Chapter 1 Introduction

1.1 History

Tuberculosis (TB), a chronic bacterial infection caused by *Mycobacterium tuberculosis* (MTB) is one of the oldest recorded human diseases and one causing one of the world's greatest public health problems (Smith, 2003; WHO, 2010). The disease was recognised many centuries ago and is traced back to the work of Robert Koch (Schluger, 2005). Before then, a French physician Rene Theophile Hyacinthe Laennec (1781-1826) known for his invention of the stethoscope also pioneered the clinical-pathological correlations of TB which marked a better understanding of the pathology and pathogenesis of TB. His landmark work '*A Treatise on Diseases of the Chest',* translated and published in London by John Forbes in 1821, provided figures illustrating tuberculous cavities as well as descriptions of the pathologic changes now referred to as caseous necrosis (Laennec, 1821).

Another forerunner of Koch, Jean-Antoine Villemin a French physician published a study in 1868 titled '*Etudes sur la tuberculosis'* (Studies on Tuberculosis) where he clearly established the infectious nature of TB. However, he was unable to identify the causative agent. Robert Koch announced his achievement at a meeting of the Physiological Society of Berlin in 1882 (Villemin, 1868). Koch contributed enormously to the study of TB.

He developed staining techniques and culture media for MTB as well as demonstrating the transmission mode. Based on his understanding of the spread of disease, the isolation of patients with TB was recommended which remains the standard today.

Koch's postulate states that; *"*To prove that TB is caused by the invasion of bacilli…and multiplication of bacilli, it is necessary to grow them in pure culture ... and by introducing isolated bacilli into animals, to reproduce the same morbid condition that is known to follow from inoculation with spontaneously developed tuberculous material" (Koch, 1882).

Several years after identifying the bacteria responsible for TB, he reported the presence of tuberculin which did not cure the disease but which demonstrated the understanding of cell-mediated immune responses now recognised as the driving forces behind the clinical manifestations of TB. A refinement of the preparation remains a key component of TB diagnostics today (Koch, 1882; Koch, 1890).

Mycobacterium tuberculosis (MTB) genus *Mycobacterium* the causative agent of TB, like other actinomycetes were initially found in soil (Stead, 1997; Smith, 2003). It is believed that some species evolved to live in mammals through the domestication of cattle. "*Specifically, it has been hypothesized that M. bovis, which causes a TB-like disease in cattle, was the hypothetical evolutionary precursor of MTB* ".

Data generated from characterisation of the genomes of the MTB complex by deoxyribonucleic acid (DNA) sequencing and related methods shows a greater than 99.9% similarity of DNA sequence among the members. The existence of synonymous single-nucleotide polymorphisms (sSNP) allows discrimination between these closely related bacteria and the results suggest that *M. bovis* evolved at the same time as MTB (Brosch *et al*., 2002; Sreevatsan *et al.,* 1997; Smith, 2005).

Consequently*, MTB and M. bovis* are thought to have possibly evolved independently from a species closely related to *M*. *canetti* based on strong evidence from a study of the distribution of deletions and insertions in the genomes of the MTB complex (Brosch *et al*., 2002; Smith, 2005).

1.2 General Description of *Mycobacterium tuberculosis* genus

The term tubercle bacillus designates two species of the family Mycobacteriaceae, order- Actinomycetales: *M. tuberculosis* and *M. bovis* ((Murray *et al.*, 2005; Todar, 2009).

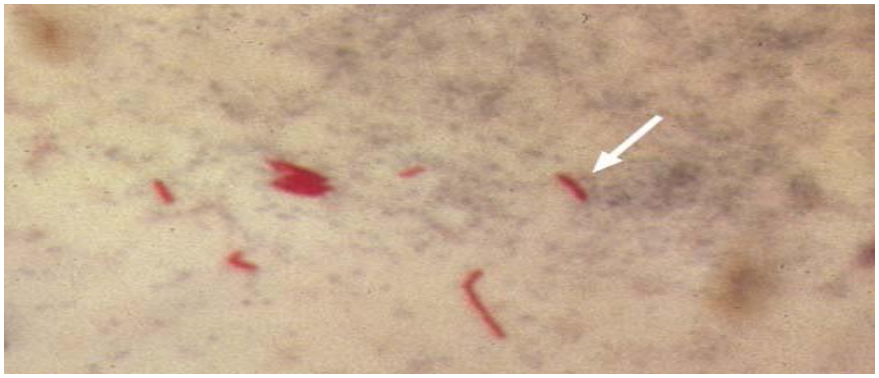


Fig.1.0 MTB acid fast bacilli (Reproduced from CDC, Centres for Disease Control and Prevention, 2005).

They differ from other mycobacterial species that share the staining characteristic referred to as acid fastness (Figure 1.0). Three other species *M*. *ulcerans*; *M. microti*, a pathogen for rodents and *M. africanum* an organism thought to be intermediate between *M. tuberculosis* and *M. bovis* are said to be a rare cause of human tuberculosis in Africa (Murray *et al.*, 2005). They are closely related and represent other members of the *M. tuberculosis* complex. Disease due to *M. bovis* is relatively rare and the terms tubercle bacillus and *M. tuberculosis* are synonymous. Humans are the only known reservoir of *M. tuberculosis*. Many non-pathogenic mycobacteria frequently form part of the normal flora (Todar, 2005).

1.2.1 Physiology and Morphology of Mycobacteria

Bacteria are classified in the genus *Mycobacterium* on the basis of their acid-fastness, the presence of mycolic acids (containing 60-90 carbons that are cleaved by pyrolysis to C22-C26 fatty acid methyl esters) and a high (61-71%) guanine plus cytosine content in their deoxyribonucleic acid (Murray *et al*, 2005).

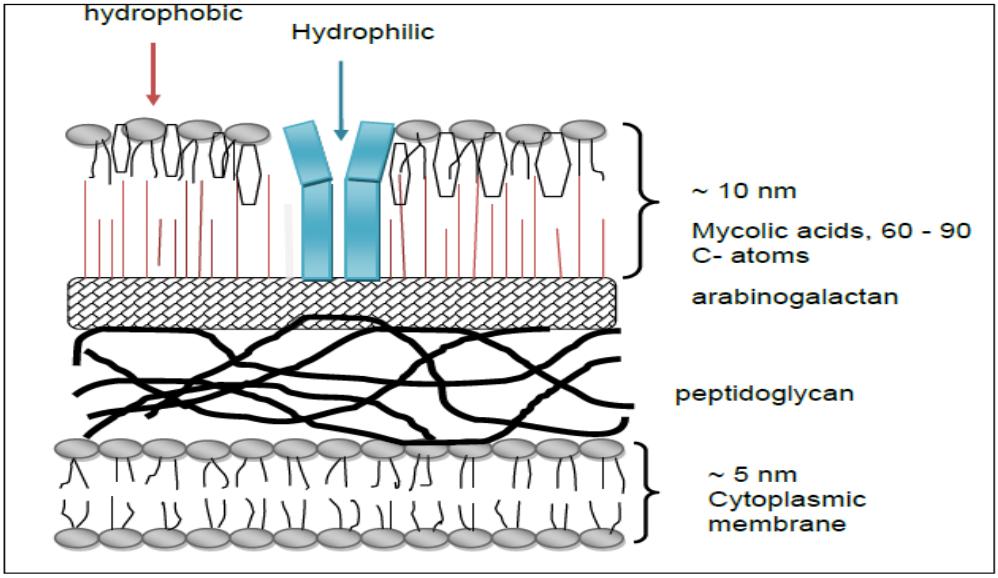


Fig.1.1 Schematic diagram of mycobacterial cell wall (adapted from Niederweis, 2011).

Species such as *Nocardia, Rhodococcus*, *and Gordonia* are partially acid-fast but have their mycolic acid chains shorter than mycobacterium. The structure of the cell wall is typical of gram positive bacteria having an inner plasma membrane overlaid with a peptidoglycan layer and no outer membrane (Fig. 1.1). The rigid core of the cell wall is composed of three macromolecules; peptidoglycan, arabinogalactan and mycolic acids. Anchored in the plasma membrane are proteins, phosphatidylinositol mannosides (PIM) and lipoarabinomannan (LAM).

The peptidoglycan layer forms the foundation upon which arabinogalactans (a branched polysaccharide consisting of D-arabinose and D-galactose) are attached. Lipids, glycolipids and peptidogycolipids are also present and altogether comprise 60% of the cell wall weight. 15% of this weight is constituted by transport proteins and porins (pore-forming proteins) which intersperse the cell layers. The purified protein derivatives (PPDs) are used as skin test reagents to measure exposure to MTB (Marwick, 1992; Daniel, 1991; Murray *et al.*, 2005).

1.2.2 Growth and cultural characteristics

Characteristic properties exhibited by MTB such as acid-fastness, slow growth, resistance to detergents, to common antibacterial antibiotics, antigenicity and clumping is attributed to the complex lipid cell wall they possess (Murray *et al*., 2005).

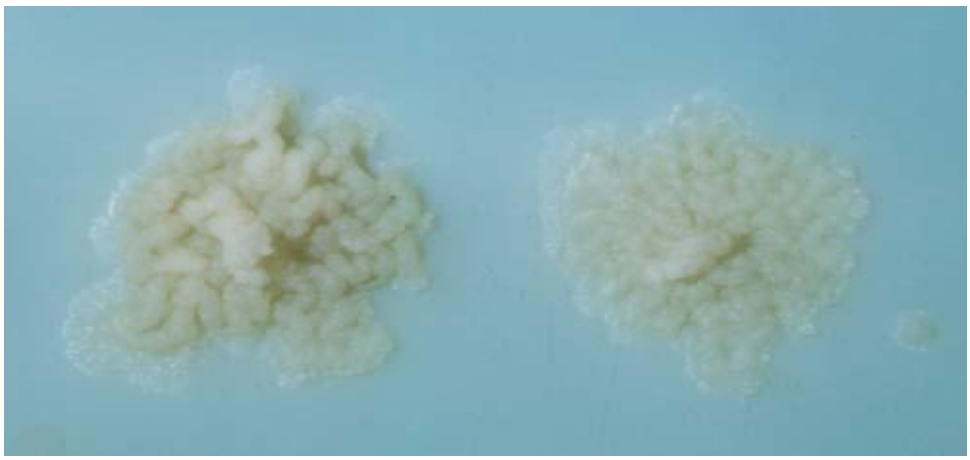


Fig.1.2 Colonies of *Mycobacterium tuberculosis* on Lowenstein-Jensen medium CDC (Todar, 2009).

M. tuberculosis grows slowly, lacks pigment, produces niacin, reduces nitrates, produces heat-sensitive catalase (inactivated by heating to 68˚C at pH 7.0) in small quantity and is usually sensitive to Isoniazid (INH). These characteristics have been used to differentiate MTB from other mycobacteria (Mandell *et al*., 2000).

INH-resistant strains are not known to produce catalase while *M. bovis* is usually niacin-negative and does not reduce nitrates. Stained smears prepared from BACTEC bottles demonstrate serpentine 'cording' which serves as a sensitive and specific marker for *M. tuberculosis* whereas mycobacteria other than tuberculosis orient randomly (Yagupsky *et al*., 1990). *M. tuberculosis* is a fairly large non-motile rod-shaped bacterium distantly related to the Actinomycetes with a high cell wall content of high molecular weight lipids.

The rods are 2 - 4 µm in length and 0.2 - 0.5 µm in width (Mandell *et al.,* 2000; Todar, 2009). *M. tuberculosis* is an obligate aerobe and it is generally accepted that MTB complexes are always found in the well-aerated upper lobes of the lungs. It is a facultative intracellular parasite normally found living in macrophages with a slow generation time of fifteen - twenty hours, compared with much less than one hour for most bacterial pathogens. Growth is slow with the generation time being between fifteen to twenty hours. Visible growth takes place from four to six weeks on solid media (Figure 1.2).

The slow generation time is a physiological characteristic that may contribute to its virulence (Todar, 2009). Two media are used to grow MTB, Middlebrook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium. MTB colonies are small and buff coloured when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing MTB (Brooks and Carroll, 2007; Todar, 2009).

1.2.3 Antigenic structure

Mycobacteria being complex unicellular organisms contain many antigenic proteins and sugars. The antigens are divided into cytoplasmic (soluble) and cell wall lipid bound (insoluble) antigens. These features have been used to classify species and type strains (Table 1.1).

Table 1.1. Description of antigenic proteins

Group	Description
i	Those common to all mycobacteria
ii	Those restricted to slowly growing species
iii	Those occurring in rapidly growing species
iv	Those unique to each individual species

The soluble antigens are divisible into those that remain within the cell cytoplasm until released by autolysis or mechanical disruption and those that are actively secreted by living whole cells (Grange, 1996). MTB epitopes (antigenic determinants) are divisible according to their distribution within the genus. Stanford and Grange (1974) described four groups of soluble antigens as shown in Table 1.1 and Figure 1.3.

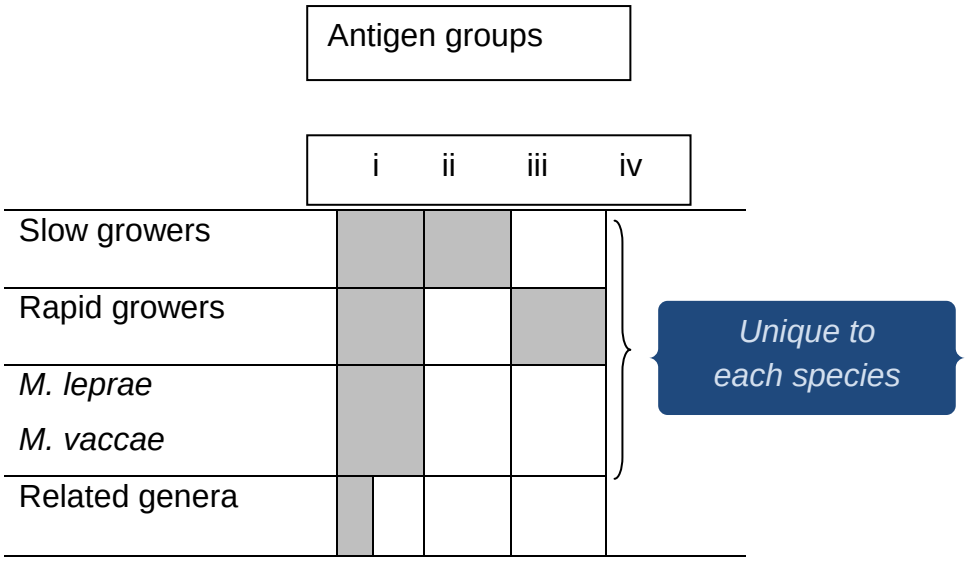


Fig.1.3 The distribution of soluble antigens in the genus *Mycobacterium* (adapted from Grange, 1996).

Several workers have attempted to isolate the species specific (group iv) antigens for use in diagnostic tests but that proved difficult in that they found that specific epitopes often occur on the same protein as shared epitopes (Grange, 1996). Another phenomenon reported was that a given determinant may be present on a range of molecules of differing physical and chemical properties. Nevertheless, A60 antigen, the major heat stable component of PPD, which forms the basis of the sero-diagnostic test for TB has been characterised by gel filtration and ion exchange chromatography (Charpin *et al*., 1990).

Serotypes have been identified in several other species but not in MTB which is rough and readily auto - agglutinates. The responsible antigens have been identified as the sugar moieties on a group of peptidoglycolipids and phenolic glycolipids collectively termed mycosides (Grange, 1996).

1.2.4 Pathogenicity and virulence determinants

According to Vera-Cabrera *et al*., 2007, the basis for MTB virulence is not completely understood. Although genetic changes in MTB was considered to occur very rarely, micro hybridisation assays in conjunction with the complete genomic sequence of MTB show that large sequences of DNA can be deleted more frequently than expected. A group of MTB isolates devoid of the phospholipase C-type enzymes plcA-plcB-plcC were characterised by polymerase chain reaction, sequencing and micro array techniques (Frothingham *et al*., 1994; Cole *at al*., 1998, Fleischmann *et al*., 2002).

Phospholipases C are well recognised virulence factors of several microorganisms, including *Clostridium* spp, *Listeria monocytogenes*, *and Pseudomonas aeruginosa etc.* They act by either destroying cellular tissues as in *Clostridium spp* or by lysing the phagolysosomal membranes and releasing intracellular microorganisms in the cytoplasm as in the case of *L. Monocytogenes* (Williamson and Titball, 1993; Tilney, 1989; Vera- Cabrera et al., 2007). Several genes encoding phospholipase C – type enzymes; plcA-plcB-plcC and plcD have been found in MTB. Types A, B and C are similar unlike plcD. This was found to be missing, or interrupted in many MTB isolates including H37Rv (a virulent strain) (Cole *et al*., 1998; Vera- Cabrera *et al*., 2007).

Studies on phospholipases by Raynaud *et al.* (2002) showed the deficiency in the ability of triple or quadruple mutants to multiply in the lungs and spleen of infected animals, with possible relevance to the survival of MTB in tissues. Investigations by other workers demonstrated less cavitary disease in humans with the more the genomic deletions in a clinical isolate of MTB (Kato-Maeda *et al*., 2001).

A study by Glickman, Cox and Jacobs (2000) on the genetic basis for cording (the formation of strand-like clumps of mycobacteria in culture) a phenotype associated with virulence; found that cording behaviour was dependent on normal functioning of the pca A gene (a novel member of a family of MTB S-adenosyl methionine- dependent methyl transferases). It has been shown to be required for α-mycolic acid cyclopropanation which is an important component of the MTB cell wall synthesis. When the gene was mutated and used to infect mice in a model which is usually lethal, the organisms did not persist or kill the animals, suggesting that the pca (pyruvate carboxylase) gene is indeed a virulence factor. Protein tyrosine phosphatase (MptpB) has also been implicated in MTB virulence (Singh *et al*., 2003; Soeller *et al.,* 2007). Based on their studies, MptpB is considered to deactivate human proteins involved in γ-interferon signalling pathway which in turn would prevent the immune system from acting against the bacteria.

1.2.5 Genetics of M. tuberculosis

The complete sequencing of MTB DNA was accomplished in 1998 by investigators at the Pasteur Institute. The sequencing of the virulent laboratory strain H37Rv revealed the genome to contain 4,411,529 base pairs encoding over 4,000 genes. A high percentage of guanine-cytosine rich sequences and large numbers of genes devoted to fatty acid synthesis were observed (Cole *et al*., 1998; Behr *et al.,* 1999; Fleischmann *et al*., 2002; Schluger, 2007).

Certain populations appear to have a high degree of vulnerability to TB. Extreme susceptibility to the disease has been observed in Eskimo populations in North America, in Yanomami Indians in the Brazilian Amazon and in black populations in the United States (Hoeppner and Marciniuk, 2000; Sousa *et al*., 1997; Stead *et al*., 1990).

It has been recognised that a substantial percentage of individuals will recover from TB without drug treatment. Historically, a tragic experience occurred in Lubeck, Germany in the year 1929 where 249 infants were accidentally injected with a stock of live, virulent MTB. This led to the death of 76 infants with 173 survivors thereby lending support to the notion of some capability for innate

resistance (Schluger, 2005). A clue to the genetic basis of resistance was provided by Gros and colleagues (1981), who identified a strain of mice with rare vulnerability to overwhelming infection with leishmania, salmonella and certain mycobacteria particularly *M. bovis*.

Genetic mapping of murine chromosome by some scientists identified a gene initially called *Bcg* but later re-named *nramp1* to be responsible for the production of a so-called natural resistance-associated macrophage protein. (Supek *et al*., 1996 ; Blackwell *et al*., 1995, 1996). Bellamy *et al.* (1998) have identified several polymorphisms in the human *nramp*1 gene and from studies conducted; there is increased relative risk for moving from latent infection to active disease associated with certain polymorphisms. However, the risk attributable to these polymorphisms is believed to be relatively small and it was shown by their investigation that resistance or susceptibility to TB is a complex genetic trait (Dorman *et al.,* 2004, 1998).

1.2.6 Clinical findings and diagnosis

MTB is transmitted primarily *via* the respiratory route. The organism expelled during coughing, sneezing or talking in the droplet form or as the desiccated airborne bacilli enters the respiratory tract and a person only need inhale a small number of these to be infected (WHO, 2010). Early symptoms of active TB can include weight loss, fever, night sweats, and loss of appetite.

Symptoms may be vague, however, and go unnoticed by the affected person. For some, the disease either goes into remission (halts) or becomes chronic and more debilitating with cough, chest pain, and bloody sputum. Symptoms of TB involving areas other than the lungs vary, depending upon the organ or area affected (NIAID, 2007).

To identify those who may have been exposed to *M. tuberculosis*, health-care providers typically inject the purified protein derivative called tuberculin under the skin of the forearm. If a red welt forms around the injection site within 72 hours, the person may have been infected however, this doesn't necessarily mean he or she has active disease.

People who may test positive on the tuberculin/mantoux test include

- Most people with previous exposure to *M. tuberculosis*
- Some people exposed to bacteria related to *M. tuberculosis*
- Some people born outside the United States who were vaccinated with the TB vaccine used in other countries

If people have an obvious reaction to the skin test, other tests can help to show if they have active TB. In making a diagnosis, doctors rely on symptoms and other physical signs, the person's history of exposure to TB and X-rays that may show evidence of *M. tuberculosis* infection. The chest X-ray may show lower zone infiltrates and unilateral or diffuse bilateral shadowing. In some cases the chest X-ray may be normal in spite of being sputum culture-positive (Woodhouse, 2003; NIAID, 2007).

The health care provider would take sputum and other samples to see if the TB bacteria will grow in the laboratory. If bacteria grow, this positive culture confirms the diagnosis of TB. Due to the fact that *M. tuberculosis* grows very slowly, it can take up to four weeks to confirm the diagnosis. An additional two to three weeks usually are needed to determine which antibiotics can be used to treat the disease.

1.2.7 Immunity and vaccination

One study in 1978, prior to the Acquired Immune Deficiency Syndrome (AIDS) epidemic showed that 85% of new TB cases were pulmonary but MTB can spread through the blood stream and the lymphatic system to the brain, bones, eyes, and skin (extra pulmonary TB) (Hopewell, 1994; Smith, 2003; Williams *et al.*,1995).

According to Wallgren (1948), the progression and resolution of the disease can be divided into four stages. The first stage involves inhaled aerosols becoming implanted in alveoli after three to eight weeks leading to dissemination by the lymphatic system to regional lymph nodes in the lung forming the primary or Ghon complex. Conversion to tuberculin reactivity occurs at this stage.

The second stage is characterised by the haematogenous spread of bacteria into many organs and in some individuals, this disseminated (miliary) TB can be acute and fatal. This lasts for about three months. The third stage lasting three to seven months may involve inflammation of the pleural surfaces causing chest pain but this stage can be delayed for up to two years. Then free bacteria or their components are thought to interact with sensitised CD4 T lymphocytes that are attracted leading to proliferation and release of inflammatory cytokines (Wallgren, 1948; Kamholz, 1996; Smith, 2003).The last stage where the disease does not progress may take up to three years.

Murray *et al.* (2005) describes the histological signs as being due to components of the host response to the infection rather than specific virulence factors elaborated by the bacteria. The helper (CD4+) T cells and cytotoxic (CD8+) T cells are stimulated as the bacteria grows leading to antibody production by the CD4+ cells. However the response elicited has been shown to be ineffective in controlling the disease because the bacteria are protected in their intracellular location. Interferon-γ and other cytokines released activate macrophages which can engulf and kill the bacteria as well as cytotoxic T cells which can also lyse phagocytic cells with replicating bacteria.

Mycobacteria are destroyed with minimal tissue damage to the host if the bacterial load is small but when the bacteria are many, the cellular immune response results in tissue necrosis. Host factors such as cytokine toxicity, ischaemia, local activation of the complement cascade are involved in the process (Murray *et al*., 2005).

The size of the locus of infection determines how easily the bacteria are eliminated while localised collections of activated macrophages (granulomas) prevent the further spread of the bacteria. Small granulomas are killed by macrophages while larger necrotic granulomas become encapsulated with fibrin which protects it. The bacteria can remain dormant in this state or be reactivated years later when the patient's immune system is compromised. This accounts for the reason why disease may not develop in those exposed to MTB until late in life (Murray *et al*., 2005)

1.3 Epidemiology (Global Incidence)

According to WHO report on TB;

- Someone in the world is newly infected with TB bacilli every second
- Overall, one - third of the world's population is currently infected with the TB bacillus
- 5 -10% of people who are infected with TB bacilli but not with HIV become sick or infectious at some time during their life. People with HIV and TB infection are much more likely to develop TB (WHO Fact sheet No. 104).

The World Health Organisation (WHO) report estimated that the largest number of new TB cases in 2005 occurred in the South- East Asia Region accounting for 34% of incident cases globally. However, the rate in sub - Saharan Africa is almost double that of South – East Asia at an estimated 350 cases per 100 000 population (Table 1.2, Figures 1.4.1- 3). WHO 2007 report estimated that 1.6 million deaths resulted from TB in 2005 equal to an estimated 4400 deaths a day.

In 2009, the report estimated 1.7 million deaths with most of the deaths occurring in Africa. The 2010 report estimated 8.8 million new cases with 1.4 million deaths including 0.35 million people with HIV.

Globally, TB is pandemic but the highest rates *per capita* are in Africa where the scourge has been fuelled by the partnership with HIV/AIDS (Table 1.2). Among people living with HIV/AIDS, TB has been found to be the leading cause of death and about 200 000 people living with HIV/AIDS die from TB every year (Maher and Raviglione, 1999; WHO, 2010).

Multi-drug resistance (MDR) has occurred in virtually all countries recently surveyed by WHO and partners. About 450 000 MDR- TB new cases are estimated to occur each year with countries in the former Soviet Union and China having the highest rates. The World Health Organization Global Task force on XDR-TB in October 2006 defined resistance to the two first line drugs, isoniazid and rifampicin as multi-drug resistant TB (MDR-TB) while resistance to at least two first line drugs plus resistance to any fluoroquinolones and any one of the second line anti -TB injectable drugs (amikacin, kanamycin or

capreomycin) i.e. three of six of the second line drugs is referred to as extensively drug-resistant TB (XDR-TB) [Figure 1.5].

XDR-TB which is extremely difficult to treat has also been confirmed in all regions of the world. This situation was highlighted as a global threat to public health by WHO in 2006, especially in high HIV-prevalent countries.

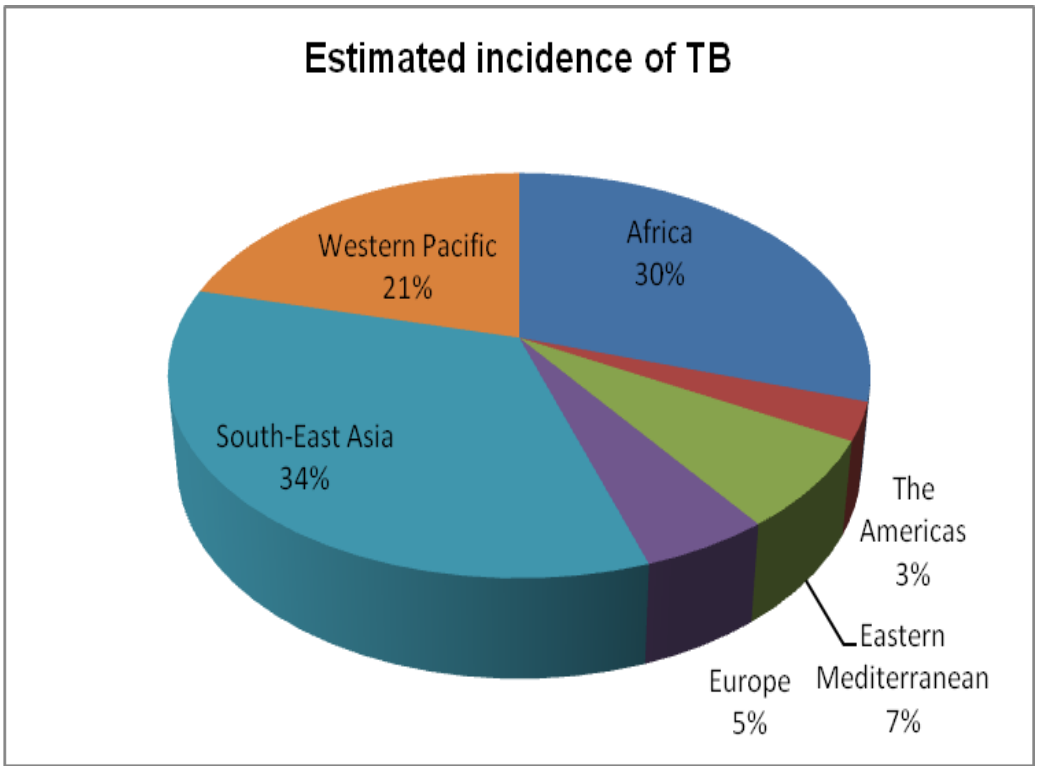


Fig.1.4.1 Chart illustrating estimated incidence of TB globally in 2008 (www.who.int/topics/tuberculosis/en)

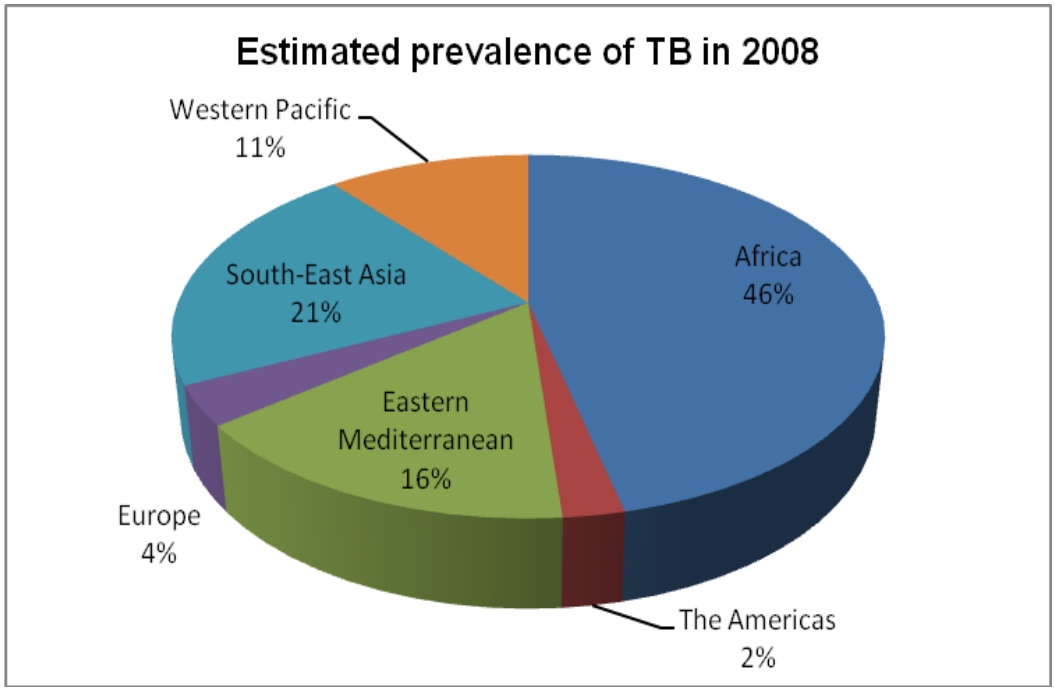


Fig.1.4.2 Chart illustrating estimated prevalence of TB in 2008 (www.who.int/topics/tuberculosis/en)

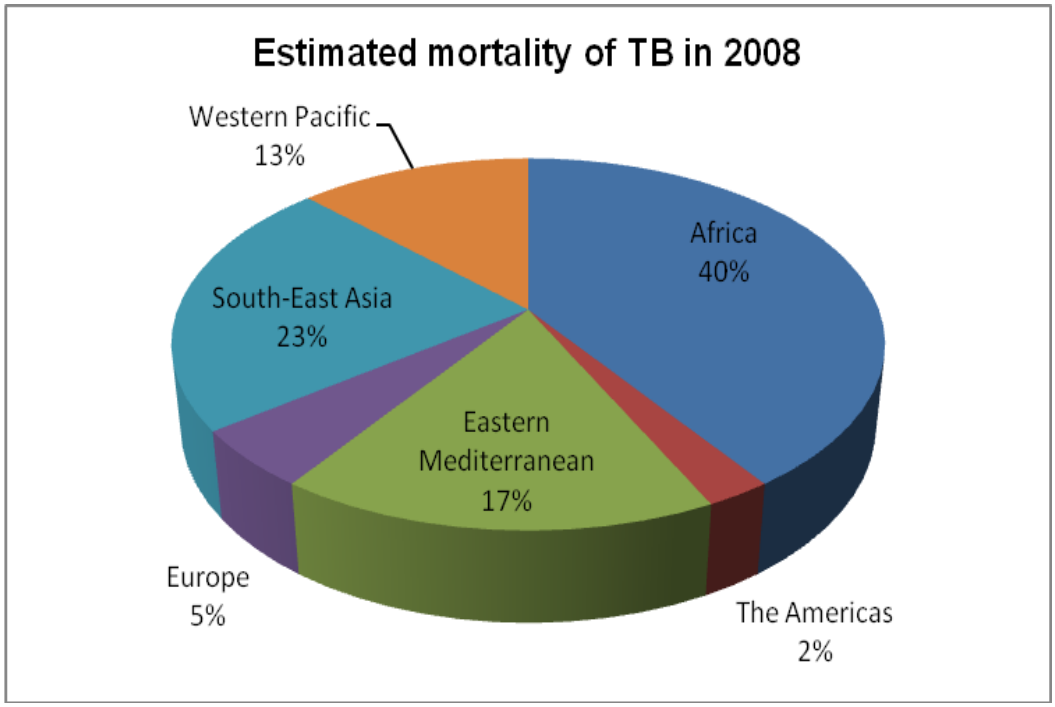


Fig. 1.4.3 Chart illustrating estimated mortality of TB in 2008 (www.who.int/topics/tuberculosis/en).

