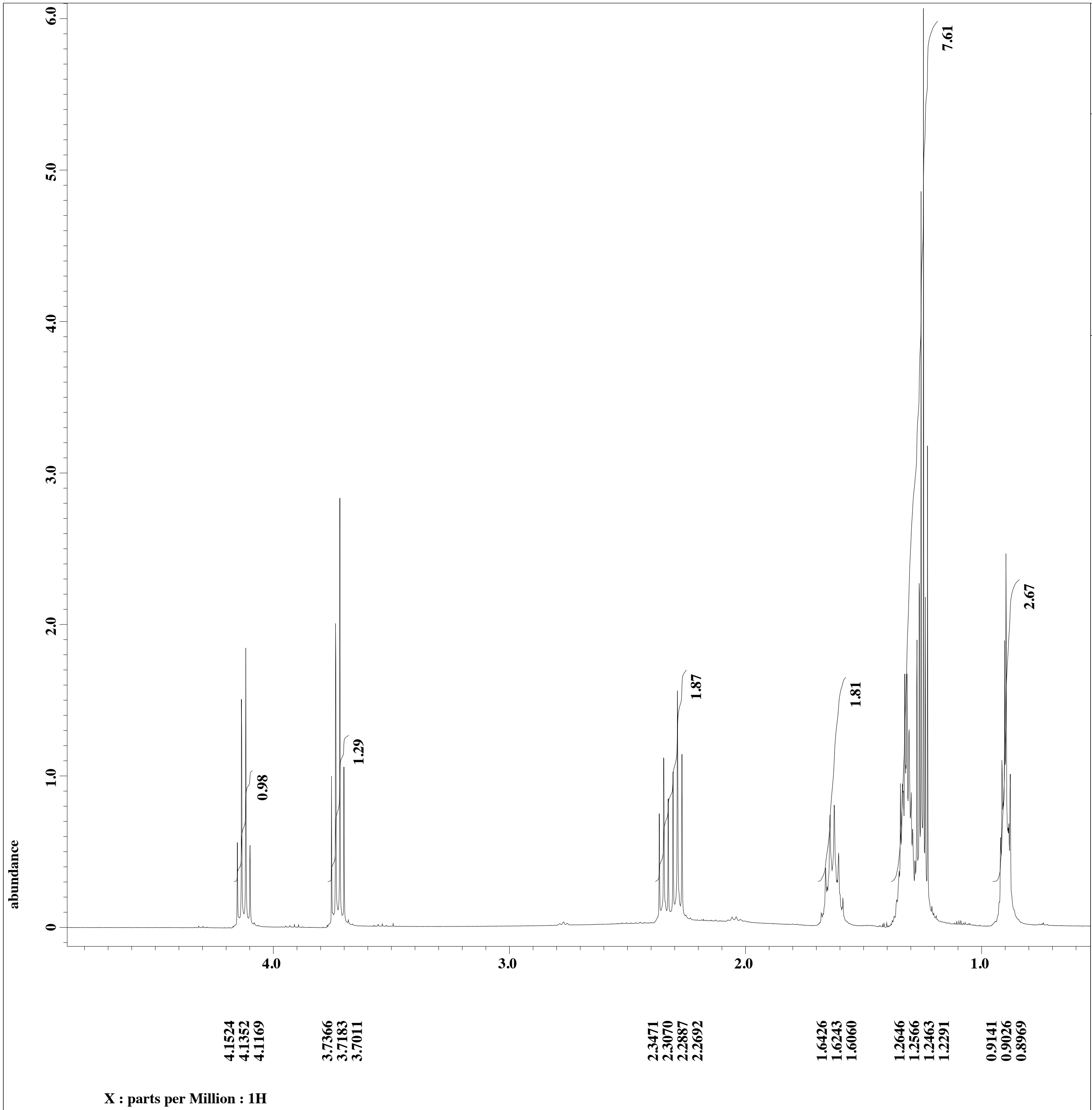

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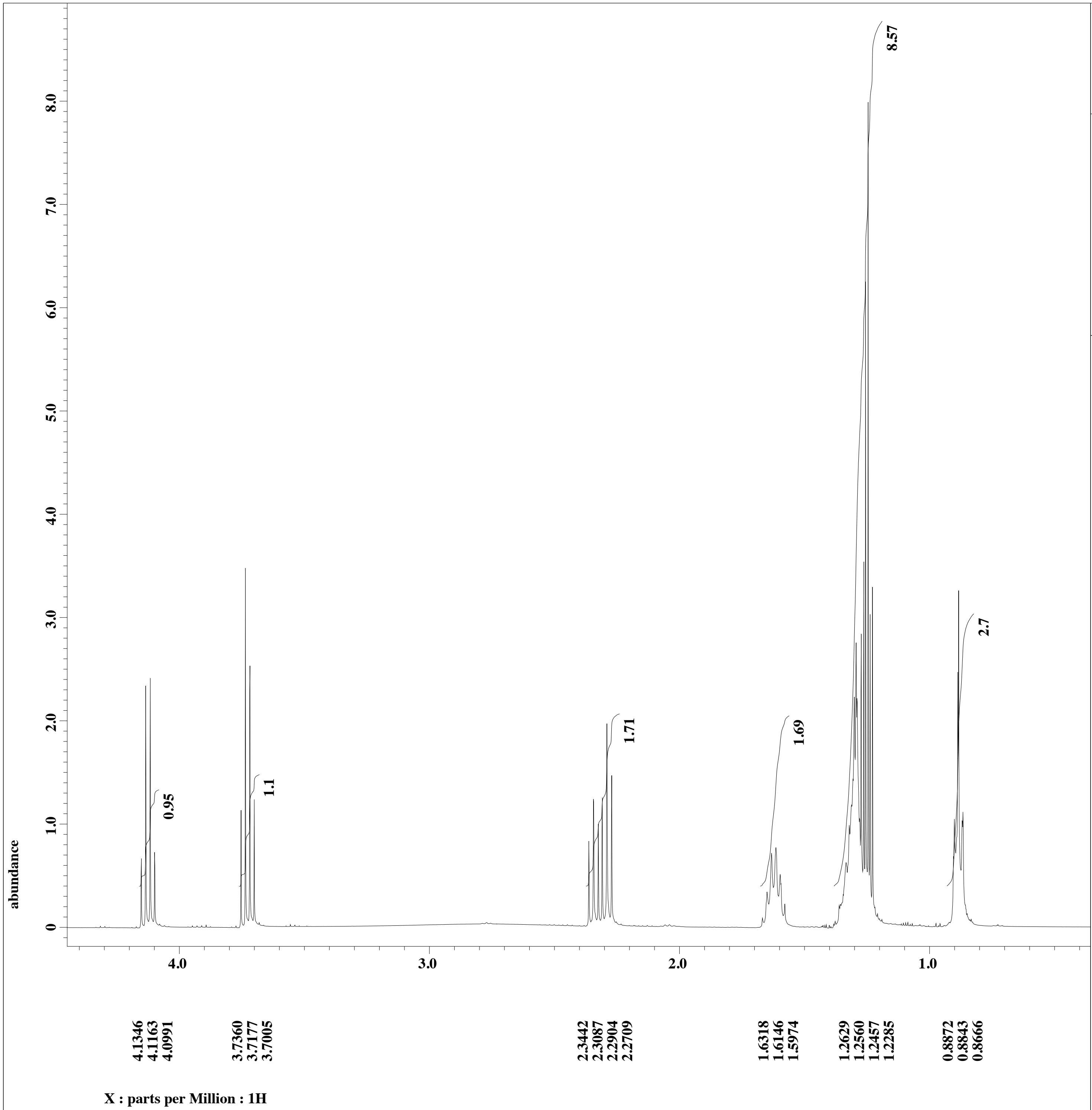
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Clipped            = FALSE
Mod_return         = 1
Scans              = 64
Total_scans        = 64

