KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

CHARACTERIZATION AND HPLC QUANTIFICATION OF XYLOPIC ACID IN THE DRIED FRUITS OF XYLOPIA AETHIOPICA

SUBMITTED BY

JAMES OPPONG KYEKYEKU

APRIL, 2010

CHARACTERIZATION AND HPLC QUANTIFICATION OF XYLOPIC ACID IN THE DRIED FRUITS OF *XYLOPIA AETHIOPICA*

SUBMITTED BY

JAMES OPPONG KYEKYEKU

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF

MASTER OF PHILOSOPHY

PHARMACEUTICAL CHEMISTRY

FROM THE DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

KUMASI, GHANA

APRIL, 2010

DECLARATION

I, James Oppong Kyekyeku, wish to declare that this thesis is my report of my own work. I further declare that, to the best of my knowledge, this work has not been previously submitted for any degree anywhere.

……...
JAMES OPPONG KYEKYEKU

(STUDENT)

Submitted on 28th day of April, 2010

……

DR. REIMMEL K. ADOSRAKU

(SUPERVISOR)

DEDICATION

Dedicated to my mum, Grace Badu

ACKNOWLEDGEMENTS

And we know that all things work together for good to them that love God, to them who are called according to His purpose.

I am grateful to the Almighty God, my Creator for the continuous gift of life.

Thanks go to my family especially my mother for their prayers and support throughout the course of the project. I really appreciate all you did and continue to do for me.

I also wish to acknowledge the immense guidance and contributions of my supervisor Dr. R. K. Adosraku.

The IR, NMR and MS data were provided by Florida A&M University College of Pharmacy through a grant from the Pharmaceutical Research Center NIH/NCRR 1 C06-RR12512-01 and RCMI. Grant No. G12 RR 03020 from NCRR.

Lastly, to all my friends who made this course bearable, I say thanks to all of them.

- James Oppong Kyekyeku, 2009

ABSTRACT

Xylopia aethiopica is an evergreen, aromatic tree growing up to **15cm** high, bark slightly ridged, native to the West, Central and Southern Africa. Flowers appear in clusters of 2-4, fruits turn red when ripe and black when dried. The dried fruit is commonly called African pepper. The dried fruits of *Xylopia aethiopica* have been used in folklore medicine. The extracts of the fruit have been shown to have antibacterial and antifungal properties.

Dried fruits of *Xylopia aethiopica* used for the project were obtained from the Kumasi Central Market and were authenticated by the Department of Pharmacognosy, KNUST.

In order to obtain pure xylopic acid crystals for use as secondary reference xylopic acid was extracted from dried fruits of *Xylopia aethiopica* with petroleum ether (40-60^{o}C) and recrystallised from ethanol. The melting point of the crystals was determined to be **261-262^{o}C** and the Rf value of xylopic acid using petroleum ether (40-60^{o}C): ethylacetate (9:1) as the solvent system to be **0.53**. NMR, infrared and mass spectroscopic analyses were carried out to characterise the isolated crystals.

HPLC was used to quantify the xylopic acid in the dried fruits using methanol: water (9:1) as the mobile phase and a phenomenex hypersil 20 micron C 18 200 x 3.2 mm reverse column. The percentage content of xylopic acid in the whole fruit was estimated to be **1.15% ± 0.08 (n=8).** The percentage content of xylopic acid in the seeds was determined to be **0.07% ± 0.02 (n=6)** and the dried pericarp of the fruit was found to contain **0.87% ± 0.20 (n=6)** xylopic acid.

The purified xylopic acid crystals were subjected to stability studies for a period of three (3) months. The conditions employed for the stability studies were temperatures of 50^{o}C, 60^{o}C and 80^{o}C. The crystals were assayed on the 20th, 50th and 70th days using the HPLC method developed for the assay of the crude fruit extract. The concentration at 50^{o}C was **87.76%, 74.68%** and **67.78%** on the 20th, 50th and 70th days respectively. The concentration at 60^{o}C was estimated to be **75.86%, 67.19%** and **47.97%** on the 20th, 50th and 70th days respectively. The concentration at 80^{o}C was found to be **68.13%, 63.10%** and **44.51%** on the 20th, 50th and 70th days respectively.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The use of plants to prevent and cure diseases has been a common practice all over the world. Spices, especially have contributed to disease prevention among the rural population of sub-saharan Africa. Medicinal plants have been used in virtually all cultures as a source of medicine. The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed **(UNESCO, 1996)**. Furthermore, an increasing reliance on the use of medicinal plants in the industrialised societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies **(UNESCO, 1998)**. Moreover, in these societies, herbal remedies have become more popular in the treatment of minor ailments, on account of the increasing cost of personal health maintenance. According to the World Health Organization (WHO), because of poverty and lack of access to modern medicine, about 65-80% of the world's population which lives in developing countries depends essentially on plants for primary health care **(Akerele O, 1993)**. The phytomedicine market has grown at an impressive rate worldwide since 1985 (from 5 to 18% a year) **(Grünwald, 1995).**

In recent years, the major pharmaceutical companies have demonstrated renewed interest in investigating higher plants as sources for new compounds and also for the development of standardized phytotherapeutic agents with proved efficacy, safety and quality. The pharmaceutical industry is researching tremendously to obtain biologically active compounds from natural sources. The isolation of bioactive compounds from natural sources is motivated by the possible destruction of rain forest or other wild habitat if whole plants are used medicinally. Because of the increasing use of medicinal plants, their future is being threatened by complacency concerning their conservation. Reserves of herbs and stocks of medicinal plants in developing countries are diminishing and in danger of extinction as a result of growing trade demands for cheaper healthcare products and new plant-based therapeutic markets in preference to more expensive target-specific drugs and biopharmaceuticals. The practise of traditional medicine is widespread in China, India, Japan, and in the United States of America.

In several industrialised societies, plant-derived prescription drugs are important in the maintenance of health. Medicinal plants are an integral component of research developments in the pharmaceutical industry. Such research focuses on the isolation and direct use of active medicinal constituents, the development of semi-synthetic drugs and the active screening of natural products to yield synthetic pharmacologically-active compounds. The development and commercialisation of medicinal plant-based bioindustries in the developing countries is dependent upon the availability of facilities and information concerning upstream and downstream bioprocessing, extraction, purification, and marketing of the industrial potential of medicinal plants.

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. It can be observed that plant secondary metabolites have been employed in a wide variety of uses. Herbal remedies have been used for centuries but more recently the active compounds have been identified. These compounds have been extracted and purified, enabling synthetic medicinal chemists to synthesise the molecules on larger scales. The technologies used in this process are quite complex. Techniques that are more refined would involve the use of chromatography to separate the different molecules present and a combination of spectroscopic techniques to correctly identify the molecules.

1.2 LITERATURE REVIEW

1.2.1 XYLOPIA AETHIOPICA

Botanical Name: *Xylopia aethiopica* (Dunal) A. Rich

Family*:* Annonaceae

Common Names:

Africa pepper, spice tree seeds, Guinea pepper, Ethiopian pepper **(Ghana herbal Pharmacopeia, 2007)**

Chemical Constituents:

Volatile oils (monoterpenoids: cineole, cuminic aldehyde, terpinone, β-pinene); diterpenoids (xylopic acid and other kaurane, trachylobane and kolavane diterpenes), oleoresins **(Ghana herbal Pharmacopeia, 2007)**, minerals-copper, manganese and zinc **(Smith, 1996).**

1.2.1.1 BOTANICAL DESCRIPTION

Xylopia aethiopica is an evergreen, aromatic tree growing up to **15cm** high, bark slightly ridged; leaves oblong-anceolate, rounded base, acuminate apex, glaucous, 4-10 cm long, 2-4 cm broad, lateral nerves 7-8; flowers in clusters of 2-4; greenish fruits with 20-30 capsules, turn red when ripe. Native to the lowland rainforest and moist fringing and deciduous forest in the savannah zones in Africa, largely located in West, Central and Southern Africa **(Ghana Herbal Pharmacopoeia, 2007)**.

1.2.1.2 USES OF XYLOPIA

In African folk medicine the dried fruits of Xylopia are used as carminative, stimulant, antifungal, broad-spectrum microbial, additive to other remedies, post-partum tonic and lactation aid **(Ghana Herbal Pharmacopoeia, 2007)**.

Figure 1.1: Dried fruits of *Xylopia aethiopica*

1.2.1.3 RESEARCH UPDATE ON XYLOPIA AETHIOPICA

Extracts of the fruit have been shown to be active against gram positive and negative bacteria **(Iwu, 1993)**. Xylopic acid has demonstrated activity against *Candida albicans* **(Boakye-Yiadom, 1977)**. Extracts of *Alepidea amatymbica* and *Xylopia aethiopica* demonstrated diuretic activity and natriuretic effects comparable to the effects of chlorothiazide, suggesting inhibition of Na^+ and K^+ reabsorption in the distal tubule **(Somova et al., 2001).** Aqueous extract of *Xylopia aethiopica* was neither a miotic nor a mydriatic, but lowered the intraocular pressure, reduced the near point of convergence and increased the amplitude of accommodation **(Igwe et al., 2003)**. The essential oils of *Xylopia aethiopica, Monodora myristica*, *Zanthoxylum xanthoxyloides* and *Z. leprieurii*, showed antibacterial and antifungal activity **(Tatsadjieu et al., 2003)**.

JUSTIFICATION

Pure xylopic acid is difficult to obtain from the international chemical market. Since pure xylopic acid was required to assay the amount in the dried fruits, xylopic acid had to be extracted and purified from the fruits. There is little information on xylopic acid (structure and bond angles of xylopic acid, **Fiagbe et al., 1979**) in the international chemical society hence the need to characterise xylopic acid and contribute to scientific literature. The fruits of *Xylopia aethiopica* have been used in folklore medicine to treat various diseases. The extracts of the dried fruits are taken with no knowledge of the amount of xylopic acid in the preparation. This work therefore seeks to quantify the amount of xylopic acid which is a major constituent in the fruits of *Xylopia aethiopica*. The method developed can be used by chemists to standardise preparations of extracts of the fruits of *Xylopia aethiopica* used by traditional medicine practitioners to treat diseases.

OBJECTIVES

- To isolate and purify xylopic acid from the dried fruits of *Xylopia aethiopica*.
- To characterize the xylopic acid crystals extracted.
- To determine the amount of xylopic acid in the dried fruits of *Xylopia aethiopica*.
- To determine the amount of xylopic acid in the seed and pericarp of the dried fruits.

1.2.3 THEORY OF EXPERIMENTAL WORK

1.2.4.1 ISOLATION AND PURIFICATION TECHNIQUES

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing other interfering materials **(British Pharmacopoeia, 2007)**.

The adoption of a particular isolation procedure will depend to a large extent on the physical and chemical properties of the product. Some guidelines for useful general approaches may however be given with regard to the physical state at ambient temperature of the crude mixture resulting from the reaction, i.e. whether it is a *one-phase* (either *solid* or *liquid)* or a *two-phase (solid/liquid* or *liquid/liquid)* system.

In the case of a solid organic product if the organic product is neutral and insoluble in water, washing with water may be used to remove soluble impurities such as inorganic salts. Alternatively the crude solid may be extracted with a suitable organic solvent, filtered, and the extract washed with water. Further washing successively with dilute aqueous acid and dilute aqueous alkali removes basic and acidic impurities. Removal of solvent after drying leads to the recovery of the purified solid for recrystallisation from a suitable solvent. Continuous extraction of the solid (e.g. in a Soxhlet apparatus) may be necessary if the required product is only sparingly soluble in convenient organic solvents.

If the crude solid product contains the required product in the form of a salt (e.g. the alkali metal salt of a phenol) and is therefore water soluble, acidification of the aqueous solution (or basification in the case, for example, of amine salts) liberates the free acidic compound (or base) which may be recovered by filtration or solvent extraction as appropriate.

The *one-phase liquid system* is more frequently encountered since many organic reactions are carried out in solution. Direct fractional distillation may separate the product, if it is a liquid, from the solvent and other liquid reagents, or concentration or cooling may lead to direct crystallisation of the product if this is a solid. However, it is often more appropriate, whether the required product is a liquid or solid, to subject the solution to the acid/base extraction procedure outlined above. This acid/base extraction procedure can be done directly if the product is in solution in a water-immiscible solvent. Knowledge of the acid-base nature of the product and of its water solubility is necessary to ensure that the appropriate fraction is retained for product recovery.

In those cases where the reaction solvent is water miscible (e.g. methanol, ethanol, dimethylsulphoxide, etc.) it is necessary to remove all or most of the solvent by distillation and to dissolve the residue in an excess of a water-immiscible solvent before commencing the extraction procedure. The removal of solvent from fractions obtained by these extraction procedures is these days readily effected by the use of a rotary evaporator and this obviates the prolong removal of large volumes of solvent by conventional distillation. A crude reaction mixture consisting of *two phases* is very common. In the case of a *solid/liquid* system, it will of course be necessary to make certain in which phase the required product resides. A simple example is where the product may have crystallised out from the reaction solvent; the mixture therefore only requires to be cooled and filtered for the bulk of the product to be isolated. The filtrate should then routinely be subjected to suitable concentration or extraction procedures to obtain the maximum yield of product. Direct filtration would also be employed when the solid consists of unwanted reaction products, in which case the filtrate would be treated as the single-phase liquid system above. Where it is evident that the product has crystallised out admixed with contaminating solid material a separation might be effected if the mixture is reheated and filtered hot.

Liquid/liquid two-phase systems are often encountered; for example, they result from the frequent practice of quenching a reaction carried out in an organic solvent by pouring it on to ice or into dilute acid. A further instance of a liquid/liquid system arises from the use of steam distillation as a preliminary isolation procedure. This is particularly suitable for the separation of relatively high-boiling liquids and steam volatile solids from inorganic contaminants, involatile tars, etc. The subsequent work-up procedure normally presents no additional problems since the phases are usually readily separable and can be treated in a manner appropriate to the chemical or physical properties of the required product by procedures already outlined.

All these preliminary procedures give solid or liquid products which are rarely of high purity; the degree of purity may be checked by chromatographic and spectroscopic methods. Purification may often be successfully accomplished by recrystallisation or sublimation for solids; fractional distillation under atmospheric or reduced pressure for liquids or low melting solids; molecular distillation for high-boiling liquids. In those cases where the use of these traditional methods does not yield product of adequate purity, resort must be made to preparative chromatographic procedures. Here knowledge of the chromatographic behaviour obtained from small-scale trial experiments will be particularly valuable.

The final assessment of the purity of a known product is made on the basis of its physical constants in comparison with those cited in the literature. In the case of a new compound the purity should be assessed and the structural identity established by appropriate chromatographic and spectroscopic methods. (**Furniss et al, 1989**)

1.2.4.2 FILTRATION TECHNIQUES

Filtration of a mixture after completion of a reaction will often be necessary either to isolate a solid product which has separated out or to remove insoluble impurities or reactants, in which case the desired product remains in solution. When substantial quantities of a solid are to be filtered from suspension in a liquid, a Buchner funnel of convenient size is employed. The use of suction renders rapid filtration possible and also results in a more complete removal of the mother-liquor than filtration under atmospheric pressure. However initial gentle suction often leads to more effective filtration than powerful suction since in the latter case the finer particles of solid may reduce the rate of filtration by being drawn into the pores of the filter paper.

Some modification of the general technique of isolation by filtration may be necessary in the light of the chemical nature of the reaction mixture, of the particle size of the solid, or of the ratio of the amount of solid to liquid material to be filtered. For example, strongly alkaline or strongly acidic reaction mixtures weaken cellulose filter papers. Acid-hardened grades which are more chemically resistant are commercially available (e.g. Whatman filter papers) but for maximum resistance to chemical attack, glass-fibre paper (e.g. Whatman) or a glass funnel fitted with a fixed sintered glass plate may be used. The filtration of very finely divided suspended material is often very tedious as a result of the filter paper pores becoming clogged. In such a case the addition of a suitable filter aid (e.g. a high grade diatomaceous earth such as Celite 545, or Whatman filter aids) to the suspension overcomes the problem; alternatively the suspension may be filtered through a bed of filter aid prepared by pouring a slurry of it in a suitable solvent into the filter funnel fitted with the required size of filter paper. The initial application of gentle suction in the filtration is in this case vital. A glass-fibre filter paper, supported on a conventional filter paper in a Buchner or Hirsch funnel, is useful for the rapid removal of finely divided solid impurities from a solution. The selection of a funnel appropriate to the amount of solid rather than the total volume of liquor to be filtered is important. **(Furniss et al, 1989)**

1.2.4.3 SOLVENT EXTRACTION

As indicated earlier the crude products of most organic reactions and extracts from natural sources are multicomponent mixtures, and a convenient initial isolation procedure, for the first stages of both the separation of such mixtures and of the purification of the components, may involve solvent extraction processes. In this process the necessity of assessing the chemical and physical nature of the components of a particular reaction mixture with regard to their solubilities in solvents, and to their acidic, basic or neutral characteristics is very crucial.

Perhaps one of the most frequent cases that is encountered is the separation of a neutral organic compound (or compounds) from a solution or suspension (as either a solid or liquid) in an aqueous medium, by shaking with an organic solvent in which the compound is soluble and which is immiscible (or nearly immiscible) with water. The solvents generally employed for extraction are diethyl ether or diisopropyl ether, toluene, dichloromethane and light petroleum. The solvent selected will depend upon the solubility of the substance to be extracted in that solvent and upon the ease with which the solvent can be separated from the solute. If prior information is not available, solvent selection should be based on some small-scale trials. In the isolation of organic compounds from aqueous solutions, use is frequently made of the fact that the solubility of many organic substances in water is considerably decreased by the presence of dissolved inorganic salts (sodium chloride, calcium chloride, ammonium sulphate, etc.). This is the so-called *salting-out effect.* A further advantage is that the solubility of partially miscible organic solvents, such as ether, is considerably less in the salt solution, thus reducing the loss of solvent in extractions.

The process of extraction is concerned with the *distribution law* or *partition law* which states that if to a system of two liquid layers, made up of two immiscible or slightly miscible components, is added a quantity of a third substance soluble in both layers, then the substance distributes itself between the two layers so that the ratio of the concentration in one solvent to the concentration in the second solvent remains constant at constant temperature. It is assumed that the molecular state of the substance is the same in both solvents.* If CA and CB are concentrations in the layers A and B, then, at constant temperature:

CA/CB = constant = K

The constant *K* is termed the *distribution* or *partition coefficient.* As a very rough approximation the distribution coefficient may be assumed equal to the ratio of the solubilities in the two solvents **(Furniss et al, 1989).**

1.2.4.4 RECRYSTALLISATION TECHNIQUES

Solid organic compounds when isolated from organic reactions are seldom pure; they are usually contaminated with small amounts of other compounds (impurities) which are produced along with the desired product. The purification of impure crystalline compounds is usually effected by crystallization from a suitable solvent or mixture of solvents.

The purification of solids by crystallization is based upon differences in their solubility in a given solvent or mixture of solvents. In its simplest form, the crystallization process consists of:

(i) dissolving the impure substance in some suitable solvent at or near the boiling point;
(ii) filtering the hot solution from particles of insoluble material and dust;
(iii) allowing the hot solution to cool thus causing the dissolved substance to crystallize out; and
(iv) separating the crystals from the supernatant solution (or mother-liquor).

The resulting solid after drying is washed and tested for purity (usually by a melting point determination, spectroscopic methods, or by thin-layer chromatography). If found impure, it is again recrystallised from fresh solvent. The process is repeated until the pure compound is obtained (i.e. the melting point is unchanged). However, confirmation by the other methods specified above is desirable. It is assumed that the impurities are present in comparatively small proportion - usually less than 5 per cent of the whole.
The most desirable characteristics of a solvent for recrystallisation are as follows;

- A high solvent power for the substance to be purified at elevated temperature and a comparatively low solvent power at the laboratory temperature below.
- It should dissolve the impurities readily or to a very small extent.
- It should yield well-formed crystals of the purified compound.
- It must be capable of easy removal from the crystals of the purified compound i.e. possess a relatively low boiling point.

It is assumed, of course, that the solvent does not react chemically with the substance to be purified. If two or more solvents appear to be equally suitable for recrystallisation, the final selection will depend upon such factors as ease of manipulation, toxicity, flammability and cost. The use of ether as a solvent for recrystallisation should be avoided wherever possible, partly owing to its great flammability and partly owing to its tendency to creep up walls of the containing vessel, thus depositing solid matter by complete evaporation instead of preferential crystallisation. Carbon disulphide, b.p. 46°C, should *never* be used if an alternative solvent can be found; it has a dangerously low flash point and forms very explosive mixtures with air.

Some common solvents available for the recrystallisation are;

Table 1.1: Common solvents for recrystallisation (Furniss et al, 1989)

SOLVENT	**b.p.(°C)**	
Water(distilled)	100	To be used whenever possible
Methanol*****	64.5	Flammable; toxic
Ethanol	78	Flammable
Industrial spirit	77-82	Flammable
Rectified spirit	78	Flammable
Acetone	56	Flammable
Ethyl acetate	78	Flammable
Acetic acid (glacial)	118	Not very flammable, pungent vapours
Dichloromethane (methylene chloride)*****	41	Non-flammable; toxic
Chloroform*****	61	Non-flammable; vapour toxic
Diethyl ether	35	Flammable, avoid whenever possible
Benzene***Ï**	80	Flammable, vapour highly toxic
Dioxane*****	101	Flammable, vapour toxic
Carbon tetrachloride*****	77	Non-flammable, vapour toxic
Light petroleum	40-60	Flammable
Cyclohexane	81	Flammable

CAUTION:** *The vapours of these solvents are toxic and therefore recrystallisations involving their use must be conducted in an efficient fume cupboard;* ***Ï *Toluene is much less*

toxic than benzene and should be used in place of the latter whenever possible. Other fractions available have b.p. 60-80, 80-100 and 100-200ºC; when the boiling point exceeds 120ºC the fraction is usually called 'ligroin'. Pentane, b.p. 36ºC, and heptane, b.p. 98ºC, are also frequently used recrystallisation solvents.

The following rough generalisations may assist in the selection of a solvent for recrystallisation, but it must be clearly understood that numerous exceptions are known in order of decreasing polarity:

1. A substance is likely to be most soluble in a solvent to which it is most closely related in chemical and physical characteristics.
2. In ascending a homologous series, the solubilities of the members tend to become more and more like that of the hydrocarbon from which they may be regarded as being derived.
3. A polar substance is more soluble in polar solvents and less soluble in nonpolar solvents. The solvents in Table 2.8 have been listed broadly in order of decreasing polar character.

1.2.4.4.1 FILTRATION OF THE HOT SOLUTION

The boiling or hot solution must be rapidly filtered before undue cooling has occurred (If a flammable solvent has been used, all flames in the vicinity must be extinguished.) This is usually done through a fluted filter paper supported in a relatively large funnel with a short wide stem; separation of crystals in and clogging of the stem is thus reduced to a minimum. The funnel should be warmed in an electric or steam oven before filtration is started, when it should be supported in a conical flask of sufficient size to hold all the solution; the conical flask is stood on an electric hotplate or steam bath and the filtrate is kept boiling gently so that the warm solvent vapours maintain the temperature of the solution undergoing filtration, and thus prevent premature deposition of crystals on the filter or in the neck of the funnel. If solid does separate out on the filter it must be scraped back into the first flask, redissolved and refiltered. The filtered solution is covered with a watch-glass, and then set aside to cool undisturbed. If large crystals are desired, any solid which may have separated from the filtered solution should be redissolved by warming (a reflux condenser must be used for a flammable solvent), the flask wrapped in a towel or cloth, and allowed to cool slowly. If small crystals are required, the hot saturated solution should be stirred vigorously and cooled

rapidly in a bath of cold water or of ice. It should be noted that large crystals are not necessarily purer than small ones; generally very impure substances are best purified by slow recrystallisation to give large crystals, followed by several rapid recrystallisations to give small crystals. If large quantities of hot solution are to be filtered, the funnel (and fluted filter paper) should be warmed externally during the filtration. The heating mantle is particularly suitable, using the lower heating element; no flames should be present while flammable solvents are being filtered through this funnel. When dealing with considerable volumes of aqueous or other solutions which do not deposit crystals rapidly on cooling, a Buchner funnel preheated in an oven may be used for filtration. The filter paper should be of close-grained texture and should be wetted with solvent before suction is applied; the solution may then be poured on to the filter. **(Furniss et al, 1989)**

1.2.4.4.2 USE OF DECOLOURISING CARBON

The crude product of an organic reaction may contain a coloured impurity. Upon recrystallisation, this impurity may dissolve in the boiling solvent and be partly adsorbed by the crystals as they separate upon cooling, yielding a coloured product. Sometimes the solution is slightly turbid owing to the presence of a little resinous matter or a very fine suspension of an insoluble impurity, which cannot always be removed by simple filtration. These impurities can be removed by boiling the substance in solution with a little decolourising charcoal for 5-10 minutes, and then filtering the solution while hot as described above. The decolourising charcoal adsorbs the coloured impurity and holds back resinous, finely divided matter, and the filtrate is usually free from extraneous colour, and therefore deposits pure crystals. The decolourisation takes place most readily in aqueous solution but can be performed in almost any organic solvent; the process is least effective in hydrocarbon solvents. It must be pointed out that boiling in a solvent with decolourising carbon is not always the most effective method of removing the colour. An excessive quantity of decolourising agent must be avoided, since it may also adsorb some of the compound which is being purified. The exact quantity to be added will depend upon the amount of impurities present; for most purposes 1-2 per cent by weight of the crude solid will be found satisfactory. If this quantity is insufficient, the operation should be repeated with a further 1-2 percent of fresh decolourising charcoal **(Furniss et al., 1989).**

1.2.4.4.3 DIFFICULTIES ENCOUNTERED IN RECRYSTALLISATION

Occasionally, substances form supersaturated solutions from which the first crystals separate with difficulty; this is sometimes caused by the presence of a little tar or viscous substance acting as a protective colloid. The following methods should be tried in order to induce crystallization:

- By scratching the inside of the vessel with a glass rod. The effect is attributed to breaking off of small particles of glass which may act as crystal nuclei, or to the roughening of the surface, which facilitates more rapid orientation of the crystals on the surface.
- By inoculating (seeding) the solution with some of the solid material or with isomorphous crystals, crystallization frequently commences and continues until equilibrium is reached.
- By cooling the solution in a freezing mixture (ice and salt, ice and calcium chloride, or solid carbon dioxide and acetone). By adding a few lumps of solid carbon dioxide; this produces a number of cold spots here and there, and assists the formation of crystals.
- If all the above methods fail, the solution should be left in an ice chest (or a refrigerator) for a prolonged period.

Occasionally, conversion into a simple crystalline derivative is applicable; subsequent regeneration of the original compound will usually yield a pure, crystalline solid. Instances will occur however when assessment by thin layer chromatography of the number of probable impurities in the isolated reaction mixture and of their relative amounts is advisable. It may then be judged whether some prior purification by suitable preparative chromatography or by solvent extraction should be performed before crystallization is attempted.

If the solvent constituting the crystallization medium has comparatively high boiling point, it is advisable to wash the solid with a solvent of low boiling point in order that the ultimate crystalline product may be easily dried. **(Furniss et al, 1989)**

1.2.4.4.4 RECRYSTALLISATON AT VERY LOW TEMPERATURES

This technique is necessary either when the solubility of the compound in the requisite solvent is too high at ordinarily obtained temperatures (refrigerator to room temperatures) for recovery to be economic, or when handling compounds which are liquid at room temperature but which may be recrystallised from a solvent maintained at much lower temperatures (say -10 to -40°C). In this latter case, after several successive low temperature recrystallisations, the compound will revert to a liquid on storage at room temperature, but the purification process by recrystallisation will have been achieved.