Table 1.2. Estimated TB incidence, prevalence and mortality, 2008

	Incidence[1]			**Prevalence** [2]		**Mortality**	
WHO region	no. in thousands	% of global total	rate per 100 000 pop[3]	no. in thousands	rate per 100 000 pop	no. in thousands	rate per 100000 pop
Africa	2 828	30%	351	3 809	473	385	48
The Americas	282	3%	31	221	24	29	3
Eastern Mediterranean	675	7%	115	929	159	115	20
Europe	425	5%	48	322	36	55	6
South-East Asia	3 213	34%	183	3 805	216	477	27
Western Pacific	1 946	21%	109	2 007	112	261	15
Global total	9 369	100%	139	11 093	164	1 322	20

[1] Incidence is the number of new cases arising during a defined period.
[2] Prevalence is the number of cases (new and previously occurring) that exists at a given point in time.
[3] Pop indicates population.

Reproduced from www.who.int/mediacentre/factsheets/

1.3.1 TB and HIV

TB and HIV form a deadly partnership each speeding up the other's progress. Someone who is HIV- positive and infected with TB bacilli is many times more likely to become sick with TB than a HIV negative person. HIV weakens the immune system and studies have shown that the natural history of HIV infection is altered by TB.

Antigen-specific type 1 CD4+ T-helper cells provide stimulatory signals such as cytokine gamma-interferon which activates TB-infected macrophages to limit intracellular replication of MTB (Woodhouse, 2003; WHO, 2010). These cells are also targeted by HIV virus and ultimately destroyed. HIV viral load is observed to rise in TB disease and this has been demonstrated *in vitro* (Goletti *et al*., 1996).

1.3.2 Principles of therapy

The goals of anti-TB therapy are to ensure a cure without relapse, to prevent death, to stop transmission of MTB and to prevent the emergence of drug-resistant disease (Blumberg and Leonard, 2002). Therapy is initiated with a multi-drug regimen to kill the bacteria rapidly in order to minimise or prevent the development of resistant strains as well as eliminate persistent organisms to prevent relapse (Blumberg and Leonard, 2002).

The treatment of TB has two phases; initiation also known as the bactericidal or intensive phase and continuation (subsequent sterilising phase). The two phases reflect the understanding of the patho-physiology of TB. Based on this premise, three sub-populations of MTB are thought to exist in the host with TB (Blumberg and Leonard, 2002). MTB is an obligate aerobe so the rapidly growing extra-cellular organisms constitute the largest sub-population which resides in well-oxygenated cavities containing 10^7 - 10^8 organisms. The second sub-population resides within poorly oxygenated caseous lesions containing 10^4-10^5 organisms. These are considered semi-dormant and undergo intermittent bursts of metabolic activity (Inderlied and Nash, 1996; Pfyffer, 2000; Blumberg and Leonard, 2002).

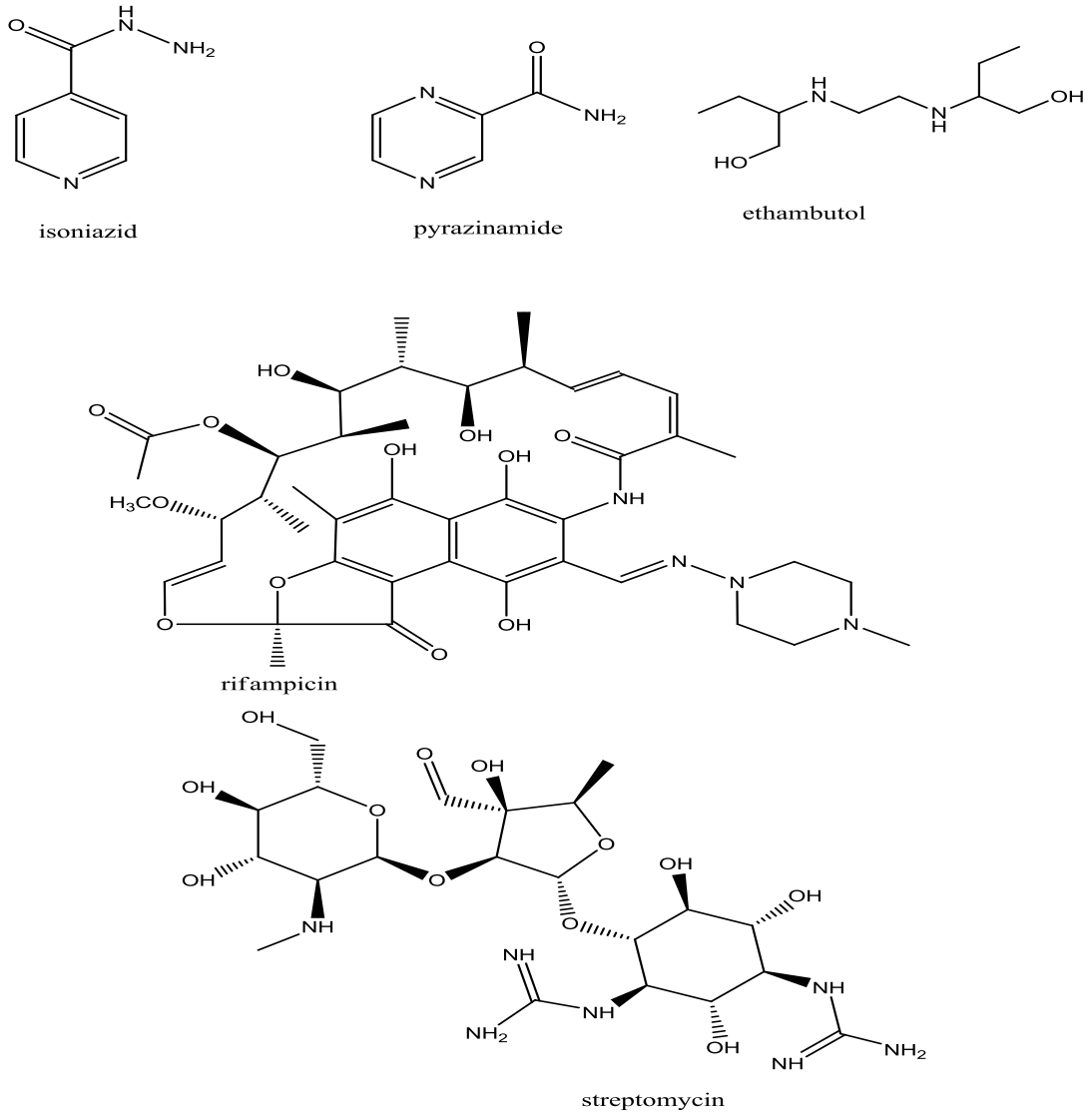


Fig. 1.5 Chemical structures of first line drugs used in therapy

The third sub-population consists of a small number of organisms fewer than 10^4-10^5 in number. They are believed to be semi-dormant within acidic environments whether intra-cellular (in macrophages) or extra-cellular within areas of active inflammation and recent necrosis (Blumberg and Leonard, 2002).

Isoniazid and rifampicin are two drugs which form the cornerstone of anti-TB therapy (Figure 1.5). Isoniazid has early bactericidal activity while pyrazinamide

(PZA) has weak early bactericidal activity within the first few weeks of therapy. The rapidly dividing cells are eliminated early in effective therapy and after two months of treatment, about 80% of patients are culture negative (WHO, 2010; Blumberg and Leonard, 2002). The second and third sub-populations are responsible for treatment failures and relapses so prolonged treatment is required. The sterilising properties of rifampicin and PZA are utilised in the continuation phase. The activity of rifampicin persists throughout therapy while that of PZA is mainly seen in the first two months (Blumberg and Leonard, 2002). Treatment of MDR-TB requires 18 - 24 months of treatment with second line drugs which are less active, more toxic and expensive (WHO, 2010).

1.3.3 Drugs used in TB treatment and mechanisms of action

The initial therapy for TB consists of a four drug regimen; isoniazid, rifampicin, pyrazinamide and ethambutol. The minimum duration for the treatment of TB with a rifampicin based regimen takes six to nine months (short-course therapy). Longer courses are required for drug resistant TB such as MDR or XDR-TB (WHO, 2010; Blumberg and Leonard, 2002; NIAID, 2007). Table 1.4 and Figures 1.6a -1.6c illustrate the drugs used and their mechanisms of action while Table 1.5 summarises the actions. Isoniazid and ethambutol act by inhibiting cell wall synthesis while rifampicin acts by inhibiting DNA dependent RNA polymerase.

The mechanism by which pyrazinamide acted was unclear but recent studies (Shi *et al*., 2011) show that when the drug converts to its active form, pyrazinoic acid it then binds to an essential protein named ribosomal protein S1 (RpsA) which in turn prevents the production of other proteins needed by the bacteria for physiological functioning (Figure 1.6b). Ethambutol is believed to act by inhibiting cell wall synthesis at the arabinogalactan layer. The proposed sites of drug activity for other drugs are shown in Figures 1.6a to 1.6d. Where drug resistance occurs to first-line drugs isoniazid and rifampicin, second–line drugs such as aminoglycosides and fluoroquinolones are added as combination therapy. With XDR-TB, treatment options are limited. However, other second line drugs which are more toxic are used and for a longer duration of therapy

(Table 1.3). Table 1.4 describes the different modes of general antibacterial actions.

Table 1.3. Drugs used in treating drug sensitive TB, options for treating MDR-TB and XDR-TB as well as new drugs under development

Drug sensitive TB	MDR-TB	XDR-TB	New TB drugs
Isoniazid (1952)	thioamides	ethambutol	pyrroles
Rifampicin (1966)	cycloserine	pyrazinamide	macrolides
Ethambutol (1961)	cyclic peptides	thioamides	oxazolidinones
Pyrazinamide (1952)	aminoglycosides	cycloserine	nitroimidazole e.g.PA-824,OPC 67683
	fluoroquinolones	PAS	diarylquinoline e.g. TMC 207
	p-aminosalicylic acid (PAS)	streptomycin	

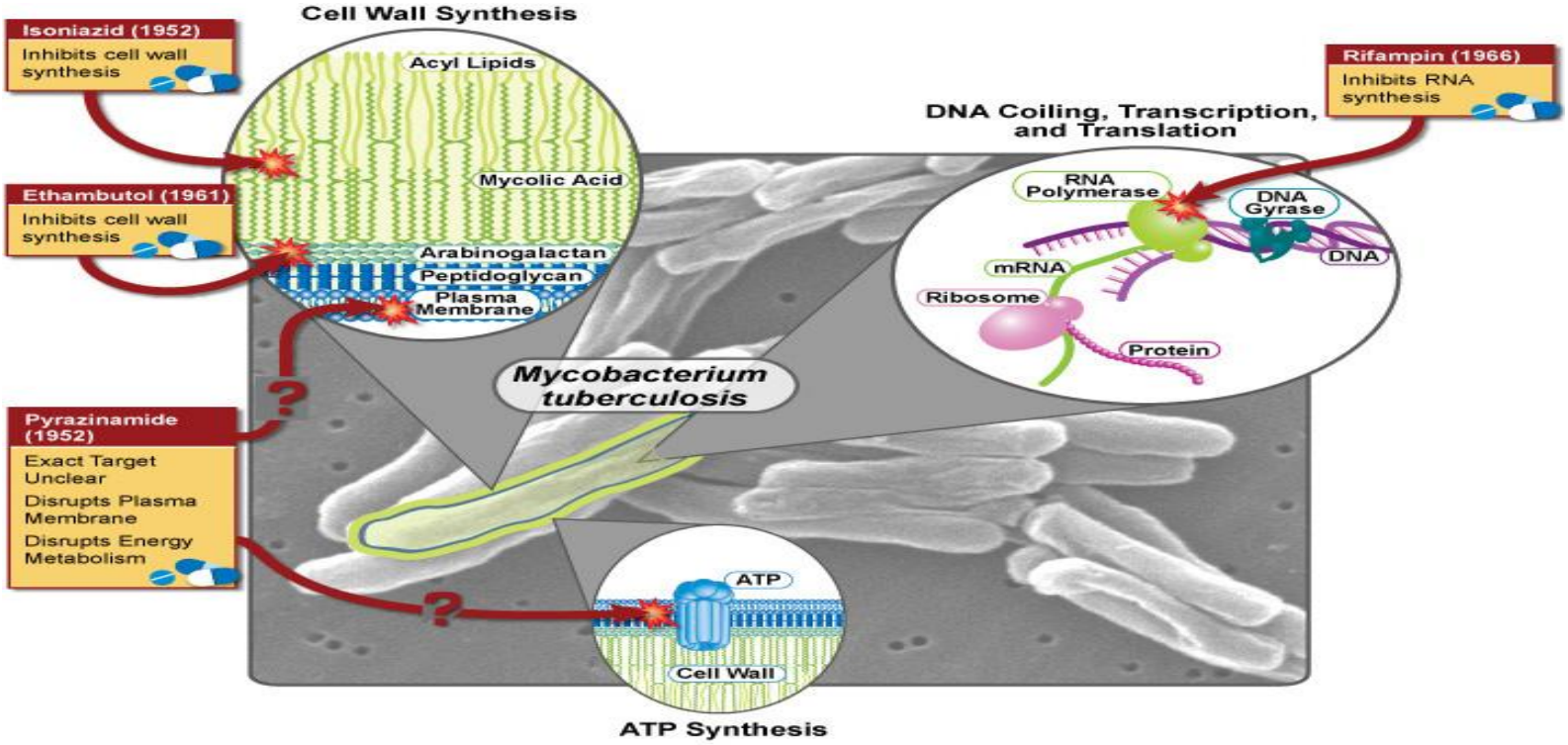


Fig.1.6a Scheme showing first-line treatment of TB for drug-sensitive TB, with proposed sites of action (NIAID, 2007).

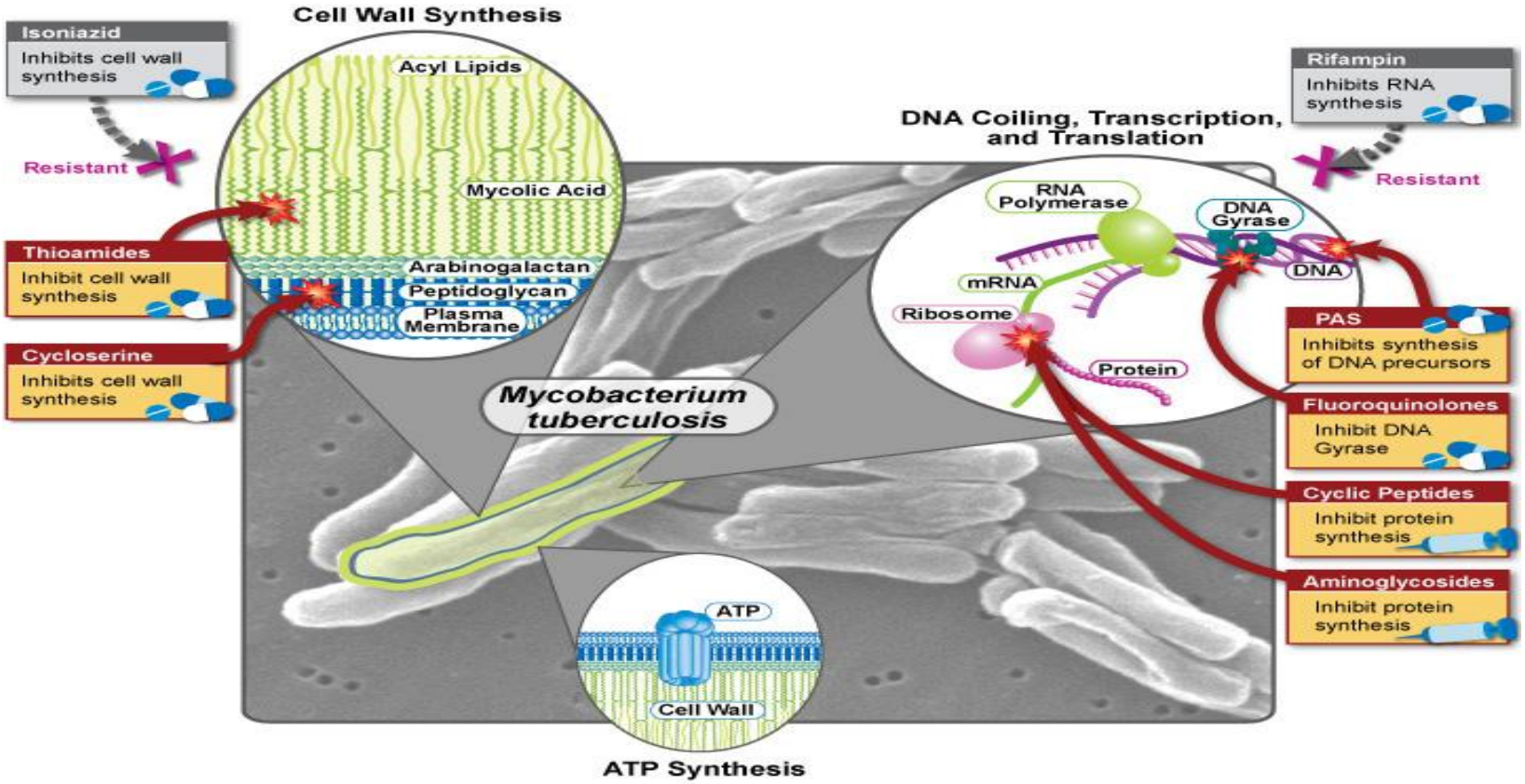


Fig.1.6b Scheme showing drugs inhibited in MDR-TB (isoniazid and rifampicin), and possible effective treatments using other drugs in combination (NIAID, 2007).

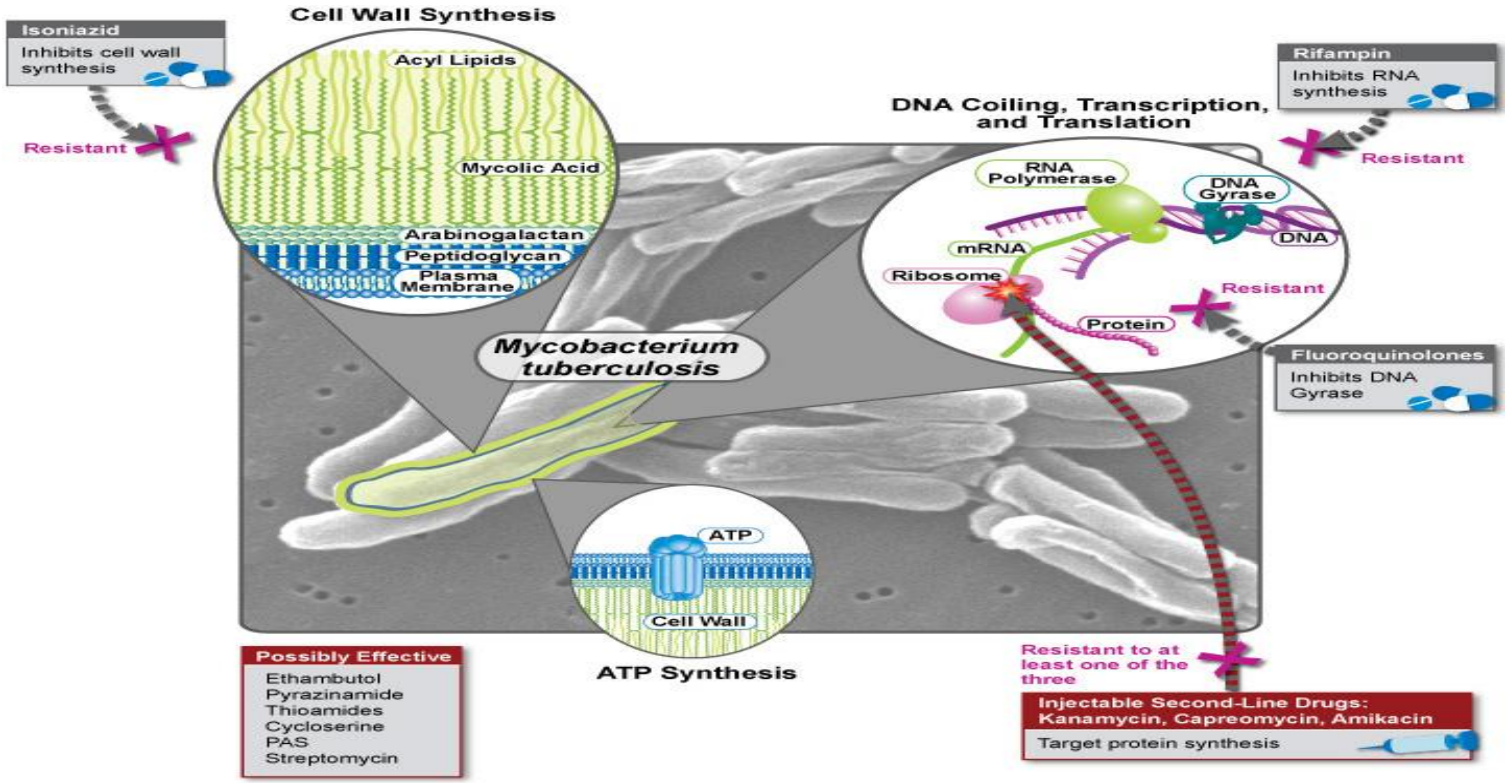


Fig.1.6c Scheme illustrates drugs inhibited in XDR-TB (isoniazid, rifampicin, fluoroquinolones and at least one of three second line injectable) with possible effective treatments using other drugs in combination though treatment options become limited (NIAID, 2007).

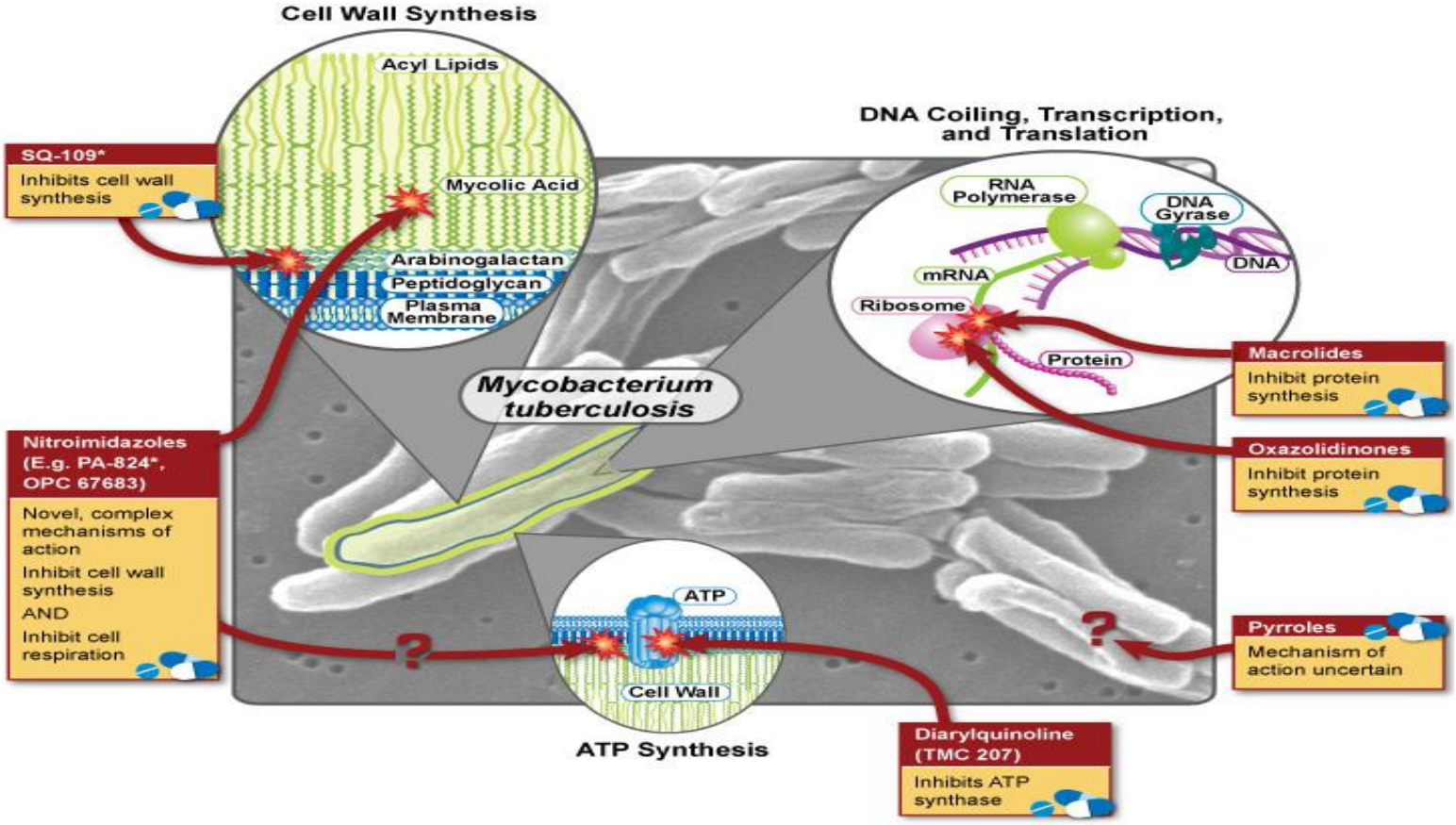


Fig.1.6d New TB drugs under development with their proposed mechanisms of action (NIAID, 2007).

Table 1.4. Summary of antimicrobial action of some clinically important antibiotics (adapted from http://www.pathmicro.med.sc.edu and Rastogi and David,1993)

SITE OF ACTION	ANTIBIOTIC	PROCESS INTERRUPTED	TYPE OF ACTIVITY
cell wall	penicillin	cell wall crosslinking	bactericidal
	cephalosporins	cell wall crosslinking	bactericidal
	cycloserine	synthesis of cell wall peptides	bactericidal
	vancomycin	mucopeptide synthesis	bactericidal
	isoniazid	mycolic acid synthesis	bactericidal
	ethambutol	arabinogalactan synthesis	bacteriostatic
cell membrane	amphotericin B	membrane function	fungicidal
	nystatin	membrane function	fungicidal
	polymixins	membrane integrity	bactericidal
	pyrazinamide	Target unclear	bactericidal
ribosomes			
50-S subunit	erythromycin	protein synthesis	bacteriostatic
	chloramphenicol	protein synthesis	bacteriostatic
	lincomycins	protein synthesis	bacteriostatic
30-S subunit	aminoglycosides	protein synthesis and fidelity	bactericidal
	tetracyclines	protein synthesis	bacteriostatic
nucleic acids	griseofulvin	cell division, microtubule assembly	fungistatic
DNA/or RNA	rifampicin	RNA synthesis	bactericidal
	quinolones	DNA gyrase (topoisomerase)	bactericidal

1.3.4 Antibiotic and molecular basis of resistance

Globally, the emergence of MDR and XDR-TB is of great concern as the treatment options are restricted. A strain of TB in KwaZulu-Natal in South Africa was found to be resistant to seven of the nine drugs tested (Wise, 2006). Inadequate drug regimens, poor patient adherence to therapy, use of immuno-suppressive drugs, homelessness, and mediocre drug quality amongst other factors have been implicated in acquired resistance (WHO, 2010; Youmans *et al*., 1946).

In contrast to other bacteria, resistance of MTB is exclusively associated with chromosomal mutations i.e. they do not involve mobile genetic elements such as plasmids seen in other bacteria and mycobacteria (Pfyffer, 2000). According to Rüsch-Gerdes (1992), resistance to drugs is the result of a spontaneous genetic event and an amplification of a natural phenomenon .Spontaneous mutations occur at a frequency of approximately 10^{-5} - 10^{-8} and cavities in pulmonary TB contain as many as 10^{7} - 10^{9} organisms leading to dual mutations being seen with a certain frequency (Inderlied and Nash, 1996).

Rifampicin, the most important component of current treatment regimens, specifically interacts with prokaryotic RNA polymerase to inhibit transcription, which leads to cell death. Specific mutations in the rpoB gene, which encodes the β-subunit of the RNA polymerase, produce drug resistance by diminishing rifampicin binding affinity for the polymerase (Telenti *et al*., 1993). More than 97% of all rifampicin- resistant TB isolates carry a mutation in the specific core region, where a total of 15 distinct mutations has been identified. Molecular research reports show that more than a single gene is responsible for drug resistance and in isoniazid it is a complex result of single or multiple mutations such as deletions, mis-sense mutations in the katG, inhA, oxyR, ahpC and/or kasA gene (Kapur *et al.,* 1994; Brennan, 1997; Telenti *et al*., 1997; Alcaide *et al*., 1997; Cole, 1995; Ramaswamy *et al*., 1998; Pfyffer, 2000; Chan *et al.,* 2007).

1.4 Need for new drugs

There is a need for the introduction of new effective TB control programmes and

novel, affordable anti-TB agents with little toxicity to replace those currently in use to which resistance has occurred (Newton *et al*., 2002). Furthermore, drugs with broader ranges of activity are also required to target emerging pathogens such as those of the mycobacterium avium-intracellulare complex (MAC) which can result in opportunistic infectious diseases (Newton *et al*., 2002; Williams *et al*., 1995). As has already been discussed, the need for new drugs has been further intensified by the global TB problem due to the rising infection rates with HIV, increased travel and the use of immunosuppressive drugs. New drugs which show no interactions on concomitant administration with HIV drugs would be of great advantage over existing drugs (Tomioka and Namba, 2006; TB alliance, 2007).

The last class of TB drugs was developed and approved in the 1960's and the long duration of treatment is hindering global TB treatment. New anti-TB drugs are needed for three main reasons; to shorten or otherwise simplify treatment of TB caused by drug-susceptible organisms and MDR-TB, improve treatment of latent TB infection and have a new site of action (Pepper *et al*., 2007). New categories of drugs that have shown promise for use in treating TB include the nitroimidazopyrans and the oxazolidinones. Experimental data has shown that, a drug that can inhibit the enzyme isocitrate lyase thought to be necessary for maintaining the latent state would be useful for treatment of latent TB infection (Tomioka *et al*., 2008).

A number of other interventions that might lead to improved treatment outcomes have been suggested. However, the drugs are still at different stages of clinical testing. These include various drug delivery systems, cytokine inhibitors; administration of "protective" cytokines such as interferon-gamma, interleukin 2 and nutritional supplements especially vitamin A and Zinc (Tomioka *et al.*, 2008).

Overall, it was suggested that commercial companies involved in drug development perceive a lack of returns from investment in TB drug research seeing that a large percentage of the disease is prevalent in developing countries which are unable to meet the healthcare needs of their populace (Tomioka and Namba, 2006).

1.4.1 Natural products in drug discovery

Natural products in the form of decoctions, teas, *etc*., have been used for millennia for the treatment of disease. The isolation of bioactive compounds from natural sources remains a major source of new medicinals especially in the area of anti-infectives and cancer (Newman and Cragg, 2007; Kingston, 2011).

Historically, plant natural products have served as one of the most significant source of new leads for pharmaceutical development. The success of natural products in drug discovery has been attributed by scientists to their high chemical diversity, the effects of evolutionary pressure to create biologically active molecules and the structural similarity of protein targets across many species (McChesney *et al*., 2007). The structural diversity seen lends support to the belief that collections of natural products are not only more varied than those made up of synthetic compounds, particularly those produced by combinatorial chemistry, but are also more similar than synthetic compounds to the 'chemical space' occupied by drug molecules (Henkel *et al*.,1999; Feher and Schmidt, 2003; Harvey 2007).

It has been generally estimated that there are approximately 300,000 species of higher plants and of this number only about 1% or roughly 3000 has been utilised for food. Out of this 3000, about 150 have been commercially cultivated. On the other hand, approximately 10,000 of the world's plants have documented medicinal properties (McChesney *et al*., 2007). McChesney *et al.* (2007) found that roughly 150-200 of plant materials are utilised in western medicine (the US, Western Europe etc).

In more recent times, natural products have continued to be significant sources of drugs and leads (Newman *et al.*, 2003; Cragg *et al.*, 2005; McChesney, 2007). Approximately 80% of medicinal products up to 1996 were either directly derived from naturally occurring compounds or inspired by a natural product (Sneader, 1996; Harvey, 2007).

Newman *et al*., (2003) in an extensive review of new drugs introduced between 1981 and 2002 found that 28% of the 868 new chemical entities were natural products or derived from natural products, with another 24% created around a

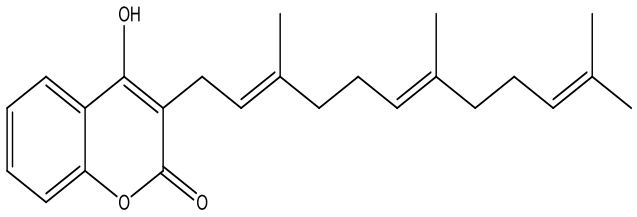
pharmacophore from a natural product. In Butler's review (2005), at least 70 natural product-related compounds were in clinical trials in 2004 in addition to launched products. The exploration of the bioactivity of natural products continues to provide novel chemical scaffold for further drug inventions (Butler, 2005; Chin *et al*., 2006; Harvey, 2007).

In the treatment of cancers, plant- derived compounds have played an important role and some of the promising and better drugs have come up from plant sources like Taxol® (Wani *et al*.,1971), campthothecin (Wall, 1998), combrestatin (Cirla and Mann, 2003), epipodophyllotoxin (Canel *et al*., 2000), and vinca alkaloids - vinblastine and vincristine (Johnson *et al*., 1963). Over 60 compounds were tested as anticancer drugs from plant sources at the National Cancer Institute in 2007 (Saklani and Kutty, 2007).

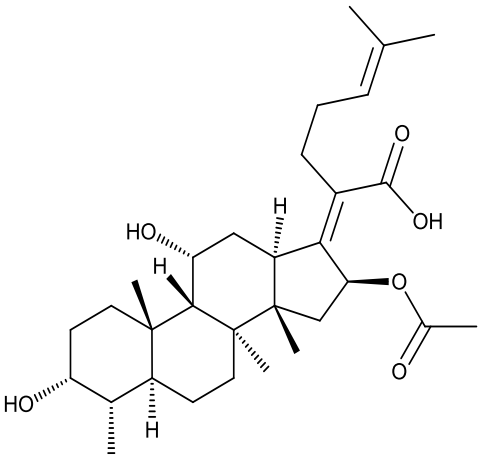
1.4.2 Plant derived anti-tubercular plant compounds

Mischer and Baker (1998) described potential anti-tubercular plant-derived compounds such as berberine, lichoisoflavone, erygibisoflavone, phaseollidin, erythrabyssin II and tryptanthrin (Gautum *et al*., 2007). Copp (2003) reviewed plant-derived antimycobacterial natural products which covered a large proportion of phytochemicals such as lipids/fatty acids and simple aromatics, phenolics and acetogenic quinones, peptides, alkaloids, terpenes (monoterpenoids, diterpenes, sesquiterpenes, sesterterpenes) and steroids.