X_90_width         = 9.75[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 0.3[dB]
X_pulse            = 4.875[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Recvr_gain         = 42
Relaxation_delay   = 4[s]
Repetition_time    = 6.18365952[s]
Temp_get           = 19.9[dC]
```



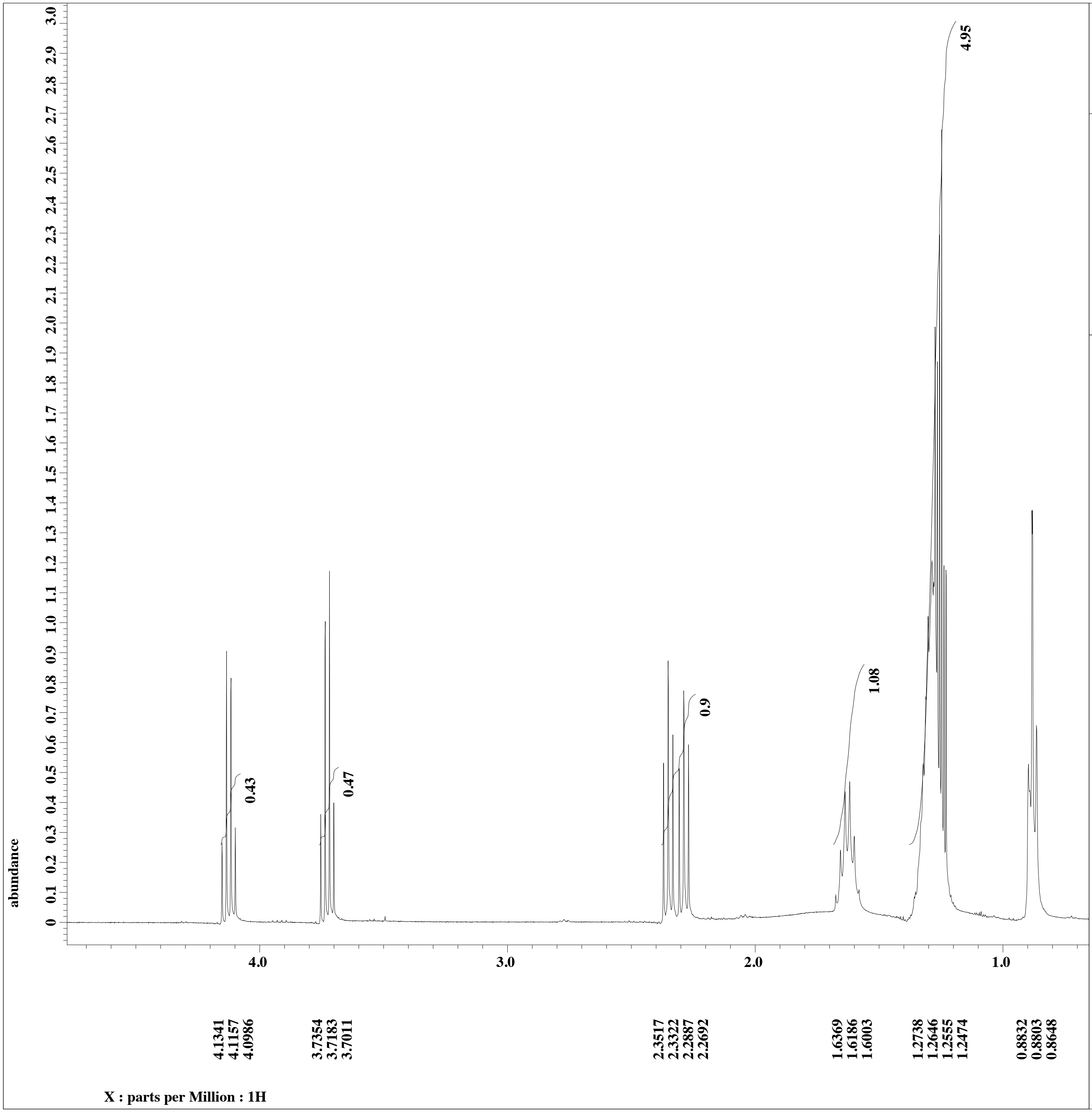
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename           = HEPT-ES-3-ANIRUDH_PRO
Author             = delta
Experiment         = single_pulse.ex2
Sample_id          = HEPT-ES-3-ANIRUDH
Solvent            = CHLOROFORM-D
Creation_time      = 12-APR-2016 17:11:47
Revision_time      = 19-MAY-2016 12:28:23
Current_time       = 19-MAY-2016 12:29:38

Comment            = ANIRUDH
Data_format        = 1D COMPLEX
Dim_size           = 13107
Dim_title          = 1H
Dim_units          = [ppm]
Dimensions         = X
Site               = ECS 400
Spectrometer       = JNM-ECS400

Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Clipped            = FALSE
Mod_return         = 1
Scans              = 64
Total_scans        = 64

X_90_width         = 9.75[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 0.3[dB]
X_pulse            = 4.875[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Recvr_gain         = 36
Relaxation_delay   = 4[s]
Repetition_time    = 6.18365952[s]
Temp_get           = 19.3[dC]
```



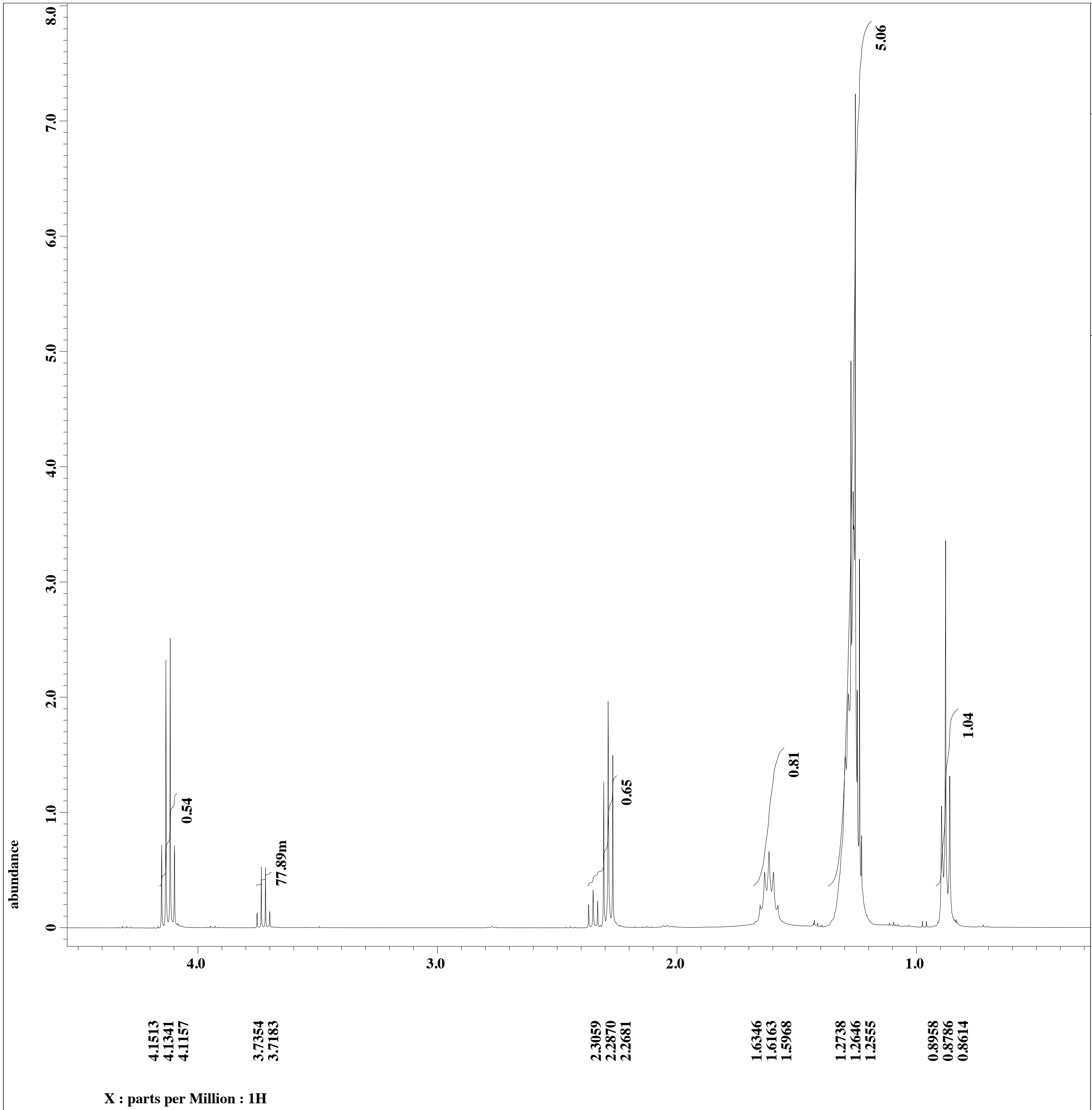
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename           = OCT-3-ANIRUDH_PROTON-
Author             = delta
Experiment         = single_pulse.ex2
Sample_id          = OCT-3-ANIRUDH
Solvent            = CHLOROFORM-D
Creation_time      = 18-APR-2016 21:46:04
Revision_time      = 19-MAY-2016 17:33:57
Current_time       = 19-MAY-2016 17:34:37