1.2.4.4.5 RECRYSTALLISATION IN INERT ATMOSPHERE

Substances that decompose, or otherwise undergo structural modification, on contact with air must be recrystallised in an indifferent atmosphere, which is usually nitrogen but may be carbon dioxide, or rarely, hydrogen. In those cases where even a short exposure to the atmosphere is harmful, the recrystallisation and filtration processes may be carried out in a nitrogen-filled manipulator glove box (available, for example, from Gallenkamp, Miller-Howe) which has been adapted to accommodate the services required for a normal recrystallisation procedure. The size of the glove box itself and the dimensions of the outlet panels will naturally limit the scale on which recrystallisation can be carried out in this manner **(Furniss et al, 1989).**

1.2.4.4.6 DRYING OF RECRYSTALLISED MATERIAL

The conditions for drying recrystallised material depend upon the quantity of product, the nature of the solvent to be removed and the sensitivity of the product to heat and to the atmosphere. With large-scale preparations of stable compounds, moist with non-toxic solvents which are volatile at room temperature (e.g. water, ethanol, ethyl acetate, acetone), the Buchner funnel is inverted over two or three thicknesses of drying paper (i.e. coarse-grained, smooth-surfaced filter paper) resting upon a pad of newspaper, and the crystalline cake removed with the aid of a clean spatula; several sheets of drying paper are placed on top and the crystals are pressed firmly. If the sheets become too damp with solvent, the crystals should be transferred to fresh paper. The crystals are then covered by a piece of filter paper perforated with a number of holes or with a large clock glass or sheet of glass supported upon corks. The air drying is continued until only traces of solvent remain (usually detected by

smell or appearance) and final drying is accomplished by placing the solid in an electric oven controlled at a suitable temperature. The disadvantage of this method of drying is that the crystallised product is liable to become contaminated with filter-paper fibre. With smaller amounts (e.g. 1-20g) of more valuable recrystallised material the filter cake is transferred to a tared watch glass, broken down into small fragments without damaging the crystalline form, and air dried under another suitably supported watch glass before being placed into a temperature-controlled oven. With low melting solids, the best method of drying is to place the crystals on a watch glass in a desiccator charged with an appropriate substance to absorb the solvent. For general purposes, water vapour is absorbed by a charge of granular calcium chloride, concentrated sulphuric acid or silica gel. Methanol and ethanol vapours are absorbed by granular calcium chloride or silica gel. Vapours from diethyl ether, chloroform, carbon tetrachloride, benzene, toluene, light petroleum and similar solvents are absorbed by a charge of freshly cut shavings of paraffin wax; since the sample may contain traces of moisture, it is advisable to insert also a dish containing a suitable desiccant **(Furniss et al, 1989).**

1.2.4.5 CHARACTERIZATION OF ORGANIC COMPOUNDS

The organic chemist is frequently faced with the problem of characterizing and ultimately elucidating the structure of unknown organic compounds. The worker in the field of natural products, for example, has the prospect of isolating such compounds from their sources in a pure state and then of determining their structures. The elucidation of the structure requires the identification of the molecular framework, the nature of the functional groups which are present and their location within the skeletal structure, and finally the establishment of any stereochemical relationships which might exist.

Preliminary investigations should be carefully carried out, particularly the study of solubility. Accurate classification of a compound as acidic, basic or neutral greatly assists the subsequent search to identify the characteristic functional group or groups present. This involves a careful consideration of the results of selected classifying chemical tests in conjunction with the recognition of adsorptions of diagnostic value in the appropriate spectra.

Having established the functionality, the spectroscopic information may reveal information of skeletal features, and the compound may then be converted into an appropriate solid

derivative, which if known, may confirm the identity of the compound, or provide further compounds on which reactivity and spectroscopic studies could be relevant.

Derivative preparation involves the application of concisely described general procedure to a specific compound, and its success may well depend upon the use of intuition and initiative in the preparative and purification stages.

1.2.4.6 PHYSICAL CONSTANTS

The most widely used physical constants in the characterisation of organic compounds are melting and boiling points **(Furniss et al, 1989)**. In general, a sharp melting point (say, within 0.5ºC) is a characteristic property of a pure organic compound. The purity should not, however, be assumed but must be established by observation of any changes in the melting point (or range) when the compound is subjected to purification by recrystallisation. If the melting point is unaffected by at least one recrystallisation, the purity of the substance may be regarded as established. Confirmation of purity may be obtained by thin layer chromatography.

1.2.4.7 CHROMATOGRAPHIC TECHNIQUES

Chromatography is essential to phytochemistry and is the key to obtaining pure compounds for structure elucidation, for pharmacological testing or for development into therapeuticals **(Marston, 2007)**. It also plays a fundamental role as an analytical technique for quality control and standardisation of phytotherapeuticals. A powerful separation method must be able to resolve mixtures with a large number of similar analytes. **(Meyer V. R., 2004)**

Table 1-2: The major chromatographic methods used in phytochemistry (Moffat et al, 1986)

Chromatographic method	Separation mechanism
Liquid–solid chromatography	Adsorption
Paper	(Adsorption), partition

Chromatographic method	Separation mechanism
Gas liquid chromatography (GLC)	Adsorption, partition
Thin-layer chromatography (TLC)	Adsorption, partition
High-performance liquid chromatography (HPLC)	Adsorption, partition
Ultra-performance liquid chromatography (UPLC)	Adsorption, partition
Supercritical fluid chromatography (SFC)	Adsorption, partition
Liquid–liquid chromatography (LLC)	Partition
Countercurrent chromatography (CCC)	Partition
Ion-exchange chromatography (IEC)	Ion exchange
Capillary electrophoresis	Charge
Ion-pair chromatography	Ion pair formation, ion interaction
Hydrophobic interaction chromatography (HIC)	(Adsorption), partition
Size exclusion chromatography (SEC)	Size of analyte
Affinity chromatography	Biological affinity

1.2.4.7.1 THIN LAYER CHROMATOGRAPHY

Thin-layer chromatography is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, metal or plastic. Solutions of analytes are deposited on the plate prior to development. The separation is based on adsorption, partition, ion-exchange or on combinations of these

mechanisms and is carried out by migration (development) of solutes (solutions of analytes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase) **(British Pharmacopoeia 2005).**

In outline the method consists of preparing, on a suitable glass plate, a thin layer of material, the sorbent, which may be either an adsorbent as used in column adsorption chromatography or an inert support which holds an aqueous phase as in column chromatography. The mixtures to be resolved are dissolved in a suitable solvent and placed as a series of spots on the film towards one end of the plate; this end is then dipped in a suitable solvent mixture and the whole enclosed in an airtight container. The solvent front is marked, the solvent is allowed to evaporate and the positions of the separated compounds are determined by suitable means. **(Evans C. W., 1989)**

1.2.4.7.1.1 STATIONARY PHASES

Silica gel is the most important stationary phase for TLC, with other inorganic oxide adsorbents, such as alumina, kieselguhr (a silica gel of low surface area) and Florisil (a synthetic magnesium silicate), of minor importance. For silica gel, silanol groups are the dominant adsorption sites.

1.2.4.7.1.2 LAYER PRETREATMENTS

Prior to chromatography, it is common practice to prepare the layers for use by any or all of the following steps: washing, conditioning and equilibration. Newly consigned precoated layers are invariably contaminated. To remove contaminants, single or double immersion in a polar solvent, such as methanol or propan–2–ol, for about 5 min is generally superior to predevelopment with the mobile phase.

Physically adsorbed water can be removed from silica gel layers by heating at about 120° for 30 min. Afterwards, the plates are stored in a grease–free desiccator over blue silica gel. Heat activation is not normally required for chemically bonded layers.

1.2.4.7.1.3 DEVELOPMENT

The principal development techniques in TLC are linear, circular and anticircular, with the velocity of the mobile phase controlled by capillary forces or forced–flow conditions. In any of these modes continuous or multiple development can be used to extend the application range.

For linear (or normal) development, samples are applied along one edge of the plate and the separation developed for a fixed distance in the direction of the opposite edge.

In continuous development the mobile phase is allowed to traverse the layer under the influence of capillary forces until it reaches some predetermined position on the plate, at which point it is evaporated continuously.

In unidimensional multiple development, the TLC plate is developed for some selected distance, then either the layer or the mobile phase is withdrawn from the developing chamber, and adsorbed solvent evaporated from the layer before repeating the development process. Multiple development provides a very versatile strategy for separating complex mixtures, since the primary experimental variables of development distance and composition of the mobile phase can be changed at any development step, and the number of steps varied to obtain the desired separation. Multiple development provides a higher resolution of complex mixtures than does normal or continuous development, can easily handle samples of a wide polarity range

For drug mixtures that span a wide retention range, some form of gradient development is required to separate all the components either in a single chromatogram or in separate chromatograms for successive developments.

In two–dimensional TLC, the sample is spotted at the corner of the layer and developed along one edge of the plate. The solvent is then evaporated, the plate rotated through 90° and redeveloped in the orthogonal direction. Using two solvent systems with complementary selectivity is the simplest approach to implement in practice, but it is often only partially successful.

1.2.4.7.1.4 DETECTION

About 1 to 10 μg of coloured substances with a quantitative reproducibility rarely better than 10–30% can be detected by visual inspection of a TLC plate. This may be adequate for qualitative methods, but for reliable quantification, in situ spectrophotometric methods are preferred, as they are more accurate and far less tedious and time consuming than excising zones from the layer for determination by conventional solution spectrophotometry. The fluorescence–quenching technique enables visualisation of UV-absorbing drugs on TLC plates that incorporate a fluorescent indicator. The zones of UV-absorbing substance appear dark against the brightly fluorescing background of a lighter colour when the plate is exposed to UV light of short wavelength. The method is not universal, since it requires overlap between the absorption bands of the indicator ($\gamma_{max} \approx 280$ nm with virtually no absorption below 240 nm) and the drug, but in favourable cases, it is a valuable and non–destructive method for zone location.

1.2.4.7.1.5 PREPARATIVE THIN–LAYER CHROMATOGRAPHY

Preparative TLC is used mainly to purify drugs or to isolate drug metabolites and impurities in amounts of about 1 to 100 mg for subsequent use as reference materials, structural elucidation, biological activity evaluation and other purposes. Scale up from analytical TLC is achieved by increasing the thickness of the layer (loading capacity increases with the square root of the layer thickness) and by increasing the plate length used for sample application.

Sample application is a critical step in preparative TLC, and if performed improperly can destroy all or part of the separation. The sample, usually as a 5 to 10 % (w/v) solution in a volatile solvent, is applied as a band along one edge of the layer to give a maximum sample load of about 5 mg/cm for each millimetre of layer thickness. Sample loads are usually lower for difficult separations and for cellulose and chemically bonded layers. A short predevelopment, of about 1 cm with a strong solvent, is often useful to refocus manually applied bands. In all cases, it is important that the sample solvent is evaporated fully from the layer prior to the start of the separation to avoid the formation of distorted separation zones. It is usual to leave a blank margin of 2 to 3 cm at each vertical edge of the layer to avoid uneven development.

After development, physical methods of zone detection are used to identify the sample bands of interest. Layers that contain a UV indicator for fluorescence quenching or the adsorption of iodine vapours are useful for this purpose. If a reactive spray reagent is used for visualisation, it should be sprayed on a small strip of the chromatogram only, so as not to contaminate the remainder of the material. Once the bands of interest are located, the zones are scraped off the plate carefully with a spatula or similar tool. A number of devices based on the vacuum–suction principle for removing the marked zones from the plate are available also. Soxhlet extraction, liquid extraction or solvent elution with a polar solvent is used to recover drugs from the sorbent. For solvent extraction, water is often added to dampen the silica gel prior to extraction with a water–immiscible organic solvent. Chloroform and ethanol (methanol is less suitable because of its higher silica solubility) are widely used for solvent elution. Colloidal silica can be removed by membrane filtration prior to vacuum stripping of the solvent. **(Moffat et al, 1986)**

1.2.4.7.1.6 Rf VALUES

The distance the material moves from the place where it was spotted is compared to the distance the solvent moves from the point where the sample was spotted. One can usually visually observe how high the solvent rises (the solvent front). The procedure is stopped before the solvent reaches the top of the plate/sheet. The ratio of how far a component in the sample moves divided by the distance the solvent moves is called the retention factor, or Rf value. The Rf value will be a specific value under the conditions of the test for each component in the sample. **(Bloch, 2006)**

1.2.4.7.2 LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a method of chromatographic separation based on the difference in the distribution of species between two non-miscible phases, in which the mobile phase is a liquid, which percolates through a stationary phase contained in a column. **(British Pharmacopoeia 2007)**

Liquid chromatography (LC) was the first type of chromatography to be discovered and, in the form of liquid-solid chromatography (LSC), was originally used in the late 1890s by the Russian botanist, Tswett, to separate and isolate various plant pigments. The coloured bands

he produced on the adsorbent bed evoked the term chromatography (colour writing) for this type of separation. Initially the work of Tswett was not generally accepted, partly due to the original paper being in Russian and thus, at that time, was not readily available to the majority of western chemists and partly due to the condemnation of the method by Willstatter and Stoll in 1913. Willstatter and Stoll repeated Tswett's experiments without heeding his warning not to use too "aggressive" adsorbents as these would cause the chlorophylls to decompose. As a consequence, the experiments of Willstatter et al. failed and their published results, rejecting the work of Tswett, impeded the recognition of chromatography as a useful separation technique for nearly 20 years. **(Scott R. P. W., 2003)**

In the late 1930s and early 1940s Martin and Synge introduced a form of liquid-liquid chromatography by supporting the stationary phase, in this case water, on silica gel in the form of a packed bed and used it to separate some acetyl amino acids. In 1941, Martin and Synge suggested the use of small particles and high pressures in liquid chromatography to improve the separation that proved to the critical factors that initiated the development of high performance liquid chromatography **(Scott R. P. W., 2003).**

The ability to separate and analyze complex samples is integral to the biological and medical sciences. Classic column chromatography has evolved over the years, with chromatographic innovations introduced at roughly decade intervals. A technique was needed which could separate water-soluble, thermally-labile, non-volatile compounds with speed, precision and high resolution Modern HPLC techniques became available in 1969; however, they were not widely accepted in the pharmaceutical industry until several years later.

It is presently used in pharmaceutical research and development:

- To purify synthetic or natural products.
- To characterise metabolites.
- To assay active ingredients, impurities, degradation products and in dissolution assays.
- In pharmacodynamic and pharmacokinetic studies.

Improvements made in HPLC in recent years include:

- Changes in packing material, such as smaller particle size, new packing and column materials.
- High–speed separation.

- Micro-HPLC, automation and computer–assisted optimisation.
- Improvements in detection methods, including the so–called hyphenated detection systems. **(Moffat et al, 1986)**

1.2.4.7.3 CHROMATOGRAPHIC PRINCIPLES

The *retention* of a drug with a given packing material and eluent can be expressed as a retention time or retention volume, but both of these are dependent on flow rate, column length and column diameter. The retention is best described as a column capacity ratio (*k*), which is independent of these factors. The column capacity ratio of a compound (A) is defined by equation (1):

$$k_A = \frac{V_A - V_0}{V_0} = \frac{t_A - t_0}{t_0} \quad (1)$$

where *V*A is the elution volume of A and *V*0 is the elution volume of a non–retained compound(i.e. void volume).

At constant flow rate, retention times (*t*A and *t*0) can be used instead of retention volumes. The injection of a solvent or salt solution can be used to measure *V*0, but the solute used should always be recorded along with reported *k* data. It is sometimes convenient to express retention data relative to a known internal standard (B). The ratio of retention times (*t*A/*t*B) can be used, but the ratio of adjusted retention times, (*t*A –*t*0)/(*t*B – *t*0), is better when data need to be transferred between different chromatographs.

Resolution is the parameter that describes the separation power of the complete chromatographic system relative to the particular components of the mixture. By convention, resolution (*R*) is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line, equation (2):

$$R = \frac{2\left(V_{R,2} - V_{R\cdot1}\right)}{W_1 + W_2} \quad (2)$$

If we approximate peaks by symmetric triangles, then if *R* is equal to or more than 1, the components are completely separated. If *R* is less than 1, the components overlap.

Sensitivity in chromatographic analysis is a measure of the smallest detectable level of a component in a chromatographic separation and is dependent on the signal–to–noise ratio in a given detector. Sensitivity can be increased by derivatisation of the compound of interest, optimisation of chromatographic system or miniaturisation of the system. The limit of

detection is normally taken as three times the signal–to–noise ratio and the limit of quantification as ten times this ratio. **(Moffat et al, 1986)**

1.2.4.7.4 CHROMATOGRAPHIC MECHANISMS

The systems used in chromatography are often described as belonging to one of four mechanistic types: adsorption, partition, ion exchange and size exclusion. *Adsorption chromatography* arises from interactions between solutes and the surface of the solid stationary phase. Generally, the eluents used for adsorption chromatography are less polar than the stationary phases and such systems are described as 'normal phase'. *Partition chromatography* involves a liquid stationary phase that is immiscible with the eluent and coated on an inert support. Partition systems can be normal phase (stationary phase more polar than eluent) or reversed–phase chromatography, referred to as RPC (stationary phase less polar than eluent). *Ion–exchange chromatography* involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. *Size–exclusion chromatography* involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, with the large molecules unable to enter the pores elute first. However, this concept of four separation modes is an over–simplification. In reality, there are no distinct boundaries and several different mechanisms often operate simultaneously. Other types of chromatographic separation have been described. *Ion–pair chromatography* is an alternative to ion–exchange chromatography. It involves the addition of an organic ionic substance to the mobile phase, which forms an ion pair with the sample component of opposite charge. This allows a reversed–phase system to be used to separate ionic compounds. *Chiral chromatography* is a method used to separate enantiomers, which can be achieved by various means. In one case, the mobile phase is chiral and the stationary phase is non–chiral. In another, the liquid stationary phase is chiral with the mobile phase non–chiral or, finally, the solid stationary phase may be chiral with a non–chiral mobile phase. **(Moffat et al, 1986)**

1.2.4.7.5 INSTRUMENTATION

HPLC instrumentation basically includes a pump, injector, column, detector and recorder or data system. The heart of the system is the column in which separation occurs. Since the stationary phase is composed of micrometer–size porous particles, a high–pressure pump is required to move the mobile phase through the column. The chromatographic process begins

by injecting the solute onto the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column and is registered as a peak on the recorder. Detection of the eluting components is important; this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyse the chromatographic data, computers, integrators and other data–processing equipment are used frequently.

1.2.4.7.6 HYPHENATED TECHNIQUES

The recent development of the so–called hyphenated techniques has improved the ability to separate and identify multiple entities within a mixture. These techniques include HPLC–MS, HPLC–MS–MS, HPLC–IR and HPLC–NMR. These techniques usually involve chromatographic separation followed by peak identification with a traditional detector such as UV, combined with further identification of the compound with the MS, IR or NMR spectrometer. MS as a detector for an HPLC system has gained wide popularity over the past several years. Advances in data systems and the simplification of the user interface have facilitated the ease of use of a mass spectrometer as an HPLC detector. The most common types of mass spectrometers used in HPLC are quadrupoles and ion traps. Tandem mass spectrometers (also called triple quadrupoles) are also commonly available and are widely used in the pharmaceutical industry for the quantitative analysis of trace concentrations of drug molecules. The process of mass analysis is essentially the same as in any other mass spectrometric analyses that utilise quadrupole or ion–trap technology. The unique challenge to interfacing an HPLC to a mass spectrometer is the need to convert a liquid–phase eluent into a gas phase suitable for mass spectral analysis. Modern mass spectrometers commonly utilise a technique known as atmospheric pressure ionisation (API) to accomplish this.

HPLC–MS–MS is commonly used in the pharmaceutical industry and in forensic science to analyse trace concentrations of drug and/or metabolite. MS–MS offers the advantage of increased signal–to–noise ratio, which in turn lowers the limits of detection and quantification easily into the sub ng/mL range.

MS–MS is also a very useful technique in the qualitative identification of previously unidentified metabolites of drugs, which thus makes MS–MS a very powerful technique in research laboratories.
Several recently published studies have utilised MS–MS as a high–throughput analytical technique in the pharmaceutical industry.

HPLC–IR has proved to be an effective method to detect degradation products in pharmaceuticals. IR provides spectral information that can be used for compound identification or structural analysis. The IR spectra obtained after HPLC separation and IR analysis can be compared to the thousands of spectra available in spectral libraries to identify compounds, metabolites and degradation products. An advantage of IR spectroscopy is its ability to identify different isomeric forms of a compound based on the different spectra that result from alternative locations of a functional group on the compound. Unlike MS, IR is a non–destructive technique in which the original compound is deposited on a plate as pure, dry crystals and can be collected afterwards if desired.

HPLC–NMR is also growing in popularity for the identification of various components in natural products and other disciplines. Although a relatively new hyphenated system, HPLC–NMR has several applications on the horizon. The miniaturisation of the system and the possibility of measuring picomole amounts of material are both areas currently attracting a large amount of attention. Also, in the future HPLC–NMR systems will be interfaced with other detectors, such as Fourier transform IR and mass spectrometers. This will provide a wide range of possibilities for further applications, which could include the analysis of mixtures of polymer additives and the ability to identify unknowns without first having to isolate them in a pure form. **(Moffat et al, 1986)**

1.2.4.8 SPECTROPHOTOMETRY

Spectroscopy is the study of the interaction of electromagnetic radiation with matter. Electromagnetic radiation makes up the electromagnetic spectrum. The electromagnetic spectrum is subdivided into regions: radio waves, microwaves, infrared (IR) radiation, visible light, ultraviolet (UV) radiation, x-rays, and gamma rays. Electromagnetic radiation can be described as a wave travelling at the speed of light. The wave can be characterized by a wavelength and a frequency. A good analogy is a series of water waves. The wavelength λ is

the distance between crests of the wave. The frequency ν, is the number of crests that pass a given point in one second or the number of times a cork floating on water bobs up and down per second. Frequency is expressed in hertz which has units of 1/sec or sec^{-1}. Frequency times wavelength equals a constant, the speed of light ($\nu\lambda$ = constant). Frequency and wavelength are inversely proportional. If the frequency increases, the wavelength decreases. Electromagnetic radiation can also be described as a stream of photons (a massless packet of energy). The energy of the photons is related to the frequency of radiation by the equation $E = h\nu = h(c/\lambda)$, where h is a constant. High energies are associated with high frequencies and short wavelengths. Low energies are associated with low frequencies and long wavelengths.

Molecules are capable of absorbing radiation in the various regions of the electromagnetic spectrum. The energy of the absorbed radiation has different effects on molecules. X-rays have sufficient energy to cause ionization (loss of electrons), UV and visible radiation promote electrons into higher energy levels (excited states), IR radiation excites vibrational modes, and microwave radiation causes the entire molecule to tumble/rotate. **(Bloch, 2006)**

1.2.4.8.1 INFRARED SPECTROSCOPY

Infrared spectroscopy measures the interaction (absorption) of infrared radiation with molecules. Bonds between atoms are usually given as specific lengths, implying rigid bonds between atoms. The bond between two atoms is also described as having the properties of a spring. The bond (spring) vibrates and the average distance between the two connected atoms (in their lowest energy ground state) is given as the bond length. The bond vibrates with a frequency that is characteristic of that specific bond. A C-H bond, a C-C single bond, and a C=C double bond have different vibrational frequencies. Electromagnetic radiation is also described in terms of a frequency. Molecules can absorb electromagnetic radiation if the frequency of the bond vibration matches the frequency of the electromagnetic radiation. Bonds vibrate in the IR frequency range and thus absorb IR radiation. **(Bloch, 2006)**

1.2.4.8.1.1 ENERGY RANGES

Most molecules absorb IR radiation in the wavelength range of 2.5 × 10–4 to 25 × 10–4 cm, which is a small segment of the IR radiation range (8 × 10–5 to 1 × 10–2 cm). This small segment corresponds to a frequency range of 12 × 1013 to 1.2 × 1013 sec–1 and an energy

range of 4.6 to 46 kJ/mol (1.1 to 11 kcal/mol). This energy range is much smaller than the energy required to break a typical single bond (about 400 kJ/mol or 100 kcal/mol) so the absorption of IR radiation does not cause bonds to break.

1.2.4.8.1.2 WAVENUMBERS

Radiation in the IR spectroscopy range is usually expressed in units of wavenumbers υ. A wavenumber usually is a frequency measurement and is equivalent to the reciprocal of the wavelength in centimeters: $\upsilon = 1/\lambda$ cm. The wavenumber range of IR spectra is from 400 to 4000 cm–1. Larger values of wavenumbers represent higher energies.

1.2.4.8.1.3 MOLECULAR VIBRATIONS

Molecules undergo two types of vibrations: stretching and bending. More energy is required for a stretching vibration than for a bending vibration. When a stretching vibration absorbs IR radiation, the amplitude of the vibration changes but the frequency of the vibration does not change. The spring stretches twice as far (the amplitude increases) but undergoes the same number of stretches-compressions per second (the frequency) as it did before absorbing the radiation. A bending vibration involves at least three atoms. This is analogous to a symmetric bending (scissoring) vibration.

1.2.4.8.1.4 SAMPLE PREPARATION

The sample to be tested can be a gas, a liquid, or a solid. The sample is put in a cell or between plates of a material that does not absorb IR radiation in the 4000 to 400 cm^{-1} wavenumber range. Cells or plates are made from salts like NaCl, NaBr, and AgCl. Window glass and plastic materials absorb IR radiation in the wavenumber range of interest, and their absorptions would appear on the spectra with those of the sample. Therefore, these materials are not used as cells or plate. Solid samples can be mixed with powdered salt (e.g. NaBr) and pressed into an optically clear pellet. A solid sample can also be ground in mineral oil to give a mull. Mineral oil contains C-H and C-C bonds that absorb IR radiation in the IR spectroscopy range and obscure these absorptions in the sample. The sample is placed in the instrument such that the IR radiation from the radiation source passes through the sample.

1.2.4.8.1.5 ABSORPTION REQUIREMENTS

A molecule will absorb IR radiation if a polar bond in that molecule is undergoing a vibration, and the vibration causes a periodic change in the dipole moment of that bond.
A polar bond can be thought of as having a positive end and a negative end. Electromagnetic radiation can also be thought of as an alternating positive and negative charge (the crest and trough of a wave). When the negative end of the molecular dipole interacts with the positive charge of the electromagnetic radiation it stretches the bond and when the negative charge of the electromagnetic radiation interacts with the negative end of the dipole it compresses the bond. Thus the interaction (absorption) of radiation causes the length (amplitude) of the bond (spring) to increase or decrease. Bonds that are nonpolar do not absorb IR radiation. The nonpolar triple bond in acetylene (HC≡CH) does not show IR absorption.