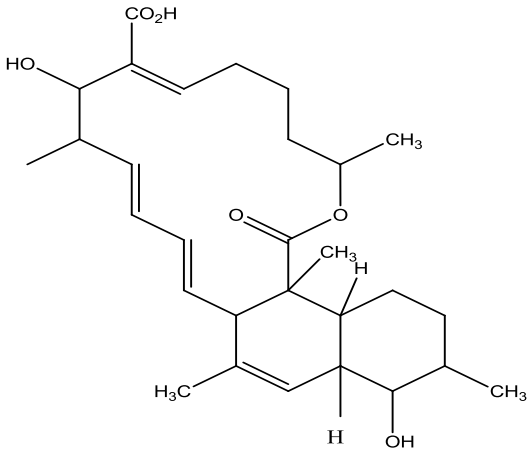
Highly active compounds from these plants included; the lactone - containing tubelactomicin A, phenolics drummondins and ferulenol, xanthone, brasiliquinones A and B, (Figure 1.6) the alkaloids celastramycin A, sampagine and the manzamines and steroids fusidic acid, saringosterol and ergosterol-5,8-endoperoxide which all exhibited antimycobacterial activity with MIC values ≤ 4 µg/mL. These compounds show the chemical diversity and complexity displayed by plants compared to first-line and second – line drugs used in therapy (Figure 1.5)



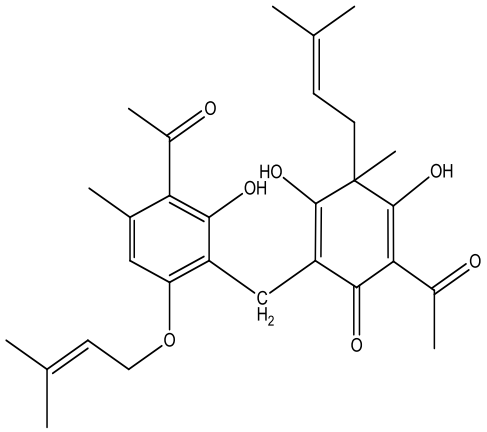
Ferulenol

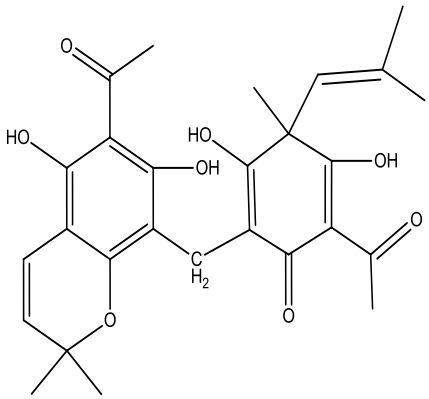


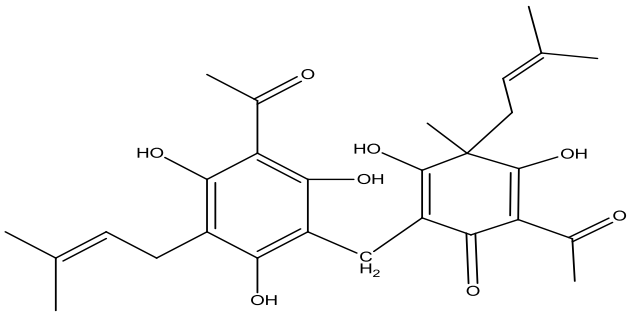
Fusidic acid



Tubelactomin A







phenolic drummondins

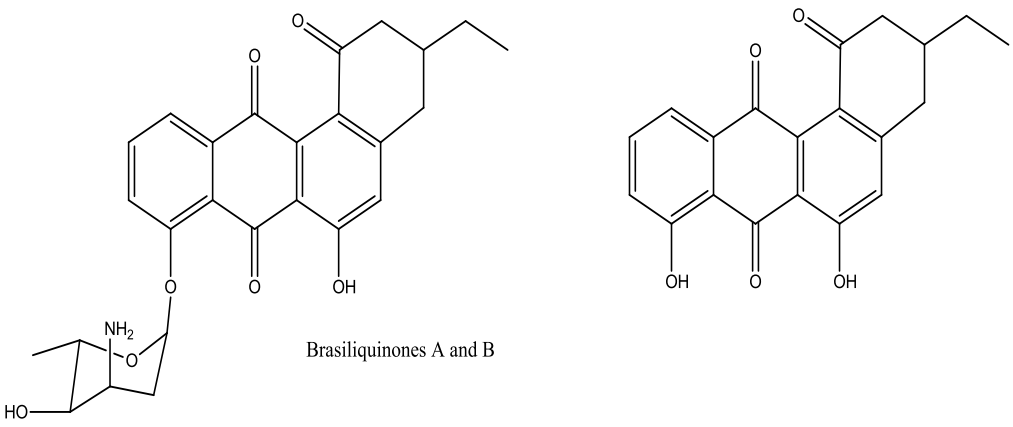


Fig. 1.7 Chemical structures of plant derived anti-tubercular compounds

Though they are not drugs *per se*, the structural skeletons could provide scaffolds or templates for the development of new anti-TB drugs (Copp, 2003).

1.4.3 Drug development from plant sources

1.4.3.1 Commercial consideration

Present day drug discovery efforts depend to a great degree on lead compounds from natural products and synthetic molecules (Nicolaou *et al*., 2011)**.** The choices of disease states being targeted are market - driven such that diseases like cancer and arthritis with enormous potential markets are considered rather than anti-malarial or anti-tubercular drugs which are greatly needed among poor and developing countries with less purchasing power (Heinrich *et al*., 2004).

The declining interest of pharmaceutical industries in developing new drugs from plant sources is also associated with the risky nature of the business. Generally, the process of drug development is recognised as a high risk or high pay-off endeavour and according to the experience of Merck Company for every 10,000 substances that are evaluated in a battery of biological assays, 20 are selected for animal testing. Of these 20, 10 will be evaluated for in humans

and only one will be approved by the Food and Drug Administration in the United States for sale as a drug. According to the report, Merck claimed that the process requires about 12 years at a cost of US$231 million (Vagelos 1991; Farnsworth, 1994).

Another important reason accounting for declining interest, was difficulty associated with sourcing authenticated plant materials as well as failure to confirm earlier activities demonstrated when they went on to development and commercialisation of the product (McChesney *et al*., 2007). There was also the issue of measurement of biological activities of complex mixtures of materials and interaction properties leading to misleading results. The strongest impediment experienced as described by some scientists, is the continuous provision and reliable supply of the active constituents for the pharmaceutical industry (McChesney and Farnsworth, 1994).

In the case of TB drugs, the gap in drug development exists for many cited reasons such as, the lack of animal models which closely resemble the disease in humans, availability of containment facilities for carrying out such studies, the slow growth of the bacteria in question and the costs involved in carrying out the research. The lack of committed investigations into new anti-tubercular drugs by drug companies has also been attributed to the difficulty associated with clinical trials involving multi-component drug therapy especially for TB which requires at least 6-month for treatment of drug-sensitive strains (Tomioka and Namba, 2006). Tomioka and Namba (2006) suggest that it would be difficult and costly to convincingly prove that newer drugs have better effects than existing ones since clinical trials would require monitoring treatment for at least six-months with follow- up studies thereafter.

Chemically, isolation of pure, pharmacologically active constituents from plants remains a long and tedious process (Hostettmann, 1997). However advances in separation science and structure elucidation technology such as coupled liquid chromatography-mass spectrometry and 2D NMR techniques have provided potent and powerful methodologies for assignment of structure to complex natural products (McChesney *et al*., 2007). Highly automated, very specific and selective bioassays are now available to carry out the evaluations of natural products efficiently and economically (Gross, 2006).

1.4.3.2 Biological consideration

M. tuberculosis is highly infectious and difficult to work with requiring containment facilities. The organism grows slowly requiring three to four weeks and mutant cells often require even more time than the wild-type (Hatfull and Jacobs, 2000).

Genetic tools to transform the *M. tuberculosis* DNA were unavailable up until 1998 when the full genomic structure was elucidated (Hatfull and Jacobs, 2000). New drugs with novel mechanisms of actions are being rationally designed using molecular tools (Tomioka and Namba, 2006). In spite of the progress being reported, it may take a while before drugs targeting specific sites are available commercially and such high technology tools are not readily accessible in countries with high TB burden.

1.4.4 Rationale for choosing plants used in research study

The World Health Organisation (2006) estimated that 80% of the population in Africa use traditional medicine for primary health-care. Since medicinal plants form the 'backbone' of traditional medicine, it strongly implies that more than 3300 million people in the less developed countries utilise medicinal plants on a regular basis. Thus for such indigenous populations generally unable to afford expensive ethical drugs, the need to study these plants for safety and efficacy and to develop galenical products that are standardised and stable is imperative (Farnsworth, 1994).

In the search for new compounds needed for TB treatment, a number of indigenous herbal remedies were screened in Nigeria against clinical isolates of *M. tuberculosis* and shown to have activities at different levels. The herbal components investigated during the research programme were selected based on the inhibitory activities observed on testing for *in vitro* drug susceptibility against *Mycobacterium tuberculosis* (the causative organism of tuberculosis) by Lowenstein-Jensen method. The recipe which consists of *Ximenia americana* (root bark) and *Pavetta crassipes* (leaf) is used as a hot water preparation in combination to treat respiratory and chest infections by traditional medical

practitioners in the Middle belt region of Nigeria (personal communication with traditional healer). In the preliminary testing, water extracts of *X. americana* and *P. crassipes* exhibited minimum inhibitory concentration (MIC) of 100 µg and 200 µg/mL respectively against clinical isolate of *M. tuberculosis* (Odumosu, *et al*., unpublished data).

Literature search on both plant materials showed no reported data on the anti-tubercular activities. The objective of the research project was therefore to evaluate the extracts chemically and biologically against *M. tuberculosis* to confirm the therapeutic value.

1.5 Research Aims and Objectives

The main aim of the study was to evaluate the anti-tubercular properties of various plant extracts of both plants extracted by sequential extraction and then to isolate and characterise the relevant and promising constituents/leads based on bioactivity directed approach using analytical chromatography and spectroscopic tools.

The secondary aim was to determine the general antimicrobial spectrum of activity against gram positive, gram negative and fungal organisms as the occurrence of opportunistic infections in TB has been reported in literature.

Chapter 2 LITERATURE REVIEW: Medicinal Plants

2.1 Introduction

2.1.1 Description - X. americana

Botanical names: *Ximenia americana* (Named after F. Ximenes, a 17th century Spanish botanist)

Family: Olacaceae

Common names: Wild Olive, Seaside plum, Tsada (Hausa, Nigeria).

Habitat: It is found in the savannah; occurring also near the coast

X. americana Linn. Family Olacaceae is a common white flowered shrub or small tree up to 5 m tall. The bark is grey to dark brown or almost black flaking off in rectangular patches. The spines are 0.25 - 0.5 inch long, straight and sharp, sometimes bearing leaves or the lateral shoots ending in a spine (Figure 2.1). Leaves are 1.5 - 2.5 inch long by 0.75 - 1 inch long. It is broad, elliptic tapering to both ends with the mid-rib slightly projecting or the apex slightly notched with 5-6 pairs of thin faint up curving lateral nerves. The stalk is 0.25-0.5 inch long. Flowers are usually in season January – May, they are white, fragrant; usually in a small cluster at the end of a common stalk 0.25 - 0.5 inch long, the whole inflorescence is 0.5 -1 inch long. Fruits are in season, April - June, ellipsoid, 0.75 -1.25 inch long, yellow, with a thin skin, sweet flesh

and hard stone; with the remains of the style forming a sharp tip. The wood is yellow or reddish-yellow, hard, heavy and fine grained (Keay *et al.,* 1964; Dalziel, 1956).

Fig.2.1 Photo of *Ximenia* plant in its habitat and as shown in Kew Gardens herbarium catalogue (http://www.kew.org)

2.1.2 Medicinal uses

The preparation of branched leaves, barks, peelings and roots is used for headaches, toothaches, mumps and conjunctivitis in frontal applications. The solutions are reserved for washing, massaging and instillations or gargles (Sofowora, 1982). The root has been used for febrile headache (local application) and venereal diseases in West Tropical Africa. The pulverised bark and root are used as a dressing for ulcers, craw-craw (pruritic popular skin eruption which may lead to ulceration, some cases caused by onchocerca) (Dalziel, 1956).

In Northern Nigeria, the root along with that of *Anona senegalensis* or *A. chrysophylla* is used in the treatment of sleeping sickness (trypanosomiasis).

In Senegal, the maceration of roots is used in treating leprosy and applied internally and externally for mental sickness. It is also a remedy for impotence.

The inhalation of dry root powder and bark mixed with other ingredients is used to treat oedema of the face (Sofowora, 1982).

In Tanzania, the root is used as a febrifuge and diarrhoea remedy (Watt and Breyer-Brundwijk, 1962; Dalziel, 1956). A decoction of the leafy twigs is given for febrile colds and cough and as a laxative in Zimbabwe (Kokwaro, 1976; Dalziel, 1956; Burkill, 1985).

2.1.3 Constituents and Pharmacology

The bark yields 16-17 per cent of tannins. Finnemore *et al*. (1958) analysed the leaves of *X. americana* in Australia and showed the presence of sambunigrin while Earle and Jones (1962) obtained positive results for alkaloids in the seeds (Sofowora, 1982). Almagboul *et al*. (1985) screened the chloroform and methanol plant extracts for antibacterial activity and observed some activity. Ogunleye and Ibitoye (2003) evaluated the ethanolic leaf extract for antimicrobial activity and it was reported to be active against *S. aureus*, *P. aeruginosa* and *C. albicans*.

Mwangi *et al*. (1994) isolated polymeric proanthocyanidin mixture, epicatechin (major flavan-3-ol), catechin, gallic acid and procyanidins from the root of *X. americana* var. caffra. A MIC of 1000 µg/mL against *Staphylococcus aureus* was obtained for crude extract of polymeric proanthocyanidins while no antimicrobial activity was observed for epicatechin and catechin. D'Agostino *et al*. (1994) investigated the chloroform – methanol extract of the roots and isolated an oleanolic acid saponin identified as 3-β-O-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)-β-Dglucopyranosyl-uronic acid] oleanolic acid 28-O- β-D-glucopyranoside.

Fatope *et al*. (2000) in their study isolated C18 acetylenic fatty acids, tariric acid and a novel fatty acid, 10*Z*, 14E, 16E-octadeca-10, 14, 16-triene-12-ynoic acid from the chloroform root extract of *X. americana.* Omer and Elnima (2003) investigated the antibacterial activity of methanol and water extracts of the root which was reported to show some activity against *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* but the chloroform fraction did not show detectable

inhibition. Mevy *et al*. (2006) analysed the volatile oil of the leaves by GC-MS and identified 33 components representing 98% of the total oil. Runyoro *et al*. (2006) screened the methanol root bark extract from Morogoro and Tanga in Tanzania using bioautographic agar overlay technique. No activity was reported against *Candida*.

Voss *et al*. (2006) identified and characterised riproximin, a new type II ribosome – inactivating protein with anti-neoplastic activity from the whole plant of *X. americana*. Araujo *et al*. (2009) isolated ximonicane, a new sesquiterpene compound and stigmastane steroid from the stem bark extract of the plant.

Table 2.1. Summary of chemical constituents reported in *X. americana* from literature.

plant part	constituents	Reference
leaf	sambunigrin	Finnemore *et al*., 1958
stem bark	tannins	Sofowora, 1982
seeds	alkaloids	Earle and Jones, 1962
whole plant	riproximin (var. caffra)	Voss *et al.,* 2006
root bark	polyphenols (var. caffra)	Mwangi *et al.,* 1994
root bark	oleanolic acid saponin	D'Agostino *et al.,* 1994
stem bark	ximonicane, stigmastane	Araujo *et al.,* 2009
root bark	C18 acetylenic fatty acids	Fatope *et al*., 2000

2.1.4 Description - Pavetta crassipes

Botanical name: *Pavetta crassipes* K. Schum

Family: Rubiaceae

Common names:Mupembe (Kenya), Rubatari (Hausa, Nigeria)

The shoots are covered with whitish corky bark. Corolla lobes are 5 - 8 mm long and the leaves has a main lateral nerve on each side of the mid-rib. The stipules and bracts are pilose inside, bracelets are stout, and the leaves are often in three subsessile, glabrous, and narrowly elongated oblong-lanceolate and rounded at the apex. The leaves are 15 - 25 cm long and 3 - 5 cm broad (Figure 2.2).

Fig.2.2 Photos of *Pavetta crassipes* in its habitat and as shown in Kew Gardens herbarium catalogue (http://www.kew.org)

The flowers are sub-sessile in clusters on very short lateral shoots. The corolla tube is about 12-18 mm long while the lobes are about 5 mm long. The Calyx is glabrous. Approximately, 91 genera and 531 species are reported to be present within the West African region (Gill, 1988).

2.1.5 Medicinal uses

The leaves are used by some people as food pounded up with other foods or boiled in slightly fermented water in which cereals have been left to steep and mixed with a corn meal. The sap is useful for coagulating rubber latex (Hutchinson and Dalziel, 1954).

Leaf extracts have been used as anti – malarial remedy against *Plasmodium falciparum* and for coughs in Tanzania and Burkina Faso. The roots are also eaten as food when cooked with chicken in soups and as anti-convulsant (Chabra *et al*., 1991; Gessler *et al*., 1994; Nadembega *et al*., 2011) while the fresh or dry fruits are eaten in East Africa as anti-helminthics (Kokwaro, 1976; Chabra *et al*., 1991).

2.1.6 Constituents and pharmacology

Constituents such as alkaloids and polyphenols have been reported to be present in *Pavetta owariensis,* a closely related species (Weniger *et al*., 2004; Baldé *et al*., 1990, 1991). Mild anti-plasmodial activity against reference clones of Plasmodium was observed for the alkaloid extracts of *P. crassipes* leaf and *P. corymbosa* aerial parts. The alkaloid extracts exhibited moderate *in-vitro* activity and weak cytotoxicity against three human cell lines (THP1, normal melanocytes, HTB-66 (Sanon *et al*., 2003, Weniger *et al*., 2004).

Sanon *et al.* (2005) reported the presence of indolomonoterpenic alkaloids, elaeocarpidin and hydroxyl-elaocarpidin, rutin, acanthospermol galactosidopyranoside in *P. crassipes* leaf. Hydroxy-elaeocarpidin was demonstrated to have good anti-malarial activity with an IC_{50} of 1.83 µg/mL and they observed synergistic effects in combination with *Mithragyna inermis.* Baldé *et al*. (2010) investigated the alkaloid fractions of the leaf with reported *in vitro* activities against bacteria, trypanosomes, leishmania, falciparum, and cancer cells.

Amos *et al.,* (1998a) investigated the effects of the aqueous extracts of *P. crassipes* on gastrointestinal and uterine smooth muscle preparations isolated from rabbits, guinea pigs and rats.

The scientists observed a concentration dependent inhibition of the spontaneous motility or elevated tone in the preparations.

Another study by Amos *et al*., (2004) demonstrated a dose-dependent decrease of spontaneous motor activity in mice with attenuated amphetamine-induced hyperactivity. They suggest that the bioactive compounds might be acting centrally through the inhibition of dopaminergic pathway or a system linked to this pathway to mediate the observed pharmacological effects. The aqueous leaf extract was also shown to have anti-inflammatory and muscle relaxant activity and hypotensive activity on rat muscles (Amos *et al.*, 1998b; 2003). Mustapha and Bala (2007) investigated the antimicrobial activity against some respiratory tract pathogens and observed moderate activity at mg levels.

Table 2.2. Summary of chemical constituents reported in *P. crassipes* from literature

plant part	constituents	Reference
Leaf	Indolomonoterpenic alkaloids	Sanon *et al.,* 2005
Leaf	polyphenols	Sanon *et al*., 2005

Following literature review of both plants material, general antimicrobial screening of the extracts was commenced.

Chapter 3 General Antimicrobial Screening of Plant Extracts Against Bacteria and Fungi

3.1 Background

Owing to the fact that opportunistic organisms have been found to co-exist with *M. tuberculosis* infection, the general focus of the studies described in this chapter was focused on the methods used in testing organisms known to be representative of the bacterial and fungal classes (Ghannoum, 1997; Vazquez, 2000; Runyoro, 2006). It was hoped that this aspect of the work would demonstrate other bacterial activities of the plant extracts in addition to the antitubercular action. This in effect would provide an overview of the general activity of the plant extracts.

3.1.1 Introduction

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine (Cos *et al.*, 2006). The effect is significant in developing countries due to the relative unavailability of medicines, cost constraints of more effective newer agents and the emergence of widespread drug resistance. Investigations on finding new antimicrobial substances needs to be continued for this reason utilising all possible avenues to finding low cost effective solutions (Okeke *et al*., 2005).

Besides synthesised small molecules arising from medicinal chemistry studies, natural products are still major sources of innovative therapeutic agents for

various conditions, including infectious diseases (Clardy and Walsh, 2004). The primary target of natural product research is on plants since they can be easily sourced and selected on the basis of their ethno-medicinal use (Verpoorte *et al.*, 2005).

It is extremely important to design *in vitro* experiments that resemble the target disease (Kariba *et al.*, 2002). Thus a plant used topically for fungal infections should be tested against dermatophytic fungi rather than the 'classic' genera such as *Aspergillus* and *Penicillium*. In the same way extracts which are claimed to have antibacterial activity should be tested against bacteria of clinical relevance (Cos *et al.,* 2006).

With better understanding of the underlying aetiology of disease states, it has become apparent that a range of different diseases may have common factors. For conditions associated with excess inflammation such as asthma, eczema, arthritis, *in vitro* tests associated with inhibition of one of the many biochemical processes related to inflammation are often used. Common examples of these are tests for lipoxygenase inhibition, inhibition of eicosanoid synthesis. Activity in one or more of these systems may indicate the anti-inflammatory property of the extract but to specify activity in a particular disease based on the response is often an extrapolation too far (Houghton *et al.*, 2007).

The traditional antibacterial and antifungal test methods are classified into three main groups, i.e. diffusion, dilution and bio-autographic methods. A relatively new method is the conductimetric assay which detects microbial growth as a change in the electrical conductivity or impedance of the growth medium (Sawai *et al.*, 2002).

Many research groups have modified these methods for specific samples, such as essential oils and non-polar extracts and these small modifications make it almost impossible to directly compare results. It is therefore a 'must' to include at least one reference compound or as many as possibly can be accessed in each assay (Cos *et al*., 2006).

3.1.2 Agar diffusion methods

In the diffusion technique, a reservoir containing the test compound at a known concentration is brought into contact with an inoculated medium and the

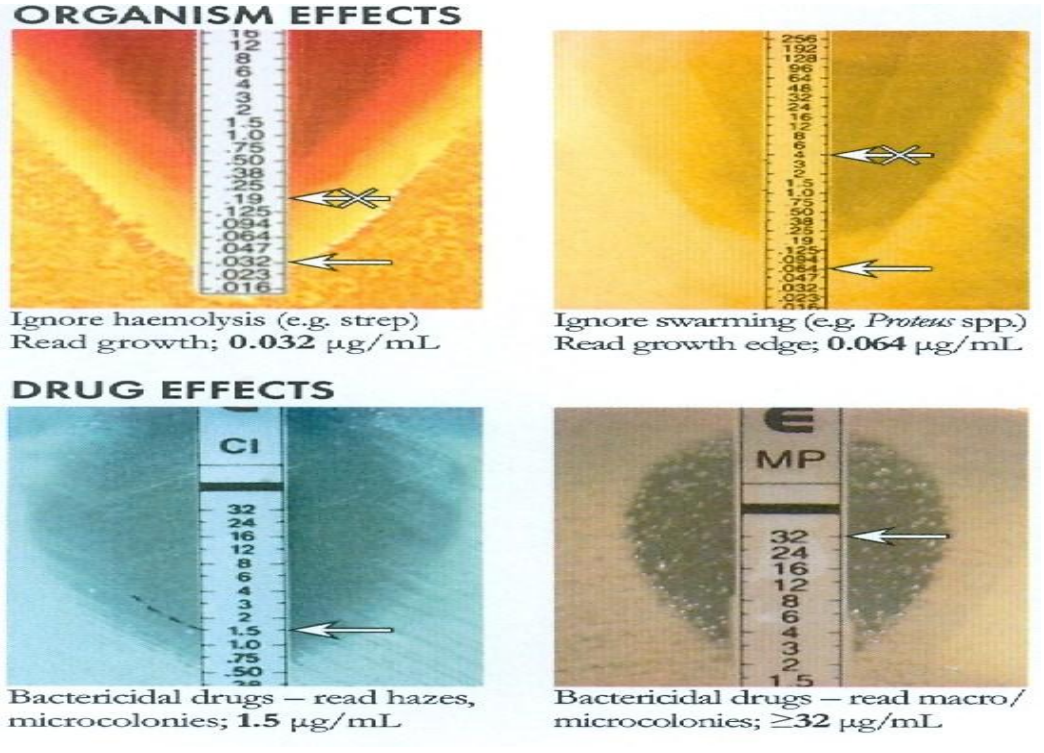
diameter of the clear zone around the reservoir (inhibition diameter) is measured at the end of the incubation period.

In order to enhance the detection limit, the inoculated system is kept at lower temperature for several hours before incubation to favour compound diffusion over microbial growth, thereby increasing the inhibition diameter (Cole, 1994). Different types of reservoirs can be used, such as filter paper discs, stainless steel cylinders placed on the surface and holes punched in the medium. The hole-punch method is the most suitable diffusion technique for aqueous extracts, because interference by particulate matter is less than with other types of reservoirs. To ensure that the sample does not leak under the agar layer, fixed agar is left on the bottom of the hole. The small sample requirements and the possibility of testing up to six extracts per plate against a single micro-organism are specific advantages of the method (Hadacek and Greger, 2000). The diffusion method is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar.

Etest® is reported to be a well-established method for antimicrobial testing in microbiology laboratories. Etest® consists of pre-defined gradient of antibiotic concentrations on a plastic strip which is used in susceptibility testing against bacterial, fungal and mycobacterial organisms. It possesses the advantage of being able to provide quantitative MIC data which is of great importance in the management of critical infections such as sepsis. Etest® is recognised as a cost effective tool for generating MICs across 15 dilutions. It has been used in resistance detection and over 100 antibiotics are available for testing aerobic bacteria, fastidious organisms, anaerobes, fungi and mycobacteria (http://www.boimerieux-usa.com). The test is used to promote rational use of antibiotics by providing results to guide individual therapy and to validate empiric drug regimens. According to the report, E test® is widely used in resistance surveillance programmes and clinical trials.

In one study (Di Bonaventura *et al*., 1998) the evaluation of E test for antimicrobial susceptibility testing of *P. aeruginosa* showed 88 and 92.5 % correlation of E test MICs with those determined by the agar dilution and disc diffusion reference methods respectively.

i)



ii)

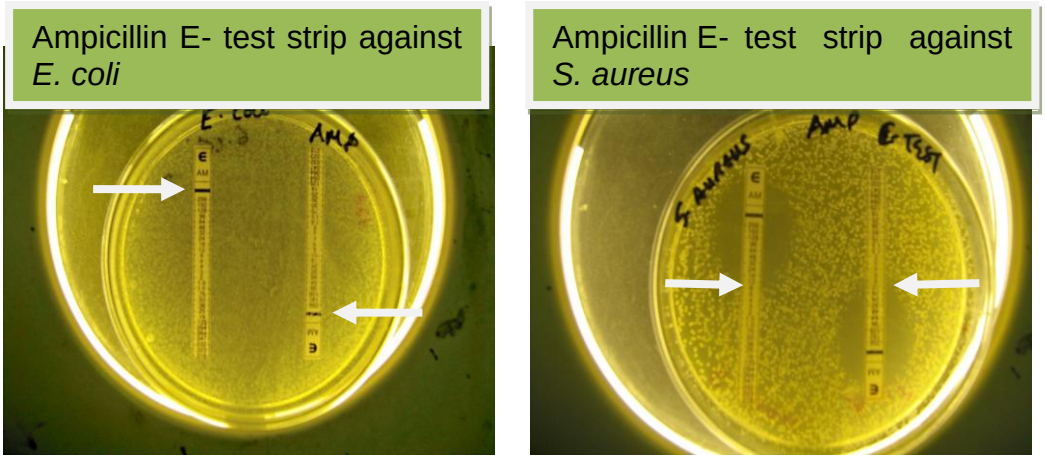


Fig.3.1 i) Guide showing how to read the MIC of antibiotic E test gradient strip (http://abbiodisk.com) ii) Examples of plates prepared experimentally showing ampicillin test strips with no inhibition against *E. coli* but inhibition with *S. aureus* (MIC of > 256 µg/mL and 8 µg/mL respectively).

In general, the relative antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility and diffusion characteristics in agar. Furthermore, agar-diffusion methods are difficult to run on high-capacity screening platforms (Cos *et al*., 2006).

3.1.3 Dilution methods

Dilution methods are used to determine the minimum Inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for susceptibility testing against which other methods, such as disc diffusion are calibrated (EUCAST, 2000). In the dilution methods, test compounds are mixed with a suitable medium that has previously been inoculated with the test organism. It can be carried out in liquid as well as in solid media and growth of the micro-organism can be measured in a number of ways (Cos *et al*. 2006).

In the agar-dilution method, the MIC is defined as the lowest concentration able to inhibit any visible microbial growth. In liquid- or broth-dilution methods, turbidity and redox-indicators are most frequently used. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 600 nm. However, test samples that are not fully soluble may interfere with turbidity readings, emphasising the need for a negative control or sterility control, i.e. extract dissolved in blank medium without micro-organisms.

The liquid-dilution method also allows assessment of whether a compound or extract has a cidal or static action at a particular concentration. The minimal bactericidal or fungicidal concentration (MBC or MFC) is determined by plating-out samples of completely inhibited dilution cultures and assessing growth (static) or no-growth (cidal) after incubation. In most investigations, the redox indicators, MTT and resazurin are frequently used to quantify bacterial (Eloff, 1998; Gabrielson *et al.*, 2002) and fungal growth (Jahn *et al.*, 1995; Pelloux-Prayer *et al.*, 1998). Resazurin has the advantage of not forming precipitates upon reduction thereby allowing for direct interpretation. Easy and reproducible measurements can be obtained with a microplate-reader, but visual reading may also be used in cases where spectrophotometry is not available.

Another assay which can be used is based on the principle that only living cells convert fluorescein-diacetate to fluorescein, producing a yellowish-green fluorescence under UV light (Chand *et al.*, 1994). However, it requires a more significant investment in equipment and validation is not easy *e.g.* Sabouraud liquid medium can quench up to 95% of fluorescence, and sodium phosphate buffers hydrolyse fluorescein-diacetate. Fluorescent constituents present in crude plant extracts may also interfere (Clarke *et al.*, 2001).

In general, dilution methods are suitable for analysing polar and non-polar extracts or compounds for determination of MIC and MBC/MFC-values. Using redox indicators or turbidimetric endpoints, dose–response effects allow calculation of IC_{50}- and IC_{90}-values, which are the concentrations required to produce 50 and 90% growth inhibition.

3.1.4 Bio-autographic methods

Bio-autography localises antimicrobial activity on a chromatogram using three approaches:

(a) Direct bio-autography, where the micro-organism grows directly on the thin-layer-chromatographic (TLC) plate

(b) Contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and

(c) agar-overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987; Rahalison *et al.*, 1991) .

Despite high sensitivity, its applicability is limited to micro-organisms that easily grow on TLC plates. Other problems are the need for complete removal of residual low volatile solvents, such as *n*-butanol, trifluoroacetic acid and ammonia and the transfer of the active compounds from the stationary phase into the agar layer by diffusion. Because bio-autography allows localising antimicrobial activities of an extract on the chromatogram, it supports a quick search for new antimicrobial agents through bioassay-guided isolation. However, this technique is not directly applicable in current high capacity screening designs. The choice of test organisms depends on the specific purpose of the investigation. In a primary screening, drug-sensitive reference strains are preferably used and should represent common pathogenic species of different classes.

Various combinations are possible, but the panel should at least consist of a gram-positive and a gram-negative bacterium. Based on literature data, it has been well-established that gram-positive bacteria are much more sensitive to drug action than gram-negative bacteria, which is reflected by a higher number

of random ‘hits’ during a screening campaign. Extracts with prominent activity against gram-positive cocci should also be tested against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), as they cause diseases which are clinically difficult to manage (Cos *et al*., 2006). ATCC strains are well-characterised and very popular for that purpose, but clinical field isolates may also be used if fully characterised by antibiogram. A small set of reference fungi is used for primary screening and includes *Trichophyton mentagrophytes* and *Epidermophyton floccosum* as representatives of the dermatophytes and opportunistic filamentous fungi, *Aspergillus niger* and *Fusarium solani* (Cos *et al.*, 2006).

3.1.5 Inoculum

The inoculums should give semi-confluent growth of colonies after overnight incubation. A semi-confluent growth can easily be recognised as shown by Figure 3.2. A heavier inoculum may result in reduced inhibition zones while a lighter inoculum may give increased inhibition zones (Andrews, 2010).