Comment            = ANIRUDH
Data_format        = 1D COMPLEX
Dim_size           = 13107
Dim_title          = 1H
Dim_units          = [ppm]
Dimensions         = X
Site               = ECS 400
Spectrometer       = JNM-ECS400

Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Clipped            = FALSE
Mod_return         = 1
Scans              = 64
Total_scans        = 64

X_90_width         = 9.75[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 0.3[dB]
X_pulse            = 4.875[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Recvr_gain         = 44
Relaxation_delay   = 4[s]
Repetition_time    = 6.18365952[s]
Temp_get           = 20.8[dC]
```



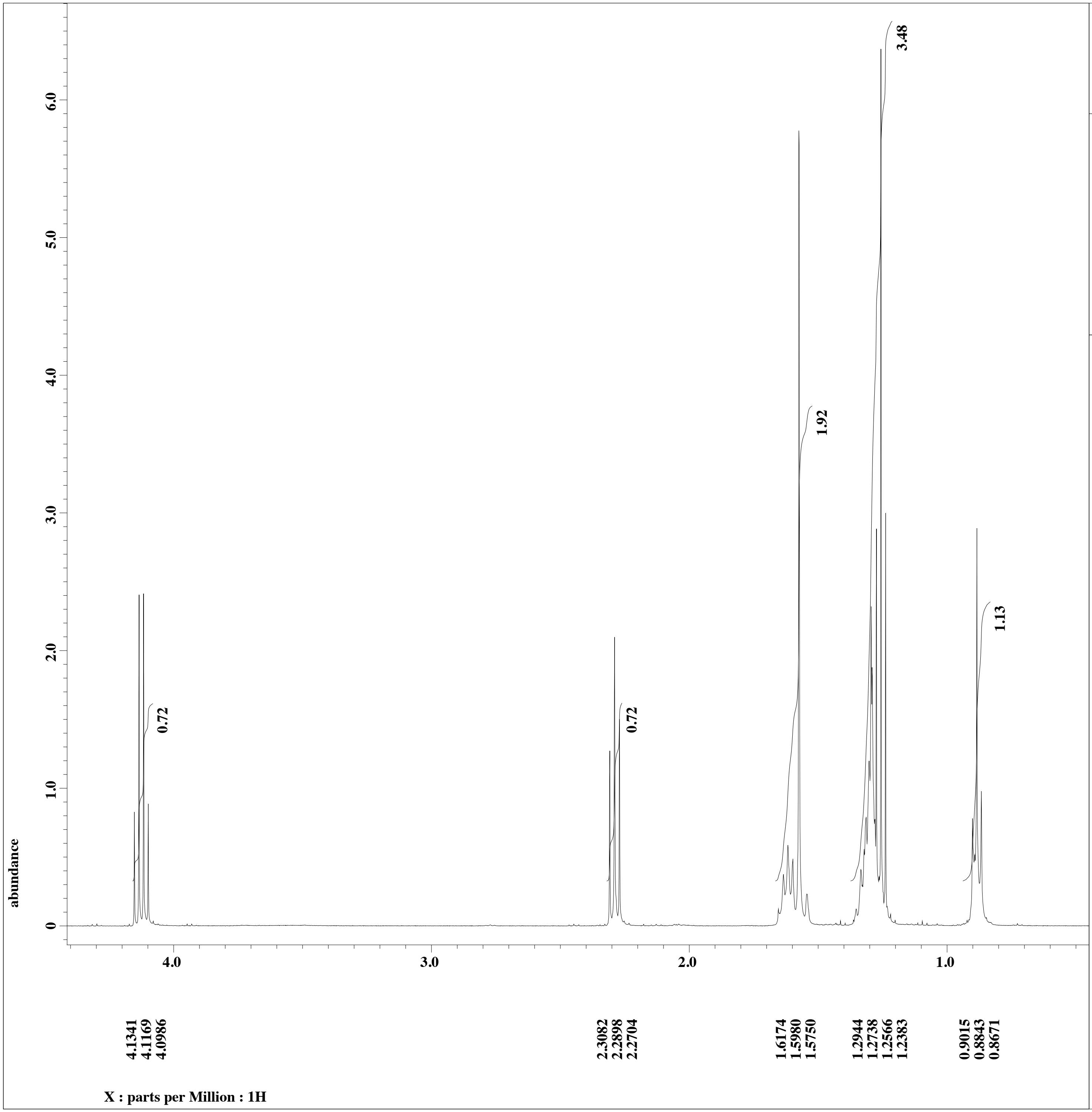
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename           = DEC-3-ANIRUDH_PROTON-
Author             = delta
Experiment         = single_pulse.ex2
Sample_id          = DEC-3-ANIRUDH
Solvent            = CHLOROFORM-D
Creation_time      = 27-APR-2016 14:57:52
Revision_time      = 19-MAY-2016 17:25:18
Current_time       = 19-MAY-2016 17:26:15

Comment            = ANIRUDH
Data_format        = 1D COMPLEX
Dim_size           = 13107
Dim_title          = 1H
Dim_units          = [ppm]
Dimensions         = X
Site               = ECS 400
Spectrometer       = JNM-ECS400

Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Clipped            = FALSE
Mod_return         = 1
Scans              = 64
Total_scans        = 64

X_90_width         = 9.75[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 0.3[dB]
X_pulse            = 4.875[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Recvr_gain         = 38
Relaxation_delay   = 4[s]
Repetition_time    = 6.18365952[s]
Temp_get           = 19.1[dC]
```



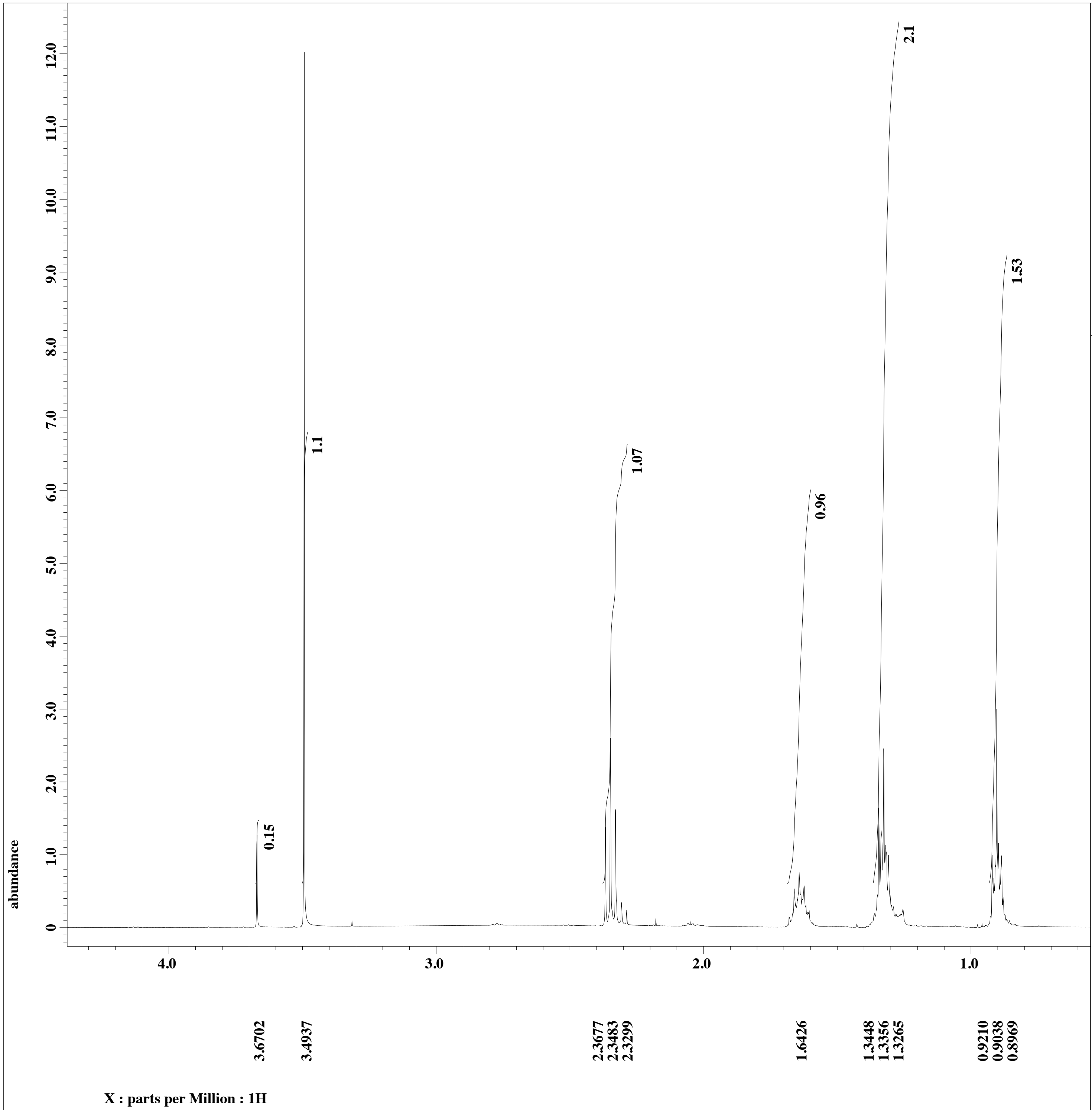
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename           = HEXPURE-ANIRUDH_PROTO
Author             = delta
Experiment         = single_pulse.ex2
Sample_id          = HEXPURE-ANIRUDH
Solvent            = CHLOROFORM-D
Creation_time      = 22-APR-2016 13:41:07
Revision_time      = 19-MAY-2016 18:01:32
Current_time       = 19-MAY-2016 18:02:04