1.2.4.8.1.6 FINGERPRINT REGION

An IR spectrum is divided into two regions. The region from 400 to about 1400 cm^{-1} is called the fingerprint region. Most of the bending vibrations and some stretching vibrations occur in this range. Nonlinear molecules with *n* atoms have 3n− 6 fundamental vibrational modes. Isobutane (C_4H_{10}), a relatively simple molecule, potentially has 3(14) − 6 = 36 vibrational modes (some modes may be infrared inactive). The region from 400 to 1400 cm^{-1} tends to have many absorption bands. Many of these bands overlap and it is often very difficult to identify specific absorptions. This region is used for matching the spectra of unknown compounds with the spectra of known compounds, as one matches fingerprints; hence the name fingerprint region. The region from 1400 to 4000 cm^{-1} has fewer absorption bands and it is relatively easy to correlate specific absorptions with functional groups. **(Bloch, 2006)**

Table 1-3: Characteristic IR absorptions (Bloch, 2006)

Bond of functional group	Absorption range, cm^{-1}	Comments
O – H stretch, alcohol	3200 – 3600 3590 – 3600	m to s, H – bonding s, no H – bonding
O - H, stretch, carboxylic acid	2500–3500	B
Alkane	2800–3000	m to s
Alkene C – H	3000–3100	M
Alkyne C – H	3000	M
C ≡ N, stretch, nitrile	2210–2260	m to w
C ≡ C, stretch, alkyne	2100–2260	w or absent
Ester	1730–1755	S
Aldehyde	1720–1735	S
Ketone	1700–1725	S
Acid	1680–1725	B
Amide	1620–1690	S
Alkene	1600–1680	w or absent
Benzene	1400–1500, 1585–1600	M
C = O, stretch, ether, ester, alcohol	1200–1250	S
N - H, stretch, amine	1180–1360	S
C - H, bend alkane, methylene methyl	 1465 1350–1475, two bands	 w w

1.2.4.8.2 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance (NMR) spectroscopy is one of the most useful tools for determining the structure of organic molecules. It gives information about the number, type, and connectivity of hydrogen and carbon atoms. The technique involves the interaction (resonance) of energy from an external energy source with the magnetic properties of a nucleus. This does not involve a nuclear reaction or a chemical reaction. The material being tested can be recovered unchanged.

1.2.4.8.2.1 NUCLEAR PROPERTIES

Nuclei are charged spinning particles that, like electrons, are considered to have spin states. Charged particles that spin generate a magnetic field. The magnetic field acts like a small bar magnet. The needle of a compass is a small bar magnet that can align with or against an external magnetic field. The needle aligns with the earth's magnetic field and points north. If we want the needle to point south, a small amount of energy would be needed to hold the needle in the opposite direction. The needle will return to pointing north (its ground state)

when it is free to move. The needle would point in random directions if the earth's magnetic field did not exist.

1.2.4.8.2.2 SPIN STATES

Nuclei are very much like a compass needle. Their magnetic component orients in random directions in the absence of a strong external magnetic field. In the presence of a strong external magnetic field, the little magnet (the nuclear spin) can orient with or against the external magnetic field. The lowest energy state exists when the nuclear spin orients with the external field. If the nucleus absorbs the appropriate amount of energy, the magnetic component can align against the external field, like the compass needle forced to point south. The magnetic component of a spin state oriented with an external magnetic field is called the alpha, α, spin state. The beta, β, spin state exists when the magnetic component is oriented against an external magnetic field.

1.2.4.8.2.3 SAMPLE PREPARATION

The sample to be tested is put in a thin glass tube, about the size of a pencil, and placed inside the coils (that supply radiowaves) in the core of the magnet. Only a few milligrams of sample are required. About 0.5 mL of solvent is used. The solvent used does not contain atoms (nuclei) that absorb radiowave energy in the range that protons absorb radiation. Solvents used include carbon tetrachloride (CCl_4), deuterated chloroform ($CDCl_3$), and deuterated water (D_2O). Deuterium (D or ^2H), an isotope of hydrogen, does not absorb radiation in the same range as does normal ^1H.

1.2.4.8.2.4 ^1H NMR SPECTROSCOPY

^1H NMR spectroscopy is referred to as proton NMR spectroscopy. A proton, by definition, is a hydrogen atom without an electron, H^+. Proton NMR is a study of hydrogen atoms covalently bonded to another atom, usually carbon, and it is not the study of free protons. However, NMR discussions use the terms proton and hydrogen atom interchangeably. For NMR to be of value as a diagnostic tool, hydrogen atoms (protons) in different chemical environments need to absorb (resonate) radiowaves at different frequencies. The nucleus of a hydrogen atom is surrounded by an electron cloud (the electron density) of its bonding electrons. The external magnetic field produces a force (torque) on the electrons, causing

them to generate a magnetic field, *H*local, that opposes the external magnetic field, *H*0. The electron density around a hydrogen nucleus is different for protons that exist in different chemical environments. Since the electron density varies, the magnetic field felt by a hydrogen nucleus (*H*eff = *H*0 – *H*local) also varies. If the magnetic field felt by the nucleus changes, the frequency at which radiation is absorbed also changes. The frequency at which radiation is absorbed gives information about the chemical environment of the protons responsible for those absorptions.

1.2.4.8.2.5 EQUIVALENT AND NONEQUIVALENT PROTONS

Protons in different chemical environments are called non-equivalent protons. They absorb radiation at slightly different frequencies measured in megahertz, MHz, values. It is more convenient to express these frequency differences relative to some standard. The standard chosen in most cases is tetramethylsilane, $(CH_3)_4Si$ (TMS). All the protons in TMS exist in an identical chemical environment and are called equivalent protons. They all absorb radiation at the same frequency. The difference in absorption frequencies from this standard is in hertz (not megahertz) values.

1.2.4.8.2.6 CHEMICAL SHIFTS

The horizontal scale of an ^1H NMR spectrum goes from 0 to 10 (right to left). The scale is in parts per million (ppm) or delta (δ) units. One δ unit equals 1 ppm. Non-equivalent protons absorb radiowave radiation at slightly different hertz values. TMS is arbitrarily given a value of 0 on the ppm (or δ) scale and other absorptions are relative to TMS. Most protons absorb at values to the left (0 to 10) of TMS on this scale. The vertical lines represent proton absorptions. The difference between the absorption of a proton and TMS is called a chemical shift. If a chemical shift occurs at 5 ppm, it is said to be downfield relative to TMS and TMS absorbs upfield relative to the absorption at 5 ppm. The terms upfield and downfield are commonly used in describing spectra.

1.2.4.8.2.7 MULTIPLETS

An absorption split into two or more peaks is called a multiplet. Two peaks are called a doublet, three peaks are called a triplet, four peaks are called a quartet, five peaks are called a quintet, and so on. A single peak is called a singlet. At room temperature, a proton exists in

its and β spin states. Each of these spin states generates a small magnetic field. One of these small magnetic fields aligns with the external magnetic field, increasing the total magnetic field strength. The other spin state generates a magnetic field that aligns against the external magnetic field, decreasing the total magnetic field strength. The net result is two slightly different magnetic field strengths are generated in the vicinity of adjacent protons. The magnetic field strength generated by a proton is much smaller than the external magnetic field strength and thus the two resulting net magnetic fields are only slightly different **(Bloch, 2006).**

1.2.4.8.2.8 SIGNAL SPLITTING: SPIN-SPIN COUPLING

Signal splitting is caused by the effect of magnetic fields of protons on nearby atoms. Signal splitting arises from a phenomenon known as spin-spin coupling. Spin-spin coupling effects are transferred primarily through the bonding electrons and are not usually observed if the coupled protons are separated by more than three sigma bonds. Signal splitting is not observed for protons that are chemically equivalent (homotopic) or enantiotopic. That is, signal splitting do not occur between protons that have exactly the same chemical shift **(Solomons, 1996).**

1.2.4.8.2.9 ^{13}C NMR SPECTROSCOPY

Further identification of a compound is obtained by analyzing a ^{13}C spectrum in addition to a ^{1}H spectrum. There are several differences between ^{13}C and ^{1}H NMR spectroscopy. The energy for spin flipping (α to β transition) occurs at a different frequency (energy) range and therefore, ^{13}C absorptions do not appear in ^{1}H spectra. Chemical shifts for ^{1}H occur primarily in a 0–10 ppm range. The range for ^{13}C spectra is about 0 to 220 ppm, which is an advantage as the ^{13}C absorptions are much further apart than ^{1}H absorptions, and there is less chance of non-equivalent peaks overlapping **(Bloch, 2006).**

1.2.4.8.2.10 FT-NMR

Almost all hydrogen atoms consist of the isotope ^{1}H. About 99% of all carbon atoms are ^{12}C and only 1% is ^{13}C atoms. Thus a ^{13}C signal is much weaker than a ^{1}H signal in a given sample. The spectrometer also records random, anomalous signals called noise. In a single scan, it is difficult to identify the desired ^{13}C signal from the noise. The advantage of FT-

NMR is that each scan takes only a few seconds, and many scans of the same sample can be taken in a few minutes. A computer stores the information from each scan and adds the scans. Since the noise is random (+ noise and − noise) it averages to about zero, giving a relatively flat baseline in the spectrum. The signals from ^{13}C are not random (all are positive values) and they are additive for each scan. The final result of many scans and computer averaging gives a spectrum with useful information about the sample. The chemical shifts for carbon atoms follow the same general pattern discussed for ^{1}H NMR spectroscopy. Electron-withdrawing atoms or groups adjacent to the ^{13}C atom of interest cause downfield shifts and electron-donating atoms or groups cause upfield shifts. Since there is such low percentage of ^{13}C atoms, the chance of two ^{13}C atoms being bonded to each other is very low and spin-spin coupling between adjacent ^{13}C atoms is not observed. Spin-spin coupling is however observed between ^{13}C and ^{1}H atoms bonded directly to each other. Weak coupling is observed between ^{13}C and ^{1}H atoms separated by two or three σ bonds. This is called long-range coupling. This coupling results in a very complex splitting pattern.

1.2.4.8.2.11 DECOUPLING

A complex splitting pattern can be simplified by spin-spin decoupling. A technique called broadband decoupling eliminates all hydrogen-carbon spin-spin coupling interactions. When a spectrum is decoupled, the multiplet is collapsed into a single peak, enhancing the signal-to-noise ratio. It is more difficult to integrate the areas of ^{13}C peaks but newer NMR spectrometers use techniques that give the relative number of carbon atoms in each peak as is done in proton NMR.

1.2.4.8.2.12 OFF-RESONANCE DECOUPLING

Selective spin-spin decoupling can be obtained so that coupling occurs only between carbon and hydrogen atoms bonded directly to each other. Eliminating long-range coupling (more than one σ bond away) between carbon and hydrogen atoms is called off-resonance decoupling. Carbon atoms now show splitting patterns conforming to the (n + 1) rule, where n is the number of hydrogen atoms bonded to that carbon atom when off-resonance coupling is employed.

1.2.4.8.2.13 DEPT

Another technique for analyzing ^{13}C spectra is called DEPT (distortionless enhancement by polarization transfer). In this process, the spectrometer varies the signal sent to the sample. Carbon atoms that are not bonded to any hydrogen atoms (quaternary carbon atoms and carbonyl, C=O, carbon atoms) do not appear in DEPT spectra. One can compare different spectra to completely identify primary, secondary, tertiary, and quaternary carbon atoms **(Bloch, 2006).**

1.2.4.8.3 MASS SPECTROMETRY

Mass spectrometry (MS) is a method for determining the molecular weight of a compound. It also gives information about chemical structure. It is used with other characterization techniques to identify the total structure of a compound.

1.2.4.8.3.1 THE MASS SPECTROMETER

The instrument used is called a mass spectrometer. The unit consists of three parts: a chamber where the sample is introduced and ionized, a long tube that directs the path of the ions, and a detection chamber. The sample is heated, if necessary, to put the sample in the vapour (gaseous) state. The spectrometer is run under a high vacuum to help vaporize the sample and remove extraneous gases. A stream of high-energy electrons, from an external source, is directed at the sample in the chamber. These electrons have energies of about 7600 kJ/mol. This corresponds to a wavelength in the far UV/X-ray region of the electromagnetic spectrum. When one of these electrons strikes a molecule of the sample being tested, it knocks an electron out of the molecule. The high-energy electron also bounces off the sample. It takes about 200 to 400 kJ/mol to knock an electron out of a molecule, and the high-energy electron (from the external source) has plenty of energy to knock an electron out of a compound like pentane resulting in a high-energy cation. The resulting high-energy cation is called a *molecular ion* **(Bloch, 2006).**

1.2.4.9 STABILITY TESTING OF DRUGS

A wide range of environmental factors such as temperature, light, and oxygen may cause drug deterioration. **(Florence and Attwood, 1988)** The effect of any particular factor on the drug

breakdown under conditions of normal storage may be evaluated from a number of experiments in which this factor is greatly exaggerated. Such testing methods, referred to as accelerated storage tests, have for many years proved an effective replacement at the development stage, for the original time-consuming practice of storing the product at room temperature for periods corresponding to the normal time the products would likely remain in stock.

1.2.4.9.1 ACCELERATED STABILITY TESTING

Instabilities in modern formulations are often detectable only after considerable storage periods under normal conditions. To assess the stability of a formulated product it is usual to expose it to high stress conditions to enhance its deterioration and therefore reduce the time required for the testing. This enables more data to be gathered in a shorter time which, in turn, will allow unsatisfactory formulations to be eliminated early within a study and will also reduce the time for a successful product to reach the market. It must be emphasized that extrapolations to 'normal' storage conditions must be made with care and that the formulator must be sure that such extrapolations are valid. It is advisable therefore to run concurrently a batch under expected normal conditions to confirm later that these assumptions are valid.

The objectives of such accelerated tests may be defined as

- the rapid detection of deterioration in different initial formulations of the same product – this is of use in selecting the best formulation from a series of possible choices;
- the prediction of shelf-life
- the provision of a rapid means of quality control, which ensures that no unexpected change has occurred in a stored product.

All these objectives are based on obtaining a more rapid rate of decomposition by applying to the product a storage condition that places a higher stress or challenge to it when compared with normal storage conditions. **(Aulton, 1988)**

1.2.4.9.2 COMMON CHALLENGES

1.2.4.9.2.1 TEMPERATURE

An increase in temperature causes an increase it the rate of chemical reactions. The products are therefore stored at temperatures greater than room temperature. The nature of the products often determines the range covered in the accelerated test. Samples are removed at various time intervals and the extent of decomposition is determined by analysis. Sensitive analytical methods should be used in all stability tests of this nature since small changes may be detected after very short storage periods. **(Aulton, 1988)**

Effect of temperature on stability

The effect of temperature on a rate constant, *k*, is indicated by the Arrhenius equation

$$k = Ae^{-EaRT}$$

$$\log k = \log A - \frac{E_a}{2.303RT}$$

From the Arrhenius equation a plot of log k versus 1/T should be linear .The rate constant at any selected storage temperature may thus be extrapolated from measurements at a series of elevated temperatures where the reaction proceeds at an accelerated rate. The time taken in conducting the stability tests is considerably reduced compared to that for a simple experiment in which the product is maintained at the required storage temperature and sampled over a period corresponding to the normal storage time **(Florence and Attwood, 1993).**

Figure 1.2: Graph showing the plot of the Arrhenius equation

An alternative method of data treatment is to plot the logarithm of the half- life, $t_{0.5,}$ as a function of reciprocal temperature since, from equation 4.10, $t_{0.5} = 0.693/k$.

Therefore $\log k = \log 0.693 - \log t_{0.5}$
And substituting into equation 1.12 gives
$\text{Log } t_{0.5} = \log 0.693 - \log A + Ea/ 2.303RT$ (1.3)

Once the rate constant is known at the required storage temperature, it is a simple matter to calculate a shelf – life for the product based on an acceptable degree of decomposition. The usual decomposition level is taken as 90 percent of the initial concentration of the drug, although this may vary, depending, for example, on whether the decomposition products produce discolouration or have undesirable side effects.

Although accelerated storage testing based on the use of the Arrhenius equation has resulted in a very significant saving of time, it still involves the time – consuming step of the initial determination of the order of the reaction for the decomposition. Whilst most investigators have emphasised the need for knowledge of the exact kinetic pathway of degradation, some have bypassed this initial step by assuming a particular decomposition model. Londi and Scott have indicated that at less than 10 per cent degradation and within the limits of experimental error involved in stability studies, it is not possible to distinguish between zero-, first- or simple second – order kinetics using curve – fitting techniques; consequently these authors have suggested that the assumption of first order kinetics for any decomposition reaction should involve minimum error. Even with the modification suggested above, the method of stability testing based on the Arrhenius equation is still time- consuming, involving as it does the separate determination of rate constants at a series of elevated temperatures. Experimental techniques have been developed which enable the decomposition rate to be determined from a single experiment. Such methods involve raising the temperature of the product in accordance with predetermined temperature-time programme and are consequently referred to as non – isothermal stability studies.

The advantages of this method over the conventional method of stability testing are that

(a) the data required to calculate the stability are obtained in a single one-day experiment, which may last for several weeks.

(b) no preliminary experiments are required to determine the optimum temperatures for the accelerated storage test, and

(c) the linearity of the plot of log f(c) against log(1 + t) confirms that the correct order of reaction has been assumed.

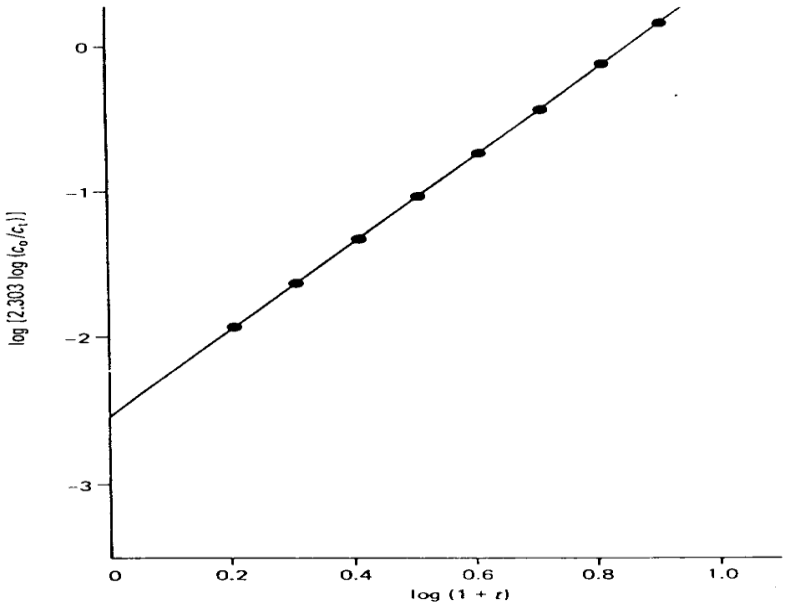

Figure 1.3: Accelerated Storage plot for the decomposition of a drug

CHAPTER TWO

EXPERIMENTAL

2.1 MATERIALS

- Buchi rotary evaporator
- Buchi recirculator chiller
- Buchi water bath
- Stuart Scientific Flask shaker, SF1
- Ceramic mortar and pestle
- Analytical Balance (Adams Instrument)
- HPLC Chromatograph- LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied biosystems),
- Integrator – Shimadzu CR501 chromatopac
- Phenomenex hypersil 20 micron C18 200 × 3.20 mm
- Melting point apparatus
- Precoated plates-silica gel Gf 254, 0.25mm Merck W. (Germany)

2.2 REAGENTS

- Methanol
- Ethylacetate
- Distilled water
- Petroleum ether
- Chloroform
- Ethanol (96%)
- Anisaldehyde

2.3 METHODOLOGY

2.3.1 EXTRACTION

Dried fruits of *Xylopia aethiopica* were bought from the market and authenticated by the department of Pharmacognosy, KNUST, Kumasi with a voucher number of FP/09/76. The dried fruits (1.085kg) were powdered and extracted with light petroleum ether (b. p. 40-60^{o}C) for 72 hours. The extract on concentration with a rotavapour at 60^{o}C deposited a green mass. The concentrated mass was allowed to stand for 48 hours to allow crystals to deposit with the addition of ethylacetate. The concentrate was decanted after the period to separate the deposited crystals from the upper oleo-resin-like mass.

2.3.2 RECRYSTALLIZATION OF THE CRUDE XYLOPIC ACID CRYSTALS

The crude xylopic acid crystals were transferred into a round bottomed flask of suitable size fitted with a reflux condenser. A quantity of distilled ethanol was added together with a few pieces of porous porcelain to prevent 'bumping'. The mixture was heated to a temperature of 70^{o}C on a water bath, and more solvent was added down the condenser until a clear solution, apart from insoluble impurities, was obtained. The hot solution was immediately filtered before rapid cooling occurred. This was done through a fluted filter paper supported in a relatively large funnel with a short wide stem. The funnel was warmed in an electric oven before filtration was started, and supported in a conical flask of sufficient size to hold all the solution. The conical flask was stood on a steam bath and the filtrate was kept boiling gently so that the warm solvent vapours maintained the temperature of the solution undergoing filtration. This was to prevent premature crystallization. The filtered solution was covered with a watch glass and then set aside to cool undisturbed. The crystallised xylopic acid was obtained by suction on a suction pump. The mass of the xylopic acid was 0.5197g (yield 0.05%$^{w}/_{w}$).

The melting point of xylopic acid was found to be 261-262^{o}C. Thin layer chromatography of xylopic acid using silica gel plate, solvent system petroleum ether (40-60^{o}C): ethylacetate (9:1) and anisaldehyde as an indicator gave the Rf value as 0.53.

2.3.3 PREPARATION OF MOBILE PHASE (1L)

900ml of methanol was measured and added to 100ml water to produce 1 L of the mobile phase

2.3.4 PREPARATION OF CRUDE FRUIT, PERICARP AND SEED EXTRACTS PRIOR TO ASSAY

A quantity of dried *Xylopia aethiopica* fruits, pericarp and seeds were accurately weighed separately and each ground to powder. The powder was transferred into a conical flask and 100ml of petroleum ether (40-60^{o}C) added. The mixture was shaken with a mechanical shaker (Stuart Scientific Flask Shaker, SF1) for 1 hour at a speed of 10. The mixture was filtered under pressure using a Buchner funnel and Whatman No. 1 filter paper. The filtrate was heated on a water-bath at 60^{o}C till all the solvent has evaporated. The residue was dissolved in the mobile phase (90% methanol: 10% water) on the water-bath at 60^{o}C. The solution was filtered to obtain the crude fruit extract, crude pericarp extract and crude seed extracts.

2.3.5 HPLC ANALYSIS OF CRUDE FRUIT EXTRACT USING ISOLATED XYLOPIC ACID

The complete chromatograph consisted of LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied biosystems) and Shimadzu CR501 chromatopac. Column used was phenomenex hypersil 20 micron C18 200 × 3.20 mm. The mobile phase consisted of 90% methanol and 10% water eluted isocratically at 0.5ml/min. 20µl portions of a suitable concentration of the isolated xylopic acid were loaded and injected unto the column after dissolving in the mobile phase at 60^{o}C. The eluent was monitored at 206 nm. Similar portions of the **crude fruit extract** were loaded and injected. The peak areas were estimated from the chromatogram and used as a measure of concentration.

2.3.6 HPLC ANALYSIS OF CRUDE SEED EXTRACT USING ISOLATED XYLOPIC ACID

The complete chromatograph consisted of LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied biosystems) and Shimadzu CR501 chromatopac. Column used was phenomenex hypersil 20 micron C18 200 × 3.20 mm. The mobile phase consisted

of 90% methanol and 10% water eluted isocratically at 0.5ml/min. 20µl portions of a suitable concentration of the isolated xylopic acid were loaded and injected unto the column after dissolving in the mobile phase at 60^{o}C. The eluent was monitored at 206 nm. Similar portions of the **crude seed extract** were loaded and injected. The peak areas were estimated from the chromatogram and used as a measure of concentration.

2.3.7 HPLC ANALYSIS OF CRUDE PERICARP EXTRACT USING ISOLATED XYLOPIC ACID

The complete chromatograph consisted of LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied biosystems) and Shimadzu CR501 chromatopac. Column used was phenomenex hypersil 20 micron C18 200 × 3.20 mm. The mobile phase consisted of 90% methanol and 10% water eluted isocratically at 0.5ml/min. 20µl portions of a suitable concentration of the isolated xylopic acid were loaded and injected unto the column after dissolving in the mobile phase at 60^{o}C. The eluent was monitored at 206 nm. Similar portions of the **crude pericarp extract** were loaded and injected. The peak areas were estimated from the chromatogram and used as a measure of concentration.

2.3.8 CHARACTERIZATION OF XYLOPIC ACID

2.3.8.1 MELTING POINT DETERMINATION

The melting point was determined with a melting point apparatus.

2.3.8.2 THIN LAYER CHROMATOGRAPHY

A chloroform solution of the isolated xylopic acid was spotted and examined using TLC precoated plates of silica gel Gf 254, 0.25mm Merck W., Germany. Solvent system used was petroleum ether (40-60^{o}C): ethylacetate (9:1). After the development, the chromatogram was observed after spraying with anisaldehyde reagent. The procedure was repeated for the crude fruit extract.

2.3.8.3 RETARDATION FACTOR (RF) DETERMINATION

The retardation factor on a thin layer chromatogram (precoated plates of silica gel Gf 254, 0.25mm Merck W., Germany) was determined by using petroleum ether (40-60^{o}C): ethylacetate (9:1) as the solvent system and anisaldehyde as the indicator.

2.3.8.4 SPECTROSCOPIC ANALYSIS

The Proton and Carbon-13 nuclear magnetic resonance spectra were obtained on Mercury-300BB Varian Spectrometer, the sample was dissolved in deuterated chloroform ($CDCl_3$). The infrared spectrum of the isolated xylopic acid was obtained on the PerkinElmer Express Version 1.02.00. Mass spectra data were obtained on LCQ Advantage MAX mass spectrometer (Thermo Electronic Inc) with ACPI probe. The sample was dissolved in methanol and introduced through an infusion pump. 30 - 35% normalized collision energy was applied on the parent mass to get the MS/MS spectra

2.3.9 STABILITY STUDIES ON THE ISOLATED XYLOPIC ACID

Purified xylopic acid obtained from the dried fruits of *Xylopia aethiopica* was assayed monthly over three months using the HPLC method developed for the assay of the crude extract. Conditions employed were temperatures of 50°C, 60°C and 80°C.

CHAPTER THREE

RESULTS/CALCULATIONS

3.1 MELTING POINT DETERMINATION

The melting point of the isolated xylopic acid was determined with a melting point apparatus to be 261-262^{o}C.