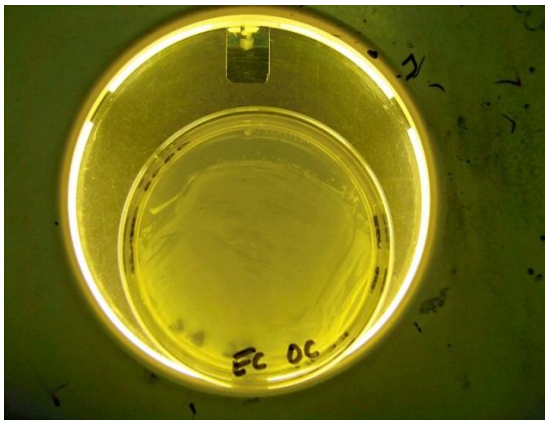
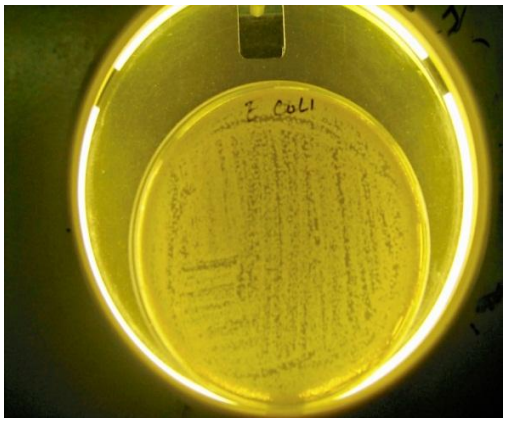
i)  ii) 

Fig.3.2 i) The plate on the left side shows an incorrect procedure while the one on the right ii) shows semi-confluent growth within an acceptable range.

3.2 Aims and Objectives

The purpose of this initial work was to determine MIC values against representative gram positive and gram negative bacteria, as well as fungal organisms in order to establish the antimicrobial spectrum of activity and to determine fractions with prominent activity against bacterial or fungal

organisms. The results would provide an indication of potential usefulness in treating opportunistic infections associated with TB disease.

3.3 Experimental

3.3.1 Materials

Iso-Sensitest agar (ISA), broth powder (CM 0473), Sabouraud dextrose agar were purchased from Oxoid, U.K. Other compounds such as dimethyl sulfoxide, gentamicin, erythromycin, miconazole, amphotericin B were purchased from Sigma Aldrich, U.K. Other compounds used in the study were griseofulvin (Acros Organics), D-glucose (BDH, Sigma), and glycerol (BDH).

3.3.2 Equipment

Class II microbiological safety cabinet was supplied by Atlas labs, U.K. while Spectrophotometer (Spectronic 20) and microplate reader (Thermo) were also utilised.

3.3.3 Bacterial and fungal strains

Control organisms which were representative of bacteria and fungi were chosen according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines. The listed typed organisms were used in the antimicrobial screening *Staphylococcus aureus* (ATCC 25823), *Bacillus subtilis* (NCIB 8054), *Escherichia coli* (ATCC 11560), *Pseudomonas aeruginosa* (ATCC 10662), *Candida albicans* (NCTC 1363), *Aspergillus niger* (UoS strain), *Penicillium chrysogenum* (UoS strain) *Trichophyton mentagrophytes* (ATCC 9129), *Propionibacterium acnes*.

3.3.4 Preparation of reagents and media

3.3.4.1 Turbidity standard for inoculum preparation- McFarland standard

The standard was prepared by adding 0.5 ml of 0.048 M barium chloride (1.17% w/v $BaCl_2.2H_2O$) to 99.5 ml of 0.18 M sulphuric acid (1% v/v) with constant stirring and transferred to a tube similar to that used in growing the broth culture. The accurate density of the turbidity standard was determined by taking the absorbance reading at 625 nm on the spectrophotometer. Absorbance values of 0.008 to 1.10 were considered to be equivalent to 0.5 McFarland

(BSAC 2010; Lalitha, 2004). The standard was vigorously agitated on a vortex mixer each time before use.

3.3.4.2 Nutrient agar/broth

One litre of deionised distilled water was added to a conical flask weighing 28 g of powder (25 g for broth) and dispersed. This was allowed to soak for about 10 minutes, swirled to mix and dispensed into sterile bottles in 9 mL portions. This was then sterilised in an autoclave at 121 ˚C for 15 minutes. For agar, the sterilised liquid was allowed to cool to 50 ˚C, mixed and poured into petri dishes. After solidification, plates were stored at 2 – 8 ˚C until use (manufacturer's instructions).

3.3.4.3 Iso-Sensitest agar/broth

One litre of deionised distilled water was added to a conical flask weighing 31.4 g of dehydrated agar powder (23.4 g for broth) and heated on a hot plate to dissolve. The solution was then distributed into tubes in 9 mL aliquots, sterilised and dispensed as described for nutrient agar.

3.3.5 Isolation and cultivation of bacterial and fungal species

E. coli, *S. aureus* and *P. aeruginosa* were routinely grown on nutrient agar at 37 ˚C and kept at 4 ˚C until required. Overnight cultures were grown on slopes or plates and used for carrying out susceptibility testing against plant extracts and reference antibiotics. *Tricophyton mentagrophytes*, *Propionibacterium acnes, Aspergillus niger, Candida albicans and Penicillium crysogenum* were grown on sabouraud dextrose agar and incubated at 30 ˚C for 7-10 days. Working suspensions were prepared from the cultures for the tests.

3.3.6 Determination of minimum inhibitory concentration by agar dilution

The dried plant powdered materials were sequentially extracted by maceration and soxhlet extraction with hexane, dichloromethane, methanol and water to obtain crude plant fractions. Crude ethanol extracts were also obtained by percolation with 80 % ethanol at room temperature to give total extraction.

The crude plant extracts were weighed in 1 g batches and diluted in dimethyl sulfoxide (DMSO) and sterile water to obtain 200 mg/mL solutions. Doubling

dilutions were done in sterile distilled water successively to obtain lower concentrations.

After diluting, 1 mL of each extract dilution was transferred to 19 mL of molten Iso-Sensitest agar in universal containers (held at 45 – 50 ˚C). This was gently mixed and poured into agar plates. This was left for about 30 minutes to solidify. Plates were incubated in air at 37 ˚C for 18 - 24 hours.

The organism was allowed to grow on the antibiotic-free control plate before readings were taken. Griseofulvin and miconazole were used as reference antifungal antibiotics to monitor positive control growth against the dermatophyte while erythromycin and gentamicin were used to monitor positive control growth against bacteria i.e. to show growth inhibition for comparison with extracts. DMSO (solvent control plate) was used to show the lack of solvent effect on the growth of the organisms.

MIC was defined as the lowest concentration of the antimicrobial agent that completely inhibited visible growth as seen with the naked eye, disregarding single colony or fine haze within the area of inoculated spot (European Committee on Antimicrobial Susceptibility testing, EUCAST 2000; BSAC 2010).

3.3.7 Antifungal susceptibility testing by agar dilution method

The crude plant extracts were weighed in 1 g batches and diluted in DMSO and sterile water to obtain 200 mg/mL solutions. Doubling dilutions were done in sterile distilled water successively to obtain lower concentrations. After the dilutions, 1 mL of each extract solution was transferred to 19 mL of molten sabouraud dextrose agar in universal containers (held at 45 - 50˚C). This was gently mixed and allowed to solidify for 30 minutes.

A suspension was prepared from a 7-10 day culture of dermatophyte grown at 30˚C using 0.45 % saline and the spore concentration was determined with the aid of a Neubauer haemocytometer to give a suspension density equivalent to 1 - 2 x 10^6 CFU/mL (Aberkane *et al.*, 2002; BSAC, 2010). An inoculum volume of 20 µL ≡ 2 x 10^4 CFU was placed on each plate and incubated at 30 ˚C for at least 48 hours. Miconazole and griseofulvin were used for determining positive control growth while agar containing no plant extract or antibiotic was used as negative control. Plates containing dilution solvents and agar alone were used

as solvent and sterility controls respectively in order to validate results obtained. Duplicate plates were observed for growth or no growth for each dilution.

3.4 Results and Discussion

The crude solvent fractions of *X. americana* and *P. crassipes* obtained from were subjected to susceptibility testing against selected control organisms representative of bacteria and fungi by the agar dilution method as recommended by BSAC (2010) guidelines (Tables 3.1 - 3.5). The plant extracts were screened against the listed organisms in order to demonstrate its activity against a wide spectrum of organisms.

Opportunistic bacteria have been reported to be present alongside *M. tuberculosis* in TB and known to exacerbate HIV infection (Ghannoum, 1997; Vazquez, 2000; Runyoro, 2006). Opportunistic microorganisms cause relatively minor disease that is not normally serious but it can become pathogenic or life-threatening when the host has a low level of immunity. TB and HIV infections are associated with low immunity with TB being a leading cause of death in HIV so testing against the microorganisms was performed to determine activities that may be beneficial in treating TB patients with mixed infections.

Both plant extracts exhibited mild antibacterial activity against representative gram positive and gram negative at mg levels. In contrast, the hexane extract of *X. americana* root was most active at 62.5 µg/mL against *P. acnes*. Two LC fractions labelled as fraction 1 and 2 obtained from the hexane extract were further tested against *P. acnes* with fraction 1 showing inhibitory activity at MIC value >100 µg/mL and fraction 2 (MIC = 100 µg/mL). Fractions 1 and 2 from the hexane extract had MIC values greater than the crude extract suggesting the possibility of synergism between the bioactive constituents. All other solvent fractions had MIC values of 125 - 500 µg/mL against *P. acnes* compared to erythromycin (MIC value of 0.195) the reference standard (Table 3.4).

The methanol, chloroform and hexane extracts of *X. americana* root had MIC values of 250 - 500 µg/mL against *T. mentagrophytes* with the hexane extract (most active) having a MIC value of 250 µg/mL compared to miconazole and griseofulvin reference standards which had MIC values > 0.2 and 3.125 µg/mL

respectively (Table 3.4). MIC values were means of three replicate measurements.

Amos *et al*., 1998 in their study reported anti-inflammatory and muscle relaxant effects of the aqueous extract of *P. crassipes* which supports the 'herbal shotgun' principle where components play different functions in achieving therapy.

Table 3.1. Antimicrobial testing of *X. americana* root extracts (MIC mg/ml)

Bacteria	hex	dcm	meth	H_2O
S. aureus	0.5	0.5	0.25	2.5
B. subtilis	0.5	0.25	1.0	> 2.5
E. coli	> 2.5	> 2.5	> 2.5	> 2.5
P. aeruginosa	> 2.5	> 2.5	> 2.5	> 2.5
Gentamicin 25 µg/mL - positive control				

Table 3.2. Antimicrobial testing of *P. crassipes* leaf extracts (MIC mg/mL)

Bacteria	etha	meth	H_2O
S. aureus	2.5	5.0	5.0
B. subtilis	2.5	5.0	2.5
E. coli	5.0	5.0	5.0
P. aeruginosa	5.0	5.0	2.5
Gentamicin 25 µg/mL - positive control			

Table 3.3. Antifungal testing of *X. americana* root extracts (MIC mg/ml)

Fungi	hex	dcm	meth
C. albicans	> 2.5	> 2.5	> 2.5
A. niger	> 2.5	> 2.5	> 2.5
P. chrysogenum	> 2.5	> 2.5	> 2.5
Amphotericin B 25 µg/mL - positive control			

Table 3.4. Anti-dermatophyte and anti-acne testing of *X. americana* root extracts MIC (µg/mL)

Organism	Erythro	Hex	$CHCl_3$	Meth	H_2O	Fr1	Fr2
P. acnes	0.195	62.5	125	500	>100	>100	100
	Micon	Hex	Chloro	Meth	Griseo		
T. mentagro -phytes	>0.2	250	250	500	3.125		

DMSO - Negative control Fr – Fraction from crude hexane extract

Table 3.5. Antimicrobial susceptibility testing of *P. crassipes* leaf extracts MIC (µg/mL) to *P. acnes*

Extract/drug	MIC (µg/mL)
hexane	> 1000
dichloromethane	> 100
Erythromycin 10 mg	0.5

Hexane and dichloromethane extracts of *Pavetta* leaf were also tested against *P. acnes* and the extracts had MIC values of 1000 and > 100 µg/mL respectively compared to erythromycin (MIC 0.195) the reference standard (Table 3.5). The screening against *P. acnes* and *T. mentagrophytes* was performed to determine the dermatological properties since information obtained from literature describes the use of the plant material in dermatological

conditions such as ringworm and craw - craw and could be of potential benefit in treating opportunistic infections in TB (Dalziel, 1956; Sofowora, 1982).

From the results of the experiments, *P. crassipes* leaf extracts were not as active when compared to the reference antibiotics or *X. americana* root extracts. The inclusion in the preparation could be justified by the principle of synergy where an extract is added to enhance the activity of the actives, such as stabilising the actives through anti-oxidant action, enhancing bioavailability rather than anti-bacterial action.

3.5 Conclusion

The results of antimicrobial susceptibility testing of both plant crude extracts revealed weak activity at concentrations (mg levels) considered to be high by literature standards. However, *X. americana* root extract exhibited moderate activity (µg levels) against gram positive bacteria such as *S. aureus* and *B. subtilis* and *P. acnes*. The extract could potentially have good activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) since it is effective against the gram positive organisms (Cos *et al*., 2006).

Essentially, the *in vitro* experiments were designed to resemble the target disease and associated symptoms so testing against representative bacteria helps in determining potential activities against organisms which may co-exist with TB infections (Kariba *et al.*, 2002).

Chapter 4 Drug Susceptibility Testing of Plant Extracts against *Mycobacterium aurum*

4.1 Background

In general, saprophytic, non-pathogenic strains of mycobacteria are used in preliminary screening assays in order to obtain quick results and/or because of biosafety concerns in working with virulent *M. tuberculosis*. The non-pathogenic strains have advantages such as rapid growth and the use of Class 1 or 2 laboratory facilities which is readily available in most institutions (Newton, 2002; Franzblau *et al*., 1998).

4.1.1 Introduction

M. aurum is a non-pathogenic strain of the genus Mycobacteriaceae. It is an aerobic, acid fast, gram positive organism (HPA, 2008). The colonies are smooth and shiny in appearance, yellow in colour, convex, entire and cells are 2 µm in length. It is easily emulsifiable and grows on glucose agar or nutrient broth at 37°C. Investigations on *M. aurum* are restricted to WHO Class 2 laboratories (HPA, 2008). *M. tuberculosis* H37Ra (avirulent) strain has also been used as a substitute or test model for the more virulent H37Rv strain in the initial assay determination for molecules with potential activity against MTB though both are slow growing (Collins and Franzblau, 1997). *M. smegmatis*, *M. bovis*, *M. chelonae* and *M. fortuitum* are other examples of common species used in preliminary anti-TB screening tests. Newer, rapid assays for *M. tuberculosis* (including MABA), however, have significantly lessened the time differential in obtaining results for slow growing and rapidly growing mycobacteria.

The vital point to be considered would appear to be the degree of similarity in drug susceptibility between any surrogate mycobacterium and virulent *M. tuberculosis* with special care needed over species or strains which might be less sensitive (Collins and Franzblau, 1997). Chung *et al.* (1995) carried out a study in which they investigated uracil uptake in *Mycobacterium aurum and M. tuberculosis* H37Rv in the bid to develop a high-through put screen for detecting anti-TB compounds. Their observations of the relative susceptibilities to anti-TB drugs showed that *M. aurum* had high predictive ability for high throughput anti-TB screening.

Reports of a study by Collins and Franzblau (1997) using an agar dilution technique suggested that rapidly growing mycobacteria such as *Mycobacterium smegmatis*, *Mycobacterium phlei*, *Mycobacterium fortuitum*, and *Mycobacterium neoaurum* were a little different to (and usually less sensitive than) H37Rv when compared to the slow-growing *Mycobacterium bovis* BCG and H37Ra with respect to MICs of fifteen compounds.

Studies by Arain *et al. (*1996) have demonstrated the similarity in drug susceptibility between *M. bovis* BCG and H37Rv. However, the observation that *M. bovis* BCG was not sensitive to pyrazinamide in the primary testing could lead to compounds with anti-TB activity being missed. It was suggested that utilising a less virulent strain of *M. tuberculosis* such as H37Ra may be a better option (Collins and Franzblau, 1997). Pascopella *et al*. (1993) in their study described how difficult it was to differentiate the physical and genetic make-up of the virulent H37Rv and avirulent H37Ra. In the study by Collins and Franzblau (1997), H37Ra appeared more sensitive than H37Rv and they suggested that the use of H37Ra for primary screening in a TB drug discovery program might result in a few false leads but would present a situation where it was less likely to miss active compounds. Thus, *M. tuberculosis* H37Ra would appear to be a suitable surrogate for the virulent H37Rv strain in a primary screening assay, thereby decreasing biosafety concerns and the required level of containment.

In summary, Visual MABA when used with *M. tuberculosis* H37Ra provides a primary anti-TB screen which can be conducted inexpensively in the most basic biosafety Level 2 laboratory. However, *M. aurum* was used in the initial screen for this study because it was readily available from the Health Protection

Agency, U.K and MTT (redox dye) was found to be a cheaper and equally good alternative.

4.2 Aims and Objectives

The aims of the work described in this chapter were to use *M. aurum* as an initial screen to demonstrate the anti-tubercular properties of the various plant extracts, to identify active fractions and determine MIC values. It was also meant to be used as a tool to monitor biological activity during the isolation and purification process of any active fractions.

4.3 Experimental

4.3.1 Materials

M. aurum NCTC 10438 was purchased from Health protection Agency, UK. Other materials used were glucose powder (Sigma and BDH), glycerol (BDH), filter paper (Whatman No 1), nutrient agar and broth powder (Lab M Ltd), dimethyl sulfoxide (Sigma Aldrich), flat bottom 96 well microplate (Iwaki, Japan), MTT-[3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] powder (Sigma) and streptomycin powder (Sigma).

4.3.2 Equipment (The equipment used was same as that described in section 3.3.1)

4.3.3 Preparation of reagents and media

4.3.3.1 McFarland standard (The preparation is described in section 3.3.4.1)

4.3.3.2 Glucose agar

1L of deionised distilled water was added to a conical flask containing 28 g of dehydrated agar powder (25 g for broth) and 10 g of glucose powder. This was allowed to soak for about 10 minutes, swirled to mix and dispensed into sterile bottles in 9 ml portions. These were then sterilised at 121˚C for 15 minutes. For agar, the sterilised liquid was allowed to cool to 50˚C, mixed and poured into petri dishes and after solidification, stored at 2-8˚C until use. The plates were used for sub-culturing and disc assays while glucose broth was used for the micro dilution assays.

4.3.3.3 Stock storage medium for bacterial culture

M. aurum was grown in glucose broth for 72 hours. This was spun and a mixture of double strength nutrient broth and glycerol (50% v/v) added to the micro-tube. This was frozen and stored at - 20 ˚C for subsequent use.

4.3.4 Isolation and cultivation of *M. aurum*

M. aurum NCTC 10438 was inoculated into glucose broth and incubated at 37˚C for 72 hours. Working suspensions were prepared from dilutions of 72-hour cultures made by comparison to 0.5 McFarland turbidity standard.

4.3.5 Determination of MIC by micro broth dilution assay

A concentration range of 31.25 -1000 µg/mL of the plant extracts were prepared directly on the multi-well plate for MIC determination. This was incubated with a bacterial suspension of *M. aurum* prepared from a fresh colony maintained on glucose agar and adjusted to a turbidity equivalent to that of a 0.5 McFarland tube. A dilution of 1 in 100 was made in broth and 100 µL transferred to wells containing 100 µL of extracts which had 2 x the final concentration.

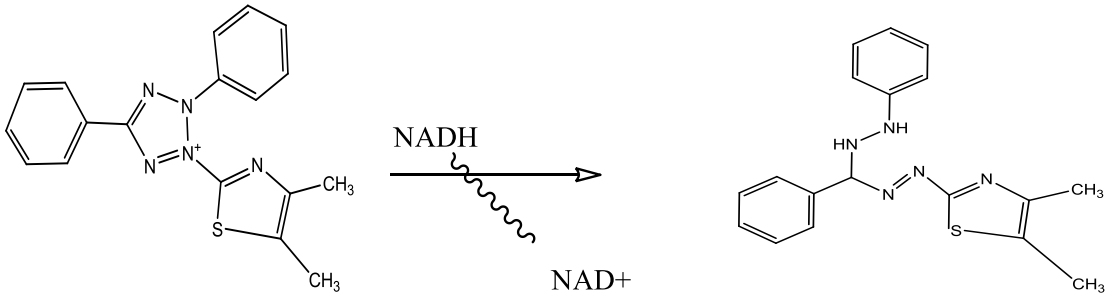


Fig. 4.1 Conversion of MTT to formazan by succinic dehydrogenase enzyme (www.roche-applied-science.com/prod_inf/manuals)

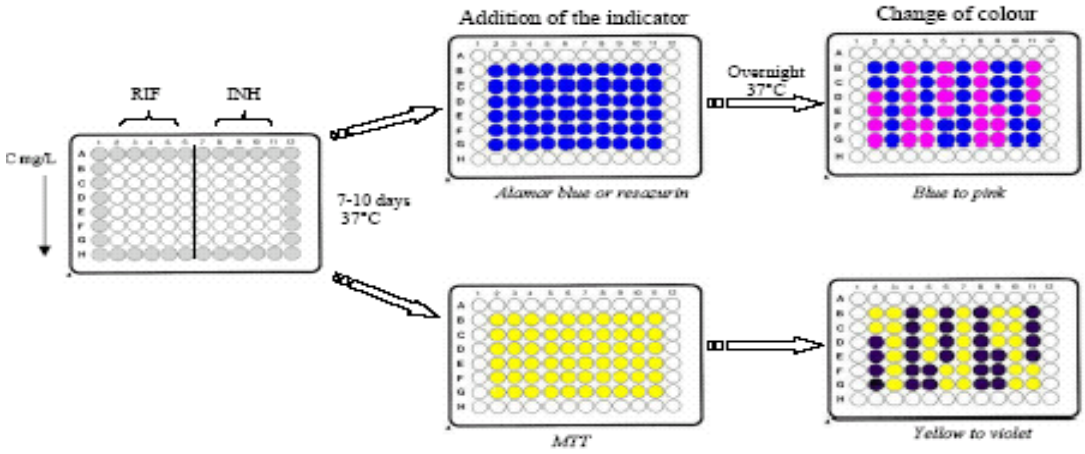


Fig.4.2 A 96 multi-well plate for conducting redox colorimetric assay (Martin *et al*., 2006)**.**

After 3 - 5 days of incubation, 20µL of MTT [3(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was added to each well and the plate re-incubated for a few hours followed by visual reading (Figure 4.2). The method is based on the reduction of a coloured indicator MTT which changes from yellow to purple. The colour change indicates the growth of bacteria and MIC was defined as the lowest concentration of the extract that prevented the change in colour.

4.3.6 Determination of drug susceptibility by disc diffusion assay

The disc method was used when the extract concentrations required were high and MICs difficult to visualise with the broth dilution method due to their coloured nature. Filter paper discs (6 mm) impregnated with plant extracts (100 – 3.125 mg/mL) were prepared by placing 10 µL volumes of dilutions to give a final concentration range of 1000 - 31. 25 µg/mL). The discs were dried on a hot plate at a mild temperature of 40 ˚C.

Agar plates were inoculated with adjusted bacterial suspensions using cotton-wool swab within 15 minutes of preparation. Sterile cotton-wool swab was dipped into the suspension and excess liquid removed by turning the swab

against the side of the tube. The swab was then used to spread the inoculum evenly across three directions. The plates were allowed to dry at room temperature before applying the discs containing plant extract dilutions firmly to the dry surface. Thereafter, plates were inverted and incubated within 15 minutes of disc application. The negative control plate was observed for semi-confluent growth and measurements of the zones of inhibition to the nearest millimetre taken with a ruler.

4.3.7 Determination of active fractions by agar overlay bio-autography

TLC was performed on fractions obtained from preparative HPLC in order to localise activity and to aid isolation of the active constituents (Section 7.5). The plates were dried to ensure complete removal of solvents. Molten glucose agar which had been maintained at 45 – 50 °C was seeded with *M. aurum* suspension (1 in 100 dilution of 0.5 McFarland turbidity standard) and poured over the TLC plate aseptically. This was allowed to solidify and the plate was then incubated overnight at 37° C for 72 hours. Bio-autograms were sprayed with 2.5 mg/mL of MTT solution and incubated for 4 hours at 37 °C. Zones of inhibition were seen as clear yellow areas while areas with microbial growth were seen as purple areas (Figure 4.5).

4.4 Results and Discussion

The results of the initial susceptibility testing of the crude extracts are presented in Table 4.1. The plant extracts were tested against *M. aurum* prior to testing of bioactive fractions against *M. Tuberculosis*. As already discussed, *M. aurum* was used as a test model system for the anti-TB screening because it is a non-pathogenic, saprophytic rapidly growing mycobacterium which has been shown to have similar drug susceptibility and genetic composition as *M. tuberculosis* although *M. smegmatis* and a number of other saprophytic organisms are also used as test models in the initial screening process (Chung *et al*., 1995; Newton *et al*., 2002). The colorimetric *in vitro* micro dilution assay method by Palomino

et al. (2002) was used to determine crude drug susceptibility (Figures 4.1 and 4.2).

The preliminary microplate assay results showed inhibitory activity against *M. aurum* at MIC of 125 µg/mL for the hexane and dichloromethane extracts of *X. americana* while the methanol extract of *X. americana* had an MIC value of 1000 µg/mL against *M. aurum*. Hexane and dichloromethane extracts of *P. crassipes* leaf exhibited no inhibitory activity at the concentration range used (125 -1000 µg/mL) while the methanol extract had inhibitory activity with an MIC value of 1000 µg/mL. The reference standard streptomycin (positive control) was initially used at a concentration of 10 µg/mL but was subsequently shown to have an MIC value of 4 µg/mL while ampicillin (100 µg/mL) was used as negative control.

Growth control (culture medium and *M. aurum* with no extract), blank (culture medium only) and solvent control (DMSO and culture medium) were also added to validate the results (Newton *et al*., 2002). The assays were carried out in triplicate to obtain MIC values. Streptomycin and ampicillin were also used as positive and negative controls in addition to a blank (culture medium only) and a growth control (control medium and bacteria without the presence of drug) to validate the results (Martin *et al*., 2006; Newton *et al*., 2000). Due to solubility problems encountered with dissolving the active non-polar fractions in DMSO by the micro dilution method, disc assay procedures were subsequently used to screen the HPLC fractions to localise the activity.

Ximenia extract in particular is highly coloured at high concentration ranges making it difficult to visualise the colour change from yellow to purple with MTT. Solutions of the extracts were made up to a concentration of 1000 µg/mL from which 100 µg was spotted on discs and subjected to drug susceptibility testing by disc assay method. Hexane and dichloromethane extracts of *Ximenia* exhibited inhibitory activity against *M. aurum* comparable to streptomycin (10 µg) at 10 times its concentration (Figure 4.3). The tests were carried out in triplicate and solvent control discs were subjected to the same treatment as the extracts.

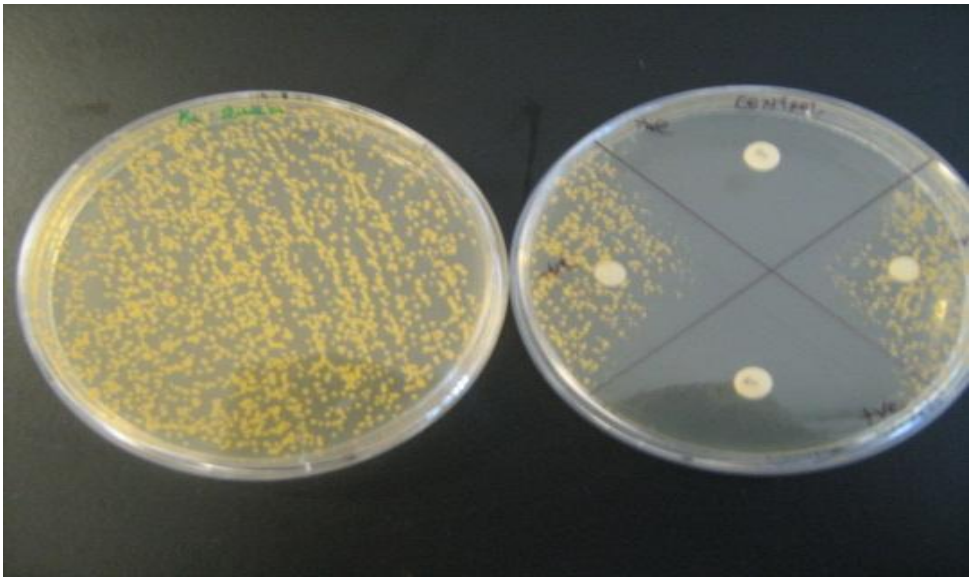
Due to the difficulties experienced with structure determination of LC fractions by NMR and mass spectrometry, further testing of fractions obtained from

preparative HPLC extracts of crude XAH and XAD to localise activity was demonstrated by disc diffusion assay and bio-autography (Figures 4.4, 4.5). From bioautograms, activity was shown to reside in the spot (R_f - 0.35) in fractions, XD3 and XD4 obtained from TLC separation as well as HPLC fractions. Efforts to characterise LC fractions were not successful so the fractions were subjected to TLC to further purify the extracts and to localise the activity. Separation details are described in chapter 7.

Table 4.1. *M. aurum* testing at test concentrations of 1000 – 125 µg/mL by micro plate agar dilution method

CODE	EXTRACT SOURCE	MIC (µg/mL)
XAH	*X. americana* hexane	125
XAD	*X. americana* dichloromethane	125
XAM	*X. americana* methanol	1000
XAW	*X. americana* water	> 1000
PCH	*P. crassipes* hexane	> 1000
PCD	*P. crassipes* dichloromethane	> 1000
PCM	*P. crassipes* methanol	250
PCW	*P. crassipes* water	> 1000
STM	Streptomycin	10
AMP	Ampicillin	100

i)



ii)

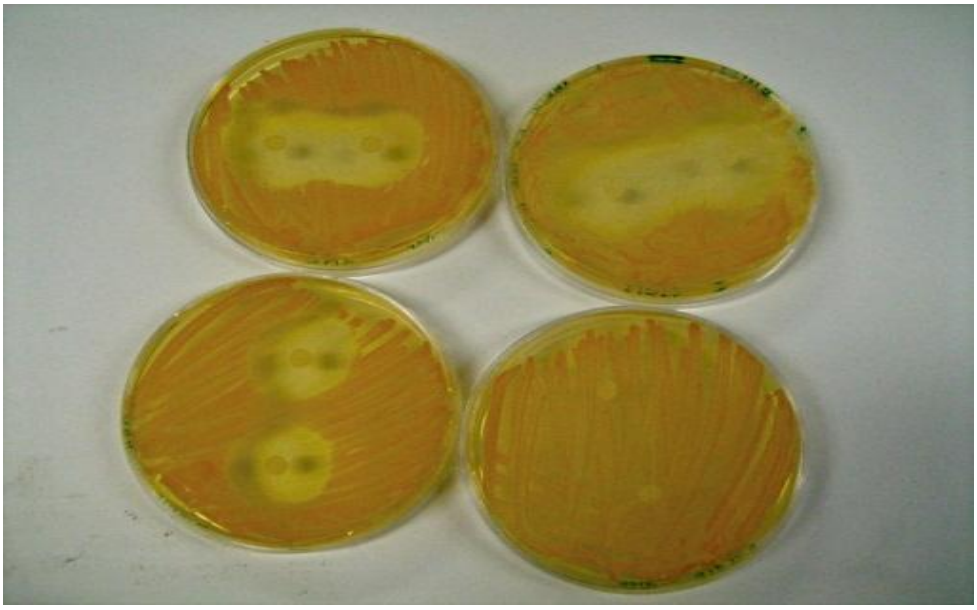
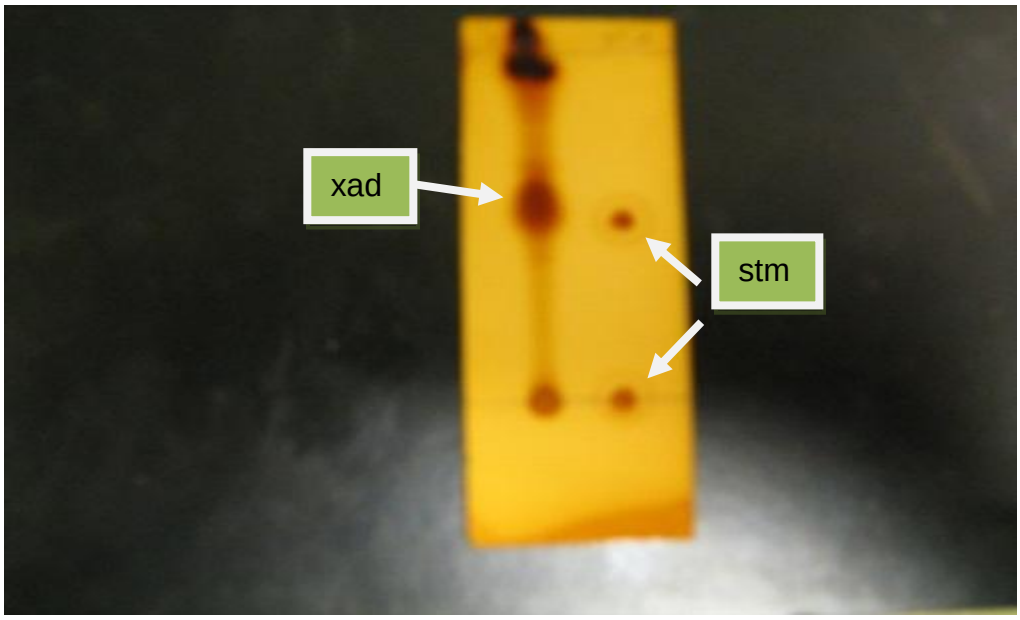


Fig. 4.3 i) Plates showing *M. aurum* control plate (growth) and discs containing 10 µg streptomycin (clear zone) and control discs (solvent). ii) Plates showing growth inhibition zones of 10 µg STM (top right), 100 µg XAD (top left), 100 µg XAH (bottom left) and solvent control disc (bottom right).

i)



ii)