Comment            = ANIRUDH
Data_format        = 1D COMPLEX
Dim_size           = 13107
Dim_title          = 1H
Dim_units          = [ppm]
Dimensions         = X
Site               = ECS 400
Spectrometer       = JNM-ECS400

Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Clipped            = FALSE
Mod_return         = 1
Scans              = 64
Total_scans        = 64

X_90_width         = 9.75[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 0.3[dB]
X_pulse            = 4.875[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Recvr_gain         = 46
Relaxation_delay   = 4[s]
Repetition_time    = 6.18365952[s]
Temp_get           = 20.1[dC]
```



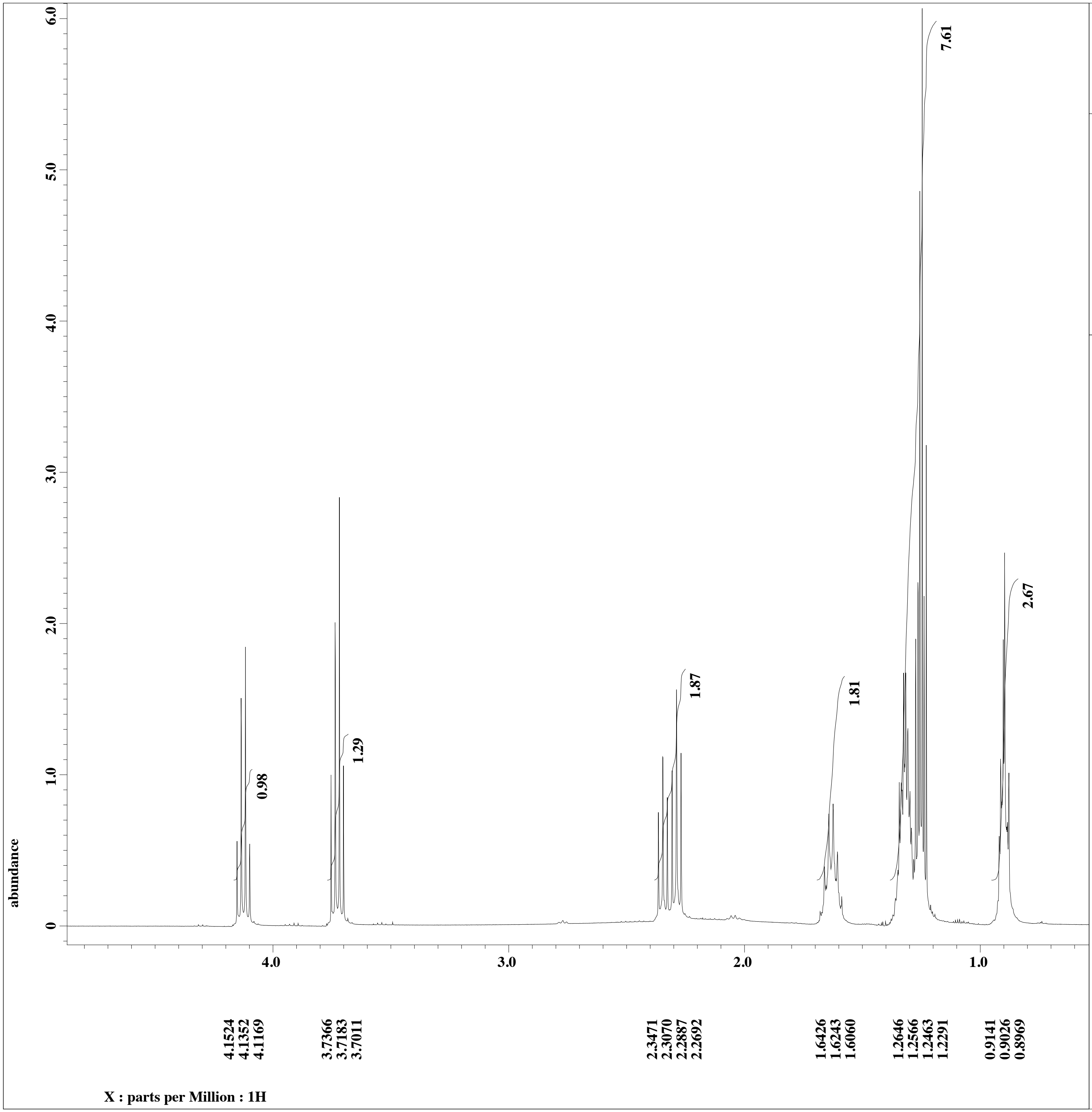
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename          = HEX-METH-ANIRUDH_PROT
Author            = delta
Experiment        = single_pulse.ex2
Sample_id         = HEX-METH-ANIRUDH
Solvent           = CHLOROFORM-D
Creation_time     =  3-JUN-2016 16:28:30
Revision_time     = 22-JUN-2016 13:10:05
Current_time      = 22-JUN-2016 13:12:06

Comment           = ANIRUDH
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

Field_strength    = 9.389766[T] (400[MHz]
X_acq_duration    = 2.18365952[s]
X_domain          = 1H
X_freq            = 399.78219838[MHz]
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.45794685[Hz]
X_sweep           = 7.5030012[kHz]
Irr_domain        = 1H
Irr_freq          = 399.78219838[MHz]
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[MHz]
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 64
Total_scans       = 64

X_90_width        = 9.75[us]
X_acq_time        = 2.18365952[s]
X_angle           = 45[deg]
X_atn             = 0.3[dB]
X_pulse           = 4.875[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 42
Relaxation_delay  = 4[s]
Repetition_time   = 6.18365952[s]
Temp_get          = 20.8[dC]
```