3.2 RETARDATION FACTOR (RF) DETERMINATION

Table 3-1: Retardation factor (Rf) values of xylopic acid

Distance moved by solvent front/cm	Distance moved by spot/cm	Rf value
5.60	2.90	0.52
5.60	2.90	0.52
5.60	3.00	0.54
5.60	2.95	0.53

Retardation Factor (Rf) = distance moved by solvent front/distance moved by sample

= 2.90/5.60

= 0.52

Retardation Factor (Rf) = distance moved by solvent front/distance moved by sample

= 3.0/5.6

= 0.54

Retardation Factor (Rf) = distance moved by solvent front/distance moved by sample

= 2.95/5.6

= 0.53

Mean Retardation Factor (Rf) = $\frac{0.52 + 0.52 + 0.54 + 0.53}{4}$

= 0.53

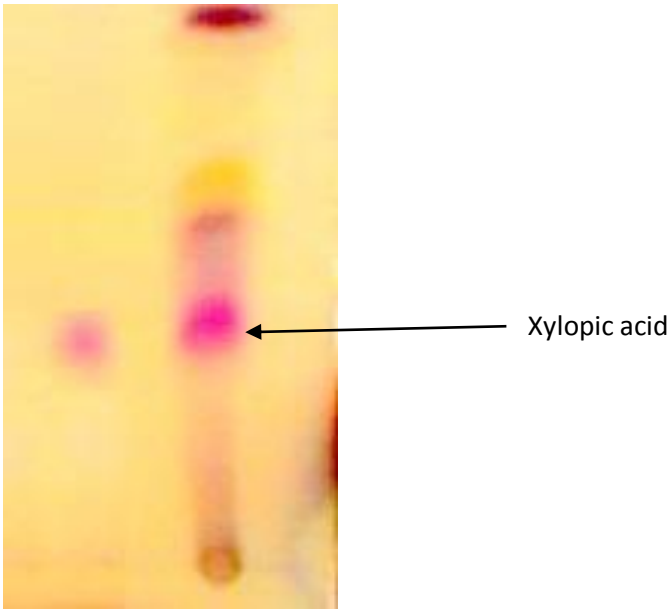

Figure 3.1: TLC chromatogram of the isolated xylopic (I) acid and crude fruit extract (II)

Table 3-2: Preliminary chemical test on the isolated xylopic acid

Test	**Observation**	**Inference**
Nature	White crystals	
Solubility	Dissolved in cold chloroform and in methanol after warming	Polar groups may be present
Litmus paper efffect	Blue litmus turned red	Acidic compound may be present
Action of methanoic NaOH solution	Soluble	Acidic group may be present
Sodium fusion	No black ppt	Sulphur may be absent
Sodium fusion	No blue stain observed on filter paper	Nitrogen may be absent
Test for phenols	No intense violet colouration	Phenol may be absent

3.4 PERCENTAGE CONTENT OF XYLOPIC ACID IN THE CRUDE EXTRACT

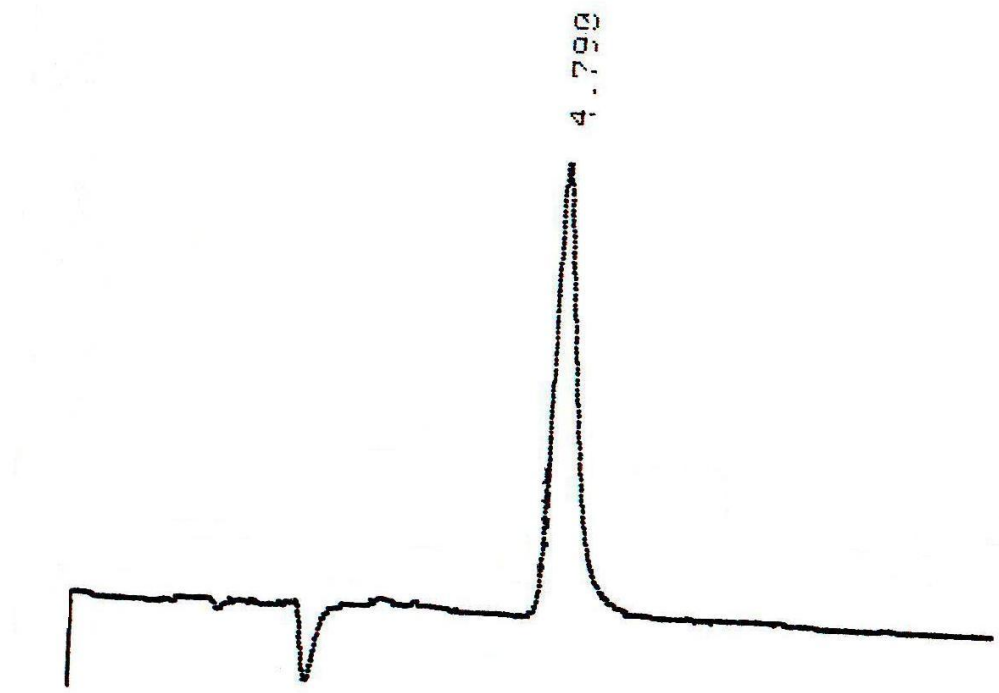

Figure 3.2: HPLC chromatogram of the isolated xylopic acid

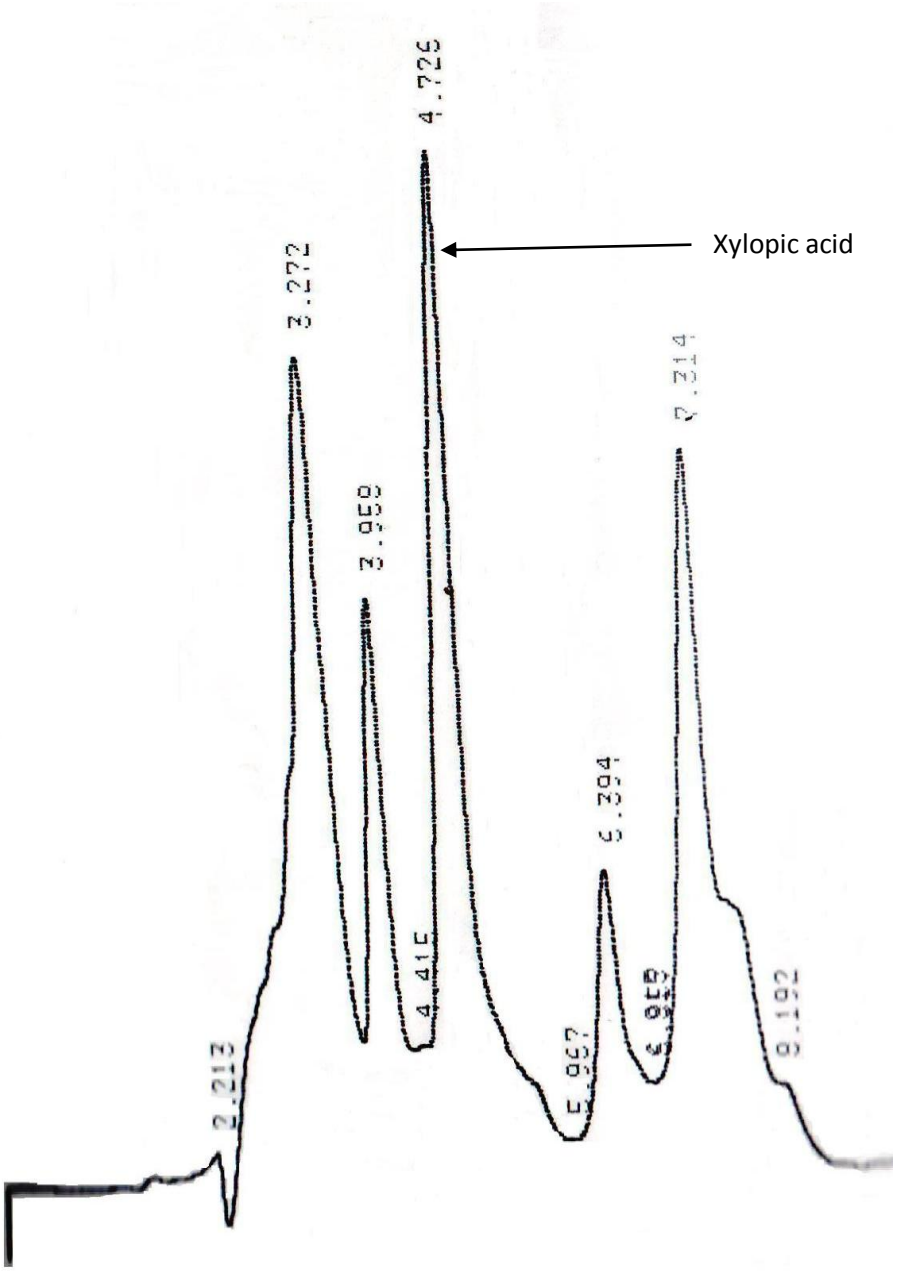

Figure 3.3: HPLC chromatogram of the crude fruit extract

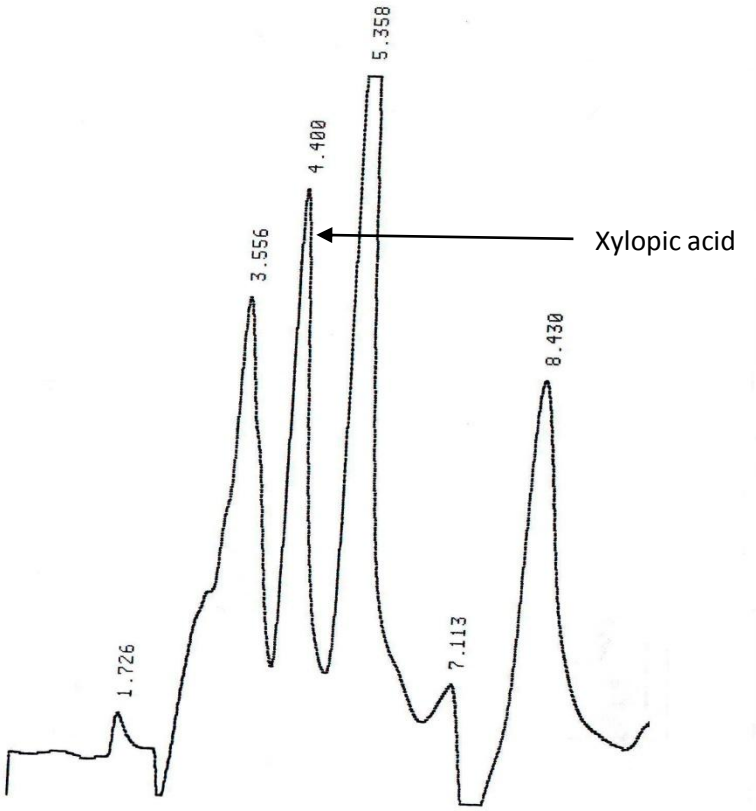

Figure 3.4: HPLC chromatogram of the crude pericarp extract

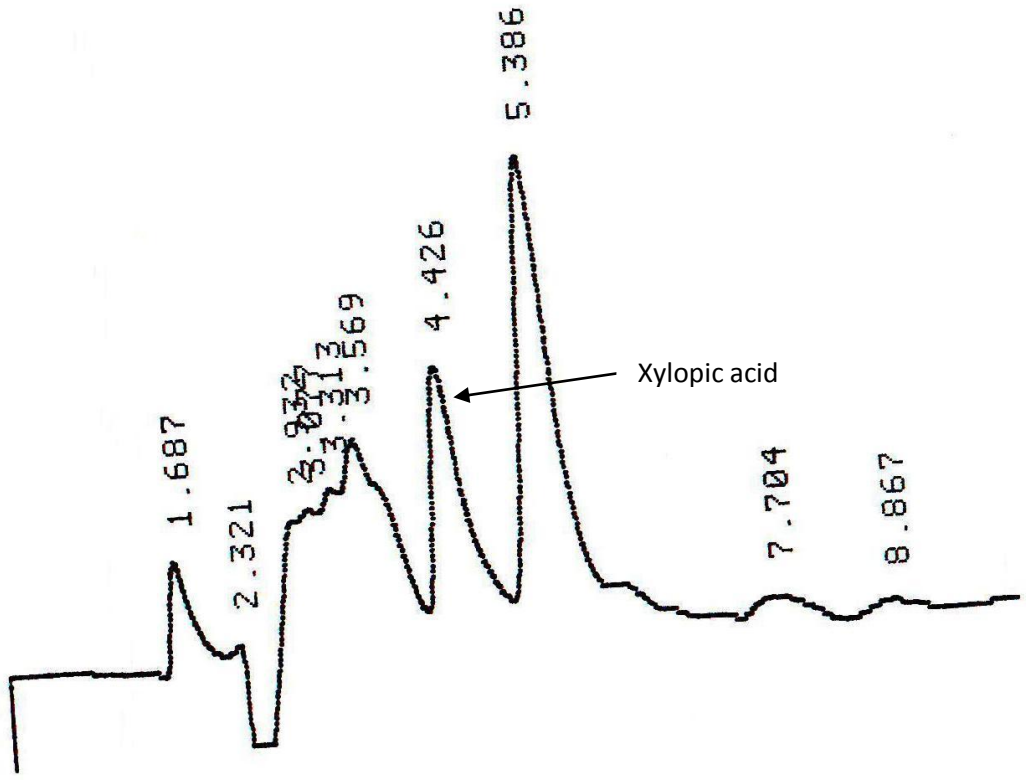

Figure 3.5: HPLC chromatogram of the crude seed extract

Table 3-3: Mean peak areas of isolated xylopic acid and xylopic acid in crude extracts on day 1

Concentration of isolated xylopic acid(%$^{w}/_{v}$)	Peak area 1	Peak area 2	Mean peak area
0.006500	3.80	3.90	3.85
0.004875	2.80	2.80	2.80
0.003250	1.80	1.80	1.80
0.001630	0.89	0.91	0.90
0.000815	0.60	0.60	0.60
Fruit extract (FE_1)	7.60	7.40	7.50
Fruit extract (FE_2)	7.10	7.10	7.10

Weight of fruit, FE_1 = 1.0494g

Weight of fruit, FE_2 = 1.0256g

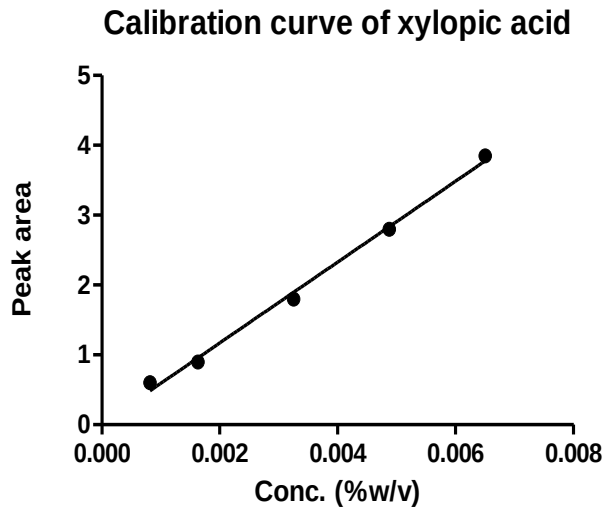

Figure 3.6: Calibration curve of isolated xylopic acid

Slope = 578.6 ± 14.17, Y-intercept = 0.014± 0.057, r^2 = 0.9952, LOQ = 1.5mg/100ml, LOD = 0.5mg/100ml

Concentration, C, in the extract (FE_1) → 7.5 = 578.6C + 0.014

= 0.01294

Percentage content of xylopic acid in fruit = 0.01294 x 100/1.0494

= **1.23% $^{w}/_{w}$**

Concentration, C, in the extract (FE_2) → 7.1 = 578.6C + 0.014

= 0.01225

Percentage content of xylopic acid in fruit = 0.01225 x 100/1.0256

= **1.19% $^{w}/_{w}$**

Table 3-4: Mean peak areas of isolated xylopic acid and xylopic acid in crude extracts on day 2

Concentration of isolated xylopic acid(% $^{w}/_{v}$)	**Peak area 1**	**Peak area 2**	**Mean peak area**
0.00600	4.80	4.80	4.80
0.0045	3.10	3.30	3.20
0.0030	2.85	2.74	2.80
0.0015	1.10	1.10	1.10
0.00075	0.70	0.50	0.60
Fruit extract (FE_1)	9.50	9.90	9.70
Fruit extract (FE_2)	10.10	9.80	9.95

Weight of fruit (FE_1) = 1.1346g

Weight of fruit (FE_2) = 1.1530g

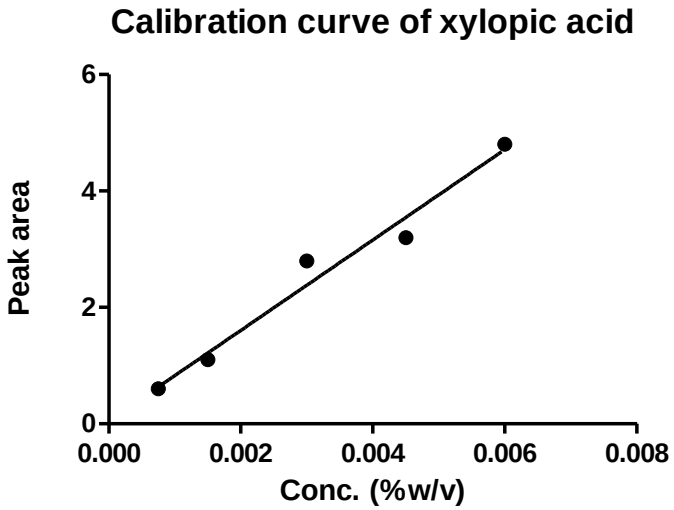

Figure 3.7: Calibration curve of isolated xylopic acid

Slope = 776.5 ± 47.76, Y-intercept = 0.053± 0.176, r^2 = 0.9706

Concentration, C, in the extract (FE_1) → 9.7= 776.5C + 0.053

= 0.01242

Percentage content of xylopic acid in fruit = 0.01242 x 100/1.1346

= **1.09% $^{w}/_{w}$**

Concentration, C, in the extract (FE_2) → 9.95 = 776.5C + 0.053

= 0.01275

Percentage content of xylopic acid in fruit = 0.01275 x 100/1.1530

= **1.11% $^{w}/_{w}$**

Table 3-5: Mean peak areas of isolated xylopic acid and xylopic acid in crude extracts on day 3

Concentration of isolated xylopic acid($\%^{w}/_{v}$)	Peak area 1	Peak area 2	Mean peak area
0.005300	14.50	13.50	14.00
0.003980	9.80	9.80	9.80
0.002650	6.80	7.00	6.90
0.001330	3.60	3.20	3.40
0.000663	1.40	1.40	1.40
pericarp extract (PE_1)	4.50	4.40	4.45
Pericarp extract (PE_2)	4.70	4.70	4.70
Seed extract (SE_1)	3.50	3.50	3.50
Seed extract (SE_2)	3.70	3.90	3.80

Weight of pericarp (PE_1) = 2.0423g

Weight of pericarp (PE_2) = 2.0647g

Weight of seeds (SE_1) = 2.0315g

Weight of seeds (SE_2) = 2.0521g

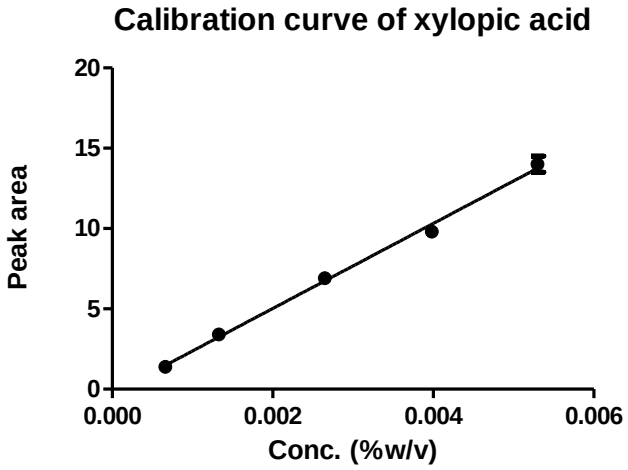

Figure 3.8: Calibration curve of isolated xylopic acid

Slope = 2646 ± 73.86, Y-intercept = -0.269± 0.241, r^2 = 0.9938

Concentration, C, in the seed extract (SE_1) → 3.5 = 2646C + -0.269

= 0.001424

Percentage content of xylopic acid in seed = 0.001424 x 100/2.0315

= **0.07%** $^{w}/_{w}$

Concentration, C, in the seed extract (SE_2) → 3.7 = 2646C + -0.269

= 0.0015

Percentage content of xylopic acid in seed = 0.0015 x 100/2.0512

= **0.07%** $^{w}/_{w}$

Dilution factor for pericarp extract = 8

Concentration, C, in the pericarp extract (PE_1) → 8[4.45= 2646C + -0.269]

= 0.01427

Percentage content of xylopic acid in pericarp (PE_1) = 0.01427 x 100/2.0423

= **0.70%** $^{w}/_{w}$

Concentration, C, in the pericarp extract (PE_2) → 8[4.7 = 2646C + -0.269]

= 0.01502

Percentage content of xylopic acid in pericarp (PE_2) = 0.01502 x 100/2.0647

= **0.73% $^{w}/_{w}$**

Table 3-6: Mean peak areas of isolated xylopic acid and xylopic acid in crude extracts on day 4

Concentration of isolated xylopic acid(% $^{w}/_{v}$)	**Peak area 1**	**Peak area 2**	**Mean peak area**
0.005300	13.90	13.90	13.90
0.003980	10.10	10.10	10.10
0.002650	7.25	7.20	7.23
0.001330	3.40	3.20	3.30
0.000663	1.90	1.90	1.90
Seed extract (SE_1)	2.50	2.20	2.35
Seed extract (SE_2)	1.80	1.70	1.75
Pericarp extract (PE_1)	4.80	4.70	4.75
Pericarp extract (PE_2)	5.20	5.00	5.10
Fruit extract (FE_1)	7.10	6.85	6.98
Fruit extract (FE_2)	7.20	7.20	7.20

Weight of seeds (SE_1) = 2.0315g

Weight of seeds (SE_2) = 2.0174g

Weight of pericarp (PE_1) = 2.0274g

Weight of pericarp (PE_2) = 2.0542g

Weight of fruit (FE_1) = 2.0790g

Weight of fruit (FE_2) = 2.0953g

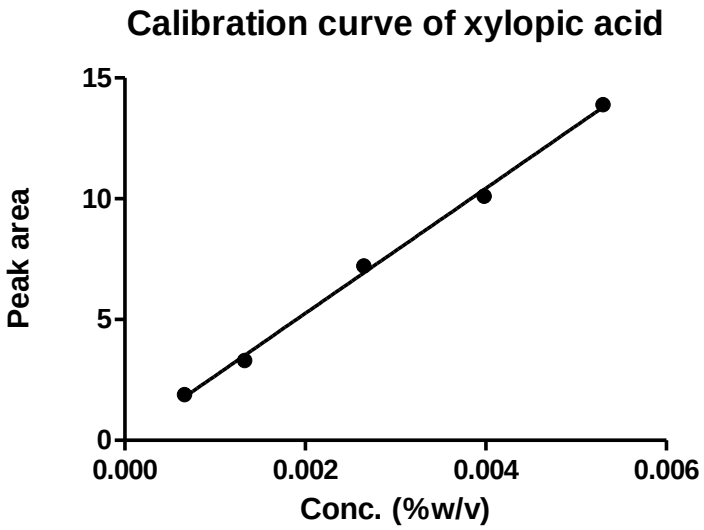

Figure 3.9: Calibration curve of isolated xylopic acid

Slope = 2587 ± 45.87, Y-intercept = -0.081± 0.15, r^2 = 0.9975

Concentration, C, in the seed extract (SE_1) → 2.35 = 2587C + -0.081

= 9.4 $x10^{-4}$

Percentage content of xylopic acid in seed (SE_1) = 9.4 $x10^{-4}$ x 100/2.0315

= **0.05% $^{w}/_{w}$**

Concentration, C, in the seed extract (SE_2) → 1.75 = 2587C + -0.081

= 7.08 $x10^{-4}$

Percentage content of xylopic acid in seed (SE_2) = 7.08 $x10^{-4}$ x 100/2.0174

= **0.04% $^{w}/_{w}$**

Dilution factor for pericarp and fruit extract = 8

Concentration, C, in the pericarp extract (PE_1) → 8[4.75 = 2587C + -0.081]

= 0.01494

Percentage content of xylopic acid in pericarp (PE_1) = 0.01494 x 100/2.0274

= **0.74%** $^{w}/_{w}$

Concentration, C, in the pericarp extract (PE_2) → 8[5.10 = 2587C + -0.081]

= 0.01602

Percentage content of xylopic acid in pericarp (PE_2) = 0.01602 x 100/2.0542

= **0.78%** $^{w}/_{w}$

Concentration, C, in the fruit extract (FE_1) → 8[6.98 = 2587C + -0.081]

= 0.02184

Percentage content of xylopic acid in fruit (FE_1) = 0.02184 x 100/2.0790

= **1.05%** $^{w}/_{w}$

Concentration, C, in the fruit extract (FE_2) → 8[7.2 = 2587C + -0.081]

= 0.02252

Percentage content of xylopic acid in fruit (FE_2) = 0.02252 x 100/2.0953

= **1.07%** $^{w}/_{w}$

Table 3-7: Mean peak areas of isolated xylopic acid and xylopic acid in crude extracts on day 5

Concentration of isolated xylopic acid($\%^{w}/_{v}$)	Peak area 1	Peak area 2	Mean peak area
0.005500	4.70	5.10	4.90
0.004130	3.53	3.67	3.60
0.002750	2.30	2.30	2.30
0.001380	1.53	1.47	1.50
0.000688	0.55	0.45	0.50
Seed extract (SE_1)	1.20	1.20	1.20
Seed extract (SE_2)	1.50	1.70	1.60
Pericarp extract (PE_1)	10.20	10.40	10.30
Pericarp extract (PE_2)	9.80	9.80	9.80
Fruit extract (FE_1)	11.10	11.00	11.05
Fruit extract (FE_2)	11.30	11.10	11.20

Weight of seeds (SE_1) = 2.0154g

Weight of seeds (SE_2) = 2.0497g

Weight of pericarp (PE_1) = 2.0646g

Weight of pericarp (PE_2) = 2.0259g

Weight of fruit (FE_1) = 2.0334g

Weight of fruit (FE_2) = 2.0826g

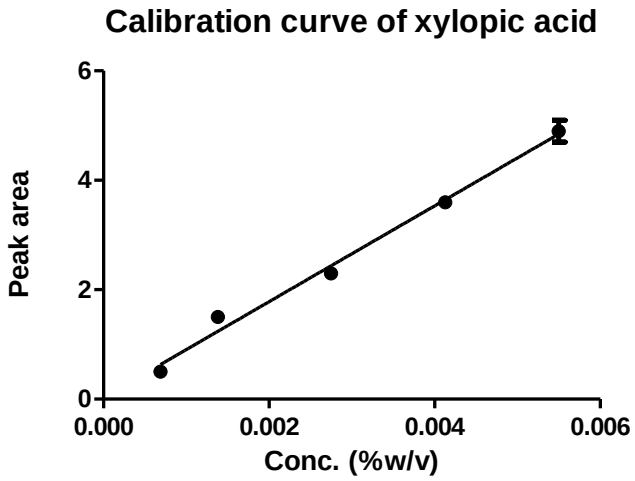

Figure 3.10: Calibration curve of isolated xylopic acid

Slope = 875.6 ± 35.74, Y-intercept = -0.03 ± 0.121, r^2 = 0.9868

Concentration, C, in the seed extract (SE_1) → 1.2 = 875.6C + -0.03

= 0.0014

Percentage content of xylopic acid in seed (SE_1) = 0.0014 x 100/2.0154

= **0.07%** $^{w}/_{w}$

Concentration, C, in the seed extract (SE_2) → 1.6 = 875.6C + -0.03

= 0.00186

Percentage content of xylopic acid in seed (SE_2) = 0.00186 x 100/2.0497

= **0.09%** $^{w}/_{w}$

Dilution factor for pericarp and fruit extract = 2

Concentration, C, in the pericarp extract (PE_1) → 2[10.3 = 875.6C + -0.03]

= 0.0236

Percentage content of xylopic acid in pericarp (PE_1) = 0.0236 x 100/2.0646

= **1.14%** $^{w}/_{w}$

Concentration, C, in the pericarp extract (PE_2) → 2[9.8 = 875.6C + -0.03]

= 0.02245

Percentage content of xylopic acid in pericarp (PE_2) = 0.02245 x 100/2.0259

= **1.11% $^{w}/_{w}$**

Concentration, C, in the fruit extract (FE_1) → 2[11.05 = 875.6C + -0.03]

= 0.02531

Percentage content of xylopic acid in fruit (FE_1) = 0.02531 x 100/2.0334

= **1.24% $^{w}/_{w}$**

Concentration, C, in the fruit extract (FE_2) → 2[11.2 = 875.6C + -0.03]

= 0.02565

Percentage content of xylopic acid in fruit (FE_2) = 0.02565 x 100/2.0826

= **1.23% $^{w}/_{w}$**

Table 3-8: Estimated amount of xylopic acid in each crude extract

Extract	**% $^{w}/_{w}$ content of xylopic acid**	**Mean % $^{w}/_{w}$ content**
Seed	0.07, 0.07, 0.05, 0.04, 0.07, 0.09	0.07% ± 0.02
Pericarp	0.70, 0.73, 0.74, 0.78, 1.14, 1.11	0.87% ± 0.20
Fruit	1.23, 1.19, 1.09, 1.11, 1.05, 1.07, 1.24, 1.23	1.15% ± 0.08

3.5 STABILITY STUDIES

The following are the percentage concentrations of the isolated xylopic acid on different days at various temperatures.