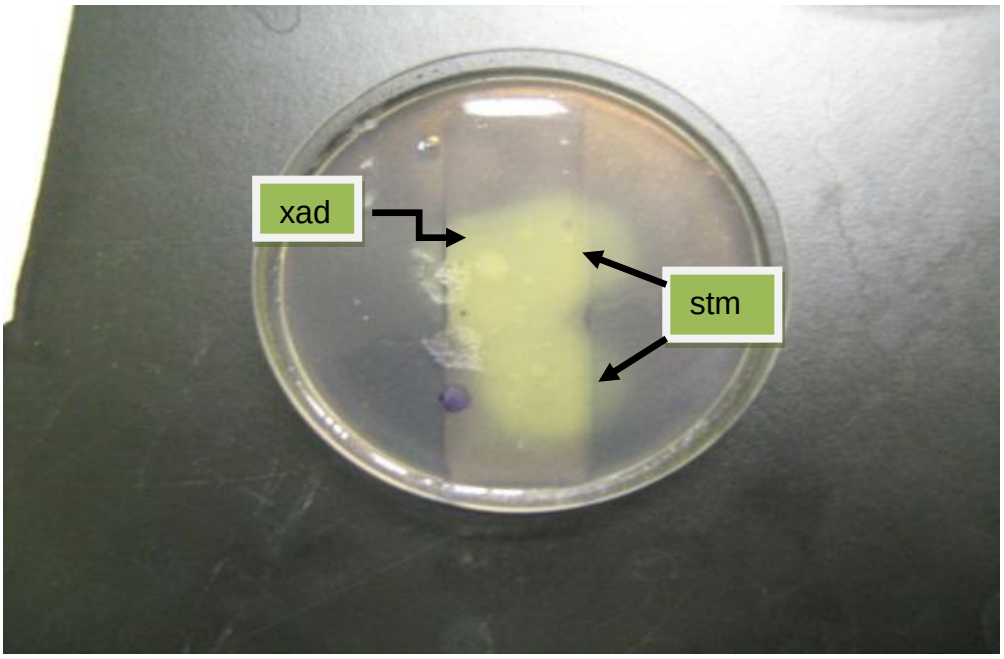
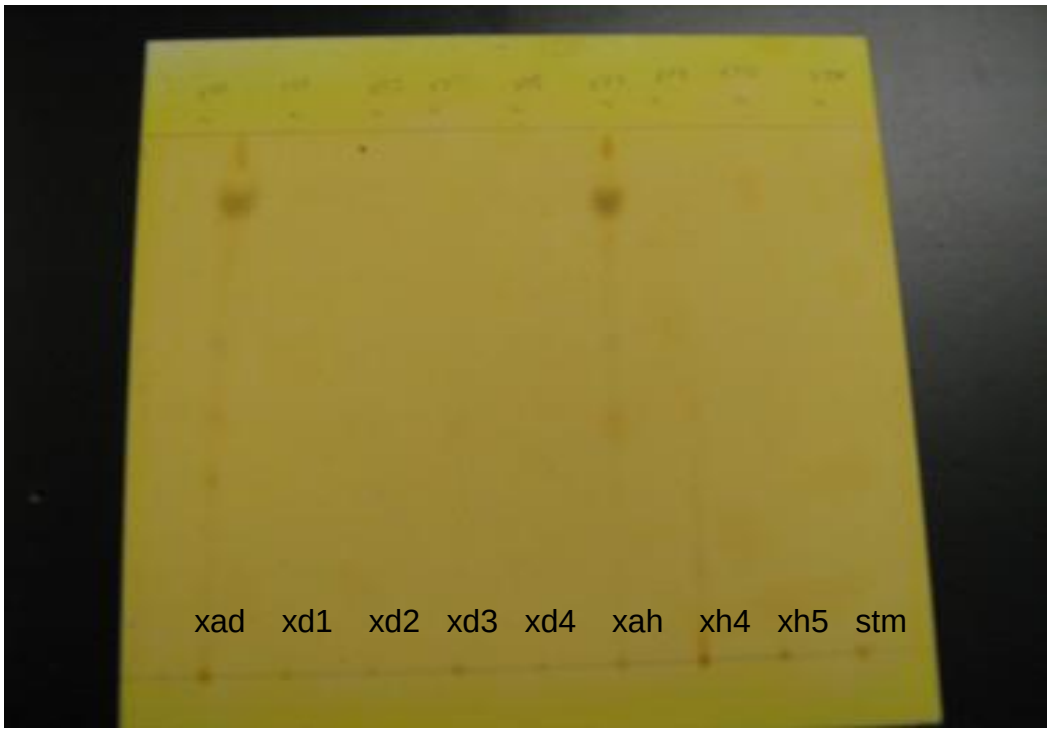


Fig.4.4 i) TLC plate (5 x 7 cm) spotted with crude XAD (most active fraction against MTB) and streptomycin (positive control) visualised with iodine after development with methanol: dichloromethane (2:98 v/v) solvent system.

ii) Duplicate TLC bio-autogram visualised with MTT solution, yellow regions show areas of inhibition while purple regions show areas with bacterial growth. The active spot had Rf value of 0.5.

iii)



iv)

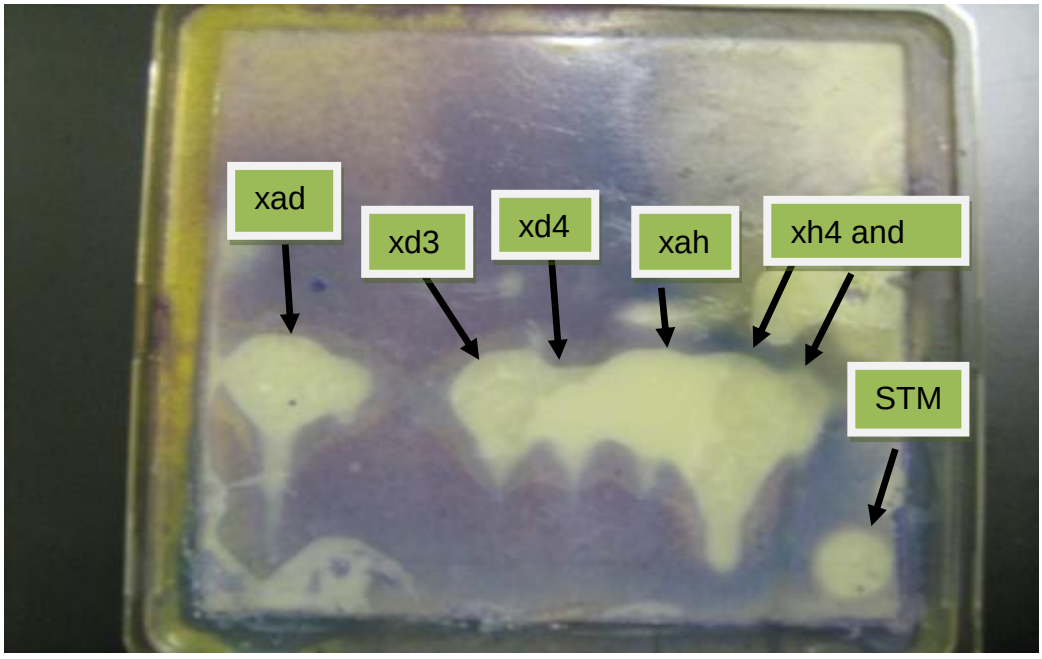


Fig.4.4 iii) TLC plate (20 x 20 cm) spotted with XAD (crude DCM extract), XD1, XD2, XD3, XD4 from TLC fractions, XAH (crude hexane extract), XH4, XH5 from LC analysis and STM. Developed with hexane: chloroform: methanol (4:4:1, v/v/v) and visualised with 2-DNP. iv) Yellow areas show inhibition zones of active fractions and reference drug streptomycin while purple areas show regions of bacterial growth on spraying with MTT.

i)

ii)

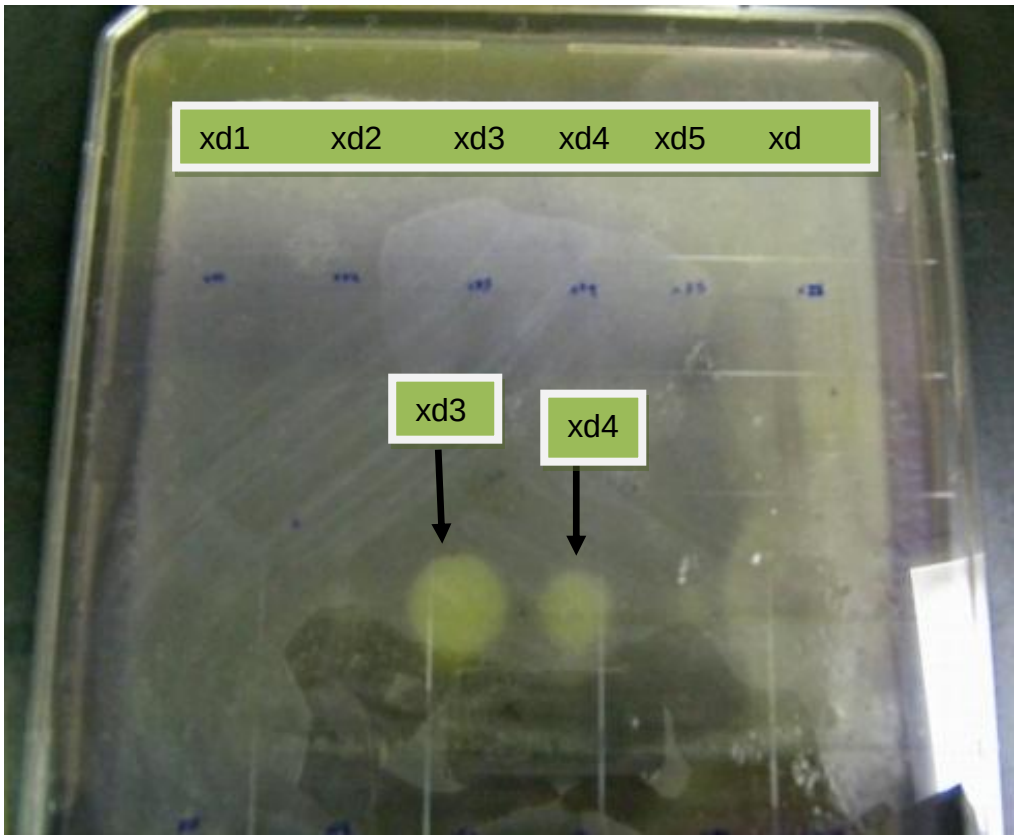


Fig.4.5 i) TLC bioautogram control plate. ii) TLC bioautogram spotted with LC fractions XD1 – 5 (section 7.4.5). Spots XD3 (36 µg) and XD4 (72µg) (Rf-0.35) show zones of inhibition.

4.5 Conclusion

M. aurum was easy to grow and maintain on glucose agar and results could later be correlated with anti-TB testing. The results obtained using the micro dilution method exhibited higher MIC values compared to the disc assay method. However, it is possible that this might result in a few false leads as suggested with some of the values (Table 4.1). The methanol fraction of *P. crassipes* initially showed some inhibitory activity at 1000 µg/mL with *M. aurum* but testing against *M. tuberculosis* at a concentration of 64 µg/mL exhibited no inhibitory activity (Table 6.1, Section 6.4). On the other hand, the hexane extract of *P. crassipes* which showed no inihibition against *M. aurum* in the initial screening had 86.7 % inhibition at 64 µg/mL on testing against *M. tuberculosis.* The advantage of subjecting the false positive extracts to further testing is that the probability of missing possible active fractions or compounds would be greatly reduced since all fractions were tested. Bioautography proved to be useful in localising active fractions from the crude extract.The active fractions from TLC separations looked promising and would be followed up with further TB testing against *M. tuberculosis*.

Chapter 5 Synergy and Efflux Studies on Methanol Extracts of *X. americana* Root and *P. crassipes* Leaf

5.1 Background

Following general antimicrobial screening with representative bacteria and fungi, it was thought necessary to conduct synergistic interaction and efflux studies since the antibacterial activity showed mild to moderate spectrum activity which was not potent enough to warrant further exploration of individual extracts as stand-alone anti-bacterial agents. Drug interaction and efflux studies were performed in order to explore efflux pump inhibitory properties of the methanol extracts and to possibly identify mechanisms of action. The methanol fractions were considered for the study because extraction yield was high for both plant materials and also because literature review of isolated and characterised constituents revealed the presence of phenolic compounds such as flavonoids which have been reported by other investigators to exhibit efflux inhibitory activities (Lechner *et al*., 2008; Gibbons *et al*., 2003).

The activity of over expressed efflux pumps in bacteria has been reported to be a major mechanism by which multi drug resistant bacteria exercise their ability to evade killing by antibiotics (Viveiros *et al*., 2008). Bacterial efflux pumps are reported to protect the organisms from antibacterial compounds or xenobiotics. They also serve as transport tools in uptake of essential nutrients as well as maintaining cell activities (Paulsen *et al.,* 2006).

The reasoning was that, if constituents were found to have synergistic or efflux inhibitory action against drug resistant bacteria, it could possibly point to the relative contribution of the fractions having no antibacterial properties and the possible reason for the inclusion in the herbal remedy.

5.1.1 Introduction

5.1.2 Synergy studies

Synergy studies have been conducted in order to explain activities seen in herbal products. The concept of synergism in phytomedicines arose out of difficulties experienced in isolating bioactive compounds from herbal products or in adducing clinical effects to single constituents in phytomedicines (Williamson, 2001).

The therapeutic efficacy of single or multicomponent herbs is thought to reside in synergistic interactions between the bioactive constituents. Often, long-lasting drug administration is required to achieve the desired clinical effect with reduction in severity of symptoms (Heinrich *et al*., 2004). This mode of drug administration can be likened to treatment of conditions such as depression, cancer, HIV in orthodox medicine where clinical effects become evident after a number of weeks (Williamson, 2001).

Another identical characteristic of phytomedicines and orthodox medicine is the need for drug combinations in treating chronic conditions. According to Heinrich *et al* (2004), many phytomedicines are yet to have their mechanisms of action elucidated. The article reports that there are examples of total herb extracts showing better effects than equivalent doses of isolated compounds. Justification for the biological effect observed has been attributed to synergy, enhanced bioavailability, cumulative effects or simply the additive properties of the constituents.

Overall, synergy is considered to be positive with the low doses used being seen as an advantage. Some ingredients which form part of herbal drug combinations have been investigated and reported to enhance bioavailability of other constituents. For instance, addition of black pepper, *Piper longum* which contains the alkaloid piperine to an anti-asthma recipe has been reported to increase the bioavailability of the mixture (Johri *et al*., 1992) and also the addition of clavulanic acid to amoxicillin used to increase susceptibility to β-lactamase producing organisms in conventional antimicrobial chemotherapy (Flores *et al*., 2005).

Bapela *et al*. (2006) investigated the activity of a combination of 7-methyljuglone, a naphthoquinone and first-line drugs, isoniazid and rifampicin in

which they reported a four to six-fold reduction in the MICs of both drugs suggestive of synergy.

In some other cases, negative or unwanted effects have also been observed to interfere with absorption of active constituents leading to decreased therapeutic concentrations. Ernst *et al.* (1999) investigated drug interactions between St John's Wort, *Hypericum perforatum* and oral contraceptives with negative effects.

5.1.2.1 Philosophy of herbal therapy

Practitioners of herbal medicines have been described as using the 'herbal shotgun' approach as opposed to the 'silver bullet' method of orthodox medicine because no particular enzyme or cellular system is targeted during therapy (Heinrich *et al*., 2004; Duke and Bodenschutz-Godwin, 1999).

Some additions to recipes are essentially not for synergy but as a supportive measure for instance, certain components which promote bowel movement are added to recipes used in the treatment of piles. The special properties of phyto-medicines are being investigated to attempt to justify the therapeutic effect of combinations of individual constituents (Williamson, 2001).

5.1.3 Synergy testing

Demonstration of synergy can be costly due to the requirement for constituents to be tested individually and then going on further to compare the effect given with the same dose in a mixture (Williamson, 2001).

The expression "polyvalent action" has been used to describe better or greater effects seen with a combination of substances than expected from single component contribution (Berenbaum, 1989). On the other hand, antagonism is defined as an opposite effect of synergy and from reports it is more easily demonstrated than the other effects.

Synergy testing by checkerboard assay technique is also called the fractional inhibitory concentration testing (Fidai *et al*., 1997). The FIC method is referred to as the checkerboard because of the 8 x 8 pattern of wells used.

The compounds tested in combination are used at concentrations above and below their MIC's determined individually against the bacteria in question. The results of a checkerboard assay after overnight incubation demonstrates a non-dynamic growth inhibition (Kamicker *et al*., 2008). The results obtained can be interpreted as synergy, indifference or antagonism depending on the FIC index calculated for the two drugs in combination. Synergy is defined as ∑FIC index ≤ 0.25, indifference 0.5 to 4.0 and antagonism as > 4.0 (Viveros *et al*., 2010; Fidai *et al*., 1997).

5.2 Aims and Objectives

The principal aim then of the next part of the research programme was to conduct synergy and interaction studies to aid prediction of possible mechanism of action, synergistic or additive activities. The study was also aimed at identifying and evaluating the relative contribution of the bioactive constituents to the antimicrobial effects exhibited by the plant extracts.

5.3 Experimentals

5.3.1 Materials (Section 4.3.1)

In addition to materials already described in Section 4.3.1, Mastring - S M13 and M14 antibiotics were purchased from Mast Diagnostics, Merseyside, UK and standard antibiotics from Sigma Aldrich, U.K.

5.3.2 Determination of minimum inhibitory concentration by broth dilution method

The overnight cultures of the bacterial micro-organisms were grown on agar plates or in broth. The inoculum was prepared by picking four or five colonies and emulsifying in saline to give a density equivalent to 0.5 McFarland standard. The suspension was visually compared to the standard and adjusted to a density approximately equivalent to 1 x 10^8 colony forming units (CFU)/mL. To aid comparison, test suspension and standard were viewed against a white background with a contrasting black line. From the 0.5 McFarland standard

culture suspension, a 1 in 100 dilution was made up in broth medium to give a final inoculum of ca. 5 x 10^5 CFU/mL. The suspension was used for the macrodilution and microdilution tests.

Macrodilution:
Antibiotic ranges of 0.5 - 256 µg/mL were prepared so that the addition of an equal volume of inoculum would give final dilution range of 0.25 – 128 µg/mL. Sterile capped tubes (75 x 12 mm) were arranged for each antibiotic and plant extract in duplicate (preparation and table of MIC values obtained are shown in Appendix A). Aliquots (1 mL) of the test organisms were added to 1 mL of the antimicrobial agents diluted in broth. The tubes were agitated with a vortex mixer and incubated at 37˚C for 18-24 hours in air. MIC endpoint was read and interpreted as the lowest concentration of antibiotic which showed no visible growth (EUCAST, 2000; BSAC, 2006).

Microdilution:
A 96 well sterile microtitre plate was labelled with the appropriate plant extract dilution and 100 µL of each extract added to 100 µL of test organism.
Inoculated and un-inoculated wells of plant extract-free broth served as growth control and sterility check to validate the results observed. The plate was covered with the lid and inoculated at 37˚C for 18 - 24 hours in air. MIC endpoint was read and interpreted as described above.

5.3.3 Determination of minimum inhibitory concentration by disc assay method (details are already described in Section 4.3.6).

5.3.4 Drug interaction assay using Mastring S antibiotic strips

Mastring S-14 (antibiotic containing strips) were placed on agar plates without plant extracts (control plates) and duplicates incorporated with plant extracts to achieve a final concentration of 50, 100, 5000 and 10,000 µg/mL. The assays were conducted as four different experiments. The plates were inoculated with *S. aureus*, *E. coli* and *P. aeruginosa* as already described in Section 4.3.6. Plates containing solvent used in sample solution and negative control plates

were added to validate the results. Inhibition zones were measured and percentage differences calculated in order to determine which antibiotics showed some interaction in the preliminary assessment (Tables 5.4a - c, Figures 5.2 a - c). Tests were carried out in triplicate.

5.3.5 Checkerboard (FIC) assay of X. americana methanol extract and streptomycin

After determining streptomycin and plant extract MICs by agar dilution (0.5 µg/mL and 3 mg/mL respectively), the checkerboard assay was then carried out in duplicate by testing solutions of *X. americana* methanol extract and streptomycin in combination. The concentration range of streptomycin and extract used in the experiment was 0.031 x MIC to 4 x MIC in an 8 x 8 format depicted in Table 5.7. Stock solutions of streptomycin (4 µg/mL) and the extract (24 mg/mL) were prepared in DMSO and further doubling dilutions done in broth before being transferred to the wells. Positive and negative control plates to assess presence and absence of turbidity were also tested (Bonapace *et al*., 2002). 50 µL of each drug dilution was added to 100 µL of organism suspension to make up a volume of 200 µL in each well. Plates were then gently mixed and incubated at 37 °C for 18 - 24 hours.

The micro- titre plate turbidity was read at 595 nm using the micro-plate reader but variable results were observed so plates were subjected to visualisation by MTT as it was found to give more consistent results. The tests were carried out in duplicate with negative and positive growth controls.

5.3.6 Computation of drug interactions

Fractional inhibitory concentration (FIC) is a term used to describe the extent of interaction or synergy between drugs tested. FIC is calculated as MIC of drug in combination divided by the MIC determined for each individual drug or compound. The sum of the FIC's of XAM and STM was calculated to express the extent of interaction. Four methods were used to interpret the synergy data.

The first method (Mean FIC) involved calculation of the FIC index using clear wells found in each row and column. The second method (full row/column)

involved calculating FIC index for each drug using row and column with lowest concentration of drug and clear wells i.e. no growth. The third method (lowest FIC index) involved taking lowest FIC index of clear well along the clear and turbid border. The fourth method (two well) defines synergy as no growth in the two wells having 0.25 x MIC of the two drugs, STM and XAM (Bonapace *et al*., 2002).

FIC index was calculated using the formula, FIC index = A/(MIC A) + B/(MIC B), where A = concentration of drug A in the micro-titre well that is the lowest inhibitory concentration of A in its row (Fidai *et al*., 1997). (MIC A) is the MIC of drug A alone. Synergy was regarded as ∑FIC value ≤ 0.5, additivity or indifference has ∑FIC value 0.5 to 4 and antagonism as ∑FIC index value greater than 4.

5.3.7 Statistical analysis

Statiscal analysis of the results obtained from drug interaction studies were subjected to evaluation by SPSS Inc. version 18 (Chicago, IL). The drug interaction studies between standard antibiotics and extracts, PCM and XAM were analysed by One - Way ANOVA (analysis of variance). Tukey was used for post-hoc testing in order to carry out multiple comparisons. The alpha value of $p < 0.05$ was considered significant. The precision of the measurements was determined by calculating the standard deviation, standard error of mean and the coefficient of variation (CV). The CV represents the ratio of standard deviation to the mean and is useful for describing the variation in a test (Zady, 1999). Standard error was calculated to obtain a measure of the precision of the sample means (Altman and Bland, 2005). The formula CV = standard deviation/mean x 100 was used to calculate the coefficient of variation as a percentage while standard error (SE) was calculated by the formula: standard deviation / square root of n where n is the number of observations.

5.4 Results and Discussion

5.4.1 Preliminary synergy testing

Several antibiotic compounds (Mastring S antibiotic strip) were tested against organisms, *S. aureus* and *P. aeruginosa*, *E. coli* by agar containing the plant

extracts. Extracts were incorporated to a final concentration of 50, 100, 5000 and 10,000 µg/mL. There were no changes in zones of inhibition with 50 and 100 µg/mL but with a final concentration of 5000 µg/mL, some antibiotics exhibited synergistic interactions. Interestingly, there was no growth with 10000 µg/mL of extracts incorporated into agar which could possibly result from toxicity leading to cell death or potency of the active constituents (Figure 5.6 i). The zones of inhibition measured for antibiotics alone and with extract (PCM and XAM) incorporated to give final concentration of 5000 µg/ mL were used to calculate the percentage difference in inhibition zone diameter. A difference of ≥ 3mm was considered as inhibitory interaction.

XAM exhibited moderate activity with streptomycin against *S. aureus* but the inhibitory effect was greater with colistin sulphate (CO) and sulphatriad (ST). Initial screening of CO and ST showed no inhibitory activity but the presence of XAM enhanced its activity by approximately 3,000 %. In comparison, PCM did not show appreciable activity against *S. aureus* (Table 5.2a, Figure 5.1a). In clinical settings, sulphatriad (an anti-metabolite) which consists of a mixture of suphathiazole, sulphadiazine and sulphamerazine is used as a combination product to reduce the risk of crystalluria associated with sulphonamide use. However, it is not commonly used in clinical practice due to the associated kidney damage from accumulation of crystals in urine (Mitscher *et al.*, 2008). The factor in favour of its continued use in certain conditions is the relative cheapness compared to newer antibiotics.

Chemically, colistin sulphate is a cyclic polypeptide antibiotic from *Bacillus colistinus* and consists of polymixins E1 and E2 which act as a detergent to disrupt bacterial cell membrane. It has been found useful for treating infections with *P. aeruginosa* but like the sulphonamides, it has been largely abandoned in the clinical setting with the development of newer penicillins and cephalosporins. Some investigators (Knox *et al*., 2011; Wishart *et al*., 2008; Wishart *et al*., 2006) reported the usefulness of colistin in treating multi-drug resistant pulmonary infection in cystic fibrosis patients. PCM had strong positive effect on antibiotics, ampicillin and cephalothin against *P. aeruginosa* and *E. coli* (cephalothin only) and to a lesser extent on tetracycline (Tables 5.1b - c, Figures 5.1b – c). Ampicillin and cephalosporin Decrease in inhibition zones

were observed with streptomycin against *E. coli and S. aureus* in the presence of PCM unlike XAM which had a positive effect.

5.4.2 Synergy test with combination of streptomycin and XAM against S. aureus

Based on the research interest on anti-tubercular activities, further experiments on synergistic interactions were conducted with a combination of streptomycin, an anti-tubercular antibiotic and XAM against *S. aureus* as a model of gram positive organism. Using four different methods as described in Section 5.3.7, the results of the checkerboard assay were interpreted as additive for methods 1 and 2 and as synergy with methods 3 and 4 (Table 5.5). The FIC index gives the level of synergistic interaction which is shown in Table 5.5. The mechanism of the interaction may be through binding to the same sites or through other receptors. Initial tests with both compounds revealed inhibitory activity against *S. aureus* which was corroborated by the checkerboard analysis.

5.4.3 Synergy testing of combination of gentamicin and methanol extracts (PCM and XAM) against E. coli SR

Initial tests of the plant extracts (5 mg /mL) in combination with streptomycin (10 µg on disc) showed antagonism with streptomycin but synergy with gentamicin against *E. coli* sensitive strain NCTC 11560 (Figure 5.2c). Both drugs belong to the aminoglycoside class of antibiotics and the objective was to see whether the presence of the extracts could enhance susceptibility of streptomycin resistant *E. coli* to streptomycin or gentamicin. However, on determining the MIC of the same extracts with streptomycin resistant strain (UoS strain) by the disc assay method, no activity was exhibited as shown in Figure 5.8. Further testing was carried out using gentamicin (10 µg disc) on agar incorporated with a serially diluted XAM and PCM extract since tests carried out earlier had shown synergy with the sensitive strain. There was a greater reduction in inhibition zone with increase in extract concentration when compared to the control plate (Table 5.6). The extracts showed no effect on the activity with streptomycin or gentamicin. The changes observed are likely due to active efflux by the streptomycin resistant *E. coli* which was not overcome by the extracts.

5.4.4 Statistical analysis

One - Way ANOVA procedure was used to test for significance between means of zone diameters of inhibition of control antibiotic gentamicin and test groups containing extracts, PCM and XAM against *E. coli* SR. The analysis showed significant differences among the groups in Table 5.6 at $p < 0.05$. Tests for significance between control groups containing colistin sulphate, sulphatriad and test groups containing extracts, PCM and XAM against *S. aureus* were determined and shown to be significant at $p < 0.05$ (details are shown in Appendix A).

Table 5.1. Mastring S M-14 antibiotic discs content with code

Code	Antibiotic	Colour	Disc content (µg)
AP	ampicillin	grey	10
KF	cephalothin	primrose	5
CO	colistin SO_4	white	25
GM	gentamicin	salmon	10
S	streptomycin	white	10
ST	sulphatriad	mauve	200
T	tetracycline	brown	25
TS	cotrimoxazole	white	25

Legend for summary of *in vitro* interaction studies charts:

OC – antibiotic alone with organism

SC - antibiotic with organism (contains solvent used in dissolving extract)

PCM - methanol extract of *P. crassipes*

XAM - methanol extract of *X. americana*

Increased zone diameter of inhibition - (change of ≥ 3mm)

Decreased zone diameter of inhibition - (change of ≥ 3mm)

Table 5.2a. Data of *in vitro* drug interaction studies using *S. aureus*. Extracts were incorporated into agar to give final concentration of 5 mg/mL. Values recorded are average of three replicates (n = 3). *SE values were ≤ 0.57

	AP	KF	CO	GM	S	ST	T	TS
OC	27*	20.3*	0	17	11.7*	0	24	19.7*
SC	26	20	0	16*	12	0	24	20.7*
OC,SC,PCM	30	30	0	15.7*	0	0	32	20
OC,SC,XAM	30	30	30	30	30	30	30	30

Zone diameter of inhibition was measured in mm. SE – standard error
MIC's of PCM = 5 mg/mL , XAM = 0.031 mg/mL

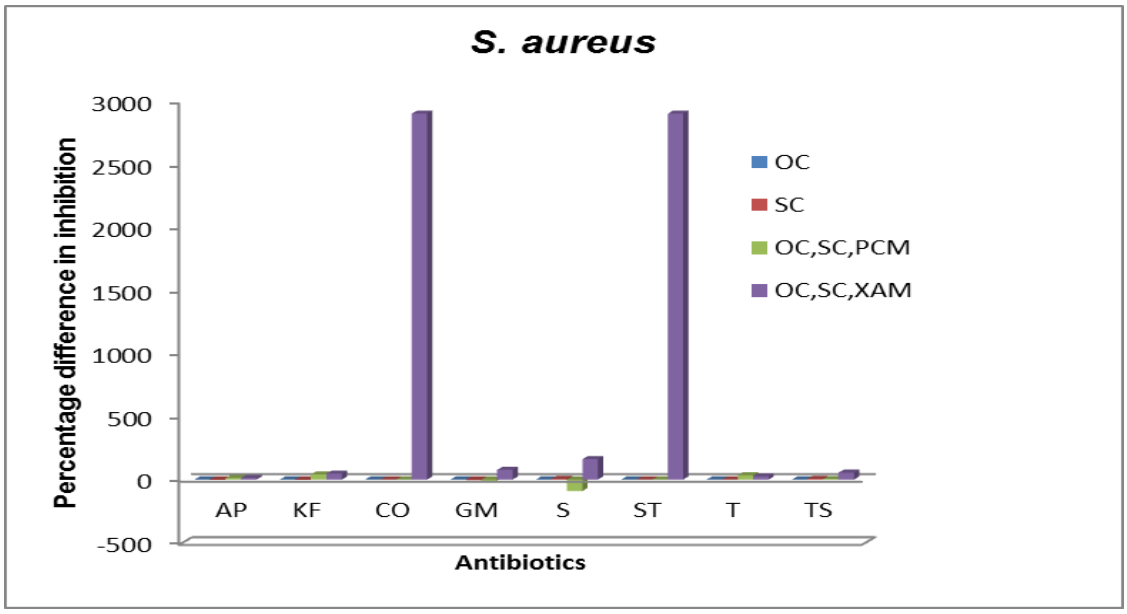


Fig.5.1a Chart showing percentage differences in zone diameter of inhibition between antibiotics alone and in combination with extracts, PCM and XAM.

Table 5.2b. Data of *in vitro* drug interaction studies using *P. aeruginosa*. Extracts were incorporated into agar to give final concentration of 5 mg/mL. Values recorded are average of three replicates (n = 3). *SE values were 0.0

	AP	KF	CO	GM	S	ST	T	TS
OC	0	0	15	16	12	17	10	12
SC	0	0	15	16	12	17	10	12
OC,SC,PCM	30	30	0	16	0	0	30	12
OC,SC,XAM	0	0	8	12	14	0	0	0

MIC's of PCM = 5 mg/mL, XAM = > 2.5 mg/mL

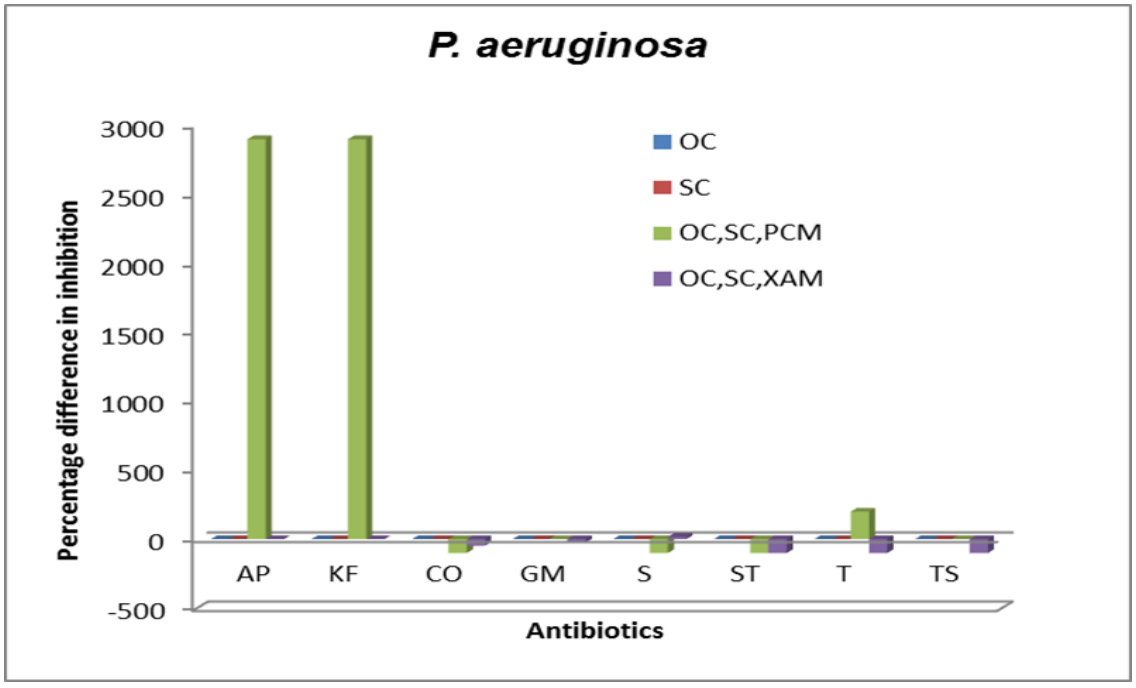


Fig.5.1b Chart showing percentage differences in zone diameter of inhibition between antibiotics alone and in combination with extracts, PCM and XAM.