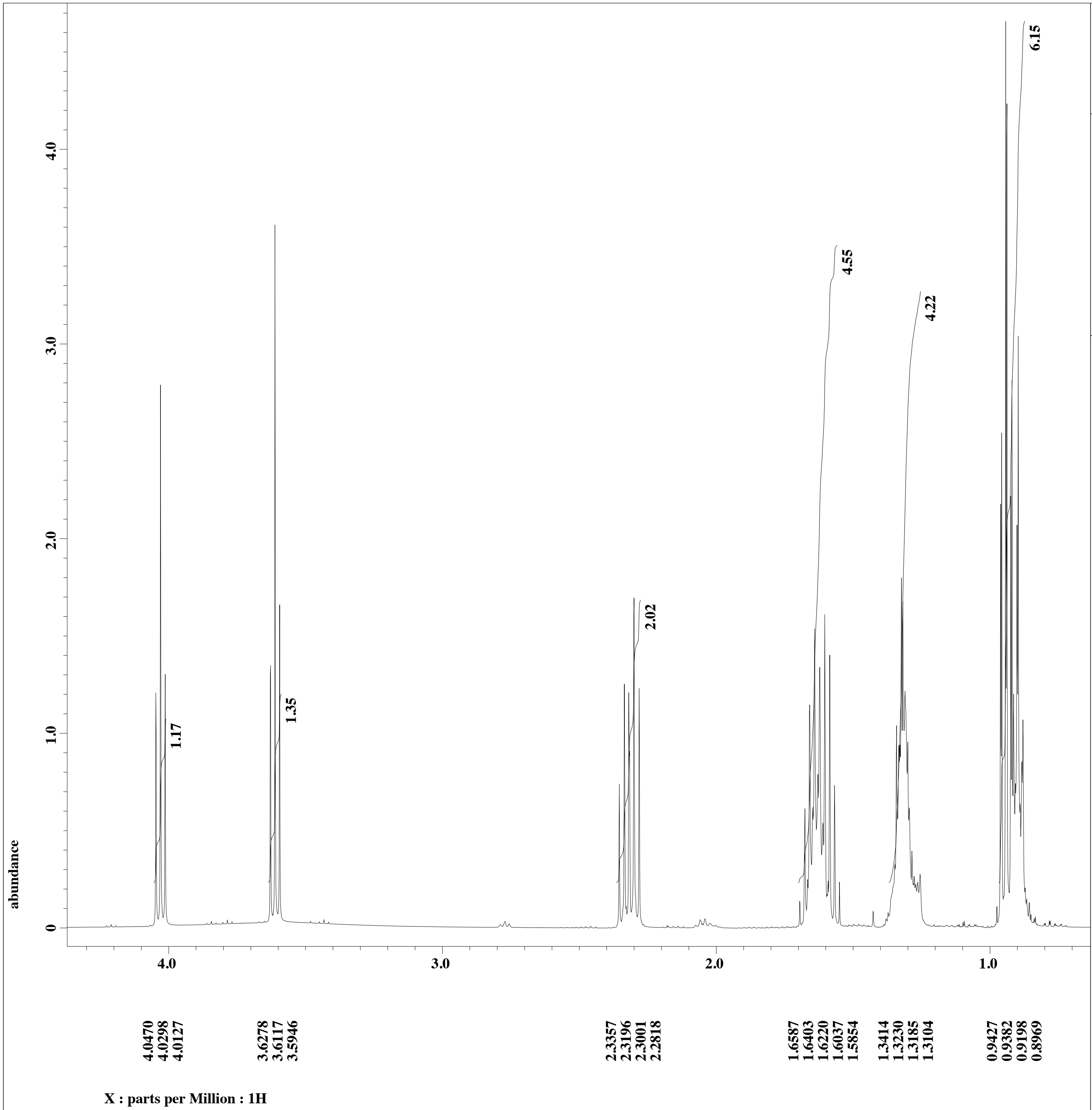
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename          = HEX-24-ANIRUDH_PROTON
Author            = delta
Experiment        = single_pulse.ex2
Sample_id         = HEX-24-ANIRUDH
Solvent           = CHLOROFORM-D
Creation_time     = 23-APR-2016 17:26:58
Revision_time     = 22-JUN-2016 12:35:22
Current_time      = 22-JUN-2016 12:36:07

Comment           = ANIRUDH
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

Field_strength    = 9.389766[T] (400[MHz]
X_acq_duration    = 2.18365952[s]
X_domain          = 1H
X_freq            = 399.78219838[MHz]
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.45794685[Hz]
X_sweep           = 7.5030012[kHz]
Irr_domain        = 1H
Irr_freq          = 399.78219838[MHz]
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[MHz]
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 64
Total_scans       = 64

X_90_width        = 9.75[us]
X_acq_time        = 2.18365952[s]
X_angle           = 45[deg]
X_atn             = 0.3[dB]
X_pulse           = 4.875[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 42
Relaxation_delay  = 4[s]
Repetition_time   = 6.18365952[s]
Temp_get          = 19.9[dC]
```



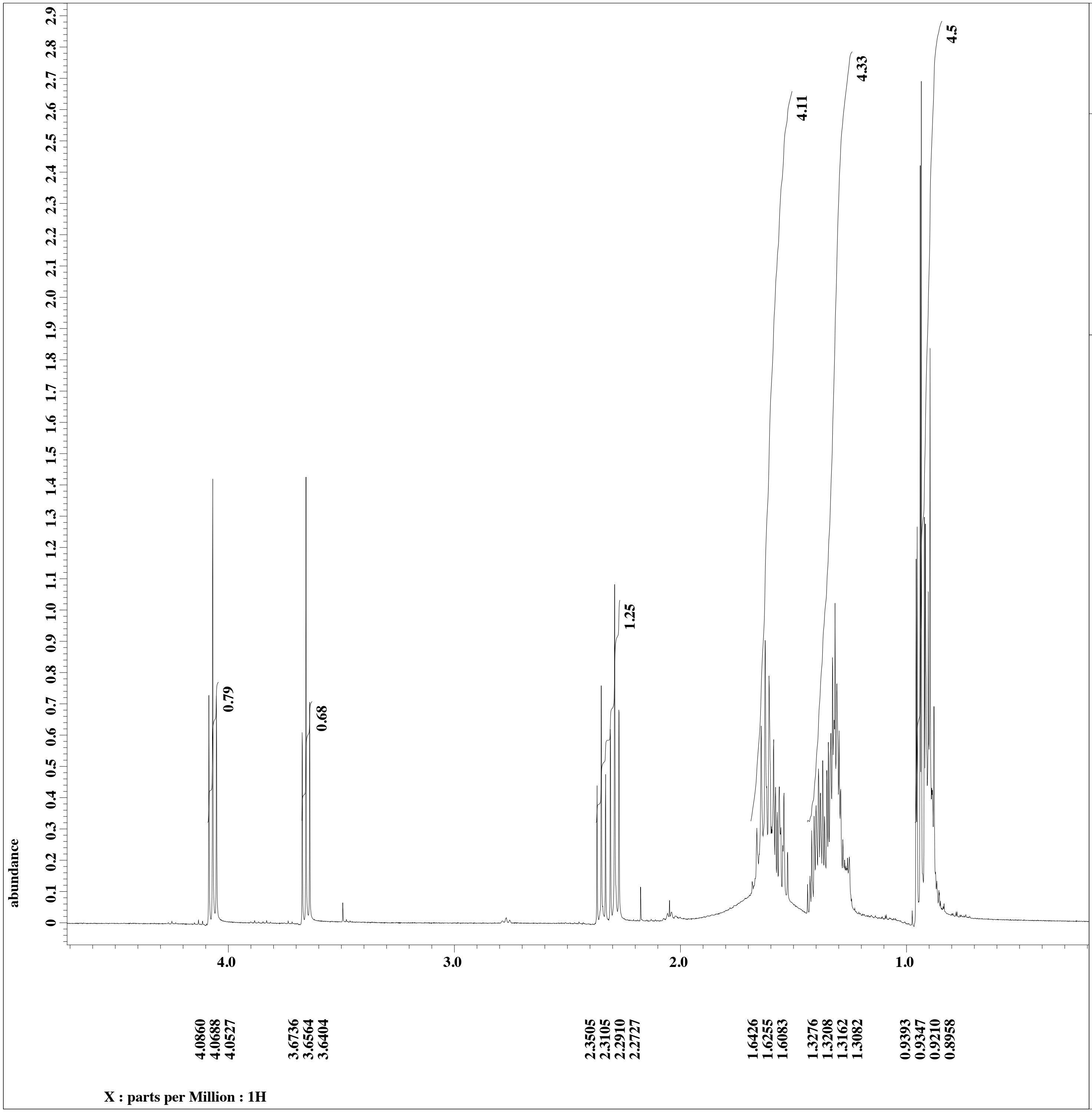
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename          = HEX-PROP-ANIRUDH_PROT
Author            = delta
Experiment        = single_pulse.ex2
Sample_id         = HEX-PROP-ANIRUDH
Solvent           = CHLOROFORM-D
Creation_time     =  3-JUN-2016 16:14:12
Revision_time     = 22-JUN-2016 13:42:45
Current_time      = 22-JUN-2016 13:43:17

Comment           = ANIRUDH
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

Field_strength    = 9.389766[T] (400[MHz]
X_acq_duration    = 2.18365952[s]
X_domain          = 1H
X_freq            = 399.78219838[MHz]
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.45794685[Hz]
X_sweep           = 7.5030012[kHz]
Irr_domain        = 1H
Irr_freq          = 399.78219838[MHz]
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[MHz]
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 64
Total_scans       = 64

X_90_width        = 9.75[us]
X_acq_time        = 2.18365952[s]
X_angle           = 45[deg]
X_atn             = 0.3[dB]
X_pulse           = 4.875[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 26
Relaxation_delay  = 4[s]
Repetition_time   = 6.18365952[s]
Temp_get          = 20.8[dC]
```