Table 3-9: Results for stability test at 50°C

Time/days	Concentration/ %^{w}/w
20	83.27
50	67.79
70	60.30

Table 3-10: Results for stability test at 60°C

Time/days	Concentration/ %^{w}/w
20	73.28
50	60.65
70	44.14

Table 3-11: Results for stability test at 80°C

Time/days	Concentration/ %^{w}/w
20	67.25
50	56.65
70	41.76

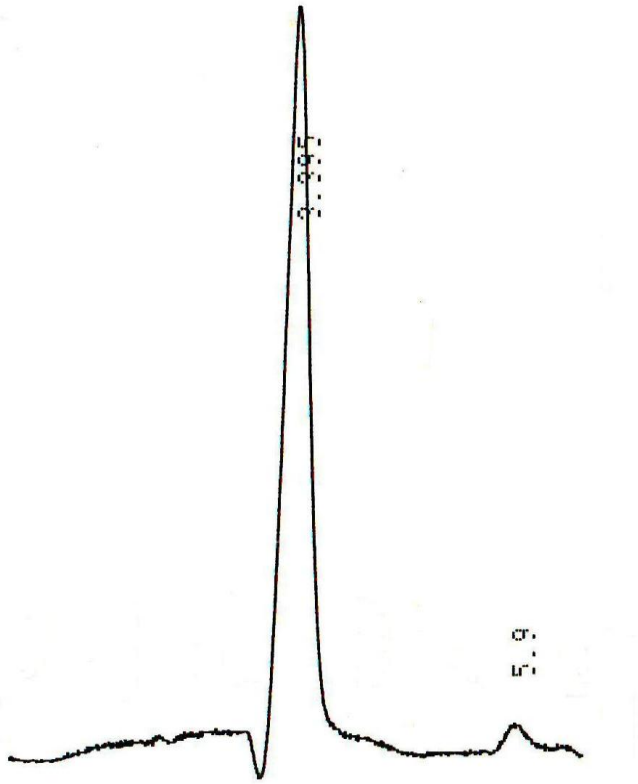

Figure 3.11: HPLC chromatogram of isolated xylopic acid after stability studies

3.6 SPECTROSCOPIC ANALYSES

3.5.1 PROTON NMR SPECTRUM

The following are ^{1}H Nuclear Magnetic Resonance and H, H COSY spectra of the isolated xylopic acid crystals;

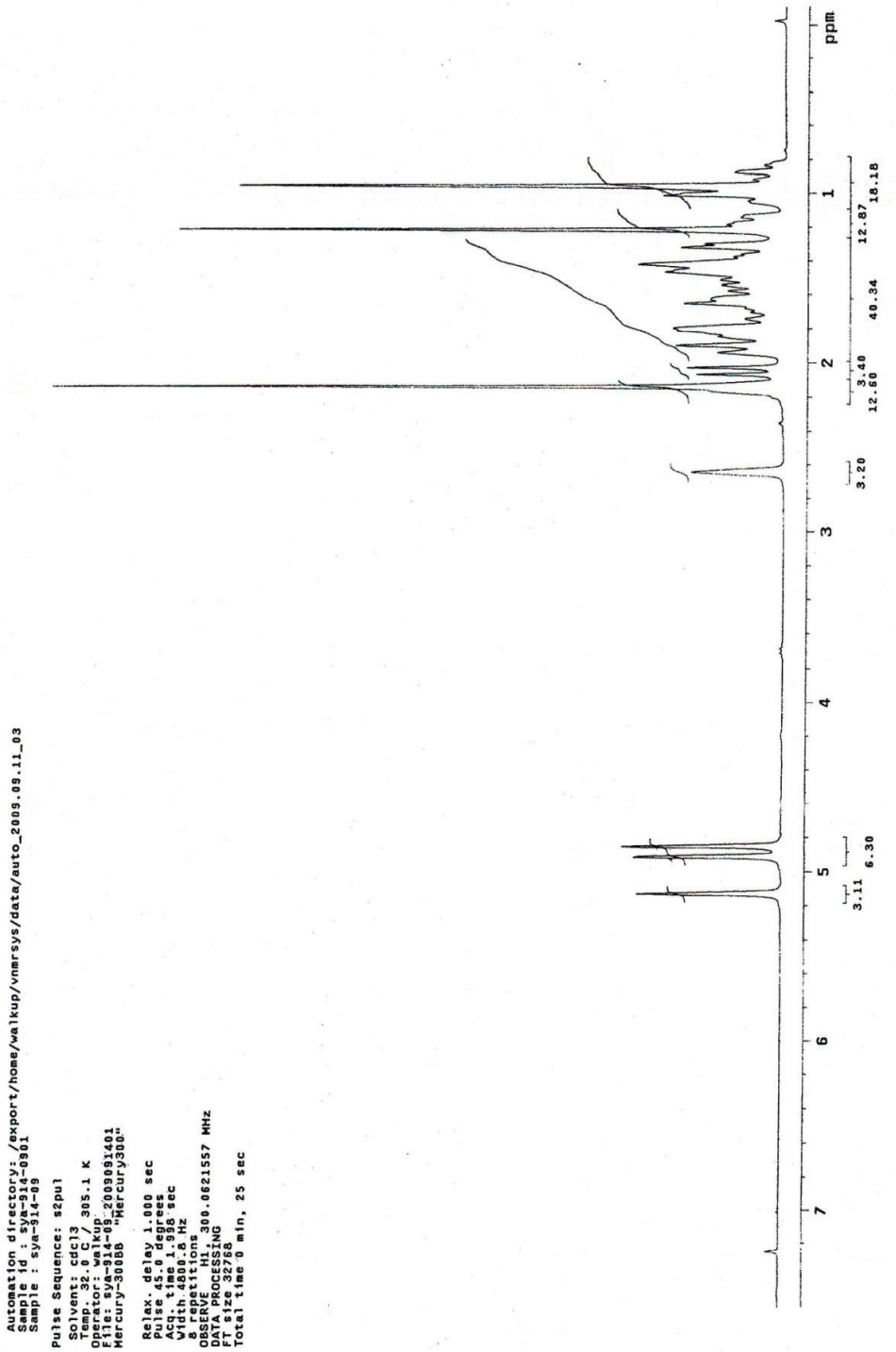

Figure 3.12: ^{1}H NMR spectrum of isolated xylopic acid

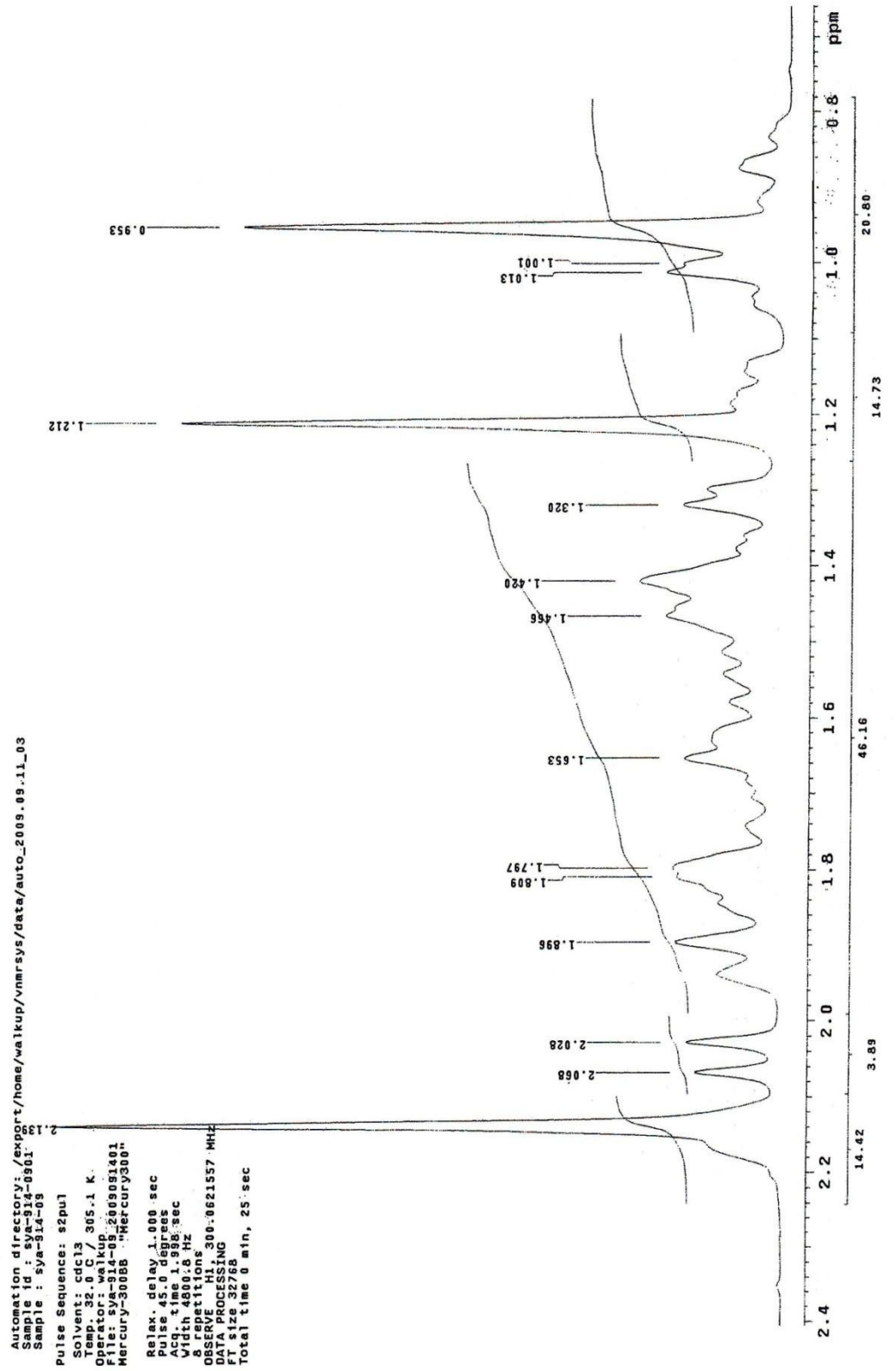

Figure 3.13: Expanded ^{1}H NMR spectrum (0.8ppm – 2.4ppm) of isolated xylopic acid

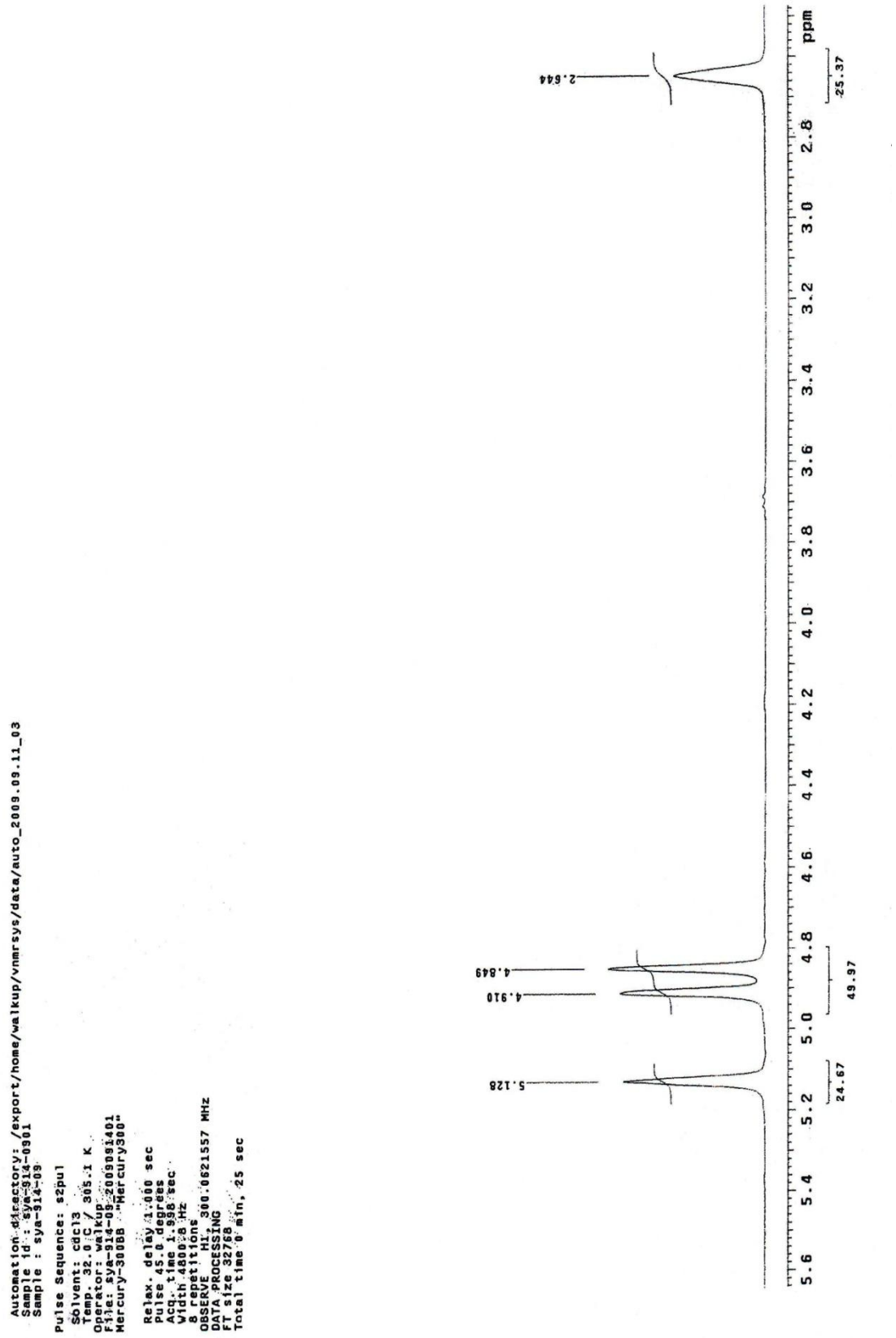

Figure 3.14: Expanded ^{1}H NMR spectrum (2.6ppm – 5.6ppm) of isolated xylopic acid

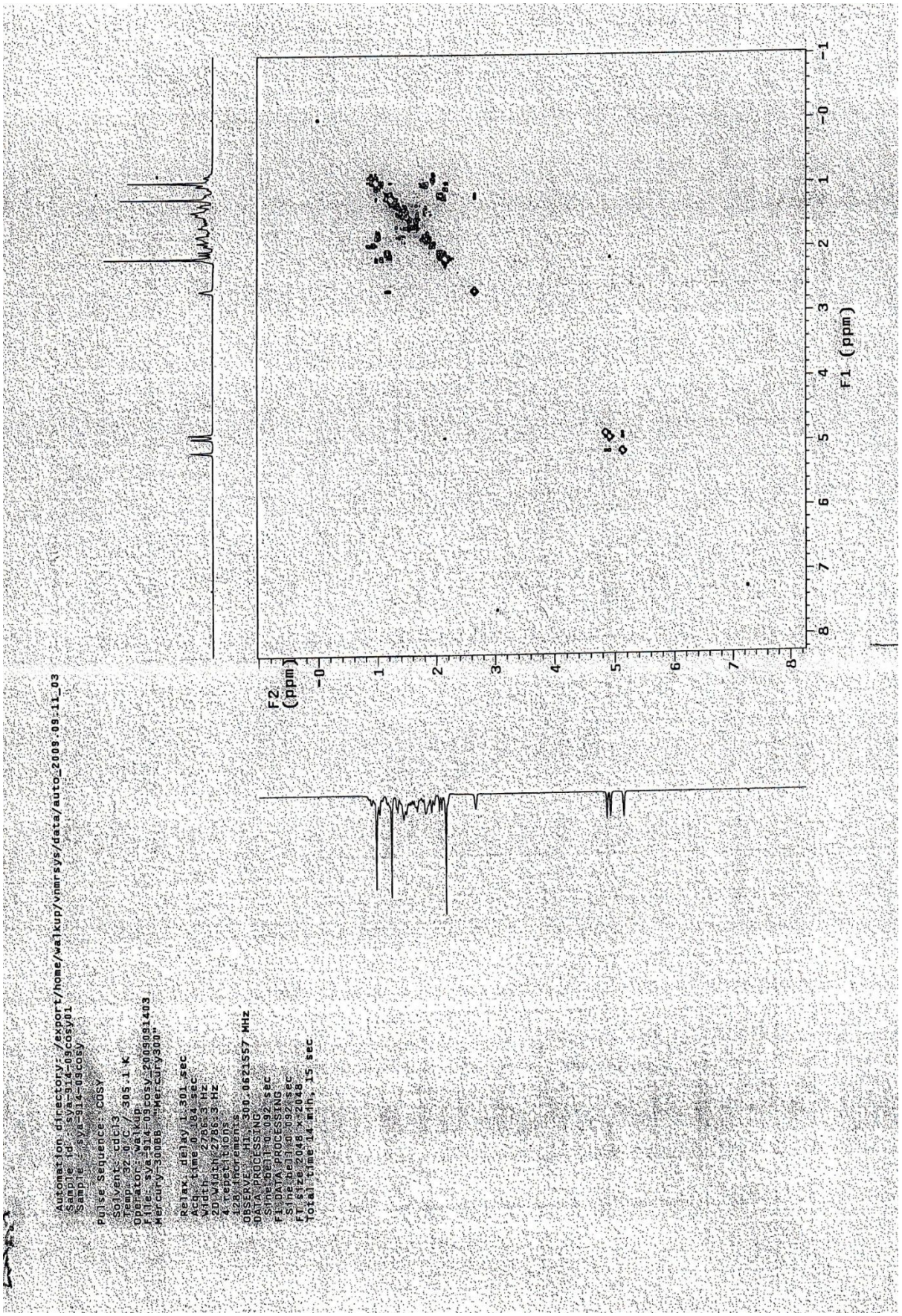

Figure 3.15: H, H COSY spectrum of isolated xylopic acid

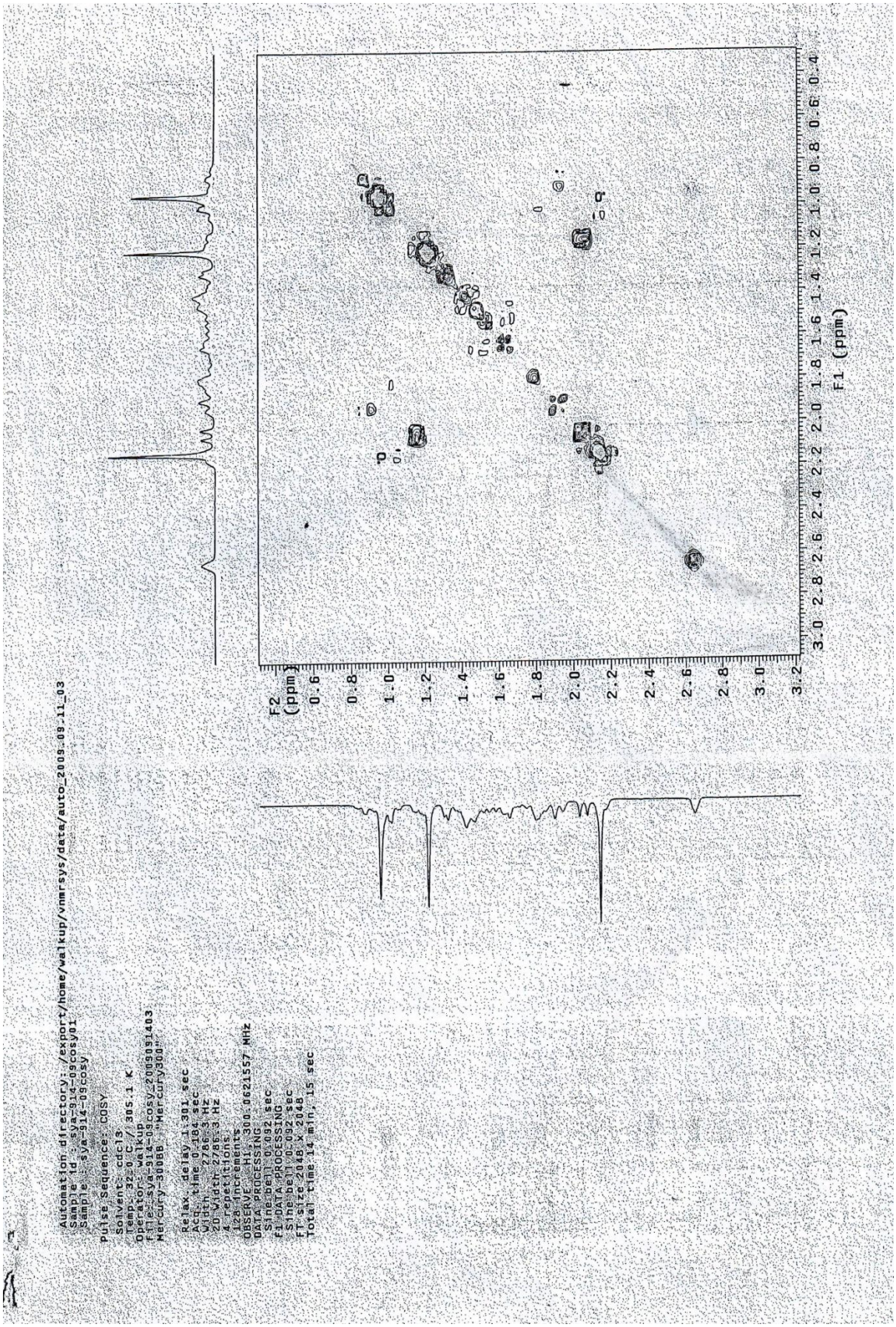

Figure 3.16: H, H COSY spectrum of isolated xylopic acid

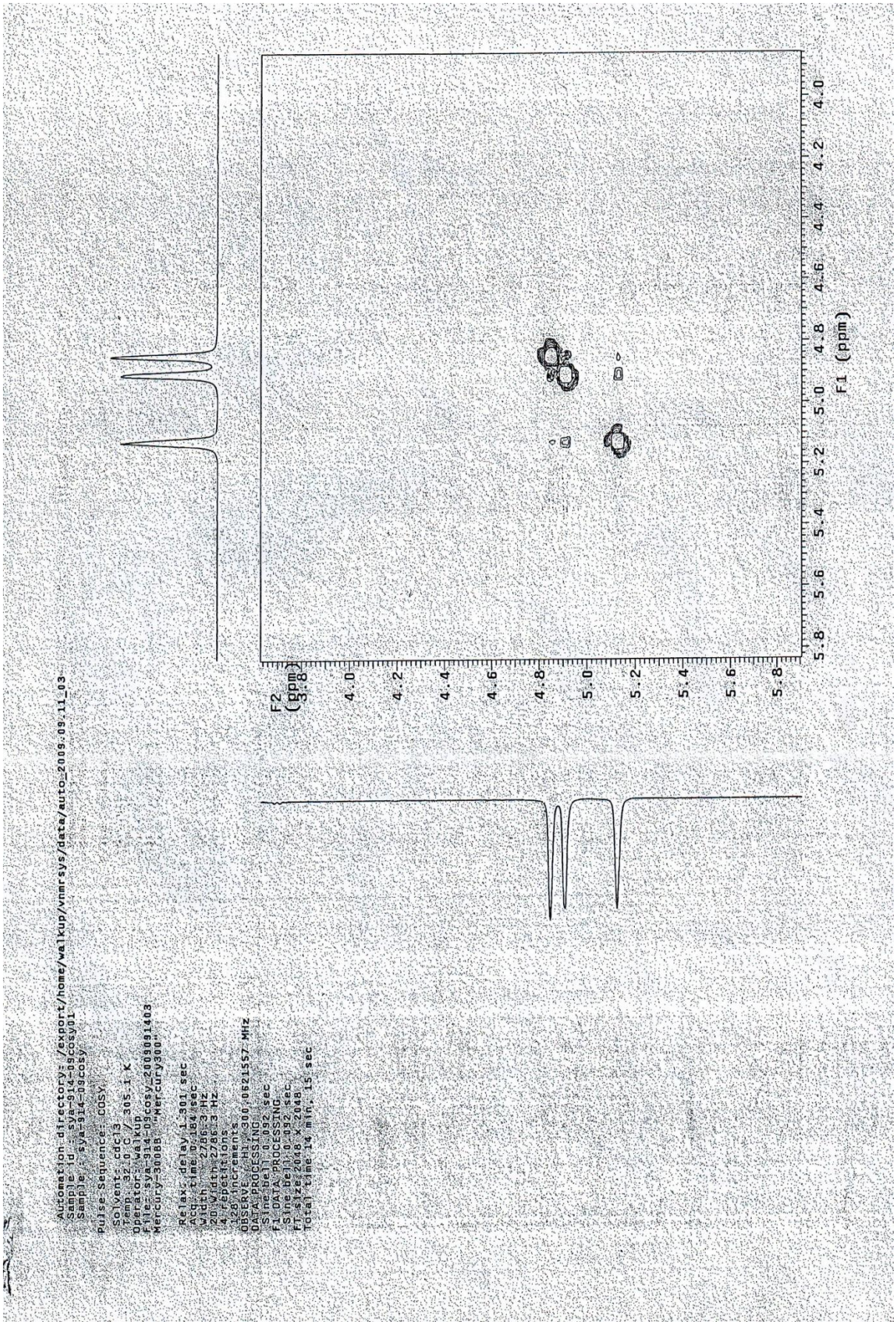

Figure 3.17: H, H COSY spectrum of isolated xylopic acid

3.5.2 CARBON-13 NMR SPECTRUM

The following are the ^{13}C Nuclear Magnetic Resonance spectra of the isolated xylopic acid crystals;