Table 5.2c. Data of *in vitro* drug interaction studies using *E. coli*. Extracts were incorporated into agar to give final concentration of 5 mg/mL. Values recorded are average of three replicates (n = 3). *SE values were ≤ 0.66

	AP	KF	CO	GM	S	ST	T	TS
OC	0	0	15	11*	9	0	8	15
SC	0	0	14.6*	12	9.7*	0	8	13.3*
OC,SC,PCM	0	10	17.6*	13.5*	0	0	0	16
OC,SC,XAM	0	0	11	19	0	0	8	18

MIC's of PCM = 5 mg/mL, XAM = > 2.5 mg/mL

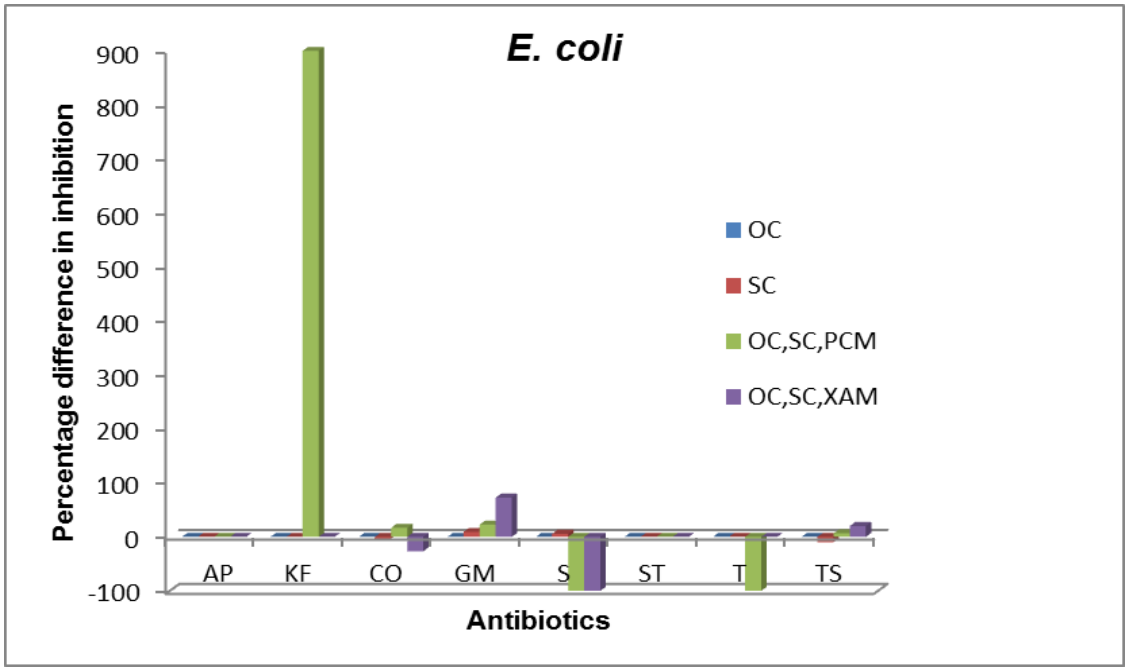


Fig.5.1c Chart showing percentage differences in zone diameter of inhibition between antibiotics alone and in combination with extracts, PCM and XAM

Table 5.3a. Summary of *in vitro* interaction studies of antibacterials with *P. crassipes* methanol extract (5 mg/mL final conc.)

Organism	Increased zone of inhibition	Inhibition cancelled	Inactive antibiotic active with extract	Indifference	Decreased zone of inhibition
S. aureus	ampicillin, cephalothin tetracycline	streptomycin		colistin SO_4, sulphatriad cotrimoxazole	
P. aeruginosa	tetracycline	colistinSO_4, streptomycin sulphatriad	ampicillin, cephalothin	gentamicin cotrimoxazole	
E. coli		streptomycin tetracycline	cephalothin	ampicillin, colistin SO_4, gentamicin, sulphatriad, cotrimoxazole	

Table 5.3b. Summary of *in vitro* interaction studies of antibacterials with *X. americana* methanol extract (5 mg/mL final conc.)

Organism	Increased inhibition zone	Inhibition cancelled	Inactive antibiotic active with extract	Indifference	decreased inhibition zone
S. aureus	ampicillin, cephalothin gentamicin, tetracycline cotrimoxazole, streptomycin		colistin $SO_{4,}$ sulphatriad		
P. aeruginosa		sulphatriad tetracycline cotrimoxazole		ampicillin, cephalothin streptomycin	colistin $SO_{4,}$ gentamicin
E.coli	gentamicin, cotrimoxazole	streptomycin		ampicillin, cephalothin, sulphatriad, tetracycline	colistin SO_4

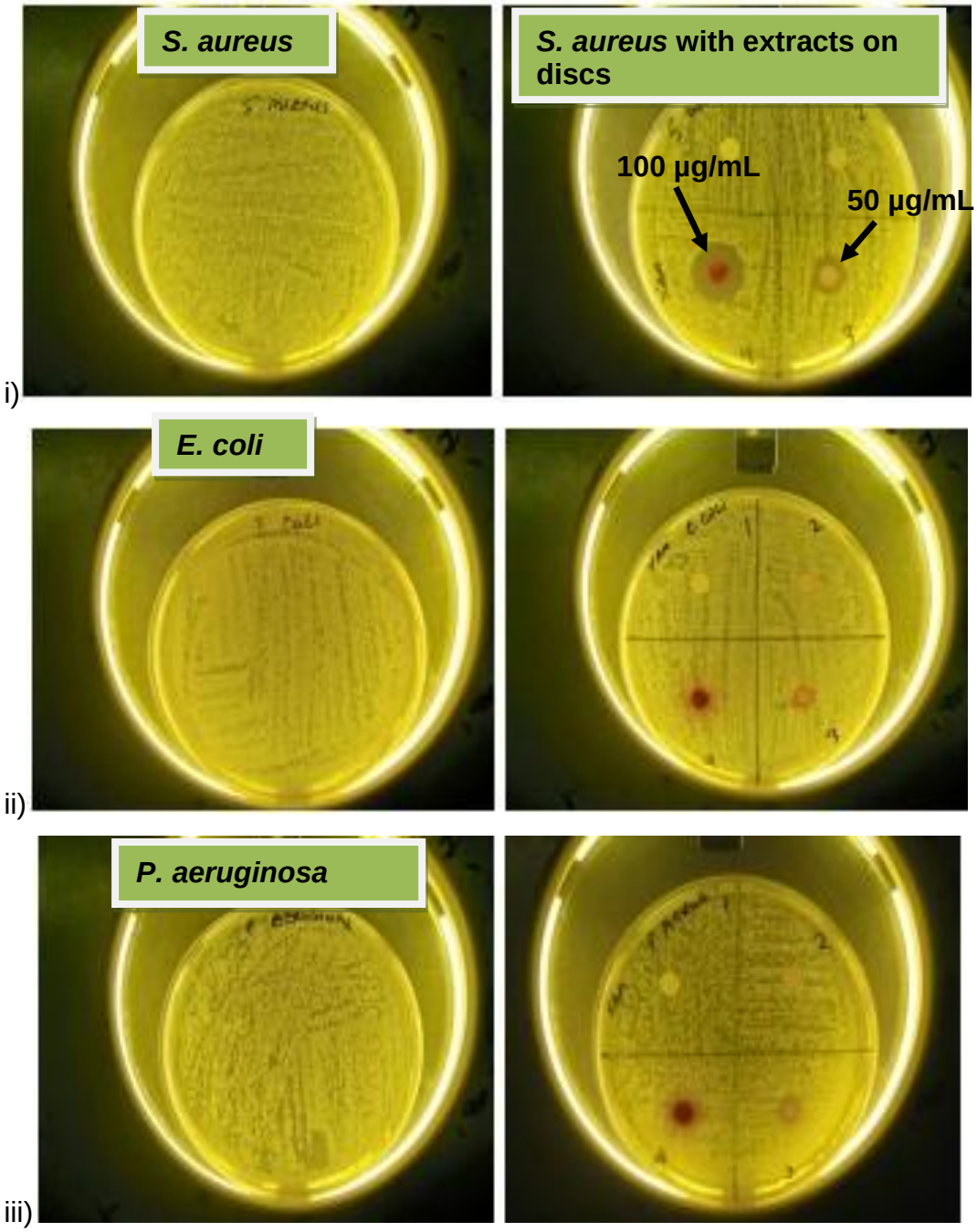


Fig.5.2 i) Plates showing *X. americana* disc assay with *S. aureus* control and with extracts on paper discs (100 - 12.5 µg/mL) showing zones of inhibition at 100 and 50 µg/mL.

ii) *E. coli* control plate and plate with extracts on discs showing no activity at 100 - 12.5 µg/mL. iii) *P. aeruginosa* control plate and with extracts showing no activity at 100 - 12.5 µg/mL.

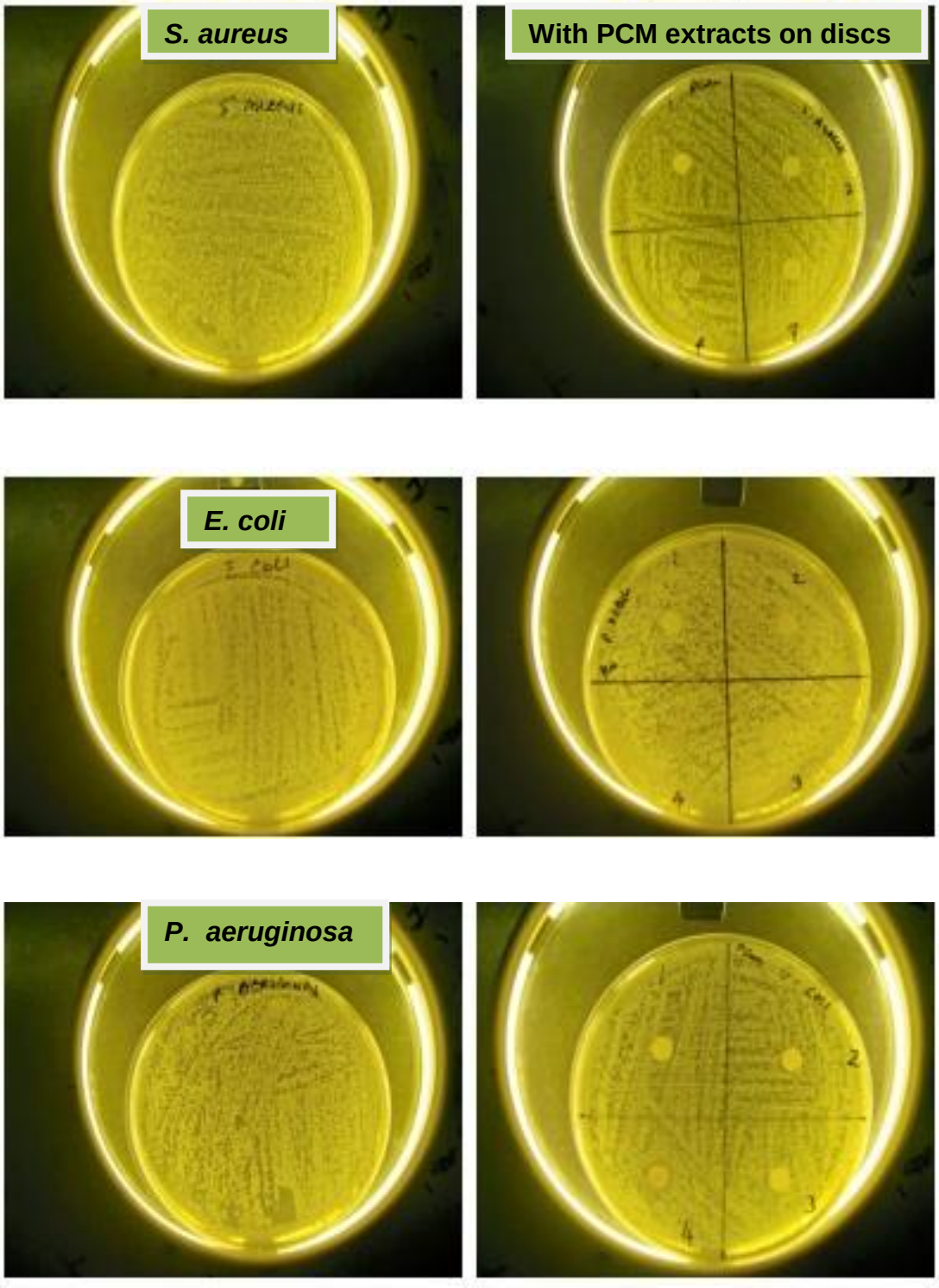
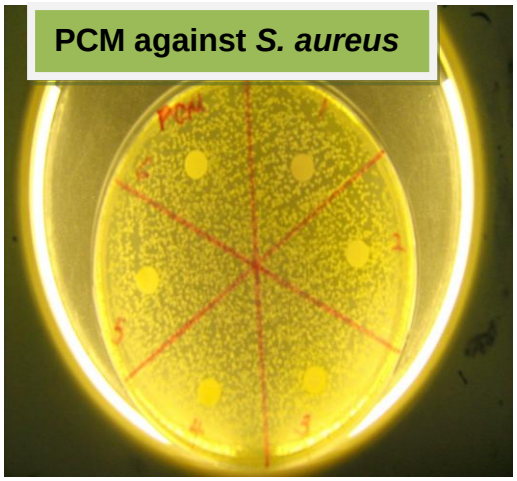


Fig. 5.3 Plates showing *P. crassipes* disc assay with *S. aureus*, *E. coli* and *P. aeruginosa*. No inhibitory activities were observed at 100 - 12.5 µg/mL for the extracts.

i) 

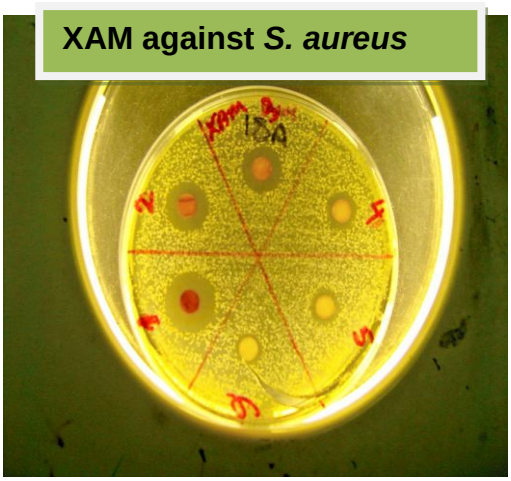
ii) 

Fig.5.4. i and ii). MIC determination of *P. crassipes* and *X. americana* methanol extracts by disc assay against *S. aureus* using higher concentration range (1 - 6, = 1000 – 31.25 µg/mL). *P. crassipes* showed no inhibition zone at higher concentrations while *X. americana* (right) exhibited inhibition at all concentration ranges.

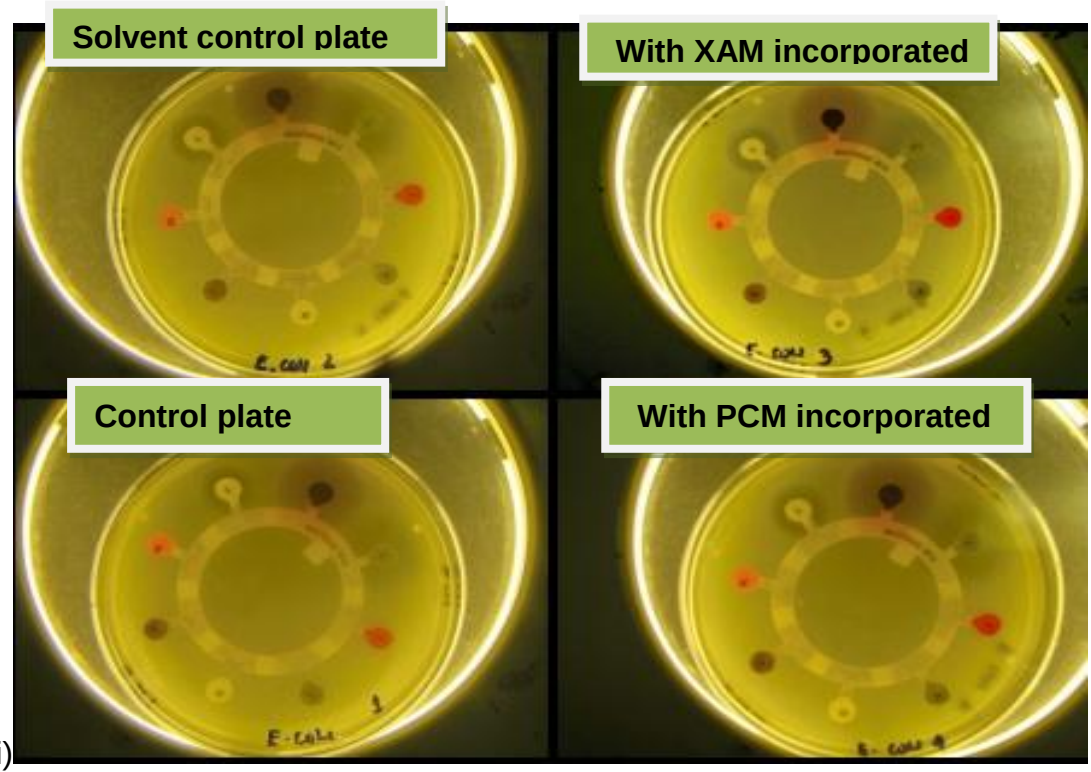
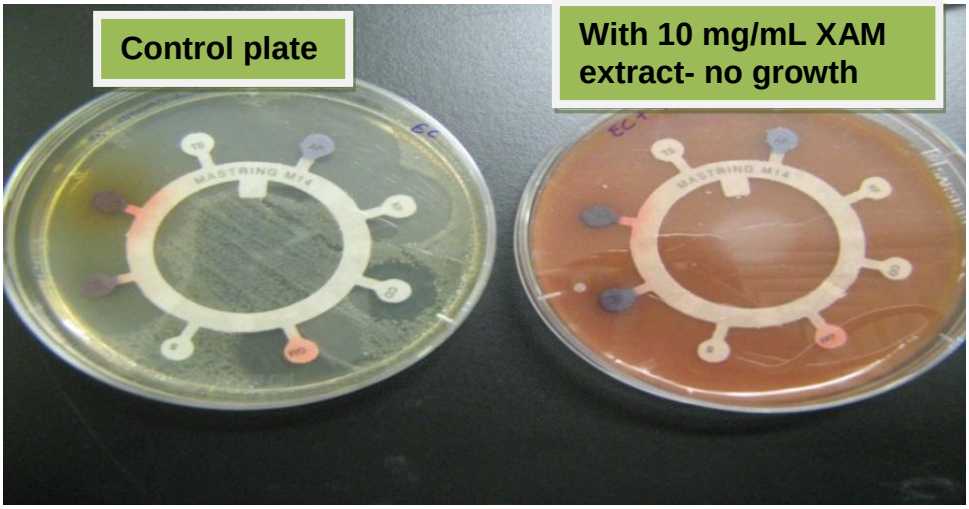
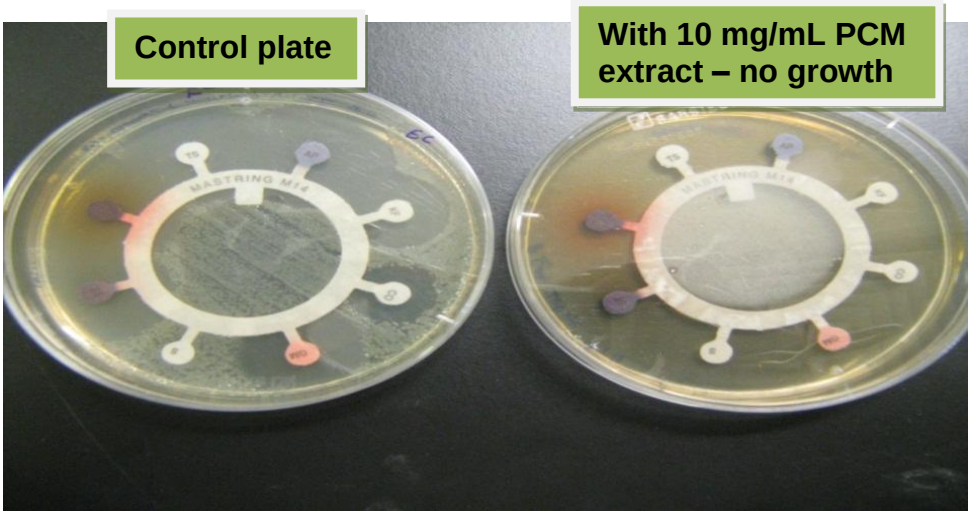
i) 

Fig.5.5 Plates show examples of *E. coli* inoculated plates containing no plant extracts, with extracts (50 µg /mL) and Mastring antibiotic strips. The preliminary tests showed no differences in inhibition zones when measured.

i)



ii)



iii)

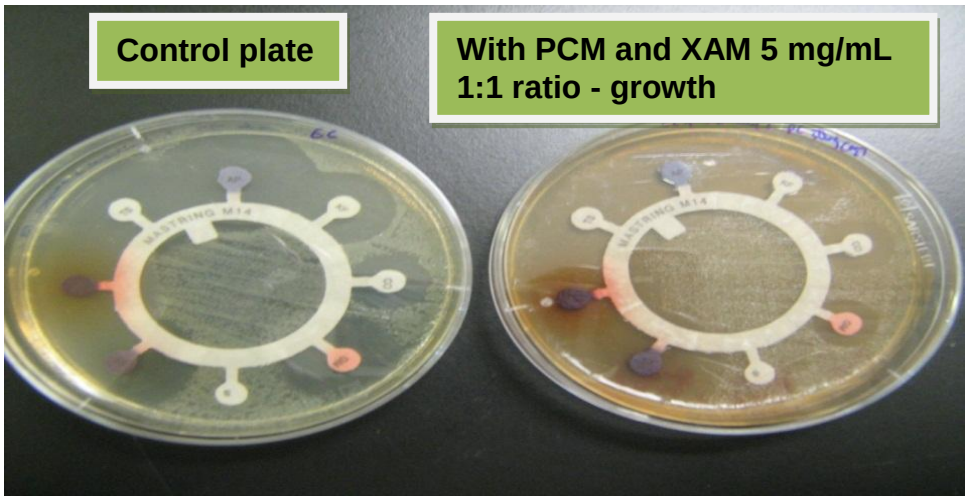
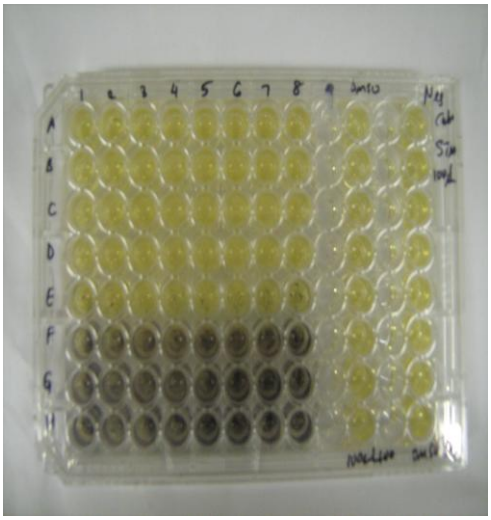
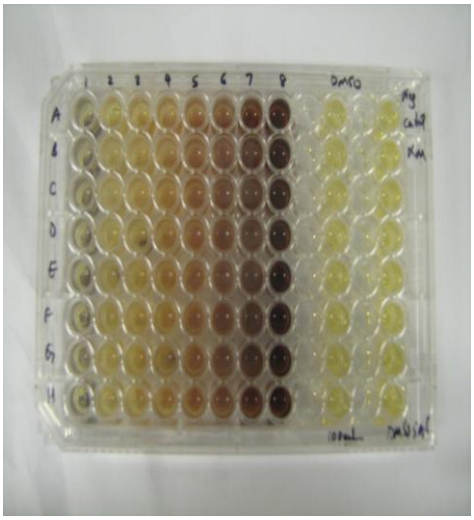


Fig.5.6 i) Plates show examples of *E. coli* inoculated control plates containing no plant extracts (control) and with 10 mg/mL XAM. ii) with 10 mg/mL PCM incorporated into agar iii) a combination of PCM and XAM in a 1:1 ratio (5 mg/mL).

i)  ii) 

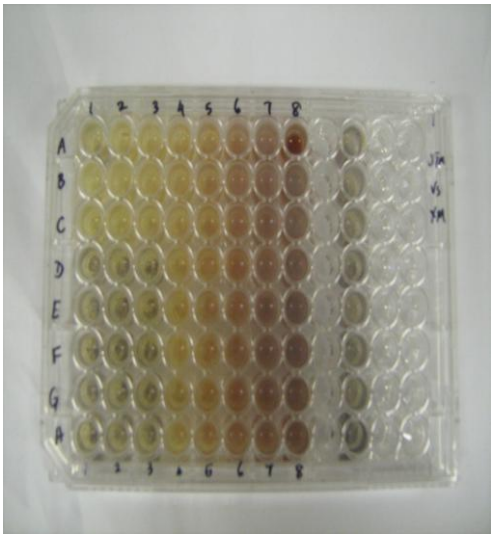
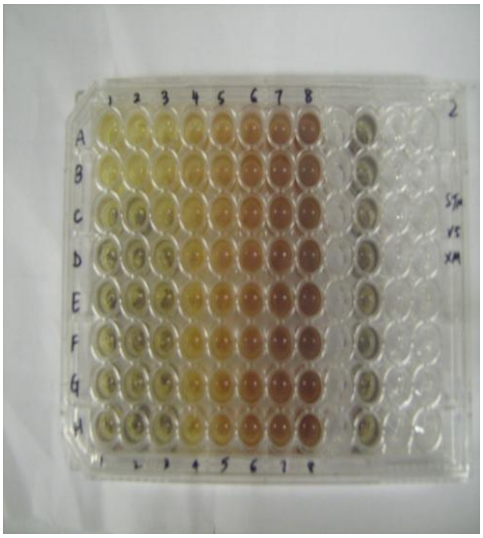
iii)  iv) 

Fig.5.7 Streptomycin vs. *X. americana* methanol extract micro dilution synergy assay (8 x 8) with *S. aureus*. Plates visualised with MTT solution. Purple wells show bacterial growth while clear wells show inhibition.

i) wells with streptomycin alone. ii) wells with *X. americana* extract alone.

iii and iv) combination of streptomycin and *X. americana* extract in duplicate plates.

Table 5.4. Schematic drawing to show methods used to calculate FIC index for each well**.** Micro-dilution synergy assay (8 x 8) involving Streptomycin (µg/mL) vs. XAM (mg/mL) - *S. aureus*.

	x MIC STM	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	Conc. in well
A	**4.00**				2					2
B	**2.00**				2					1
C	**1.00**	1, 2	1, 2	1, 2	2	2	2	2	2	0.5
D	**0.50**				1, 2					0.25
E	**0.25**				1,2,4					0.13
F	**0.13**				1, 2					0.06
G	**0.06**				1, 2					0.03
H	**0.03**				1,2,3					0.015
	x MIC XAM	**0.03**	**0.06**	**0.13**	**0.25**	**0.50**	**1.00**	**2.00**	**4.00**	
	Conc. in well	0.09	0.19	0.38	0.75	1.5	3.0	6.0	12.0	

Gray wells represent wells with turbidity (growth).

Numbers in wells 1- 8, A - H represent methods used to calculate FIC index

Table 5.5. Checkerboard results showing differences among methods of interpretation (average values calculated for plates in duplicate).

method	FIC index	interpretation
1	0.68	additivity
2	1.25	additivity
3	0.28	synergy
4	NA - Two wells clear	synergy

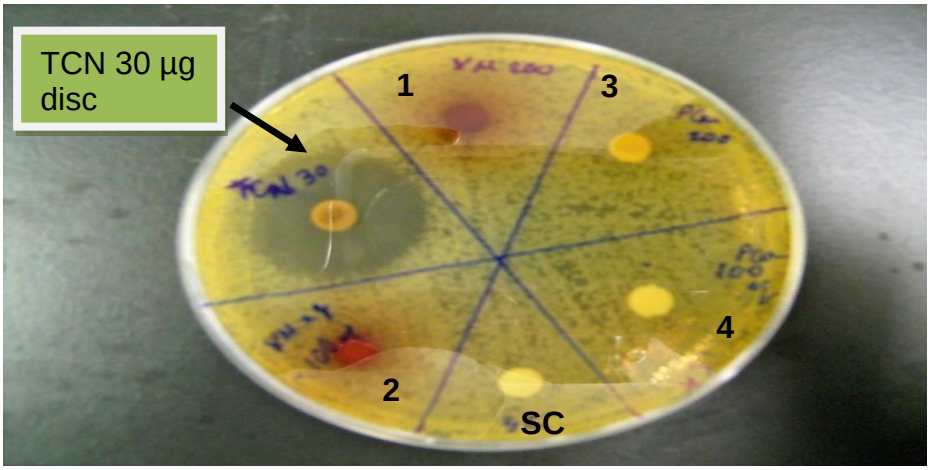
NA- Not applicable

Table 5.6. Table of values showing inhibition zone diameters (mm) for *P. crassipes* and X. *americana* against *E. coli* streptomycin resistant strain at a final concentration range of 5 – 0.625 mg/mL. Each sample was combined with gentamicin 10 µg. The values displayed are for six replicates. Individual MICs of PCM and XAM were > 2 mg/mL against *E. coli* SR.

	CONTROL	**SOLVENT CONTROL**	**PCM 5.0**	**PCM 2.5**	**PCM 1.25**	**PCM 0.63**	**XAM 2.5**	**XAM 1.25**	**XAM 0.63**
	28	28	24	24	27	27	15	20	20
	28	28	24	24	26	27	17	19	20
	28	28	24	25	26	27	17	19	20
	28	28	23	24	25	27	15	19	20
	28	28	23	24	26	27	15	17	20
	28	29	23	24	26	27	15	17	20
Mean	**28.0**	**28.2**	**23.5**	**24.2**	**26.0**	**27.0**	**15.7**	**18.5**	**20.0**
SD	**0.0**	**0.4**	**0.5**	**0.4**	**0.6**	**0.0**	**0.9**	**1.1**	**0.0**
SE	**0.0**	**0.2**	**0.2**	**0.2**	**0.2**	**0.0**	**0.4**	**0.5**	**0.0**
%CV	**0.0**	**1.4**	**2.1**	**1.7**	**2.3**	**0.0**	**5.7**	**5.9**	**0.0**
% change	0.0	0.6	-16.6	2.8	7.6	3.8	- 42.0	18.1	8.1

SD - Standard deviation, SE - standard error, % change – percentage decrease or increase between control and test samples

i)



ii)

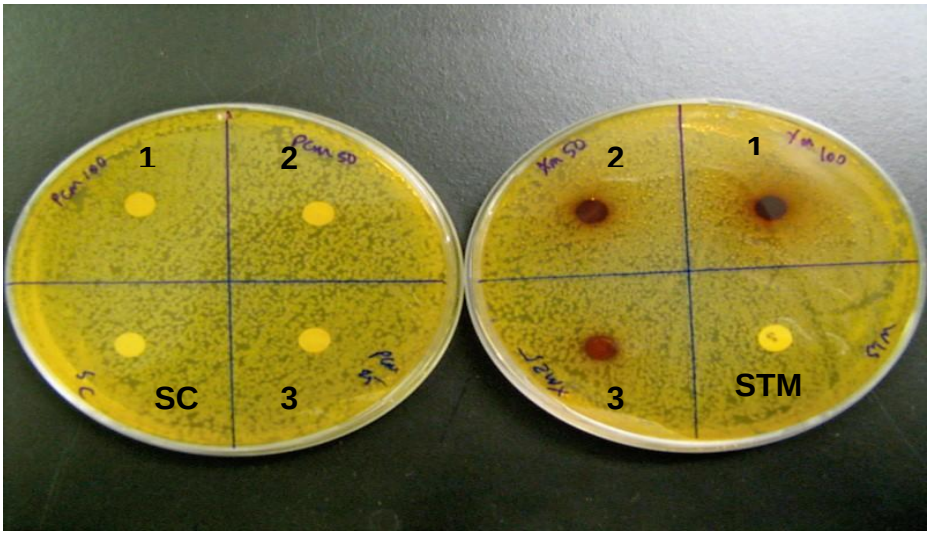


Fig.5.8 i) Plate shows XAM and PCM MIC determination at high concentrations (**1** - XAM 2 mg/disc, **2** - XAM 1 mg/disc, **3** – PCM 2 mg/disc, **4** – PCM 1 mg/disc, **SC** – solvent/disc) against *E. coli* SR (UoS strain). Tetracycline (30 µg disc) was used as positive control. ii) MIC was determined for PCM and XAM using extract concentration range of 1000 -125 µg (**1- 3**) on discs).

Based on the results obtained, *X. americana* methanol extract (XAM) exhibited strong synergy with colistin sulphate and sulphatriad against *S. aureus* (Tables 5.2 – 5.3 and Figures. 5.1- 5.6) while *P. crassipes* methanol extract (PCM) had strong synergistic effects with ampicillin and cephalothin against gram negative organisms, *P. aeruginosa* and *E. coli.* Negative effects were observed with both plant extracts against *E. coli* SR. Analysis by One-Way ANOVA for the groups show significance at $p < 0.05$. The synergy results suggest that some constituents in the non -TB active fraction may have additive effects on active

fractions. This part of the work was then followed up with efflux inihibitory studies to evaluate potential efflux properties.