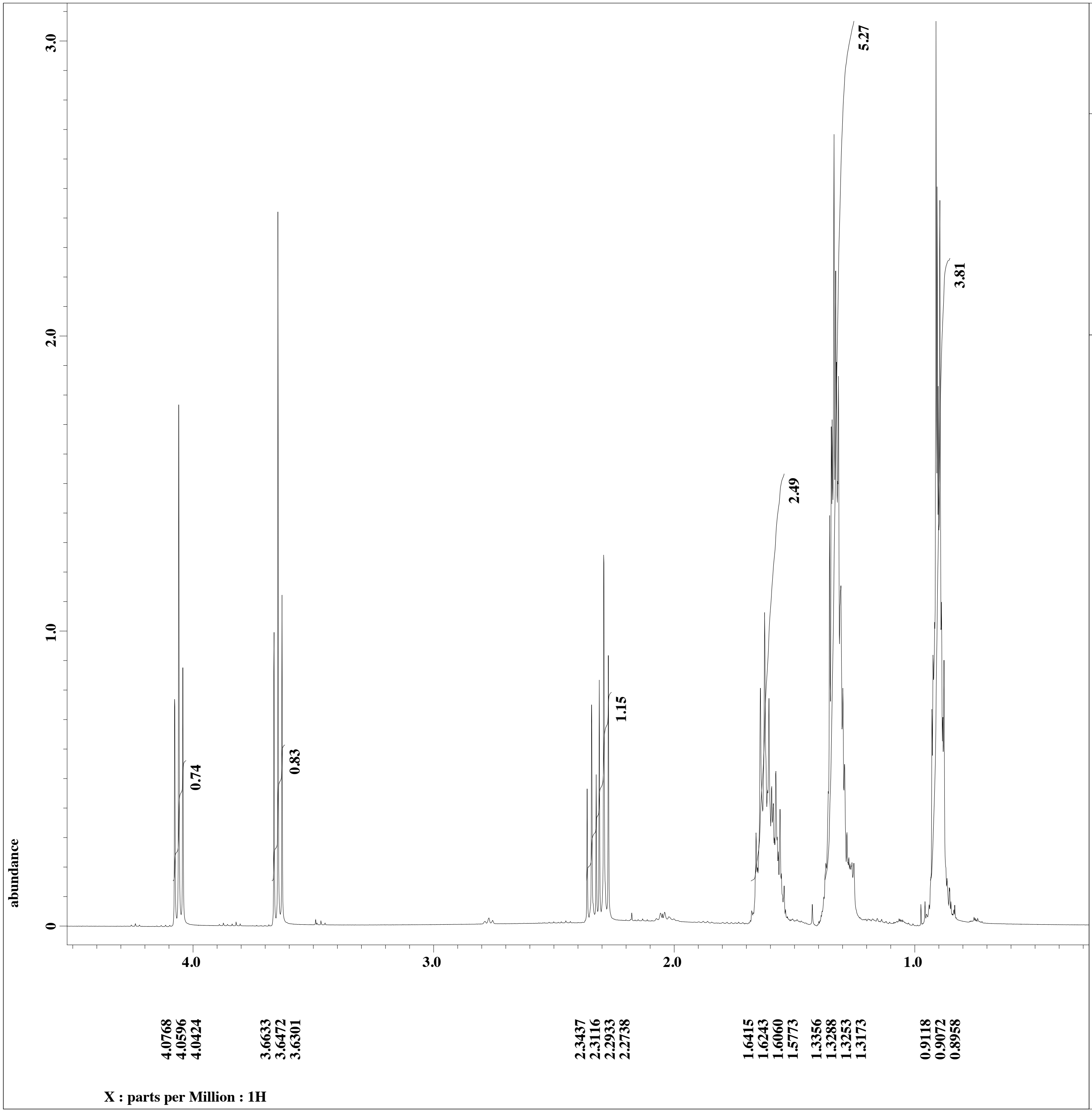
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename          = HEX-BUT-ANIRUDH_PROTO
Author            = delta
Experiment        = single_pulse.ex2
Sample_id         = HEX-BUT-ANIRUDH
Solvent           = CHLOROFORM-D
Creation_time     =  3-JUN-2016 15:45:14
Revision_time     = 22-JUN-2016 12:47:23
Current_time      = 22-JUN-2016 12:47:47

Comment           = ANIRUDH
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

Field_strength    = 9.389766[T] (400[MHz]
X_acq_duration    = 2.18365952[s]
X_domain          = 1H
X_freq            = 399.78219838[MHz]
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.45794685[Hz]
X_sweep           = 7.5030012[kHz]
Irr_domain        = 1H
Irr_freq          = 399.78219838[MHz]
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[MHz]
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 64
Total_scans       = 64

X_90_width        = 9.75[us]
X_acq_time        = 2.18365952[s]
X_angle           = 45[deg]
X_atn             = 0.3[dB]
X_pulse           = 4.875[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 44
Relaxation_delay  = 4[s]
Repetition_time   = 6.18365952[s]
Temp_get          = 20.9[dC]
```



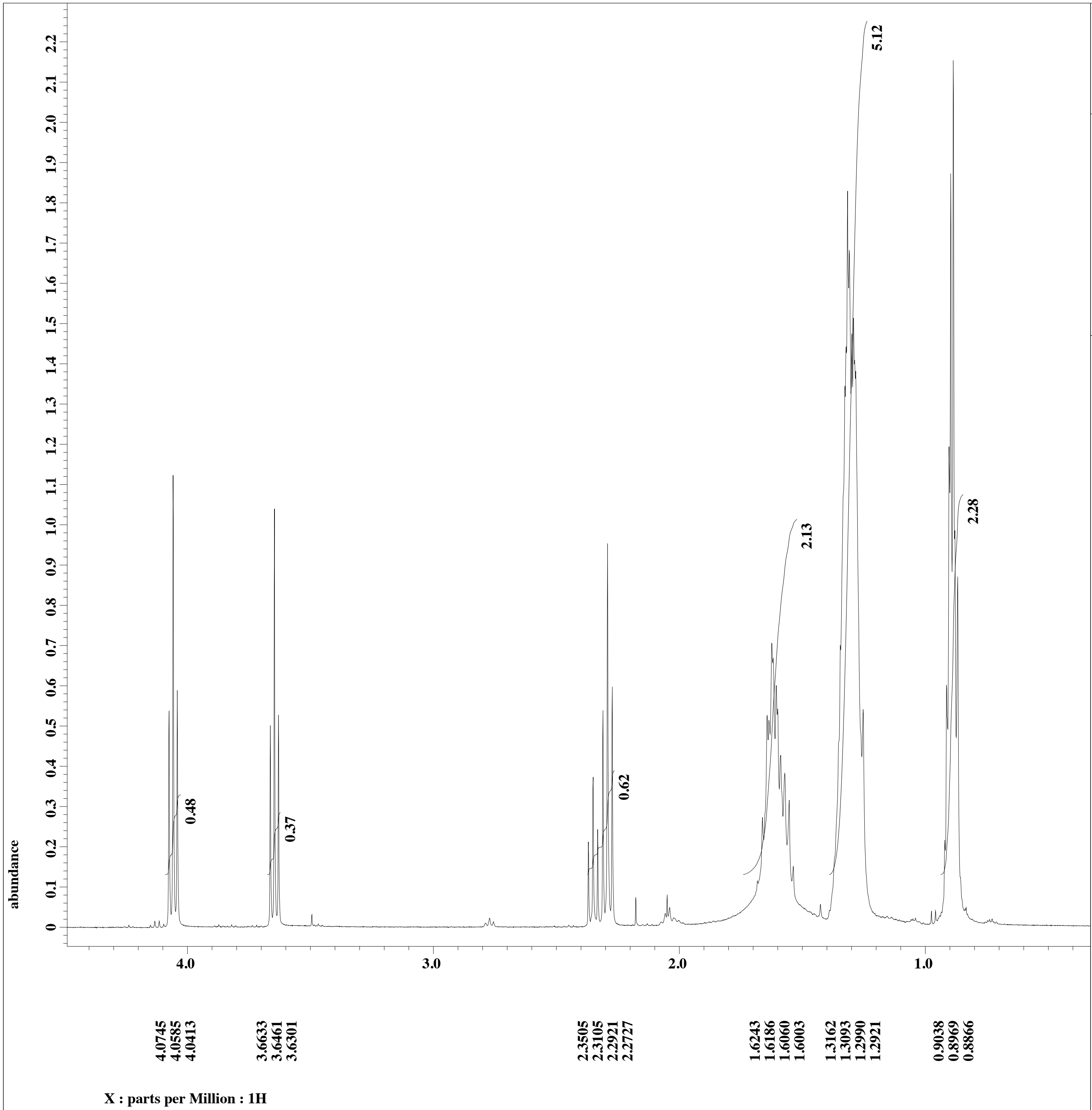
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename          = HEX-PENT-ANIRUDH_PROT
Author            = delta
Experiment        = single_pulse.ex2
Sample_id         = HEX-PENT-ANIRUDH
Solvent           = CHLOROFORM-D
Creation_time     =  3-JUN-2016 15:59:47
Revision_time     = 22-JUN-2016 13:39:18
Current_time      = 22-JUN-2016 13:39:43

Comment           = ANIRUDH
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

Field_strength    = 9.389766[T] (400[MHz]
X_acq_duration    = 2.18365952[s]
X_domain          = 1H
X_freq            = 399.78219838[MHz]
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.45794685[Hz]
X_sweep           = 7.5030012[kHz]
Irr_domain        = 1H
Irr_freq          = 399.78219838[MHz]
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[MHz]
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 64
Total_scans       = 64

X_90_width        = 9.75[us]
X_acq_time        = 2.18365952[s]
X_angle           = 45[deg]
X_atn             = 0.3[dB]
X_pulse           = 4.875[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 34
Relaxation_delay  = 4[s]
Repetition_time   = 6.18365952[s]
Temp_get          = 20.9[dC]
```