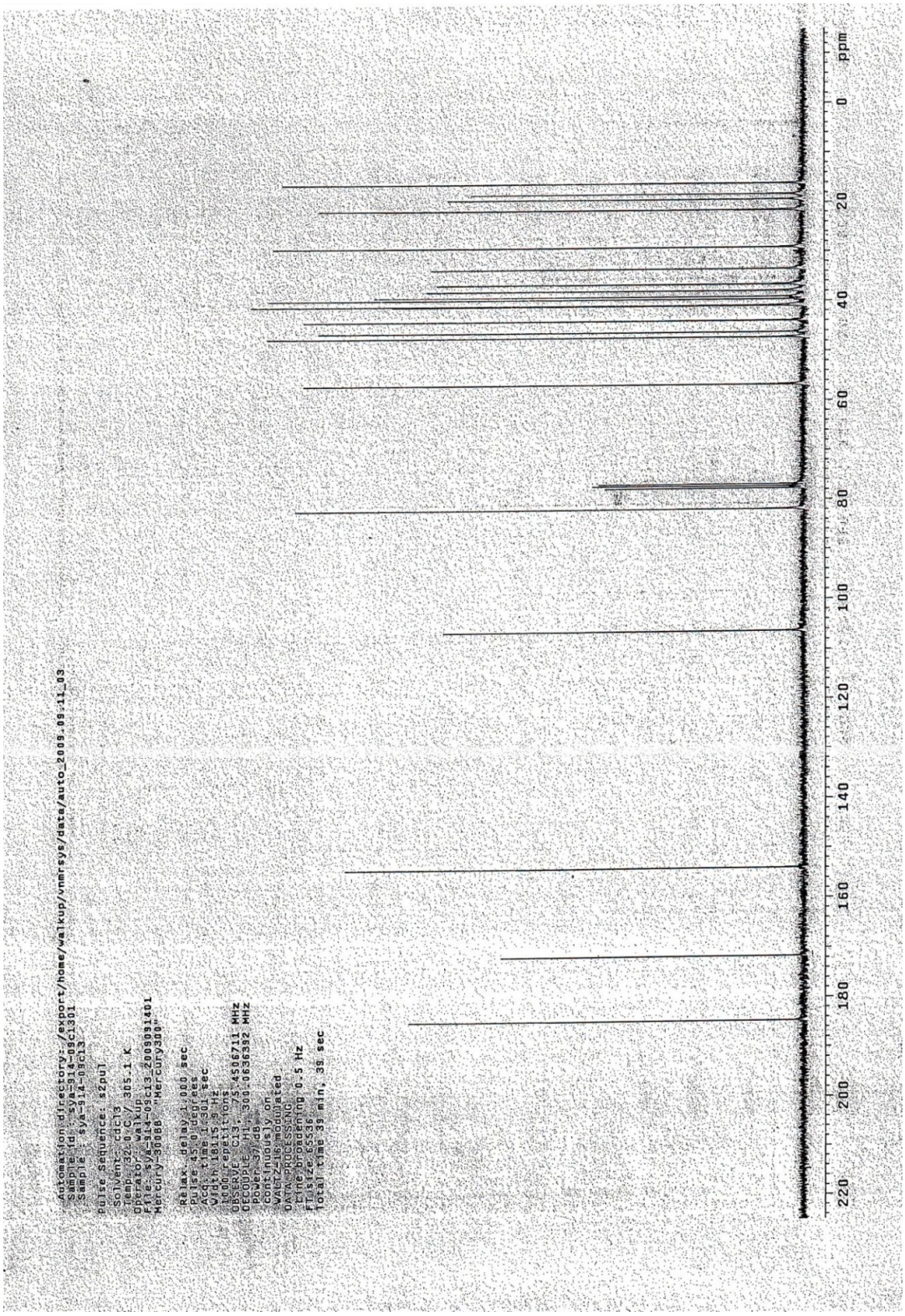

Figure 3.18: ^{13}C NMR spectrum of isolated xylopic acid

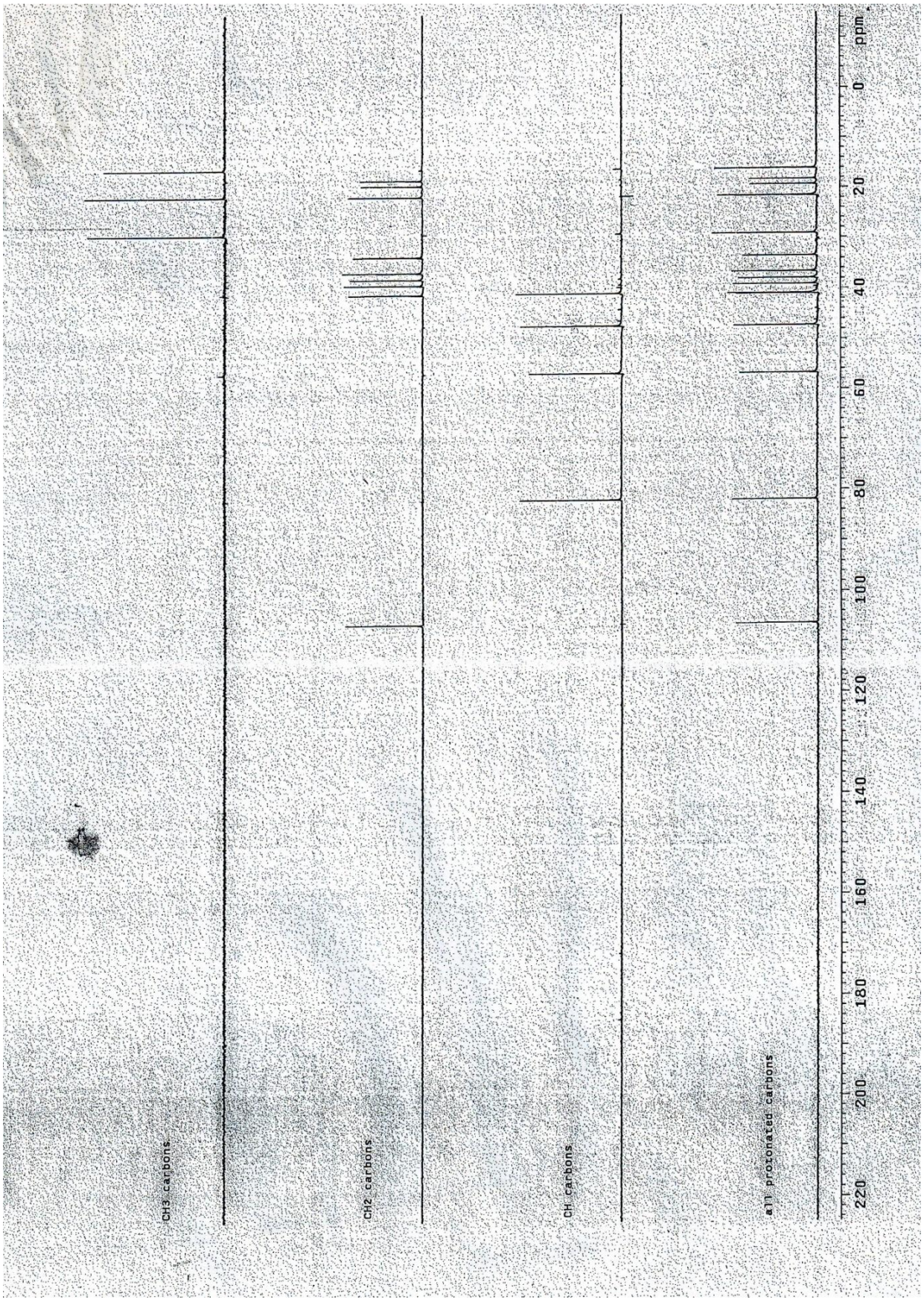

Figure 3.19: ^{13}C NMR spectrum of isolated xylopic acid

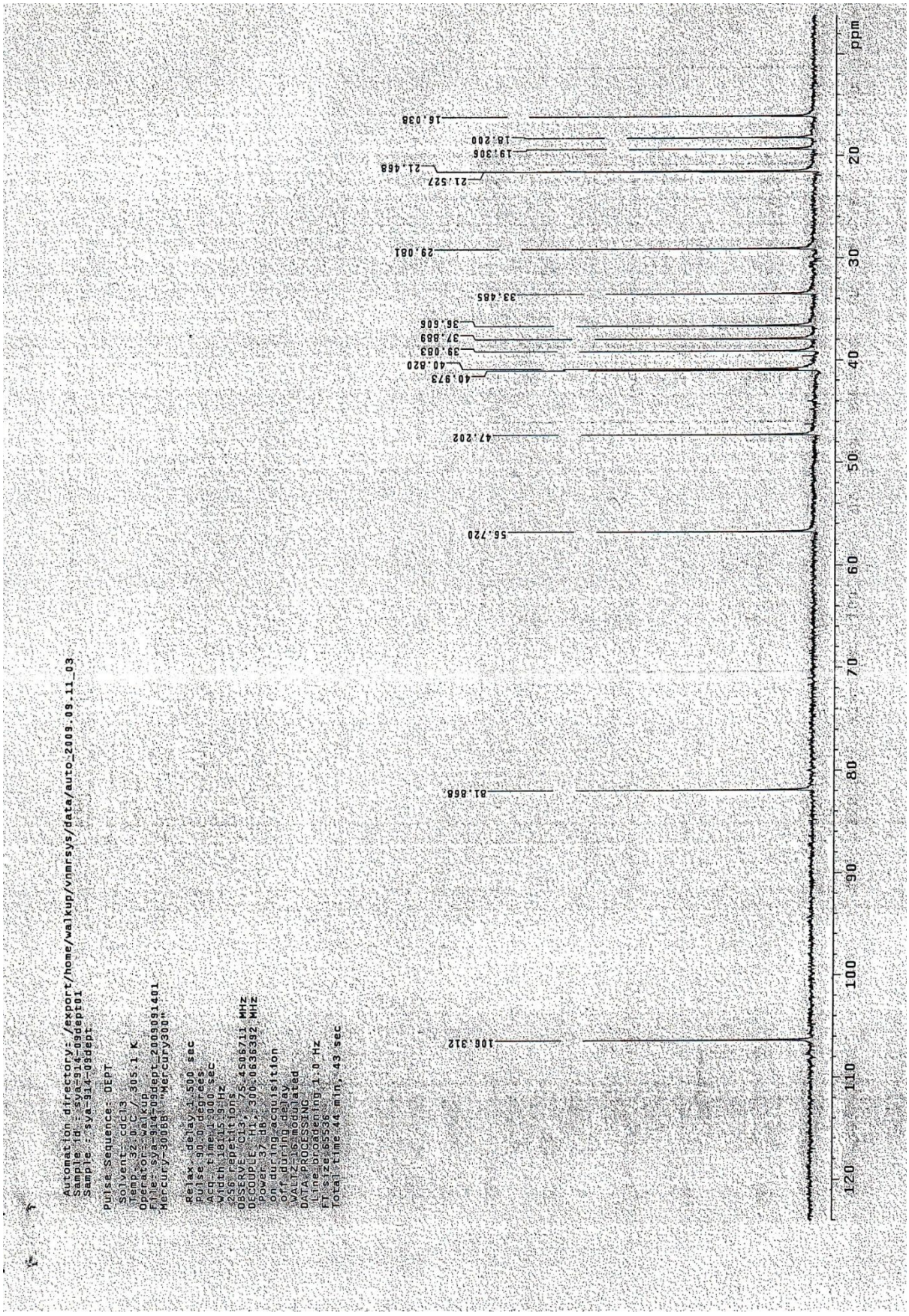

Figure 3.20: Expanded ^{13}C NMR spectrum of isolated xylopic acid

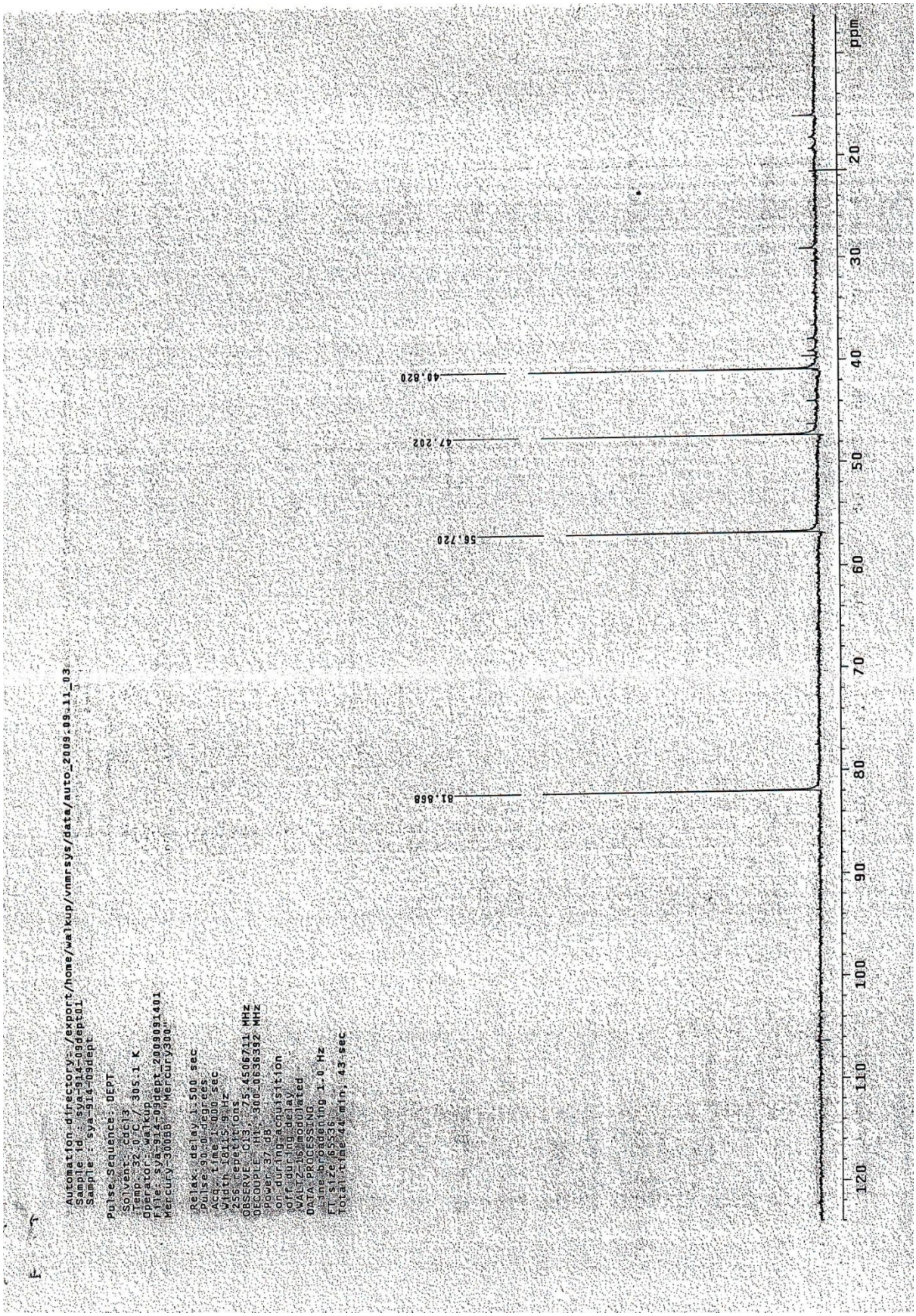

Figure 3.21: Expanded ^{13}C NMR spectrum of isolated xylopic acid

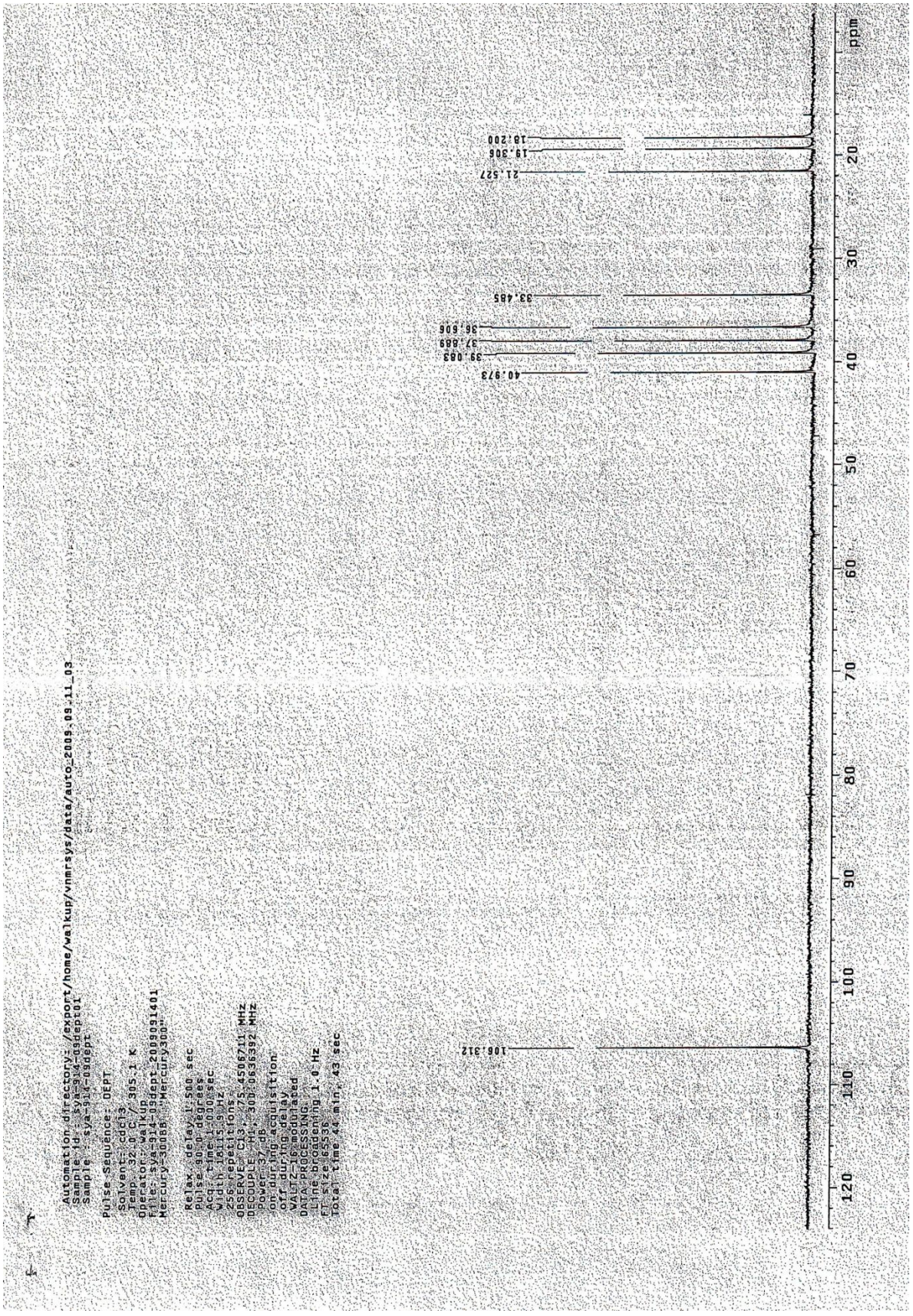

Figure 3.22: Expanded ^{13}C NMR spectrum of isolated xylopic acid

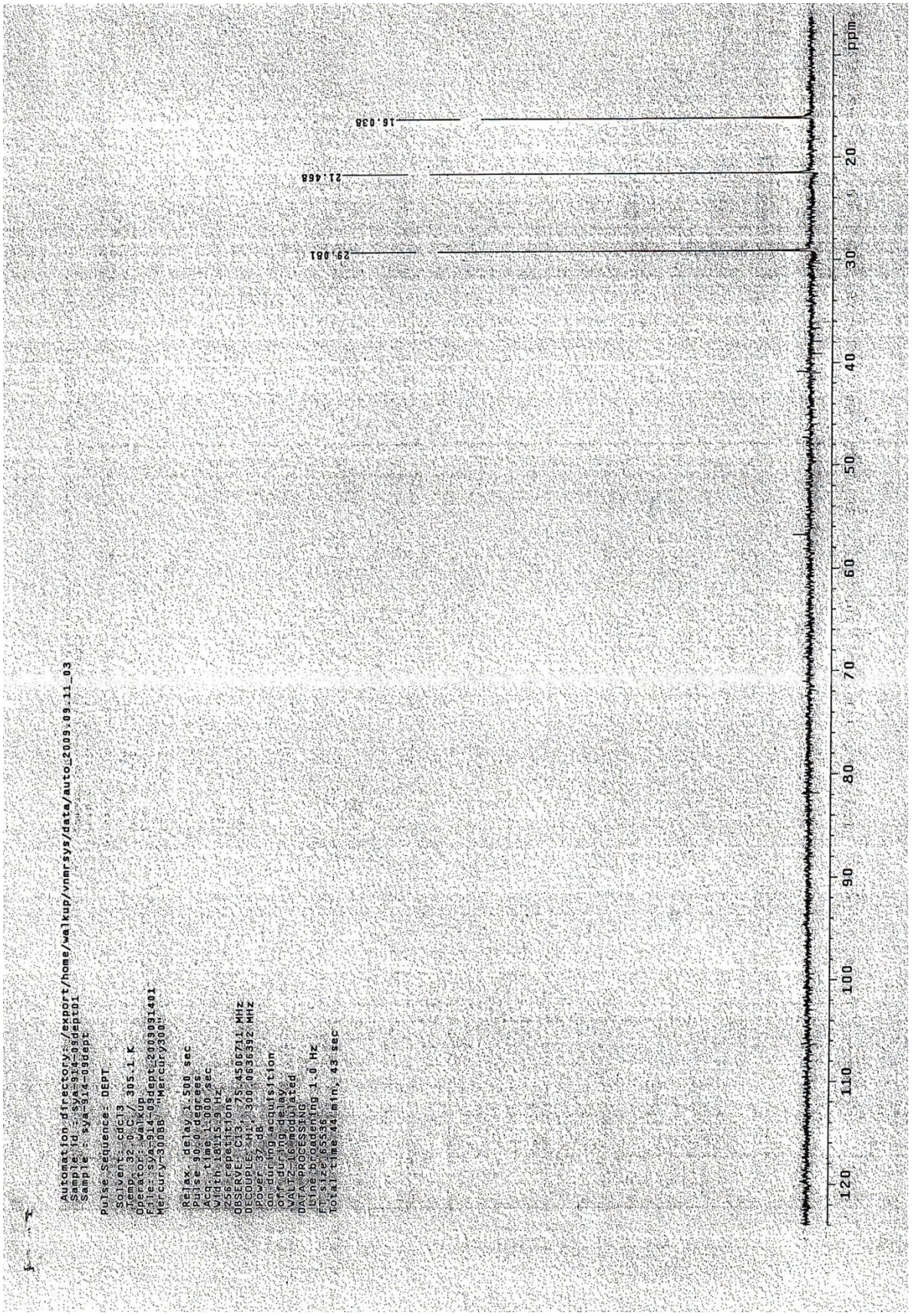

Figure 3.23: Expanded ^{13}C NMR spectrum of isolated xylopic acid

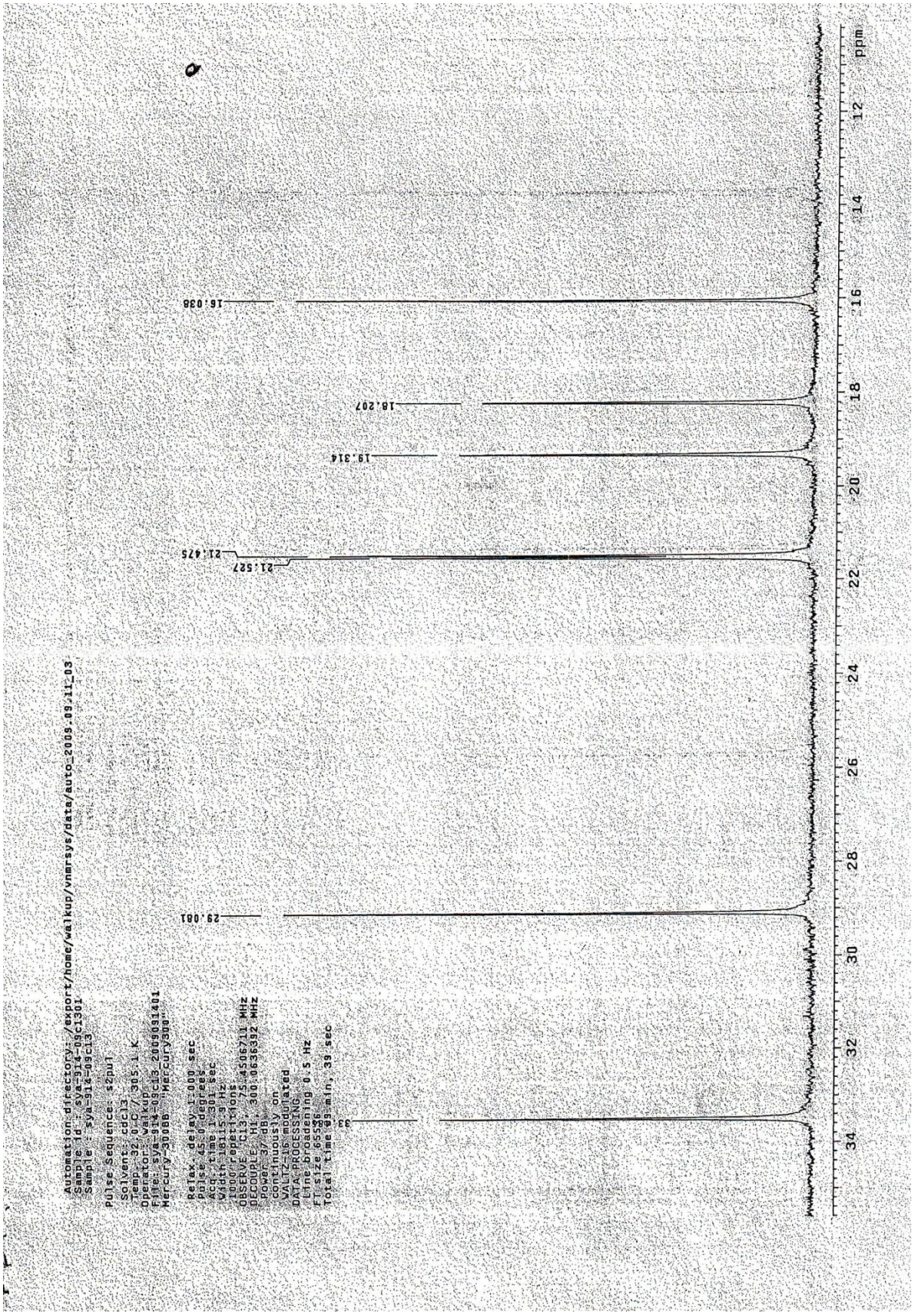

Figure 3.24: Expanded ^{13}C NMR spectrum of isolated xylopic acid

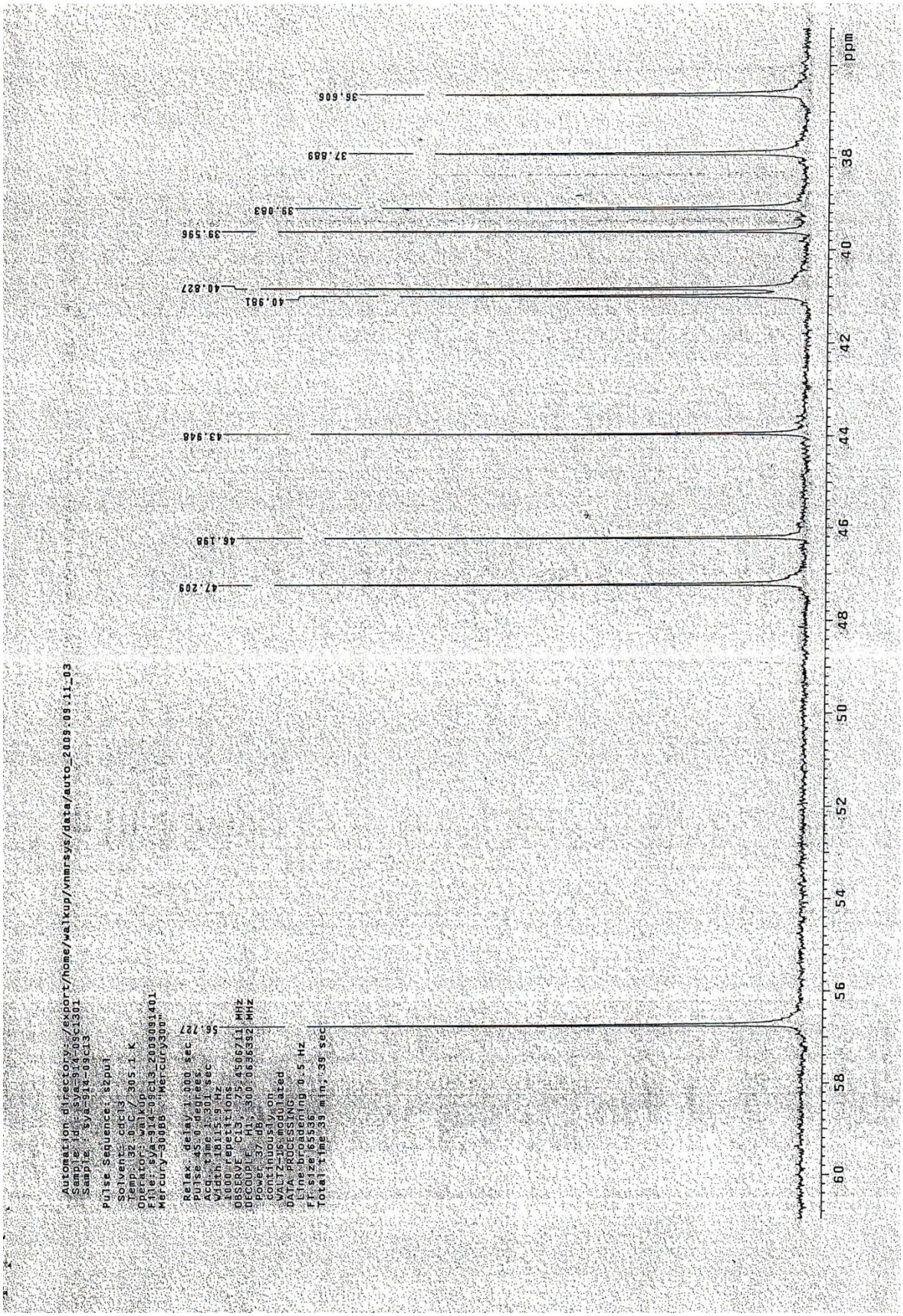