5.5 Evaluation of efflux pumps inhibitory property by Rotor - Gene 3000

Microorganisms have been reported to have the ability to protect themselves from xenobiotic by efflux pumps. They also serve as mechanisms for maintaining normal physiological functions and uptake of essential nutrients (Fernandes *et al*., 2003; Lynch, 2006; Paulsen *et al*., 1996). A major mechanism of resistance observed in both microbes and cancer cells is the membrane protein-catalysed extrusion of drugs from the cell.

Resistant cells utilise proton-driven anti-porters and/or ATP- driven ABC (ATP-binding cassette) transporters to extrude cytotoxic drugs that usually enter the cell by passive diffusion. Although some of these drug efflux pumps transport specific substrates, many are transporters of multiple substrates. These pumps are often termed H^+ anti porters and can be categorised into a number of families such as major facilitator superfamily (MFS), small multidrug resistance family (SMR), resistance nodulation cell division family (RND), and drug/metabolite efflux family (DME – The family is an addition which is reported to be poorly defined). The proteins in DME have been shown to possess some similarity to the SMR family (Borges-Walmsley *et al*., 2003).

The predominance of these H^+ anti porters in bacteria is shown clearly by *E. coli*, which is predicted to have 19, 7 and 3 drug transporters belonging to the MF, RND and SMR families respectively but only 7 belonging to the ABC (ATP-binding cassette) transporter family. All of these well known drug transporters have been investigated and shown to be expressed in a strain of *E. coli* that is hypersensitive to drugs, to test whether they could confer drug resistance. It was found that 21 of the proton anti porters, including 11 MF, 6 RND, and 2 SMR transporters, conferred drug resistance, but only one ABC transporter conferred drug resistance (Nishino and Yamaguchi, 2001). The tripartite AcrAB-TolC system which consists of three sections; inner membrane transporter (AcrB), a protein (AcrA) in the middle and outer membrane channel (TolC) has been well characterised in *E. coli* as an intrinsic MDR efflux pump system. The presence of a number of resistance pumps provides *E. coli* with the ability to

pump out a wide variety of substrates (Viveiros *et al*., 2008; Amaral *et al*., 2007).

One of the major antibiotic resistance mechanisms utilised by more than 15 species of gram - negative bacterial cells is RND efflux pump which eliminates several classes of antibiotics such as penicillins and cephalosporins, macrolides, aminoglycosides, fluoroquinolones, and tetracyclines (Kamicker *et al*., 2008). Compounds which inhibit efflux pumps are identified by measuring the accumulation of ethidium bromide (EtBr) in bacterial cells. Ethidium bromide, an aromatic compound with a phenanthridine core acts as a substrate for efflux pump (Figure 5.9). It has a structure resembling DNA base pair and this similarity enables it to insert itself into a DNA strand.

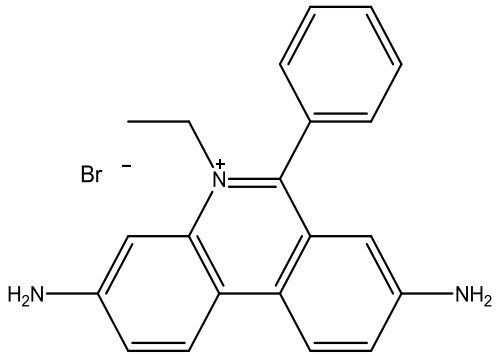


Fig.5.9 Chemical structure of ethidium bromide.

The fluorescent nature of the complex formed with nucleic acids makes it useful as a nucleic acid fluorescent tag in various techniques. Ethidium bromide can be detected using excitation wavelength of 300 nm and 520 nm with an emission peak of 600 nm. EtBr's intense fluorescence results from the hydrophobic environment found between base pairs as a result of binding. The movement into this environment and away from water molecules allows the EtBr cation to shed any water associated molecules and to fluoresce (Berthold product insert, www.berthold-jp.com).

In synergism studies, antagonism or indifference can be established in a bacterial organism by using a combination of an antibiotic and a generally accepted inhibitor. *H. Influenzae* has been used as a model organism to study

efflux systems because it has the advantage of containing single efflux pump unlike *P. aeruginosa* and *E. coli* which have multiple pumps. The ability to circumvent the resistance to antibiotics through treatment in conjunction with efflux inhibitors has implications for setting new drug therapies for bacterial infections (Kamicker *et al*., 2008). EtBr is believed to be efficiently effluxed by the pumps and can only accumulate in the presence of an efflux pump inhibitor (EPI).

Potential EPI's are tested for antimicrobial susceptibility in order to rule out intrinsic activity and synergy due to combined antibacterial activity (Kamicker *et al.*, 2008). Furthermore, compounds with no intrinsic antimicrobial activities are then tested for synergy with a range of antibiotics against organisms with efflux pumps using a single concentration of inhibitor. According to Kamicker *et al.* (2008), any compound showing reduced MIC values or synergy of eight-fold in combination with a single EPI concentration can then be subjected to more rigorous investigation by fractional inhibitory concentration (FIC) method.

As part of the on going assay, the FIC assay is followed by the protonophore assay which involves using radiolabelled lactose which accumulates in the cell via the proton motive force. Any compound which allows this accumulation is believed to act through disruption of the proton motive force (PMF) and not through efflux pump mechanisms. This test is also used to exclude compounds thought to be potential EPIs. As a final step in the *in vitro* investigation of active EPI, kinetic kill studies over 24 hours are finally carried out to show the antibacterial effects in the presence of antibiotic alone and in combination with the EPI.

5.6 Aims and Objectives

Since PCM and XAM extracts were shown to have no intrinsic activity against *E. coli* SR the efflux experiment was aimed at determining the potential efflux properties of the extracts using *E. coli* as a bacterial model. The accumulation and efflux of EtBr as an efflux pump substrate was monitored by real-time fluorimetry using the semi-automated Rotor-Gene 3000 PCR analyser.

5.7 Experimental

5.7.1 Materials

E. coli SR broth (grown to mid – log phase, O.D of 0.6), microtubes (200 µL), 0.22 µm syringe, filters, cuvettes, pipettes, sterile tubes, PBS, ISB, 0.8 % v/v glucose in PBS, distilled water, ethidium bromide stock solution (1 mg/mL), Chlorpromazine (CPZ) in distilled water filtered with 0.22 µm syringe filters, streptomycin, MTT (5 mg/mL in PBS filtered with 0.22 µm filter). All chemicals were obtained from Sigma – Aldrich except otherwise stated.

5.7.2 Equipment

The Rotor - Gene 3000 PCR analyser (Corbett Research Sydney, Australia) incorporating the following diodes and filters [excitation source: LED high power diodes, 470 nm, 530 nm, 585 nm and 625 nm; detection Filters: 510 nm (FAM), 555 nm (JOE), 610 nm (ROX) and 660 nm (Cy5)] was used to monitor the fluorescence on a real-time basis. Spinning and absorbance readings were performed using micro - centrifuge (Centaur) and spectrophotometer.

5.7.3 Determination of MIC by E test® and *macro-dilution*

Plastic strips containing pre-defined gradient of antibiotic concentrations were placed on agar plates inoculated with bacterial organisms to determine their MICs. Inoculum preparation, inoculation and incubation were carried out as described under disc diffusion assay. The MIC determination was carried out for ampicillin, gatifloxacin and rifampicin antibiotic strips.

Ampicillin was tested against *S. aureus*, *E. coli* and *P. aeruginosa* while gatifloxacin and rifampicin were tested against *M. aurum* in duplicate. MIC values were determined for compounds, EtBr, STM and CPZ against *E. coli* (streptomycin resistant) by the macro-dilution method to determine the intrinsic activity before conducting the efflux experiments as described in Section 5.8.7.

5.7.4 One concentration synergy testing

It is expected that potential efflux pump inhibitors would have no intrinsic antimicrobial action, so a concentration range of 128 - 0.5 µg/mL of

streptomycin was tested against *E. coli* (SR) in the presence of a single concentration of CPZ (standard reference EPI), XAM and PCM. A compound which has efflux pump inhibitory properties would allow an antibiotic to have a lower MIC value in its presence. An eight – fold decrease or larger in the MIC values would lead to further testing by checkerboard method.

5.7.5 EtBr concentration curve

A serial dilution of EtBr was made up in PBS to obtain a concentration range of 1- 6 µg/mL. The fluorescence was read using Rotor-Gene 3000 analyser in order to determine the concentration of EtBr which gives maximal absorption (Viveiros *et al*., 2010; Rodrigues *et al*., 2008).

5.7.6 EtBr accumulation assay

E. coli (streptomycin resistant) was grown to mid- log phase ($O.D_{600nm}$ of 0.6) from an overnight culture by placing 1 mL of inoculum into 50 mL ISB in a flask on a shaker at 37° C. This was monitored by taking aliquots and measuring the absorbance. The OD_{600nm} of 0.6 was attained in about 4 – 4.5 hours. 10 mL of the broth culture was centrifuged at 4,000 rpm for 10 minutes.

The supernatant was discarded and the pellets washed twice with the same volume of PBS. The cellular suspension was then adjusted to $O.D_{600nm}$ of 3.0. Glucose (0.8 % v/v) was added to give a final concentration of 0.4% v/v from which 95 µL of the suspension was added to 200 µL micro tubes. This was then made up to 100 µL volume by adding 5 µL of EtBr to obtain final concentration range of 1- 6 µg/mL. The instrument was programmed to obtain 30 cycles in 30 minutes according to the instruction manual. The excitation and emission wavelengths were selected by using Joe (source 530 and detector 555) channel with a gain of 5.

Thereafter, the micro tubes were placed in the Rotor-Gene 3000 analyzer to monitor the fluorescence at 37 $^{\circ}$C for 30 minutes. These conditions are designed to maximise the level of fluorescence as the EtBr equilibrates in the periplasmic space of the cells where its fluorescence is enhanced.

5.7.7 EtBr efflux assay

After carrying out the accumulation procedure, 2 µg/mL EtBr solution was selected to be tested with a concentration of CPZ (8 µg/mL) to give maximal absorption under conditions which promote maximal accumulation such as the lack of energy source (glucose), and a temperature of 25 °C. The cells were incubated for 60 minutes after which the tubes were centrifuged and re-suspended in EtBr - free PBS. The tubes were placed on ice to prevent efflux before monitoring fluorescence. Glucose was added to a final concentration of 0.4 % and tubes with PBS containing glucose, without glucose and with CPZ were used as controls. Graphs show real-time fluorescence of the tubes.

5.8 Results and Discussion

5.8.1 MIC determination against E. coli streptomycin resistant strain

The MIC values were determined for ethidium bromide (8 µg/mL), STM (> 128 µg/mL), Chlorpromazine, CPZ (16 µg/mL) to ensure that the concentrations used in further experiments would not affect the growth of the bacteria. Half the MIC of each compound was used for the procedure. MIC determination of XAM and PCM exhibited values of > 1000 µg on disc (Figure 5.8).

The test against *M. aurum* using gatifloxacin (MIC 0.008 µg/mL) and rifampicin (1.5 µg/mL) was conducted in the hope that the same extracts in combination would be tested to determine synergy and efflux by Rotor-Gene 3000 but this was not followed up due to economic and time constraints.

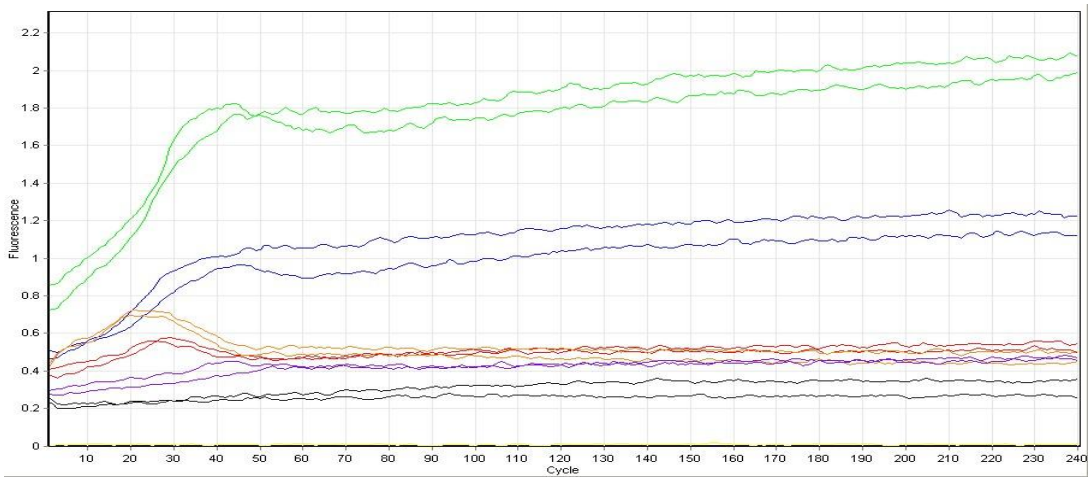
5.8.2 One concentration synergy testing

The MIC of streptomycin (256 - 0.5 µg/mL) was determined in the presence of a single concentration of CPZ (24 µg/mL) and observed to have MIC of 32 µg/mL in combination. Streptomycin by itself showed no inhibition at 256 µg/mL. XAM and PCM at an arbitrary single concentration of 64 µg/mL had no effect on the MIC of streptomycin against *E. coli* SR.

5.8.3 EtBr concentration curve

EtBr at different concentrations were assayed to determine the concentration which gives maximal absorption. According to Paixão *et al* (2009), a minimum concentration of 1 µg/mL EtBr can be used for accumulation assay where one is unable to carry out a concentration assay. The graph shows a concentration-dependent response in fluorescence (Figure 5.10).

a)



b)

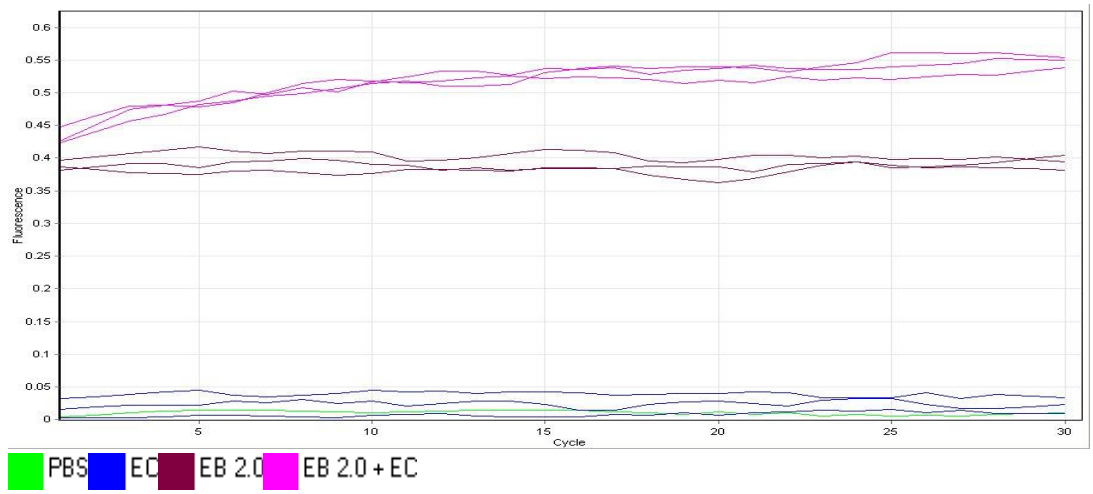
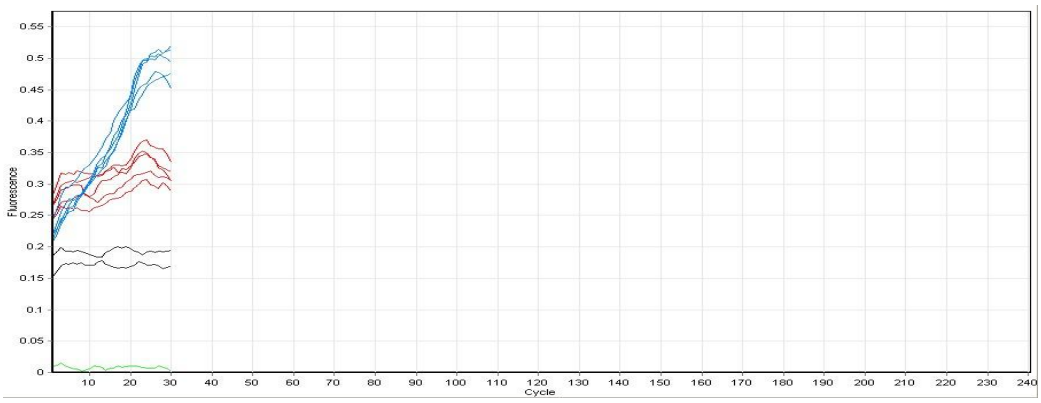


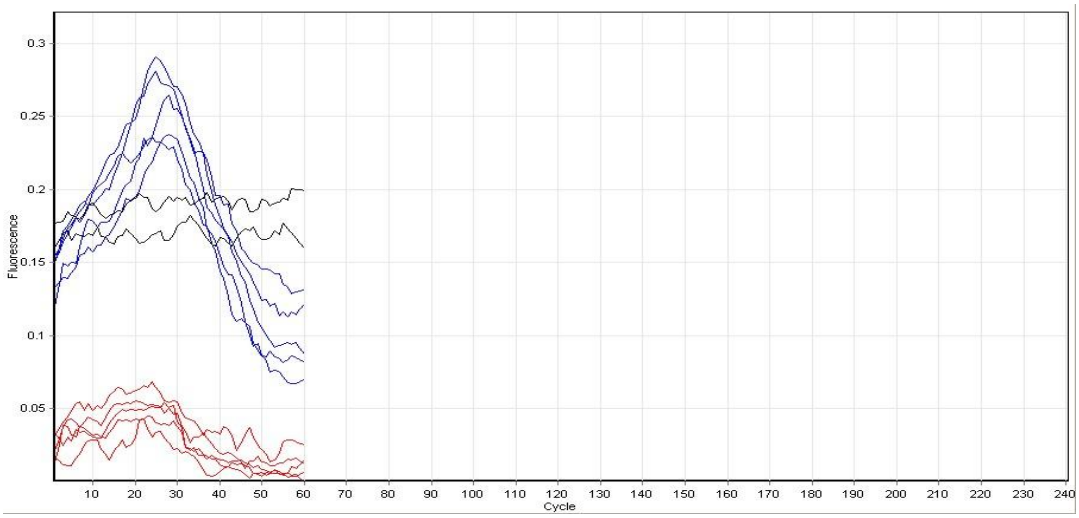
Fig. 5.10 Graph (a) shows the different concentrations of ethidium bromide with different ratios of *E. coli* and glucose control. Graph (b) shows controls, EtBr and EtBr with *E. coli.* Results display traces of two replicates for EtBr tests (a) and three replicates for *E. coli* tests and control (b).

5.8.4 Demonstration of accumulation of EtBr

Accumulation of EtBr was demonstrated by comparing *E. coli* cells suspended in PBS and glucose alone and in the presence of CPZ after incubation at 25 °C for 60 minutes (Figure 5.11). Fluorescence was monitored at 37 °C. In the presence of glucose, fluorescence decreased when compared to the sample in PBS as shown in Figures 5.10a and b.



a) 1 EtBr +glu +EC 1 EtBr + PBS+EC 1 EtBr +PBS+Glu Glucose control

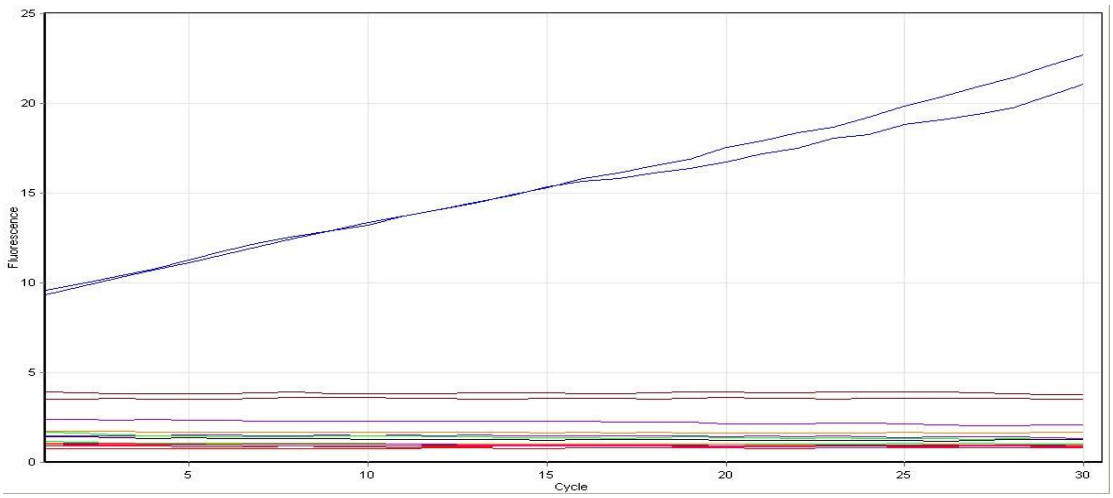


b) 1 EtBr +glu +EC 1 EtBr + PBS+EC 1 EtBr +PBS+Glu

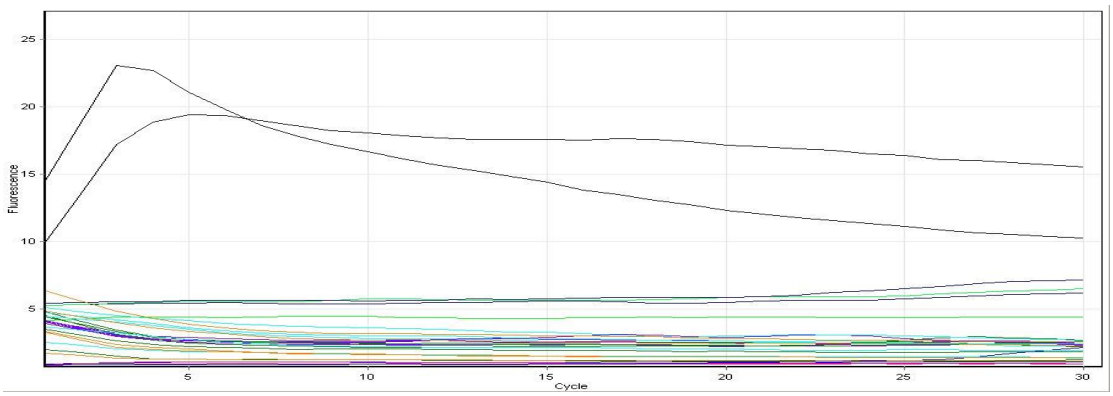
Fig.5.11 a) Graph showing *E. coli* cells loaded with EtBr (1 µg/mL) in the presence of PBS and glucose with controls (30 min cycle). b) Graph showing *E. coli* cells loaded with EtBr (1 µg/mL) in the presence of PBS and glucose with controls (60 min cycle). Results display traces for five replicates for test samples and two replicates for control.

5.8.5 Demonstration of efflux of EtBr mediated by *E. coli* efflux pumps

The absence of glucose results in an increase in EtBr accumulation within the bacterial cells as shown in Fig. 5.11. A greater increase in fluorescence occurs with the addition of 8.0 µg/mL of CPZ after incubation at 25°C indicating that efflux is inhibited (Figure 5.12a) however, fluorescence was reduced for samples at 37°C in glucose though differences in fluorescence are not easily distinguished for cells in glucose with different concentrations of CPZ (Figure 5.12 a and b).



a) EC in PBS | EC +EB 2.0 + CPZ 8.0 | EB 2.0 + CPZ 8 | washed EC + CPZ 8.0- no glu | washed EC + CPZ 1.0 glu | washed EC + CPZ 2.0 glu | washed EC + CPZ 4.0 glu | washed EC + CPZ 8.0 glu

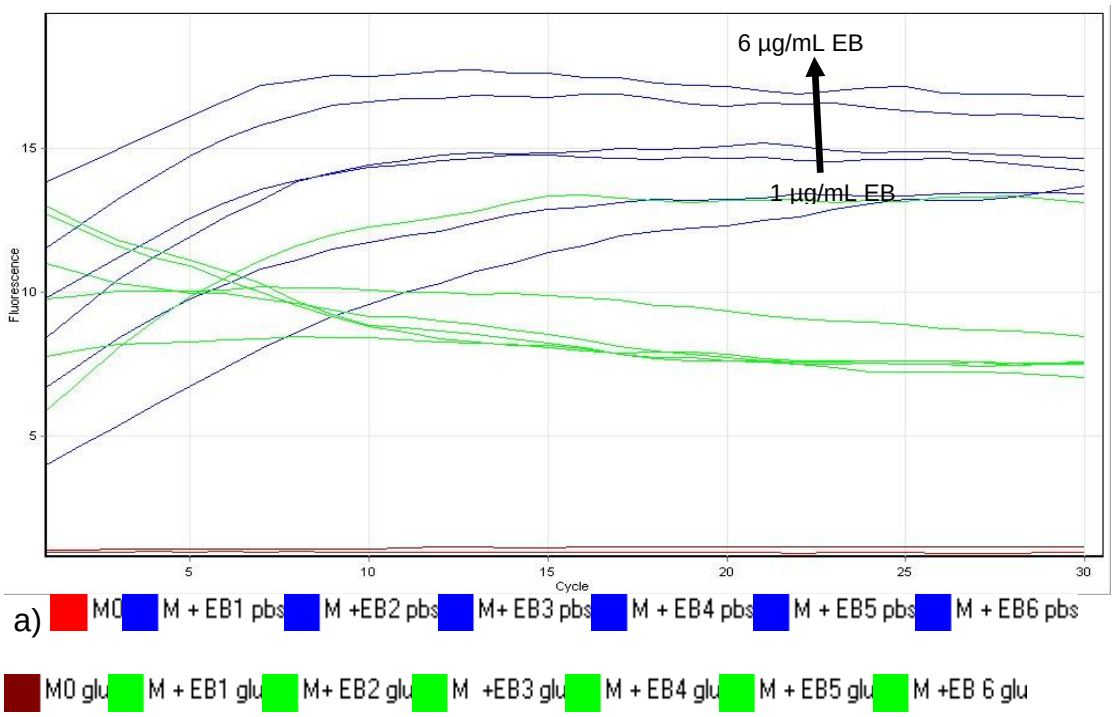


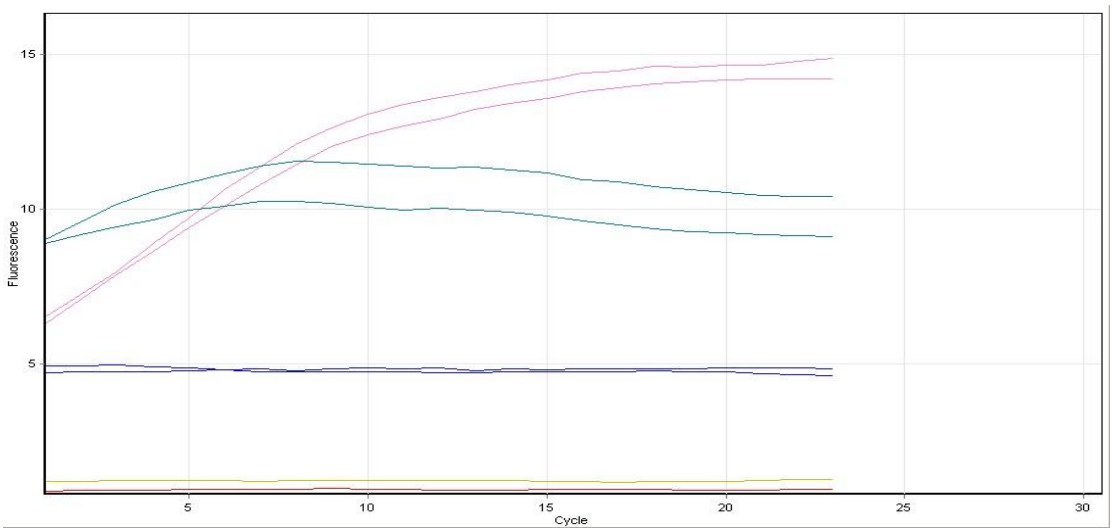
b) EC | EC + EB 2 | EB 2 | EB 2.0 + CPZ 8 | EC + EB 2.0 + CPZ 8 in PBS | EC + EB 2.0 + CPZ 8 in Glu | EC + EB 2.0 + CPZ 8 -pbs | EC + EB 2.0 + CPZ 8 -glu

Fig.5.12 a) Graph of efflux experiment showing the influence of different concentrations of EPI (CPZ) on fluorescence and b) graph showing steep reduction in fluorescence when compared to cells loaded with EtBr alone. Results display two replicates of test samples and controls.

5.8.6 Demonstration of accumulation and efflux in *M. aurum*

Accumulation and efflux was demonstrated in *M. aurum* by Figure 5.12. The accumulation of EtBr was greater with the higher the concentration of EtBr but a concentration of 3 µg/mL EtBr gave reasonable fluorescence so this was subsequently used in the experiment. There was a reduction in fluorescence in the presence of CPZ (8 µg/mL) when cells were loaded in glucose compared to cells in PBS.





b) MA + EB 0 MA + EB 0 glu MA +EB3 MA +EB3 + CPZ 8 pbs MA +EB3 + CPZ 8 glu

Fig.5.13 a) Graph showing *M. aurum* cells loaded with EtBr (1- 6 µg/mL) in PBS and in glucose. b) *M. aurum* cells loaded with 3 µg/mL EtBr in PBS and glucose. Results display duplicate traces for test samples and controls.

5.9 Conclusion

E. coli and *M. aurum* cells loaded with efflux pump inhibitor, CPZ in PBS exhibited greater inhibition of EtBr efflux indicated by the level of fluorescence compared to cells loaded with glucose (Figures 5.11; 5.12). The presence of glucose and temperature of 37 °C provided an energy source for cells to extrude EtBr leading to a decrease in fluorescence. Real-time fluorescence analysis is shown by these results to be a valid and sensitive method to determine the potential of materials to show efflux inhibition of antimicrobial compounds. Trials with *E. coli* were conducted to standardise the procedure and indicated that cells accumulated ethidium bromide to an equilibrium level within 30 to 40 minutes as shown in Figures 5. 10a and b. Cells incubated with ethidium bromide and glucose demonstrated lower fluorescence than cells incubated with ethidium bromide in PBS buffer. The influence of glucose agrees with that found by other workers and supports the view that glucose as a source of cellular energy aids the efflux of antimicrobial agents (Paixão *et al*., 2009; Viveiros *et al*., 2010).

The effect of glucose is further shown when a standard efflux inhibitor, CPZ, is added to *E. coli* cells with ethidium bromide (Figure 5. 11). In the absence of glucose, levels of fluorescence rise rapidly while in the presence of glucose they remain at much lower levels suggesting that glucose is assisting efflux. The real time fluorescence analysis produces similar results when applied to *M. aurum* as shown in Figure 5.12. Here, the incubation of cells with ethidium bromide produces increases in fluorescence as the dye is absorbed into the cells. In the presence of glucose, levels of fluorescence are reduced (Figure 5.12 a). The inclusion of CPZ increases the level of fluorescence considerably.

The results from this study show that *E. coli* SR contains an efflux pump sytem which extrudes EtBr and is influenced by CPZ. This analysis could thus be used as a rapid test to determine whether extracts have efflux inhibition and could be applied to *M. aurum* for the extracts considered in this report.

Chapter 6 Drug Susceptibility Testing of Plant Extracts against *M. tuberculosis*

6.1. Background

The main aim of the study is to evaluate the anti-tubercular properties of the plant extracts. However, *M. aurum* test model was utilised as a monitoring tool in the initial screening to enable chemical characterisation to go on while appropriate arrangements were being made for the testing against the target organism. The biological testing against *M. tuberculosis* H_{37}Rv (virulent strain) could not be carried out at the University of Sunderland due to the infectious nature of the bacterium and the inaccessibility of the appropriate bio safety level 3 facilities required for laboratory activities in the propagation and manipulation of cultures of *M. tuberculosis.*

6.1.1. Introduction

The most suitable target organism in an anti-TB discovery effort is the actual etiologic agent, *M. tuberculosis*, a well characterized virulent strain available from the American Type Culture Collection (ATCC, Rockville, MD) denoted as *M. tuberculosis* H37Rv (ATCC 27294). It has a drug susceptibility profile which is fairly representative of the majority of drug susceptible clinical isolates (those which have not developed drug resistance as a result of prior treatment with one or more clinical TB drugs).

Testing against drug-resistant and MDR strains of *M. tuberculosis* is not critical in primary screening, because these strains are not ‘‘superbugs’’, which are resistant to multiple antibiotics by virtue of a single mechanism, such as the effusion pumps found in other bacteria, but are instead the result of specific

step-wise mutations to individual drugs. Thus, they would be expected to be susceptible to any novel compound, which does not act at the same site as existing TB drugs (Pauli *et al*., 2005).

However, because *M. tuberculosis* H37Rv is a virulent strain, investigations involving it should only be handled in a Biosafety Level 3 laboratory (BL-3) requiring negative air pressure relative to an anteroom and hallway, a pass-through autoclave and a Class 2 biosafety cabinet. Personnel working within the BL-3 lab must wear protective gear, most importantly a respirator, which will minimize the risk of infection by aerosolized *M. tuberculosis*. Since few institutions have a BL-3 laboratory, most investigators either collaborate with an institution, which does possess such a facility or work with avirulent surrogate organisms (Pauli *et al*., 2005).

Many natural products researchers have chosen to work with rapidly growing, avirulent, saprophytic mycobacteria. One such species is *M. smegmatis* (ATCC 607), which has been extensively used and been referred to erroneously as *M. tuberculosis* 607 in several publications. These organisms, however, only possess a limited degree of similarity to *M. tuberculosis* with regard to drug susceptibility. Another alternative to working with virulent strains of *M. tuberculosis* is to use the slow growing, avirulent strain known as *M. tuberculosis* H37Ra (ATCC 25177) or the commonly used vaccine strain, *M. bovis* BCG (ATCC 35743).

These organisms are reported to be more closely related to *M. tuberculosis* H_{37}Rv than the rapid-growing mycobacteria with respect to both drug susceptibility profile and genetic composition. It is suggested that working with these organisms requires only the use of a Class 2 biosafety cabinet and sound microbiological technique (Pauli *et al*., 2005).