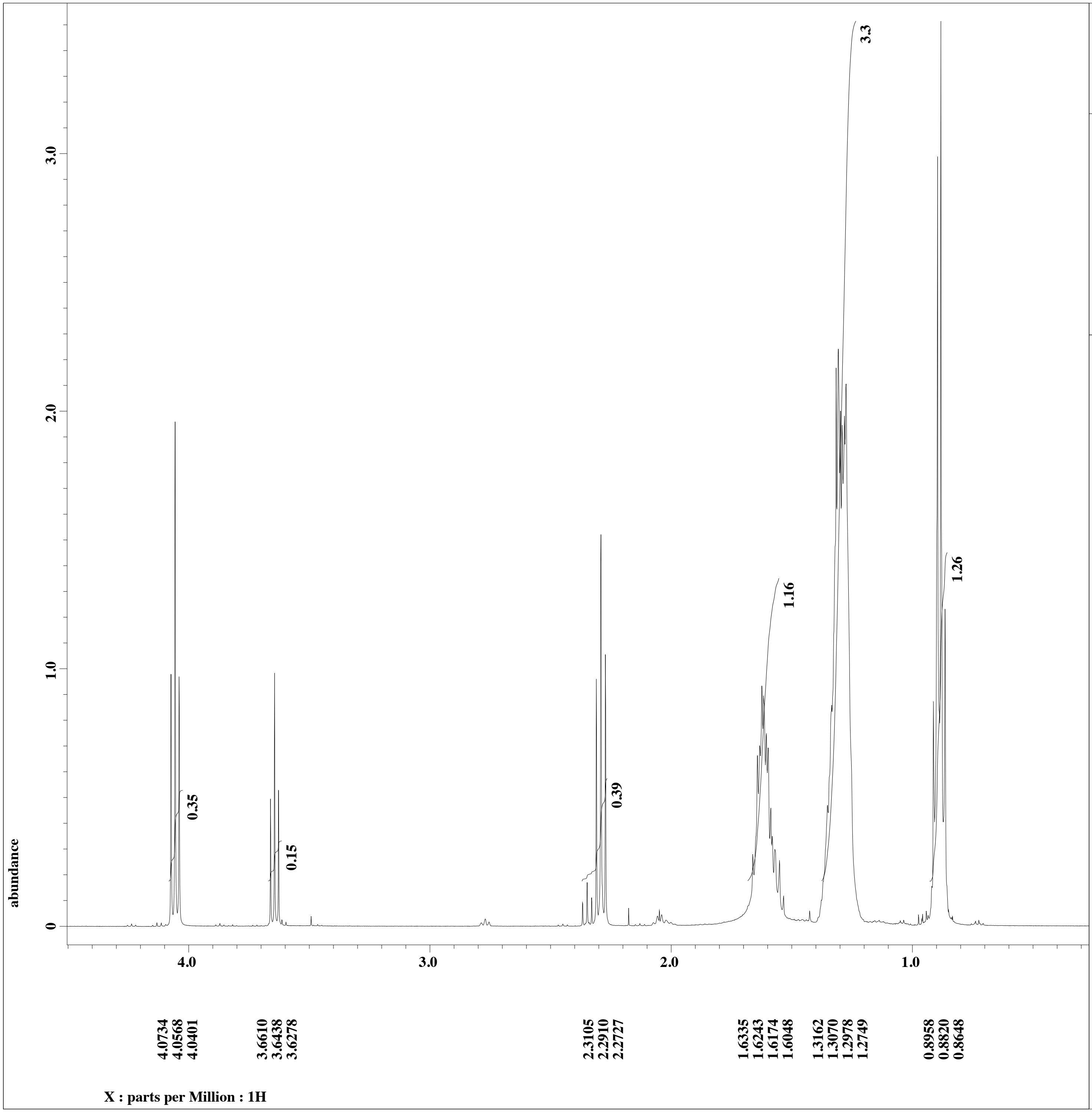
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename          = HEX-HEPT-ANIRUDH_PROT
Author            = delta
Experiment        = single_pulse.ex2
Sample_id         = HEX-HEPT-ANIRUDH
Solvent           = CHLOROFORM-D
Creation_time     = 13-JUN-2016 14:07:51
Revision_time     = 22-JUN-2016 12:59:28
Current_time      = 22-JUN-2016 13:00:01

Comment           = ANIRUDH
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

Field_strength    = 9.389766[T] (400[MHz]
X_acq_duration    = 2.18365952[s]
X_domain          = 1H
X_freq            = 399.78219838[MHz]
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.45794685[Hz]
X_sweep           = 7.5030012[kHz]
Irr_domain        = 1H
Irr_freq          = 399.78219838[MHz]
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[MHz]
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 64
Total_scans       = 64

X_90_width        = 9.75[us]
X_acq_time        = 2.18365952[s]
X_angle           = 45[deg]
X_atn             = 0.3[dB]
X_pulse           = 4.875[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 42
Relaxation_delay  = 4[s]
Repetition_time   = 6.18365952[s]
Temp_get          = 20[dC]
```



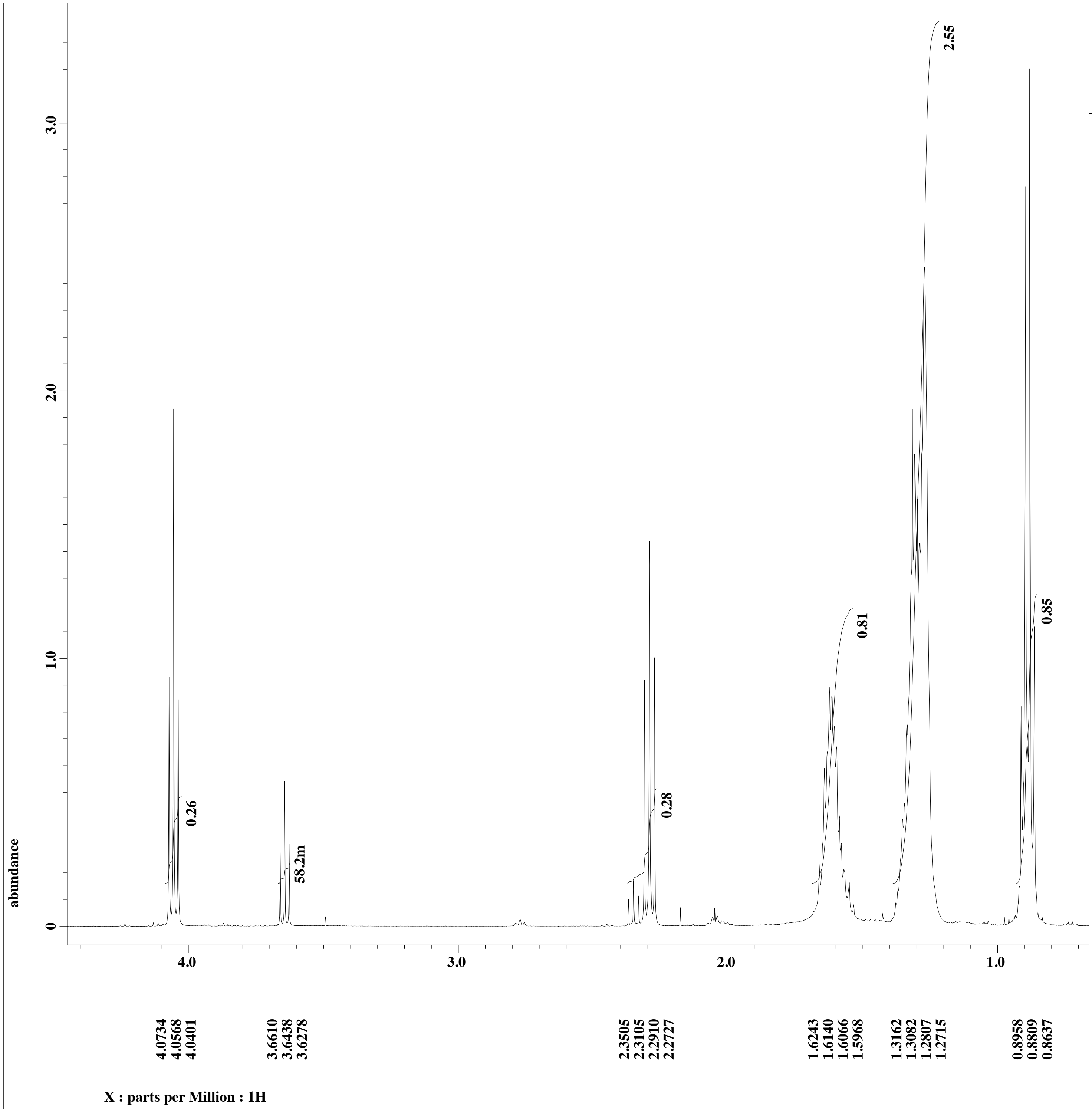
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename          = HEX-OCT-ANIRUDH_PROTO
Author            = delta
Experiment        = single_pulse.ex2
Sample_id         = HEX-OCT-ANIRUDH
Solvent           = CHLOROFORM-D
Creation_time     = 13-JUN-2016 14:22:36
Revision_time     = 22-JUN-2016 13:32:46
Current_time      = 22-JUN-2016 13:33:17

Comment           = ANIRUDH
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

Field_strength    = 9.389766[T] (400[MHz]
X_acq_duration    = 2.18365952[s]
X_domain          = 1H
X_freq            = 399.78219838[MHz]
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.45794685[Hz]
X_sweep           = 7.5030012[kHz]
Irr_domain        = 1H
Irr_freq          = 399.78219838[MHz]
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[MHz]
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 64
Total_scans       = 64

X_90_width        = 9.75[us]
X_acq_time        = 2.18365952[s]
X_angle           = 45[deg]
X_atn             = 0.3[dB]
X_pulse           = 4.875[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 38
Relaxation_delay  = 4[s]
Repetition_time   = 6.18365952[s]
Temp_get          = 20[dC]
```