Figure 3.25: Expanded ^{13}C NMR spectrum of isolated xylopic acid

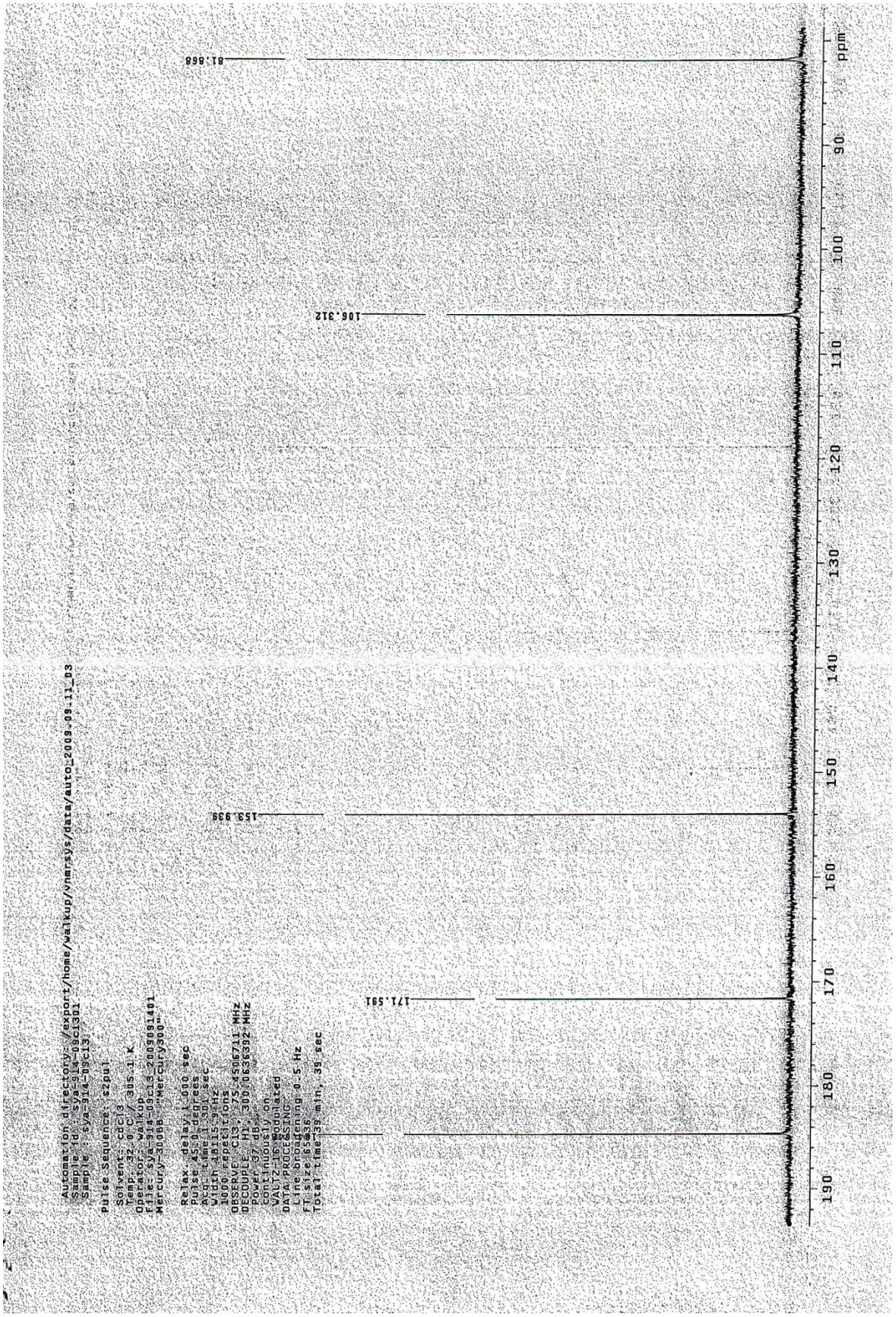

Figure 3.26: Expanded ^{13}C NMR spectrum of isolated xylopic acid

3.5.3 INFRA RED SPECTRUM

The following are the Infra red spectra of the isolated xylopic acid crystals;

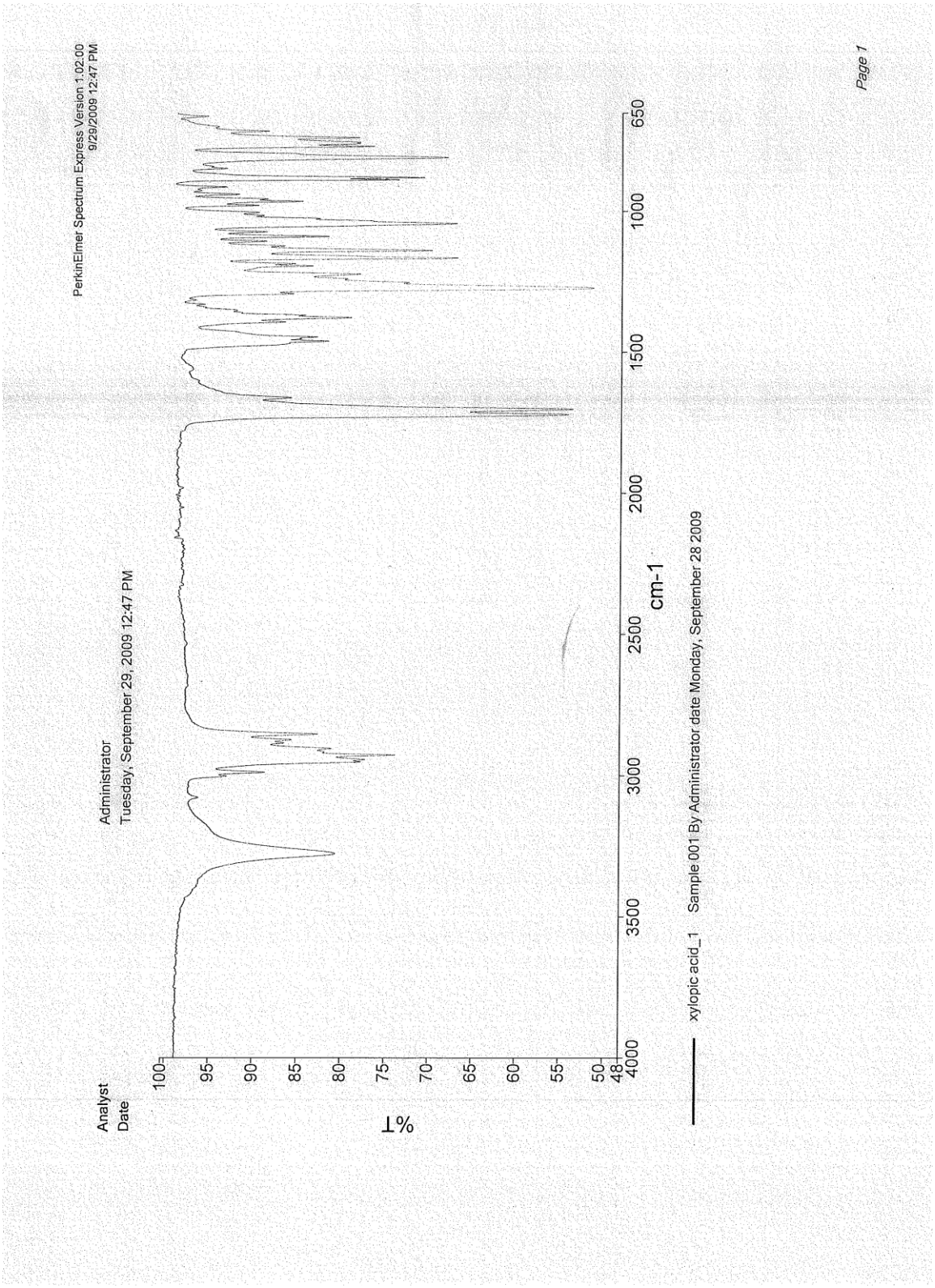

Figure 3.27: IR spectrum of isolated xylopic acid

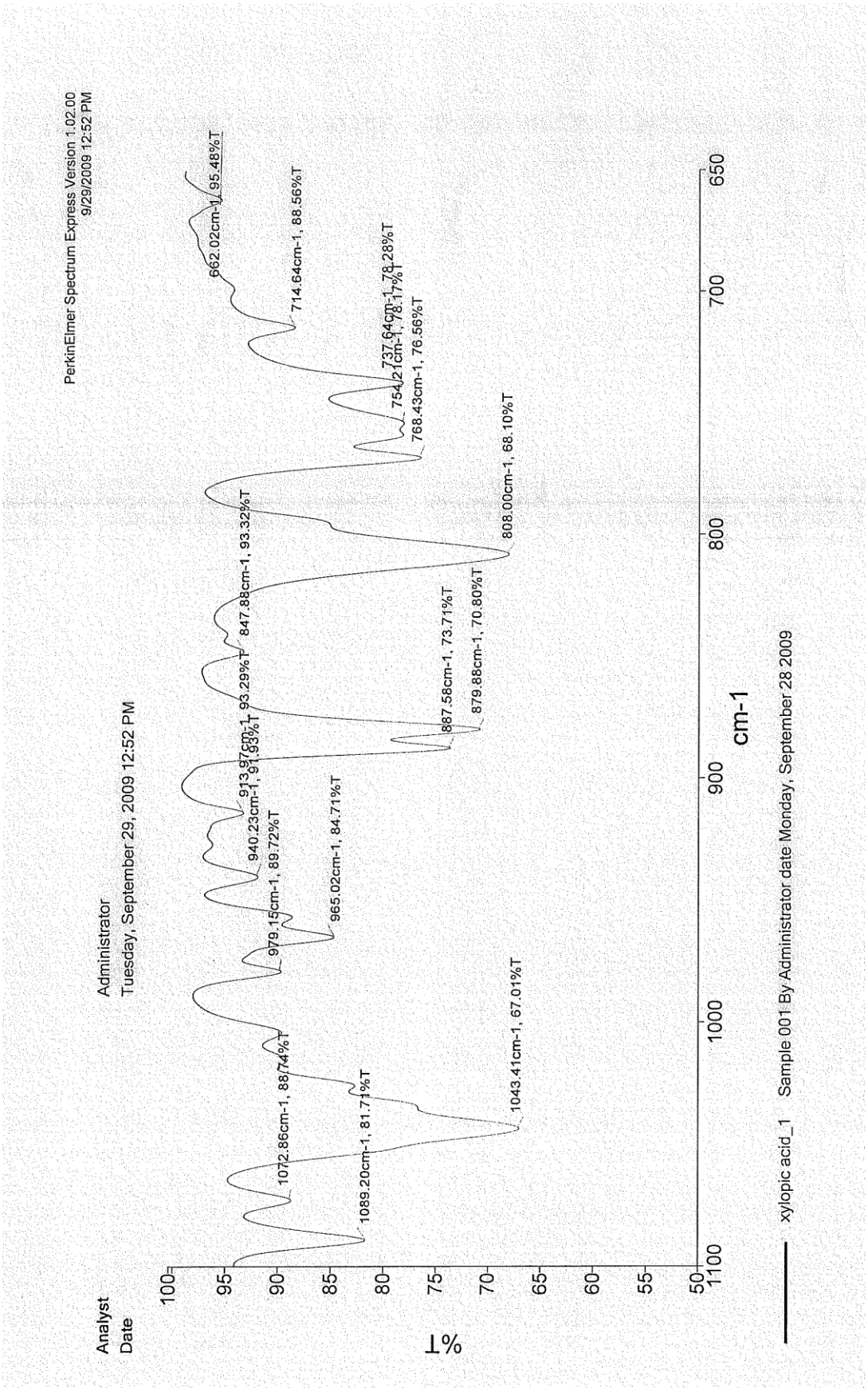

Figure 3.28: Expanded IR spectrum (650cm^{-1} – 1100cm^{-1}) of isolated xylopic acid

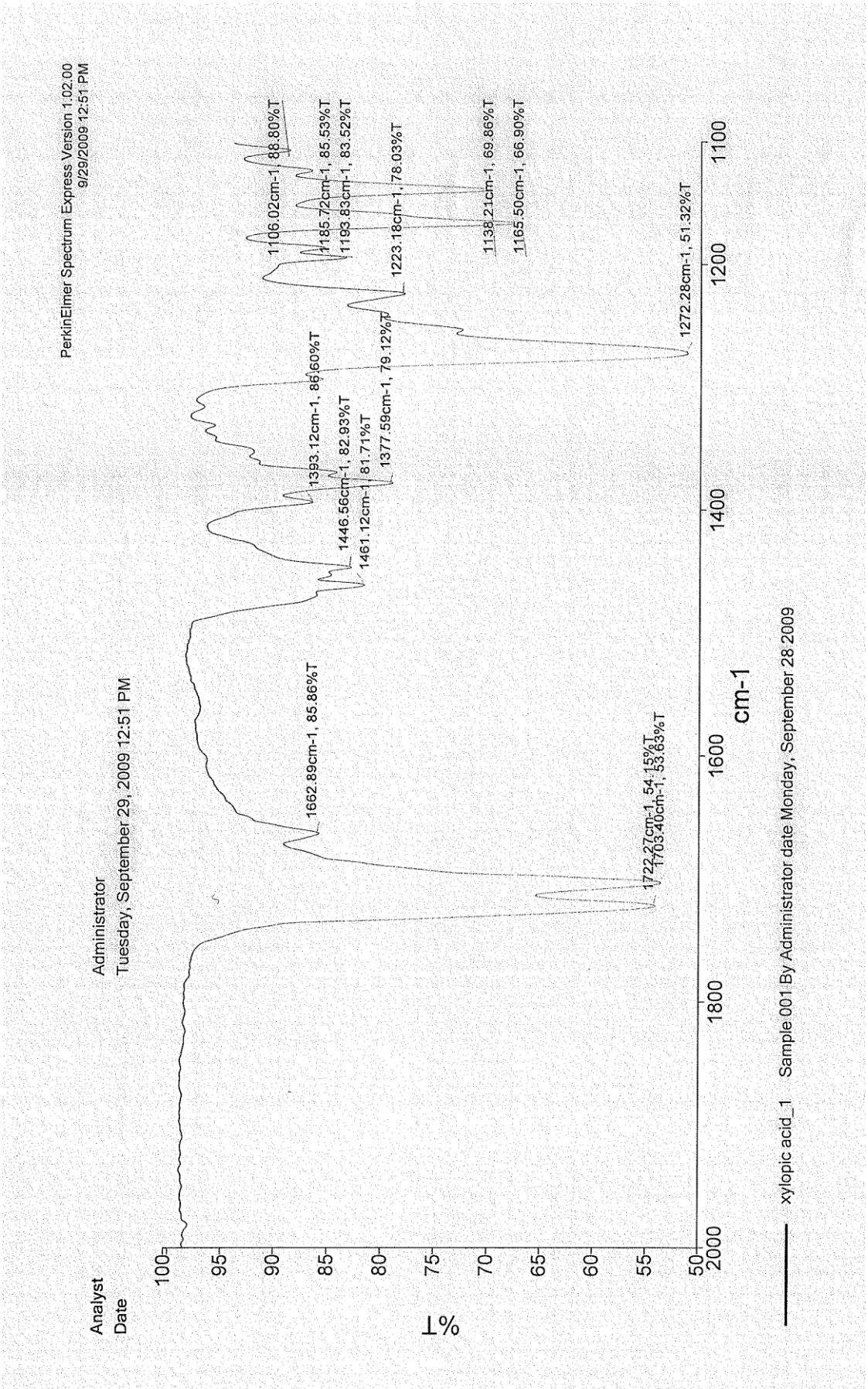

Figure 3.29: Expanded IR spectrum (1100cm^{-1} – 2000cm^{-1}) of isolated xylopic acid

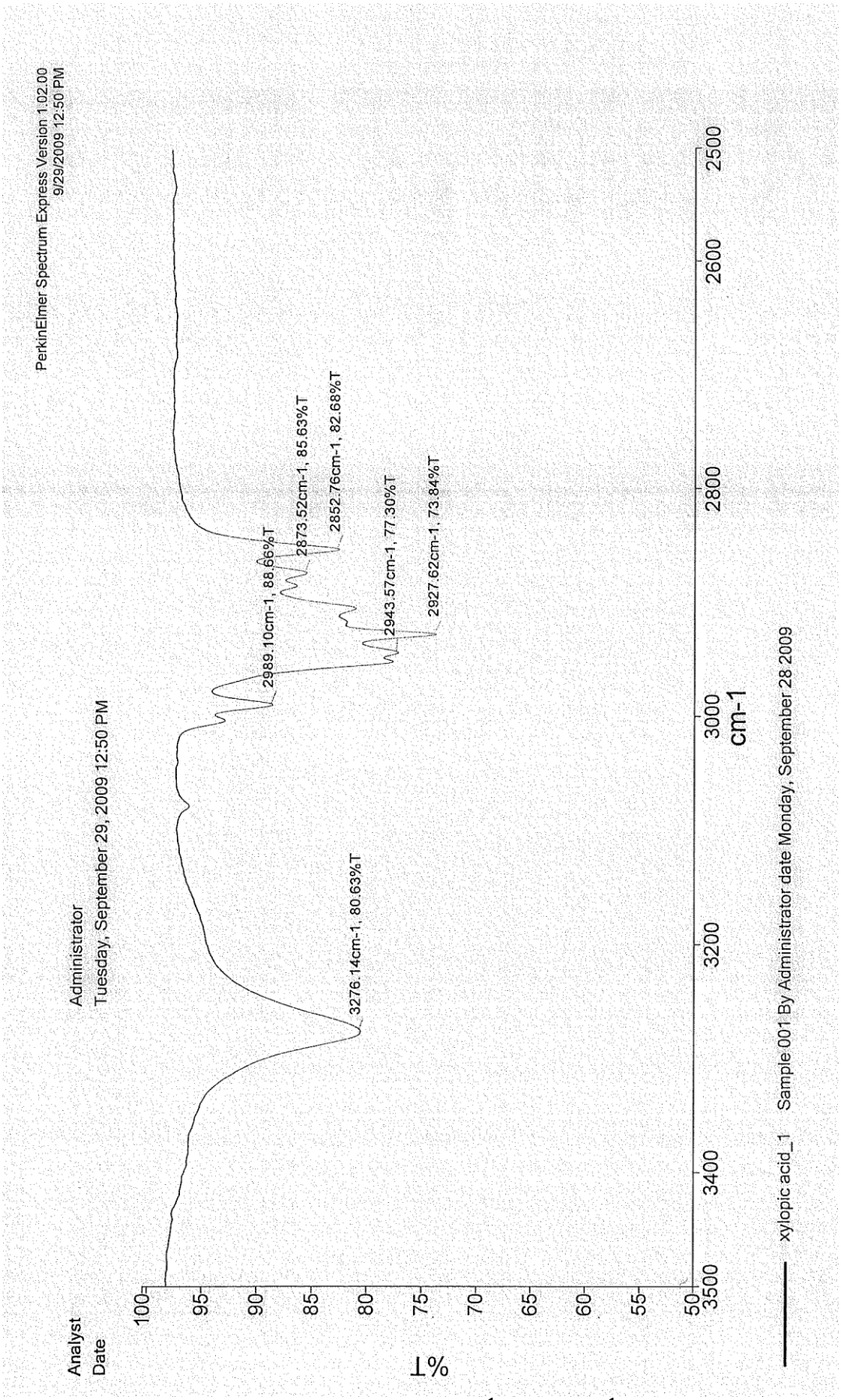

Figure 3.30: Expanded IR spectrum (2500cm^{-1} – 3500cm^{-1}) of isolated xylopic acid

3.5.4 MASS SPECTRUM

The following are the Mass spectra of the isolated xylopic acid crystals;

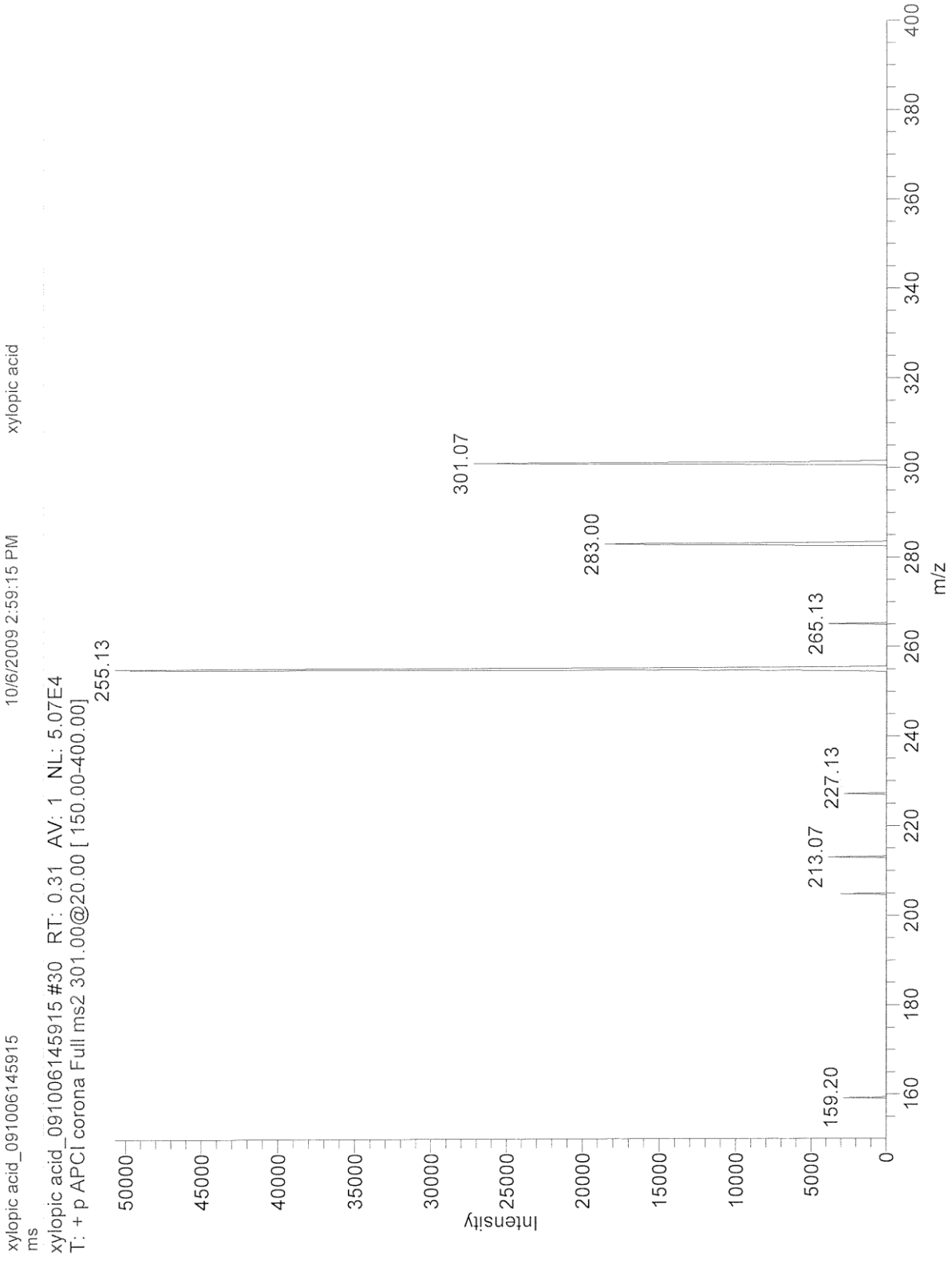

Figure 3.31: Mass spectrum of isolated xylopic acid

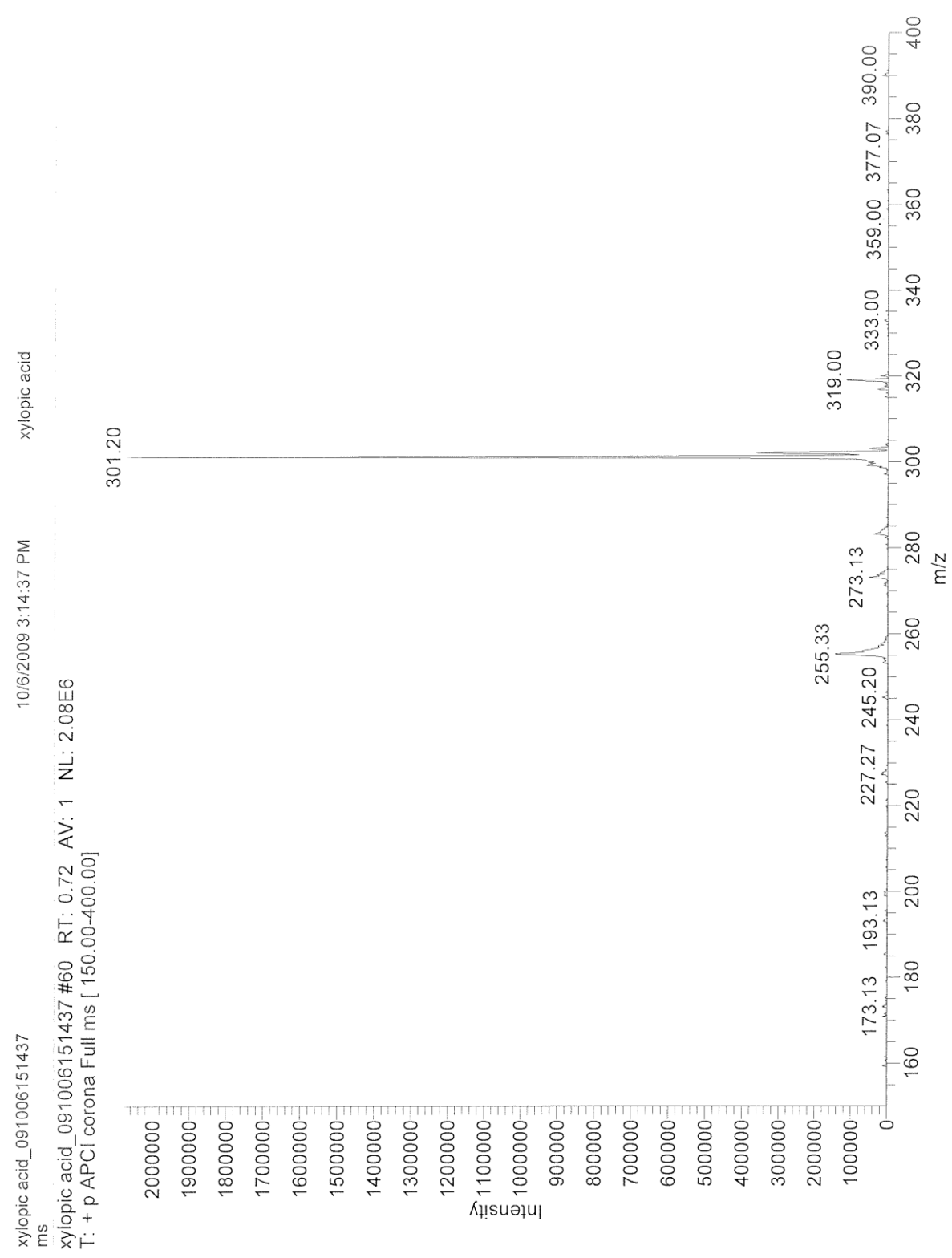

Figure 3.32: Mass spectrum of isolated xylopic acid

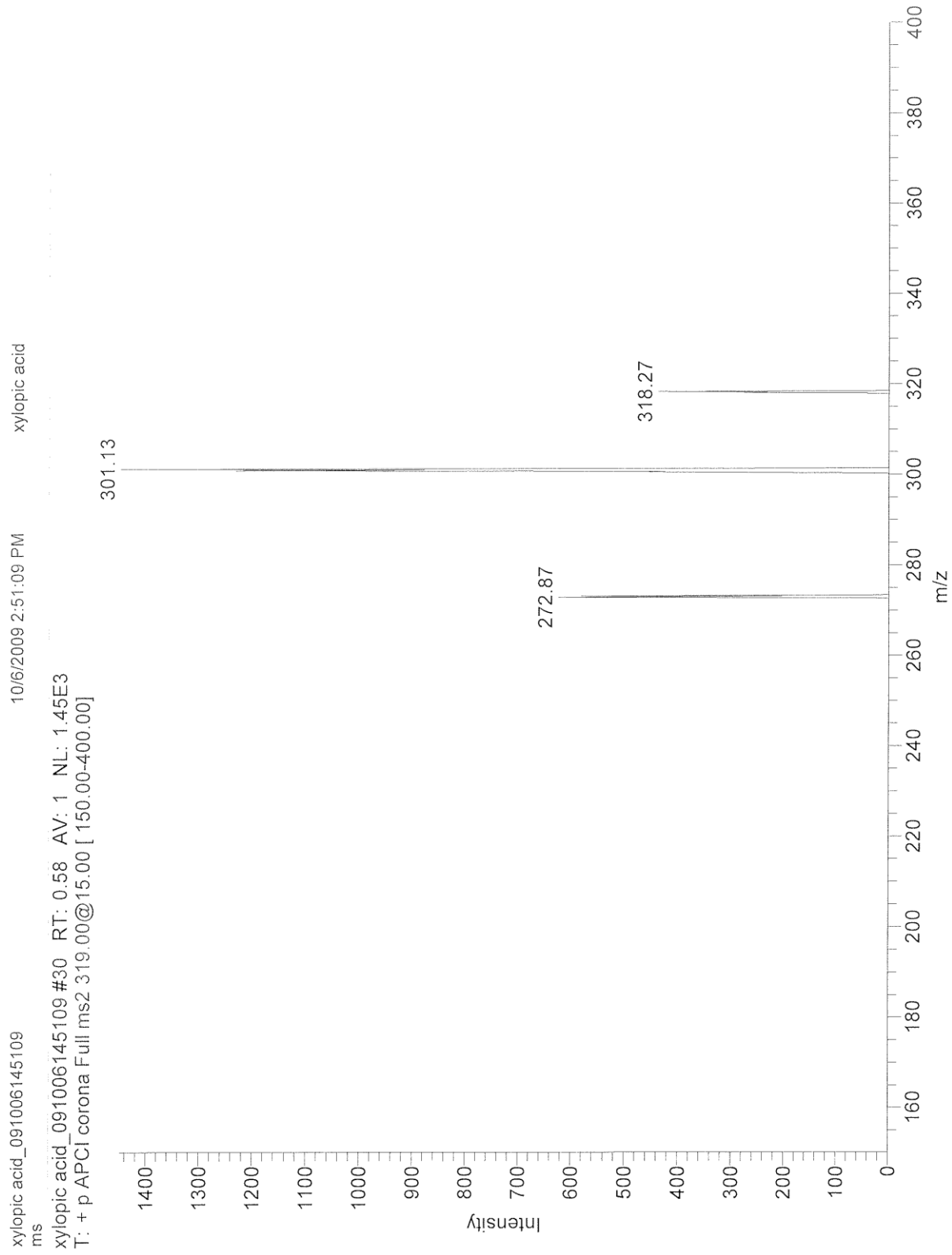

Figure 3.33: Mass spectrum of isolated xylopic acid

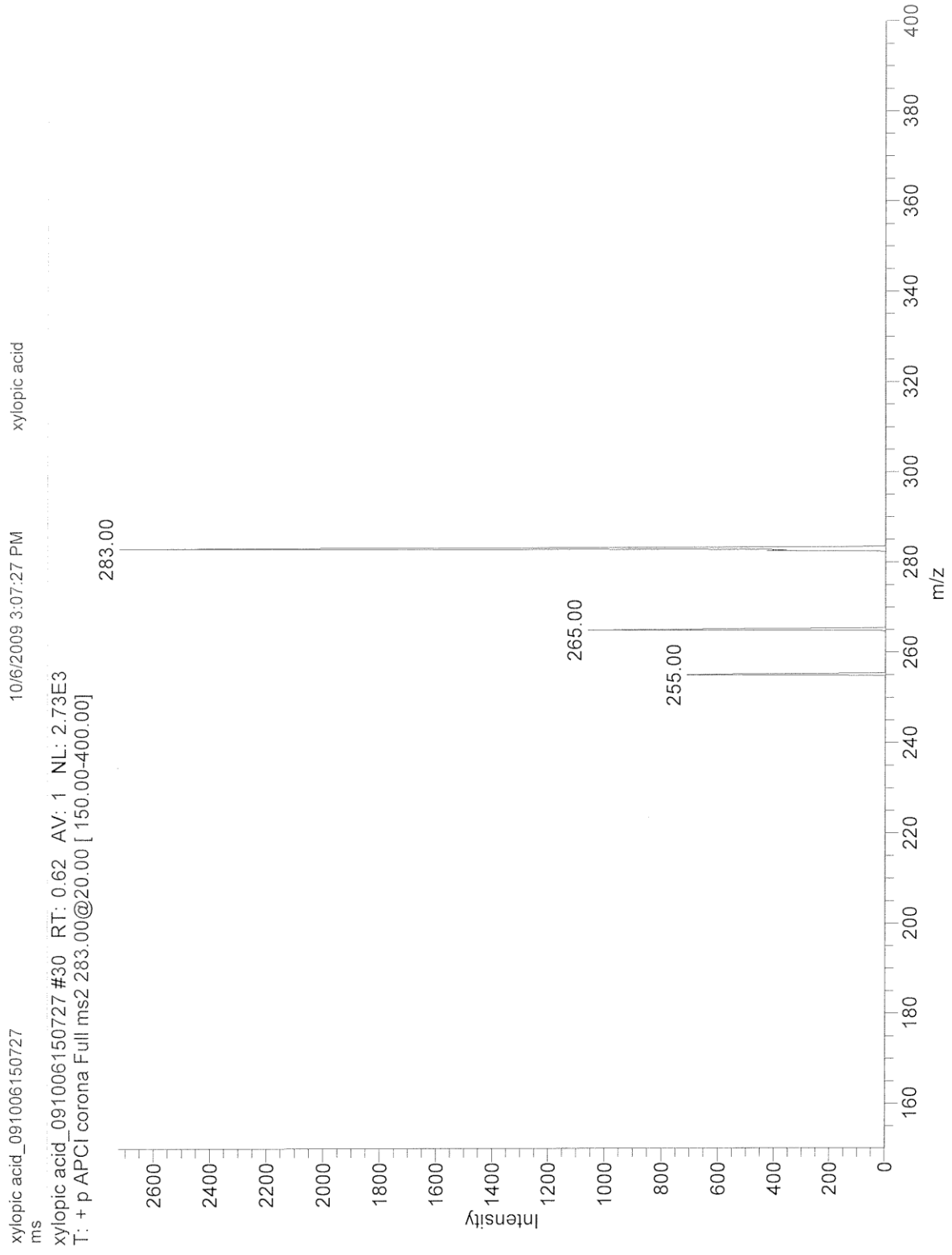

Figure 3.34: Mass spectrum of isolated xylopic acid

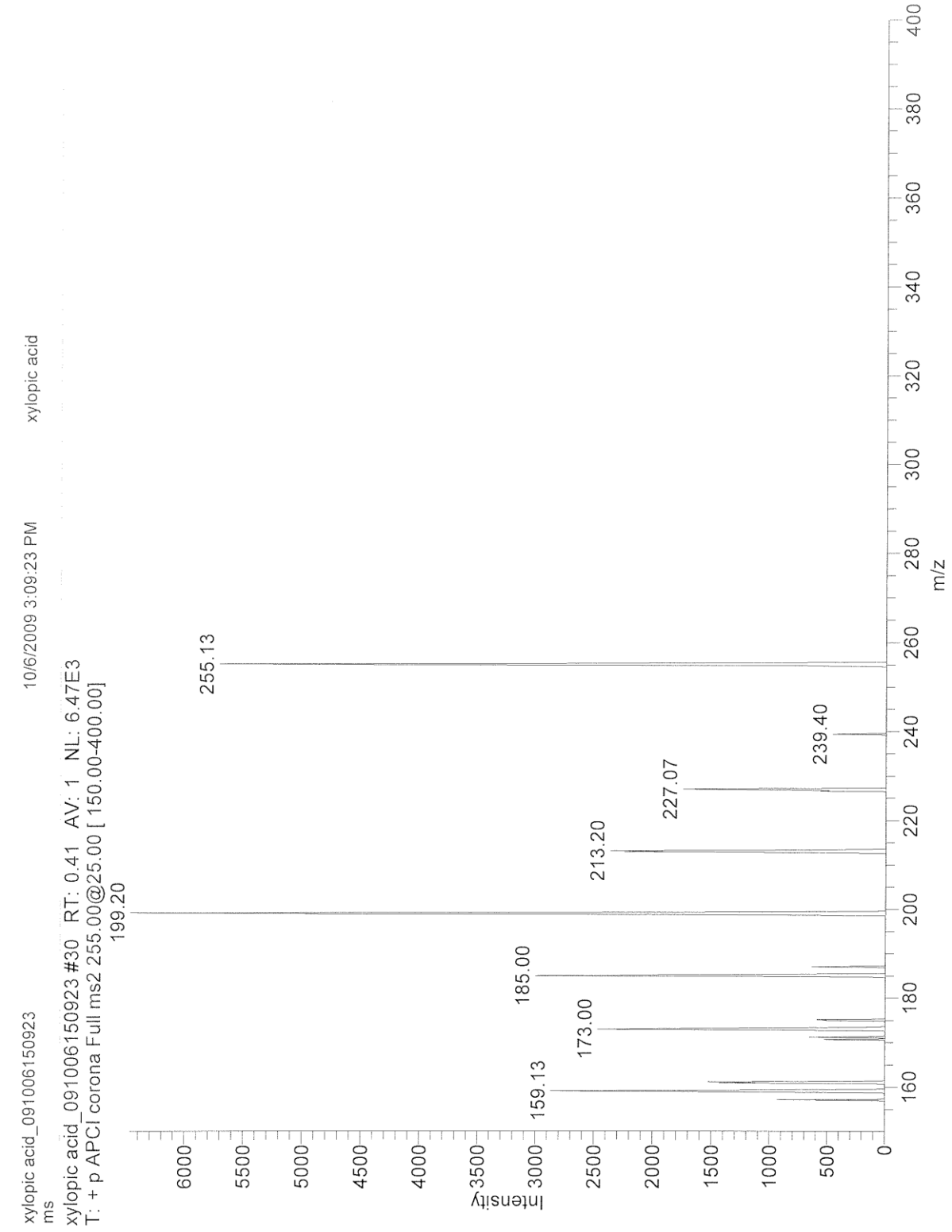

Figure 3.35: Mass spectrum of isolated xylopic acid

CHAPTER FOUR

DISCUSSION AND CONCLUSION

The dried fruits of *Xylopia aethiopica* have been used as a spice among the folk in Ghana. The medicinal properties of this spice have been evaluated by many authors. The fruit contains essential oils **(Tairu A. O. et al, 1999)**, kaurane diterpenes including xylopic acid **(Ekong and Organ, 1968)**, oleoresins **(Ghana Herbal Pharmacopoeia, 2007)** and minerals such as copper, manganese, zinc **(Smith et al, 1996)**. The dried fruits were powdered and the xylopic acid was isolated with petroleum ether (b.p. 40-60^{o}C) with a percentage yield of **0.05%.**

4.1 CHARACTERIZATION OF XYLOPIC ACID

The xylopic acid crystals were recrystallised from distilled ethanol to obtain purified xylopic acid crystals with percentage purity of approximately 95%. The melting point was determined to be **261-262^{o}C** which is similar to the **259-260^{o}C** reported by Ekong and Organ (**Ekong and Organ, 1968)**. The crystals were insoluble in cold methanol but readily dissolved in methanolic NaOH indicating it may be an acidic compound. A solution of the crystals gave a positive test for acidic compound by turning a wet blue litmus paper red. The sodium fusion test carried out on the isolated crystals gave a negative test for Sulphur and Nitrogen. The TLC chromatogram showed only one spot as compared to the many spots showed by that of the crude fruit extract **(fig 3.1)**. This therefore indicates that the purity of the recrystallised xylopic acid isolated is high. The retardation factor (Rf) of the isolated xylopic acid was determined to be **0.53 ± 0.01(Table 3-1)** using petroleum ether (40-60^{o}C): ethylacetate (9:1) as the solvent system and anisaldehyde as the indicator.

4.1.1 SPECTROSCOPIC ANALYSES

Spectroscopic analyses were carried out to further characterise the isolated and purified xylopic acid crystals.

4.1.1.1 PROTON NMR SPECTRUM

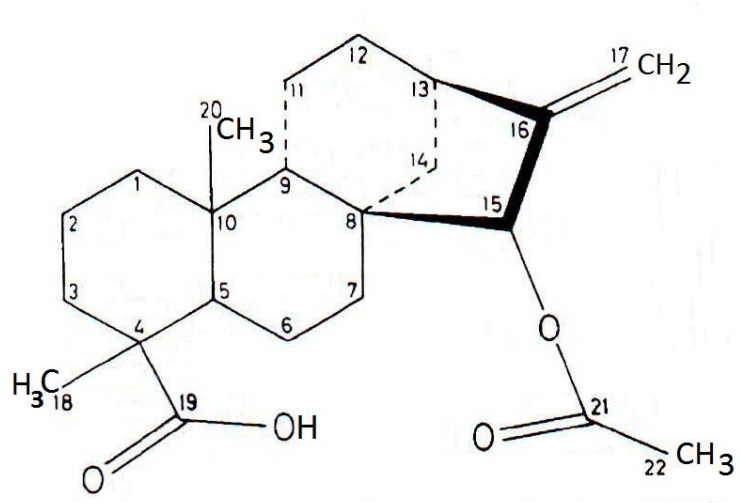

Figure 4.1: Chemical structure of xylopic acid [15β-Acetoxy-(-)-kaur-16-en-19-oic Acid]

The structure and bond angles of xylopic acid **(Fiagbe et al., 1979)** present a variable amount of proton couplings. The singlet signal at 2.139ppm may correspond to the methyl protons at position 22 of xylopic acid chemical structure **(Furniss et al., 1989)**. The possible couplings are shown in the COSY pulse sequence spectrum **(fig 3.14).** The doublet signal at 4.910ppm and 4.849ppm couples with the singlet occurring at 5.128ppm from the spectrum of the COSY pulse sequence, this show clearly that the protons giving rise to these signals are close enough to allow coupling. Furthermore, the singlet at 2.644ppm also couples with the multiplet at 1.0ppm. The signals between 1.320ppm and 2.068ppm may correspond to the protons attached directly to the ring system which couple with one another hence the multiplicity.

4.1.1.2 CARBON-13 NMR SPECTRUM

The signals at 16.038ppm, 21.468ppm, and 29.081ppm corresponding to CH_3 carbons from the spectrum of the Carbon-13 NMR analysis **(fig 3.19)** confirms the three(3) CH_3 at positions 18, 20 and 22 of xylopic acid chemical structure. The number of CH_2 carbons is nine (9) and this is shown by the signals at 18.2ppm, 19.306ppm, 21.527ppm, 33.485ppm, 36.606ppm, 37.889ppm, 39.083ppm, 40.973ppm and 106.312ppm. The bands at 40.82ppm, 47.202ppm, 56.72ppm and 81.868ppm correspond to CH carbons which are carbons 5, 9, 13 and 15 of the chemical structure. Thus the number of all protonated carbons as shown in the spectrum is 16 as there are in xylopic acid. The signals at 153.939ppm, 171.591ppm and 184.648ppm correspond to carbons 17, 19, and 21 which are three of the non-protonated

carbons. However, the signals of the three other non-protonated carbons are absent because in Distortionless Enhancement by Polarization Transfer (DEPT) experiment, quaternary carbons are not detected **(Terence and Burkhard, 2007)**. The quaternary carbons are carbons 4, 8 and 10. The triplet signal at 77ppm is due to the solvent $CDCl_3$ because of the coupling between carbon and deuterium. This triplet signal is however absent when the pulse sequence is DEPT **(Terence and Burkhard, 2007)**.

4.1.1.3 INFRA RED SPECTRUM

The stretching vibrations of carbonyl groups always give rise to intense absorption peaks in the infrared spectrum, and a carbonyl stretching vibration is almost always one of the strongest absorptions in a spectrum **(Lewis D. E., 1996).** The strong absorption bands at 1722.27cm^{-1} and 1703.40 cm^{-1} which occurred together clearly demonstrate the presence of two carbonyl groups in the molecule **(fig 3.27).** The feature which distinguishes carboxylic acid from all other carbonyl compounds is the broad absorption band which occurred at 3276.14 cm^{-1}. This band is the result of strongly hydrogen bonded O-H stretching vibrations **(Furniss et al., 1989)**. The weak band at 940.23cm^{-1} corresponds to O-H out-of-plane deformation (wag) vibration which is a characteristic feature of dimeric acid species **(Furniss et al., 1989)**. The C-O stretching vibration in esters results in very strong bands in the 1300-1100cm^{-1} region which is evident from the strong absorption band appearing at 1272.28cm^{-1} **(Furniss et al., 1989)**. The aforementioned features are consistent with xylopic acid which has carboxylic acid and ester functional groups.

Methyl and methylene groups have asymmetric and symmetric C-H stretching vibration modes, giving rise to absorption bands just below 3000cm^{-1}. The C-H stretching vibrations of unstrained ring systems of cycloalkanes give rise to bands in the same region of the spectrum as acyclic compounds. Thus absorption bands at 2989.10cm^{-1}, 2943.57cm^{-1}, 2927.62cm^{-1}, 2873.52cm^{-1} and 2852.76cm^{-1} correspond to CH_2 and CH_3 vibration modes **(Furniss et al., 1989)**. Xylopic acid is a cycloalkane.

4.1.1.4 MASS SPECTRUM

Much of the structural information in a mass spectrum comes from the fragmentation pattern: the way in which the molecular ion breaks apart to lose its excess energy, involving simple

cleavage and rearrangement **(Lewis D. E., 1996)**. The molecular weight of xylopic acid is approximately 360g. The mass spectrum **(fig 3.31)** of the isolated xylopic acid obtained shows a very weak peak at m/z 360 which is the molecular ion. However, the intense peaks at m/z 301.20 and m/z 301.13 are attributed to the fragment ion after the cleavage of the ester group ($OCOCH_3$). The fragment ion at m/z 213.20 corresponds to the three fused rings without the two methyl (CH_3), the methylene (CH_2), the ester ($OCOCH_3$) and carboxylic acid (COOH) groups whilst the loss of one of the methyl (CH_3), the methylene (CH_2), the ester ($OCOCH_3$) and carboxylic acid (COOH) groups gives a fragment ion at m/z 227.13. The cleavage of the carboxylic acid (COOH) group generates the fragment ion at m/z 318.27. The peak at m/z 272.87 comes about after the loss of the two methyl (CH_3), the methlyene (CH_2) and the carboxylic acid (COOH) groups in the molecule.

4.2 PERCENTAGE CONTENT OF XYLOPIC ACID IN THE CRUDE EXTRACTS USING ISOLATED XYLOPIC ACID AS SECONDARY REFERENCE

Little work has been carried out by researchers to determine the amount of xylopic acid in the dried fruits of *Xylopia aethiopica*. The isolated and purified xylopic acid was used as the reference **(fig 3.2)** for the assay of the crude extracts of the fruit, seed and pericarp. The retention time of the pure xylopic acid was noted and used to identify the peak corresponding to xylopic acid in the crude fruit extract **(fig 3.3).** Calibration curves were plotted on each day before the assay to minimise any random errors. The calibration curves were used to estimate the percentage content of xylopic acid in the fruit, pericarp and seed. The peaks areas of the extracts from the HPLC chromatograms are depicted in **Table 3-3**. The percentage content of xylopic acid in the dried fruit was estimated to be **1.15%$^{w}/_{w}$ ± 0.08 (n=8).** The percentage content of xylopic acid in the dried seeds was determined to be **0.07%$^{w}/_{w}$ ± 0.02 (n=6)** and the dried pericarp of the fruit was found to contain **0.87%$^{w}/_{w}$ ± 0.20 (n=6)** of xylopic acid as shown in **table 3-8**. It can therefore be deduced that most of the xylopic acid content in the fruit is concentrated in the pericarp of the dried fruit with relatively small amount in the seeds. However, both the pericarp and the seed contribute to the total content of xylopic acid in the fruit.

4.3 STABILITY STUDIES

Xylopic acid has an ester functional group which is susceptible to breakdown over time. The isolated and purified xylopic acid crystals were therefore subjected to stability studies to

investigate the decomposition pattern and storage conditions of xylopic acid. The crystals were exposed to temperatures of 50^{o}C, 60^{o}C and 80^{o}C and followed for a period of three months. The HPLC chromatogram (**fig 3.11**) of the crystals after the stability studies shows no peak apart from that for xylopic acid at all the temperature conditions. This may be due to the inability of the broken-down products to absorb UV radiation at a wavelength of 206nm hence not detected by the detector. The concentration of xylopic acid decreased from the 20th day through to the 70th day at all the temperature conditions. At 80^{o}C the concentration of xylopic acid was **67.25%$^{w}/_{w}$**, **56.65%$^{w}/_{w}$** and **41.76%$^{w}/_{w}$** on the 20th, 50th and 70th days respectively. Concentration of **73.28%$^{w}/_{w}$**, **60.65%$^{w}/_{w}$** and **44.14%$^{w}/_{w}$** of xylopic acid was determined on 20th, 50th and 70th days respectively at 60^{o}C. At 50^{o}C, the concentration of xylopic acid was found to be **83.27%$^{w}/_{w}$**, **67.79%$^{w}/_{w}$** and **60.30%$^{w}/_{w}$** on the 20th, 50th and 70th days respectively. The above results depict that xylopic acid crystals are less stable at higher temperatures. It can therefore be deduced that the crystals may be stored at room temperature (approximately 30^{o}C) with relatively low rate of breakdown. Xylopic acid should also be stored in less humid areas because of the ester functional group which can easily be hydrolysed in the presence of water.

4.4 CONCLUSION

Xylopic acid crystals have been isolated from the dried fruits of *Xylopia aethiopica* with a percentage yield of **0.05%$^{w}/_{w}$**. Recrystallization of the crude xylopic acid crystals in distilled alcohol at a temperature of 70^{o}C produced crystals of a purity of approximately **95%**. The melting point of the isolated and purified xylopic acid was determined to be **261^{o}C – 262^{o}C** and the retardation factor (Rf) was determined to be **0.53 ± 0.01** on a precoated silica gel plates. The crystals were further characterised by Nuclear Magnetic Resonance, Infra Red and Mass Spectroscopic analyses.

The amount of extractable xylopic acid in the dried fruits of *Xylopia aethiopica* was determined to be **1.15%$^{w}/_{w}$ ± 0.08 (n=8)** of the fruit weight upon direct analysis by a developed HPLC method(C_{18} stationary phase, mobile phase of 90% methanol:10% water, monitored at 206nm) using isolated xylopic acid as reference. The same method was employed to estimate the amount of xylopic acid in the pericarp and seeds of the dried fruits to be **0.87%$^{w}/_{w}$ ± 0.20 (n=6)** and **0.07%$^{w}/_{w}$ ± 0.02 (n=6)** respectively. The knowledge of the quantity of xylopic acid in the fruit will assist traditional medicine practitioners to prescribe exact doses of xylopic acid to their patients. The method developed can be used by chemists

to standardise preparations of extracts of the fruits of *Xylopia aethiopica* used by practitioners to treat diseases. The stability of xylopic acid crystals was investigated at various temperatures. The decomposition of xylopic acid crystals was highest at 80^{o}C and lowest at 50^{o}C and can thus be stored at room temperature.

4.5 RECOMMENDATONS

- The amount of xylopic acid in the fruit must be investigated seasonally to ascertain the best time to harvest the fruit.
- The method of extraction of xylopic acid from the dried fruits must be investigated to improve the yield.
- Determination of the true order of the thermal degradation of xylopic acid must be done since the stability studies performed was not sufficient to predict the order of kinetics.
- Stability studies with other challenges such as light have to be performed and the decomposition pattern compared to that of the thermal challenge for similarities and differences.
- Suitable methods have to be developed for detection of possible degradation products and acceptable limits set for any toxic components that are discovered.

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