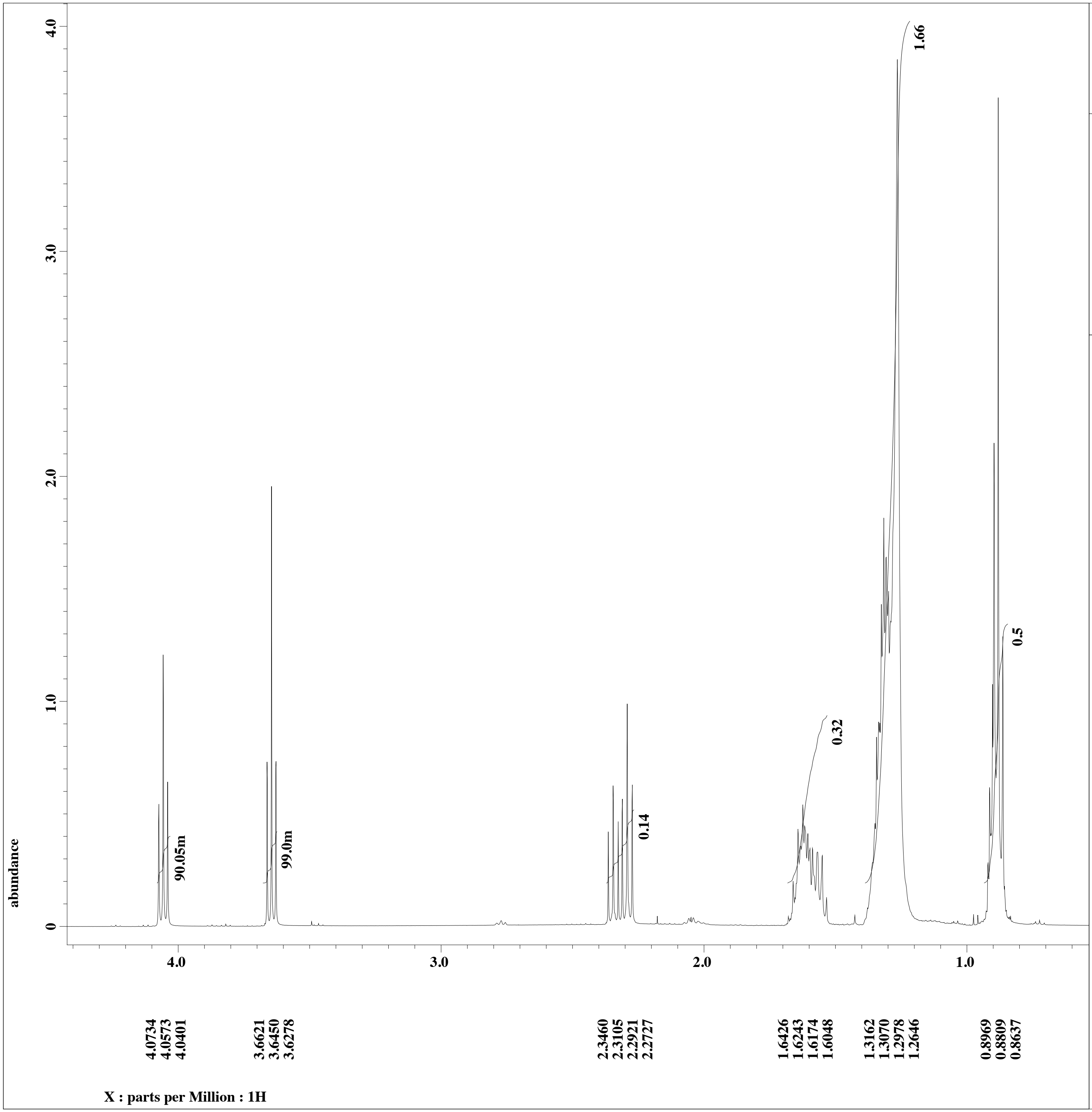
```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename          = HEX-NAN-ANIRUDH_PROTO
Author            = delta
Experiment        = single_pulse.ex2
Sample_id         = HEX-NAN-ANIRUDH
Solvent           = CHLOROFORM-D
Creation_time     = 13-JUN-2016 13:38:59
Revision_time     = 22-JUN-2016 13:21:00
Current_time      = 22-JUN-2016 13:21:32

Comment           = ANIRUDH
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

Field_strength    = 9.389766[T] (400[MHz]
X_acq_duration    = 2.18365952[s]
X_domain          = 1H
X_freq            = 399.78219838[MHz]
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.45794685[Hz]
X_sweep           = 7.5030012[kHz]
Irr_domain        = 1H
Irr_freq          = 399.78219838[MHz]
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[MHz]
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 64
Total_scans       = 64

X_90_width        = 9.75[us]
X_acq_time        = 2.18365952[s]
X_angle           = 45[deg]
X_atn             = 0.3[dB]
X_pulse           = 4.875[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 38
Relaxation_delay  = 4[s]
Repetition_time   = 6.18365952[s]
Temp_get          = 20[dC]
```



```
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp_auto : 0.2
trapezoid3 : 0[%] : 80[%] : 100[%]
fft : 1
machinephase
dc_correct
ppm
thresh : 5[%] : 1
peak_pick : 0[Hz] : 0.1[ppm] : Both : 0[
auto_reference : 5[%]
```

```
Filename           = HEX-DEC-ANIRUDH_PROTO
Author             = delta
Experiment         = single_pulse.ex2
Sample_id          = HEX-DEC-ANIRUDH
Solvent            = CHLOROFORM-D
Creation_time      = 13-JUN-2016 13:53:27
Revision_time      = 22-JUN-2016 12:55:02
Current_time       = 22-JUN-2016 12:56:01

Comment            = ANIRUDH
Data_format        = 1D COMPLEX
Dim_size           = 13107
Dim_title          = 1H
Dim_units          = [ppm]
Dimensions         = X
Site               = ECS 400
Spectrometer       = JNM-ECS400

Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Clipped            = FALSE
Mod_return         = 1
Scans              = 64
Total_scans        = 64

X_90_width         = 9.75[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 0.3[dB]
X_pulse            = 4.875[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Recvr_gain         = 32
Relaxation_delay   = 4[s]
Repetition_time    = 6.18365952[s]
Temp_get           = 20[dC]
```