Several methods have been used to screen samples for MTB in biological samples and for drug susceptibility. Many investigators in the area of TB research have utilised drug susceptibility testing methods such as the absolute concentration method, resistance ratio and proportion methods in diagnosis, treatment and resistance surveillance of TB universally (Drobniewski *et al*., 2007). However, these methods require two to four weeks to obtain results and with some other specialised methods, it requires expensive equipment which is not readily available to most laboratories.

The method (MABA) used in determination of minimum inhibitory concentration for this study is a sensitive, rapid, inexpensive, and non-radiometric which offers the potential for screening a large number of compounds against slow growing mycobacteria (Franzblau *et al*., 1998).

The Alamar blue oxidation-reduction dye is a general indicator of cellular growth and/or viability; the blue, non-fluorescent, oxidized form becomes pink and fluorescent upon reduction. Growth is easily measured by the use of fluorometers, spectrophotometer or visually (deFries and Mitsuhashi, 1995; Collins and Franzblau, 1997). The proposed mechanism by which the dye detects living cells involves metabolic-based reduction via reactions of the respiratory chain (Voytik-Harbin *et al.,* 1998).

The method has also been used in the assay of lymphocyte proliferation, detection of methicillin resistant *Staphylococcus aureus* (MRSA) and a variety of other assays (deFries and Mitsuhashi, 1995; Pfaller and Barry, 1994; Zabransky, Dinuzzo, and Woods, 1995). Collins and Franzblau (1997) in their extensive research study suggested that fluorimetric MABA facilitates data acquisition for large-scale efforts while visual MABA is a very inexpensive alternative, providing nearly identical results.

6.2 Aims and Objectives

This aspect of the study was directed at screening and determination of the MIC values of the different plant fractions against *M. tuberculosis* H37Rv ATCC (virulent strain) using the micro plate Alamar Blue assay (MABA) in collaboration with Professor S.G. Franzblau (Institute for TB Research, Chicago, Illinois).

6.3 Experimental

6.3.1 Materials

M. tuberculosis H_{37}Rv ATCC 27294, Middlebrook 7H12 medium (Difco Laboratories, MI, USA), Middlebrook 7H11 agar (Difco), Middlebrook 7H9 broth, (Difco Laboratories, MI, USA), Alamar blue (Trek Diagnostic, Westlake, OH), Tween 80 and the reference drug compounds were purchased from Sigma.

6.3.2 Equipment

BioStatQ fermentor by Braun Biotech was used for simulating a low oxygen medium.

6.3.3 Preparation of reagents and media

Reagents and media used are described in Lilienkampf *et al.,* 2009.

6.3.4 Methods

6.3.4.1 Isolation and cultivation of M. tuberculosis H37Rv

All mycobacterial species were grown in Middlebrook 7H9 broth (Difco Laboratories, MI, USA) supplemented with 10% albumin–dextrose complex and 0.05 % (v/v) Tween 80 or on Middlebrook 7H12 agar (Difco Laboratories) supplemented with 10 % oleic acid–albumin–dextrose complex (Collins and Franzblau, 1997, Falzari *et al.,* 2005, Franzblau *et al.,* 1998). Stock solution of MTB H37Rv was prepared by comparison to McFarland No. 1 turbidity standard and diluted to yield 2 x 10^5 CFU/mL.

6.3.4.2 Determination of MIC against M. tuberculosis H37Rv

All crude fractions of *X. americana* and *P. crassipes* were evaluated for MIC against *M. tuberculosis* H37Rv (ATCC 27294) using the Micro-plate Alamar Blue assay (MABA) in 7H12 medium (Collins and Franzblau**,** 1997). Stock solutions of the various plant extracts were prepared in DMSO and two – fold dilutions of each stock extract solutions were prepared in Middlebrook 7H12 medium in a volume of 100 µL in 96 well micro-plates (black view plates). MTB H_{37}Rv (100 µL of 2 x 10^5 CFU/mL) was added to make up 200 µL in the micro-plates.

Positive control drugs were diluted in deionised water or DMSO as appropriate. Compounds were tested in ten 2-fold dilutions, typically from 128 - 0.25 µg/mL for crude extracts and 100 - 0.19 µg/mL for pure compounds. Plates were incubated at 37 $^{\circ}$C for 7 days. On the 7^{th} day, 12.5 µL of 20 % Tween 80 and 20 µL of Alamar Blue were added to the test plates. The plates were then incubated at 37 $^{\circ}$C for 16 – 24 h and fluorescence measured at excitation and emission wavelengths of 530 and 590 nm (Lilienkampf *et al.,* 2009). The MIC

value against MTB H37Rv was defined as the lowest concentration effecting a reduction in fluorescence (or luminescence) of ≥ 90% relative to bacteria only controls. Reported values are means of two replicate measurements.

6.4 Results and Discussion

The crude non-polar and polar fractions of *X. americana* and *P. crassipes* were evaluated for *in vitro* drug susceptibility against *M. tuberculosis* H37Rv (virulent strain) ATCC 27294 using the Micro-plate Alamar Blue Assay (MABA) on 7H12 Middlebrook medium (Franzblau *et al*; 1998). Hexane and dichloromethane extracts of *X. americana* displayed good anti-TB activity of over 94% inhibition at 60.6 and 30.5 µg/mL respectively at the test concentration range of 128 - 0.5 µg/mL (Table 6.1). In contrast, *P. crassipes* fractions demonstrated weak activity (below 90%) against *M. tuberculosis* H37Rv at an MIC value 128 µg/mL except for the hexane fraction which had an inhibition of 86.7% at 64 µg/mL. The results from the testing of crude extracts show that the fractions possibly contain compounds with potential activity against MTB H37Rv compared to reference drugs RMP, INH, MOX and PA- 824 which had MIC values between 0.06 – 0.77 µg/mL. The results from MTB testing show correlation with findings from initial screening of aqueous plant materials against clinical isolate of *M. tuberculosis* in which *X. americana* and *P. crassipes* displayed MIC values of 100 and 200 µg/mL based on the Lowenstein- Jensen method (Metchock *et al*; 1999) and with *M. aurum* results (Table 6.1, Figure 4.2). Activity was found to be in the lipid fractions and this may be due to the ability of the extracts to penetrate the cell wall in high enough concentration to have an effect. The methanol fractions containing polyphenols such as 5-O-caffeoyl quinic acid and rutin with anti-oxidant properties showed mild or no effects *in vitro* against MTB H37Rv but may be acting to stabilise or enhance the actives. Some polyphenolic compounds have been reported by other investigators to have anti-oxidant, efflux pump inhibitory, muscle relaxant, anti-inflammatory, resolving pleural effusion, and lymphoedema (Amos *et al*., 1998; Michalet *et al*., 2007; Kopko, 2005).

TB in the lungs (common manifestation) is characterised by symptoms such as cough lasting 3 weeks or more, pain, unexplained weight loss, fever , fatigue so

the other constituents may be working in concert or synergy with the anti-tubercular compounds to achieve resolution of symptoms and successful therapy (WHO, 2010). According to Pauli *et al., (*2005), the MIC of a crude natural extract may or may not be a reliable inidicator of the chances for success in isolating a potent anti-mycobacterial agent from that extract.

The possibility exists that an extract with a relatively low MIC (high activity) may contain large quantities of only very few moderately active major constituents, while moderately active crude materials could lead to minor compounds with high activity. This was observed with an anti-TB compound ergosterol-5, 8-endoperoxide isolated from the methanol extract of *Ajuga remota* which had an MIC of approximately 100 µg/mL against MTB H37Rv in the initial study with the crude extract but was subsequently demonstrated to have an of MIC 1 µg/mL on further purification (Cantrell *et al*., 1999). Also, compounds with weak antibacterial activity have been reported to be significantly enhanced by other constituents in the same species (Michalet *et al*., 2007).

Table 6.1. Table of results showing the anti-TB activity of plant fractions

Extract	Stock (mg/mL)	Test Conc. (µg/mL)	%Inhibition at highest concentration	MIC (µg/mL)
PCH	6.4	64 - 0.25	86.7	> 64
XAH	6.4	64 - 0.25	**94.3**	**60.6**
PCD	12.8	128 - 0.5	52.1	> 128
XAD	6.4	64 - 0.25	**96.4**	**30.5**
PCM	12.8	128 - 0.5	0.0	> 128
XAM	12.8	128 - 0.5	0.0	> 128
PCW	12.8	128 - 0.5	0.0	> 128
XAW	6.4	64 - 0.25	5.4	> 64
PCE	12.8	128 - 0.5	79.5	> 128
XAE	12.8	128 - 0.5	71.3	> 128

RMP				**0.06**
INH				**0.20**
MOX				**0.77**
PA824				**0.49**

Legend:

PCH, PCD, PCM, PCW, PCE - HEXANE, DICHLOROMETHANE, METHANOL, WATER EXTRACTS OF *P. crassipes*
XAH, XAD, XAM, XAW, XAE - HEXANE, DICHLOROMETHANE, METHANOL, WATER EXTRACTS OF *X. americana*
RMP, INH, MOX, PA824 – RIFAMPICIN, ISONIAZID, MOXIFLOXACIN, NITRO-IMIDAZOPYRAN

6.5 Conclusion

Hexane and dichloromethane extracts of *X. americana* were most active with 94.3 and 96.4 % inhibition at 60.6 and 30.5 µg/mL respectively against *M. tuberculosis* H_{37}Rv (virulent strain). The hexane extract of *P. crassipes* had 86.7 % inhibition at 64 µg/mL and could possibly have better activity at higher concentrations.

Herbal therapy is characterised by multi-component constituents directed at resolving signs and symptoms of diseases i.e. holistic as opposed to conventional medicine which emphasizes target - directed therapy. The *in vitro* test results lend some credence to the use of the plants in traditional medicine.

Consequently, chromatographic and spectroscopic analyses were focused on the hexane and dichloromethane fractions of *X. americana* after biological testing.

Chapter 7 Chromatographic and Spectral Characterisation of Plant Extracts

7.1 Background

Chemical characterisation of the plant extracts was performed using a variety of tools particularly high performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and gas chromatography (GC) to separate and isolate fractions after testing the crude fractions to check that there was activity against *M. tuberculosis* the target organism was retained and to find out which isolates were active. Molecular characterisation to attempt to fully elucidate the structures was performed using mass spectrometry (MS) and nuclear magnetic resonance (NMR).

Other fractions such as the methanol fractions which had some activity initially against *M. aurum* but no effect at concentrations tested against *M. tuberculosis* were also investigated by HPLC to provide LC fingerprint chromatograms which could be potentially useful for subsequent quality control work especially bearing in mind that the possibility of synergistic effects had not been completely ruled out. Another consideration was that well-known polyphenolic constituents previously identified by other researchers from both plant materials had been reported to have useful activities in other assay systems e.g. rutin with reported anti-oxidant and anti-inflammatory properties (Guardia *et al*., 2001). Based on published literature, classes of chemically diverse compounds from natural products have been investigated and shown to have anti - tubercular activities thus it was necessary to look at all potential compounds.

7.1.1 Introduction

Chromatography is a versatile tool utilised in separating and isolating constituents in mixtures of compounds for identification, structure determination

and drug development purposes (Marston, 2007). It has been used in routine analysis for quality control, toxicological and clinical studies as well as for standardisation of phytomedicines (Scott, 2008; Beesley, 2010). HPLC has the capacity to separate, identify and quantitate compounds in solution. However, because liquids are viscous, pressure is needed in the columns to enable separation (Figure7.1).

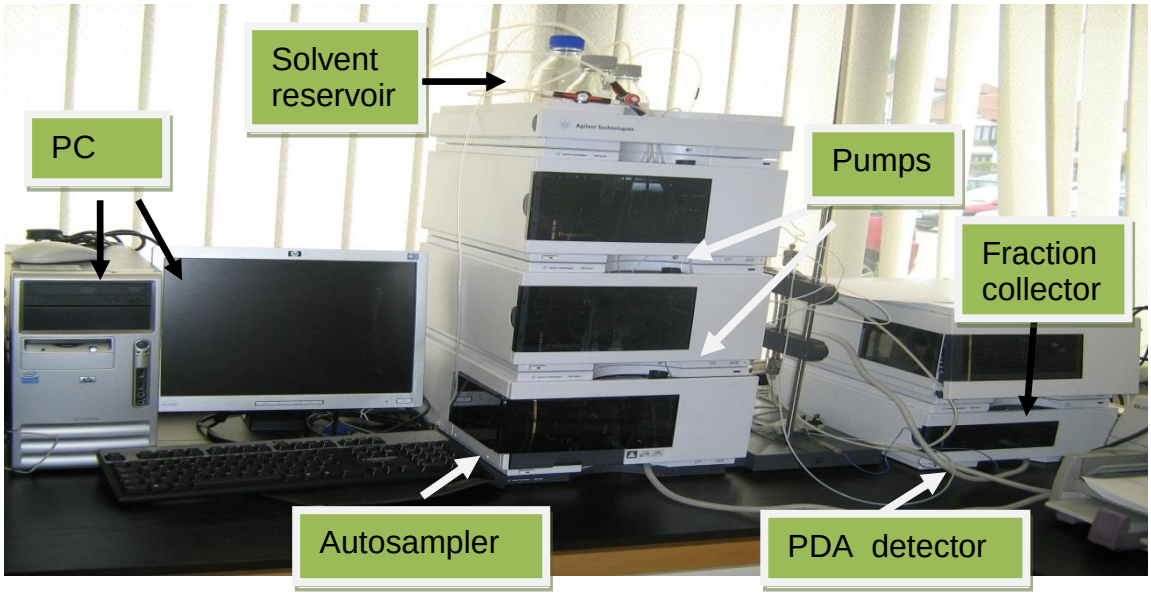


Fig.7.1 Agilent 1200 Preparative LC system with ChemStation data processing unit.

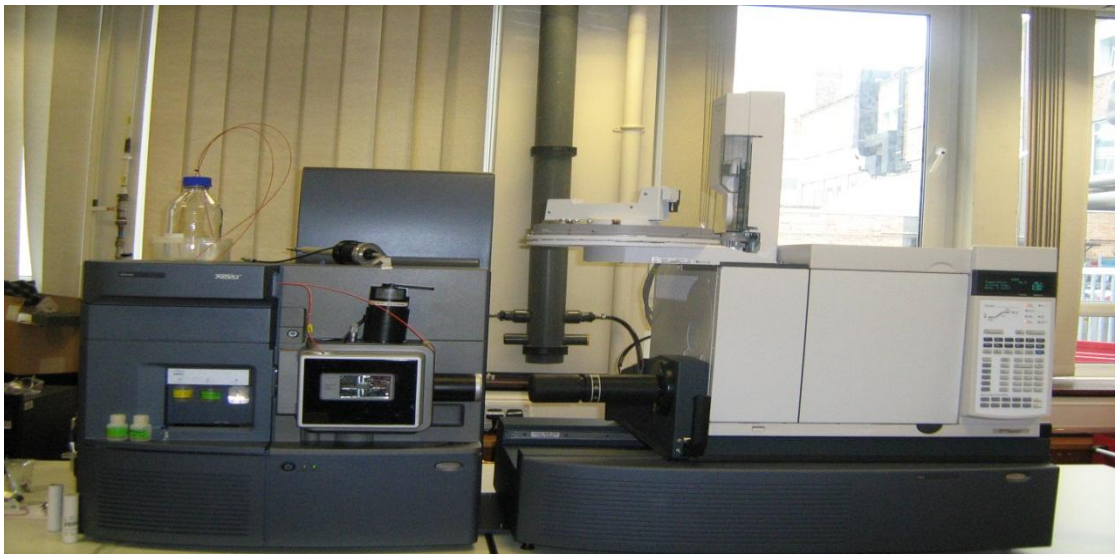


Fig.7.2 Xevo QToF Mass Spectrometer.

Also, advances in NMR spectroscopy and MS have made it possible to elucidate structures of natural products in minute amounts of material. A multidisciplinary approach is utilised in the elucidation of structure determination in natural product chemistry (Hostettmann *et al*., 1997). Apart from LC, GC and supercritical fluid chromatography (SFC) have also found use as techniques for assays of natural products and pharmaceuticals. However, without derivatisation to improve volatility, GC is restricted to low molecular weight and thermo-stable compounds while LC/UV or SFC/UV is restricted to compounds containing a chromophore. Since capillary SFC employing carbon dioxide as mobile phase is compatible with flame ionization detection, it is possible to analyse many solutes at trace levels with high chromatographic efficiency. Supercritical carbon dioxide offers higher solute diffusivity compared with the inert carrier gas conventionally used in gas chromatography and has a lower viscosity than the liquid solvents used in HPLC. Thus, glycerides of fatty acids can be separated and eluted at a lower temperature and with shorter analysis time in SFC (Giron *et al*., 1992). With LC and SFC, a universal detector compatible with these separation methods in both isocratic and gradient mode is necessary.

Universal detectors such as flame ionization (FID), refractive index (RI) detectors and mass spectrometers may be used as alternative detectors (Figure 7.2). FID is frequently coupled with capillary SFC but its use with LC and packed SFC is difficult to perform and nowadays, the commercial availability of such instrumentation is very limited. The RI detector has been described in the literature to be useful for carbohydrate detection but it has been reported to have poor sensitivity with gradient elution and easily affected by temperature (Kohler *et al.,* 1997). This detector can also be coupled to liquid chromatography and packed supercritical fluid chromatography. It has been reported that polysaccharides, not detected by spectrophotometry at 280 nm or higher wavelengths can be detected by this method (Barreira, 2010; Kohler *et al*., 1997). The evaporative light scattering detector allows direct detection of all non-volatile compounds regardless of their functional group or chromophore because the principle of operation is based on measurement of scattered light and has been found useful as a detector in structure determination of compounds with poor UV chromophores. Also mobile phase systems used in an

LC/ELSD separation method can be used for LC/MS detection especially in structure determination of compounds (Jarrell, 2009; Kohler *et al*., 1997).

7.1.2 Hyphenated techniques

The advantage of coupling various techniques lies in the rapid and efficient elucidation of known compounds which would prevent the often tedious process of isolating common constituents. Hyphenated techniques such as LC/MS or LC/MS/MS and LC/NMR have been used for the structural determination of small quantities of plant material (Hostettmann *et al*., 1997). It must be noted though that, even today, such expensive online technology is not routinely available even in relatively well-equipped laboratories.

MS is a powerful and sensitive tool for identifying unknown compounds by virtue of its ability to "weigh" ion fragments and relate them back to the compound of interest. In GC/MS utilises an energetic ionisation method (such as electron ionisation), which causes the parent compound to break into multiple fragment ions (Figure 7.2). The mass spectrum of the compound as well as its mass and location in the chromatogram can be used for its identification. This is accomplished by comparing the fragment pattern with a library database of compounds e.g. National Institute of Standards and Technology (NIST) which helps to identify the chemical formula and possibly the chemical structure. In LC/MS, the common ionisation approach (electrospray) is quite gentle and typically only produces a single parent ion.

MS/MS can be used in a tandem system to help identify the compounds, or if more expensive equipment is accessible, accurate mass measurements alone can identify likely formula candidates. With MS/MS, the fragment ions can be identified which assists the deduction of structural information (Kuehl and Wang, 2007; Atkins and Jones 1999). Various ionisation methods are utilised in MS depending on the nature of the compounds being analysed. In electron ionisation (EI), the extensive fragmentation enables the elucidation of the structure though it has been reported to have the disadvantage that the characteristic molecular ion could be absent and heat generated could lead to chemical breakdown of the sample (Hill, 1972). With chemical ionisation, fragmentation is not as extensive compared to EI but it is said to produce a more intense molecular peak.

NMR as an analytical tool has been found to be useful in elucidating structures though when compared to other spectroscopic methods; it is considered to be relatively insensitive although the advent of high field instruments has increased its versatility (Williams and Fleming, 1995). NMR has the advantage of being non-destructive and non-selective for molecules in solution. Moreover, the sample solution can be dried off and recovered for further analyses or alternatively, solutions in deuterated dimethylsulphoxide (DMSO) may be used directly for biological testing (Corcoran and Spraul, 2003). The NMR spectra of compounds are assigned chemical shifts and coupling patterns which are then compared to model compounds in characterising and quantitating molecular systems. Where molecular complexity occurs, higher magnetic field strengths are used to resolve overlapping multiplet patterns thereby allowing for interpretation of more first-order like spectra. Multi - dimensional NMR in the form of J - spectroscopy offers a way to resolve even highly overlapping resonances into readily interpretable multiplets and permits chemical shift assignments to be made in a simple and direct manner (Gray, 1994).

7.2 Aims and Objectives

The aim of this phase was to attempt to separate and isolate the constituents of plant extracts of *X. americana* and *P. crassipes* using appropriate analytical tools such as high performance liquid chromatography, thin-layer and gas chromatography. This would provide molecular and LC fingerprints of the extracts which could be used for identification and serve as possible markers to aid quality control of the herbal products in particular with respect to reproducibility of extracts produced. A further aim was to use NMR and mass spectral data to aid structural elucidation of isolated active constituents.

7.3 Experimental

General laboratory glassware was supplied by Schott (Germany), Pyrex and E-MILboro (Englanda. Reagents and chemicals were of analytical reagent grade (Sigma) while solvents were HPLC grade (Sigma Aldrich, UK). Water used in

preparing solutions was purified by PURELAB® Bioscience purification system (Siemens).

7.3.1 Materials

Sample vials were obtained from Agilent, while PTFE (polytetrafluoroethylene) Acrodisc syringe filter (0.2 µm) were used for sample filteration. HPLC columns used in the study were obtained from Hichrom Ltd, U.K.

7.3.2 Equipment and accessories

The HPLC systems used for reversed phase analyses employed Hewlett Packard HP 1090 LC system, an Agilent 1290 (analytical) and Agilent 1200 preparative systems equipped with detectors such as photodiode diode array, refractive index, evaporative light scattering and controlled by ChemStation data processing software. The HPLC system used for normal phase screening employed a Shimadzu (Milton Keynes, UK) LC-6A pump and SPD-6AV detector. In each case, a manual Rheodyne (Kotati, Ca, USA) 7125 loop injection valve, fitted with a 20 µL loop, was used for loading samples. Data was collected using a Dionex (Leeds, UK) data system. The sonicator used to degas mobile phases was from GS Group-ULTRAWAVE Ltd, Cardiff. The Shimadzu LC system was initially used for normal phase LC before transferring the methods to the Agilent 1290 systems.

7.3.3 Reagents for TLC

a) Sulphuric acid reagent (50 % H_2SO_4 in methanol): general spray reagent

After spraying and drying, plates were heated at 105 °C for 10 minutes. Various compounds show different colourations.

b) Dragendorff's reagent for alkaloids: Solution A: 0.85 g basic bismuth nitrate dissolved in 10 mL glacial acetic acid and 40 mL of water with heating. Solution B: 8 g potassium iodide in 20 mL water.

Stock solution: Solutions A and B are mixed in equal proportions before use. After spraying, plates were observed in daylight for orange colouration when alkaloids are present (Stahl, 1969).

c) Brady's reagent (2, 4 - dinitrophenylhydrazine; 2, 4 - DNP): for aldehyde and ketone groups. 2 g of diphenylhydrazine was dissolved in 100 mL of methanol. Thereafter, 4 mL of concentrated H_2SO_4 was added carefully to the mixture. TLC plates were sprayed and heated in the oven. The presence of red-orange colouration indicates the presence of a carbonyl group.

d) Fehling's reagent: Fehling's solution I and II are added to the sample extract in equal volumes to form a deep blue solution. The formation of a brick - red precipitate indicates the presence of reducing sugars.
i) Fehling's solution I: Prepared by dissolving 7 g of hydrated copper (II) sulphate in 100 mL of water.
ii) Fehling's solution II : prepared by dissolving 35 g of potassium sodium tartrate and 10 g of sodium hydroxide in 100 mL of water.

e) Lieberman Burchard's test: A few drops of acetic anhydride were added to samples dissolved in chloroform and the tube containing the mixture was boiled. On cooling, concentrated sulphuric acid was added carefully down the sides of the tube. The formation of a brown ring at the junction of the two layers was suggestive of a steroidal nucleus.

7.3.4 Methods

7.3.4.1 Collection and preparation of plant materials

X. americana root and *P. crassipes* leaves obtained from Nigeria were authenticated by Prof. Olorede of the Department of Biological Sciences, University of Abuja and identities further confirmed by Dr Martin Cheek in comparison with voucher specimens (K000411874 and K000657947) at Royal Botanic Gardens, Kew, Surrey, London. The plant parts were pulverised and 100 g of each was sequentially extracted by cold maceration to obtain crude

hexane (yield - 1.14%), dichloromethane (yield - 0.3%), methanol (yield - 10%) and water (yield - 5%) extracts.

The extracts were also subjected to extraction by Soxhlet apparatus but due to the fact that no difference was observed during the biological testing cold maceration was subsequently used for extractions. Water samples were freeze dried and organic extracts concentrated using a Buchi rotavapor under reduced pressure at less than 40˚C and stored in a refrigerator at - 20 ˚C until use. This procedure was carried out a number of times to obtain fresh crude fractions for biological testing and other chemical analyses. Crude extracts of hexane and dichloromethane were submitted for NMR and mass spectral analyses based on positive results of initial screening against *M. aurum*, the test model system for TB.

7.3.4.2 Preparation of mobile phases

Mobile phase solvents were degassed by helium out-gassing for analyses on Agilent 1200 preparative and Hewlett Packard 1090 LC systems and by membrane in-line degassing on the Agilent 1290 system. Analytical work on Shimadzu LC systems required solvents to be degassed by sonication at room temperature.

7.3.4.3 UV analysis

The crude extracts of both plant materials were individually dissolved in the appropriate solvents for hexane, dichloromethane, methanol, and water extracts. The double beam UV-Vis spectrophotometer was used to obtain the UV profile of the extracts (Figure 7.3).

7.3.4.4 HPLC analysis

Normal phase HPLC analysis was performed on Hewlett Packard 1090 system using three analytical columns; Luna $NH_{2,}$ ACE 5 CN and ACE 5 SILICA, 250 x 4.6 mm. Initially dichloromethane was used to elute two fractions from the hexane fraction on an ACE 5 CN, 250 x 10 mm i.d column and analysed by

NMR to confirm purity. Hexane and dichloromethane crude extracts exhibited similar chromatograms and NMR spectra so they were both subjected to the same experiments. After a number of experiments were carried out using various ratios of mobile phase and columns with different selectivity to optimise separation, methanol: dichloromethane (2:98, v/v) mobile phase system was used on an ACE 5 silica column to elute the crude hexane and DCM extracts of *X. americana* (Figure 7.3). Detection was by means of a Hewlett Packard diode array detector at 245, 254 and 280 nm.

Analytical and preparative reversed phase HPLC was performed on the Hewlett Packard HP1090 LC system and the Agilent 1200 series systems equipped with a photodiode array detector with ChemStation data processing software, using three analytical columns; Luna NH_2, Luna SCX and ACE 5 C18 silica. Several ratios of mobile phases were tested using methanol – water and with ammonium acetate buffer on ACE 5 C18 silica column and Luna NH_2, and Luna SCX columns. Methanol and water fractions of *X. americana* and *P. crassipes* were subjected to the same reversed phase experiments. Based on optimisation, a gradient system of methanol and water (A-15, B - 85) was finally used to elute *P. crassipes* crude methanol extract to obtain eight fractions. The fractions were analysed by NMR and two fractions by accurate mass spectrometric measurements. The crude methanol extract was also subjected to LC/MS using the same gradient profile.

7.3.4.5 Fractionation of X. americana crude extracts and isolation of active constituents by HPLC

Fractionation of the crude hexane extract of *X. americana* was performed using repetitive semi-preparative HPLC (ACE 5 CN; 250 x 10 mm i.d column). NMR spectra of the two fractions obtained could not be used in structure determination so further experiments were performed on crude hexane and DCM fractions with methanol: dichloromethane (2:98 v/v) mobile phase to obtain four fractions. The fractions were subjected to NMR and mass spectral (MS) analysis. However, MS and NMR did not match suggested structures

based on the data. TLC analysis was also used to obtain isolates as an alternative procedure.

7.3.4.6 Isolation of active fractions from X. americana hexane and dichloromethane LC fractions by thin layer chromatography (TLC)

Thin layer chromatographic analysis of crude hexane, DCM and LC fractions was carried out on Silica gel 60Å F_{254nm} TLC 20 x 20 mm analytical plates for comparative purposes. Preparative TLC was carried out on crude hexane and DCM extracts using 20 x 20, 500 µm plates (Merck). Solvents used in developing TLC were HPLC grade and purchased from Sigma Aldrich. β-sitosterol and stigmasterol were used as TLC reference standards based on the NIST data base search of the extracts MS data.

7.3.4.7 Gas chromatography of X. americana hexane and dichloromethane extracts

Initial GC measurements were carried out using Hewlett Packard 5890 series II system and DB - 624 capillary column (30 m x 0.32 mm, 1.8 µm). Further GC analysis employed Agilent 7820 GC system and HP- 5 capillary column. The following conditions below were utilised in conducting the experiments using β-sitosterol, stigmasterol, cholesterol and stearic acid as reference standards.

General details:

HP- 5 capillary column: 30 m (L) x 0.32 mm (i.d) x df 0.25 µm (Agilent J and W)

Flow: 5.5528 mL/min

Pressure: 23.03 psi (constant pressure)

Average velocity: 76.106 cm/sec

Hold up time: 0.65697

Run time: 102 minutes

Oven:

Initial temperature: 100 °C for 1 min, 2 °C/min to 300 °C

Equilibration time: 1 min

Max. Oven temperature: 325 °C

FID:

Heater: 300 °C

H_2 flow: 30 mL/min

Airflow: 400 mL/min

Make-up flow (N_2): 25 mL/min

Constant column + make-up: 30.553 mL/min

Back signal (FID): 50 Hz/0.004 min

7.3.4.8 HPLC analysis using refractive index (RI) detector

NMR spectra of the active fraction obtained were not resolvable but results from mass spectral measurements were suggestive of a sterol-like fragment which had a base peak with the same molecular mass as stigmast -3, 5 - diene and sitosterol. Due to the poor UV absorbing properties of some spots on the TLC plate developed with IPA-hexane (5:95 v/v) the RI detector was then considered as an alternative in addition to detection by ELSD. Agilent 1290 infinity series LC system equipped with an RI detector was used to separate hexane and DCM extracts in comparison to reference standards, β-sitosterol and stigmasterol using methanol-dichloromethane (2:98 v/v) mobile phase system on ACE 5 SIL column, 250 x 4.6 mm. *X. americana* total water extract was also analysed by the same system using IPA:hexane (2:98 v/v) on ACE 5 CN column, 150 x 4.6 mm and UV detection at 210 and 254 nm for comparative purposes.

7.3.4.9 HPLC analysis of XD3 and XD4 (sub-fraction of M. tuberculosis active fraction monitored with M. aurum)

Due to the difficulties encountered in identifying components of the active fractions separated by HPLC and analysed by NMR and MS, bioautography

was utilised in order to localise the activity and to further purify the fraction by TLC. Bio-autographic analysis of dichloromethane LC fractions of *X. americana* showed that the anti- tubercular activity was localised in band 3 and 4 zones on the TLC plate with R_f 0.35 (chapter 4, Figure 4.4 iii). Water - methanol (30:70 v/v) (isocratic) and gradient analysis (methanol: water (15/85 v/v) was used to separate the constituents by Hewlett Packard HP 1090 equipped with PDA detector. The mobile phase gradient profile is detailed below;

Column: ACE 5 C18, 250 x 4.6 mm (Hichrom Ltd, U.K)

Mobile phase: water - methanol (30:70 v/v)

Flow rate: 1 mL/min

Injection volume: 20 µL

Wavelengths: 210, 230, 254, 280 nm

Run-time: 30 minutes (isocratic), 45 minutes (gradient)

Temperature: ambient

Gradient time-table:

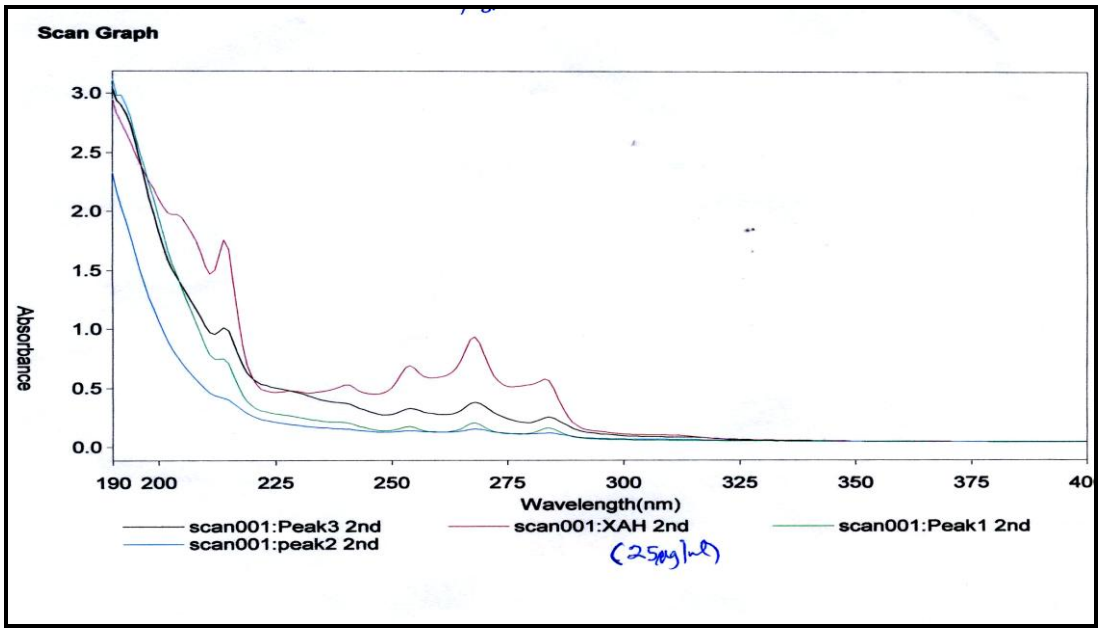
% A (15) – methanol (Time in minutes)	% B (85) - water
0	90
15	70
20	70
21	80
25	80

7.4 Results and Discussion

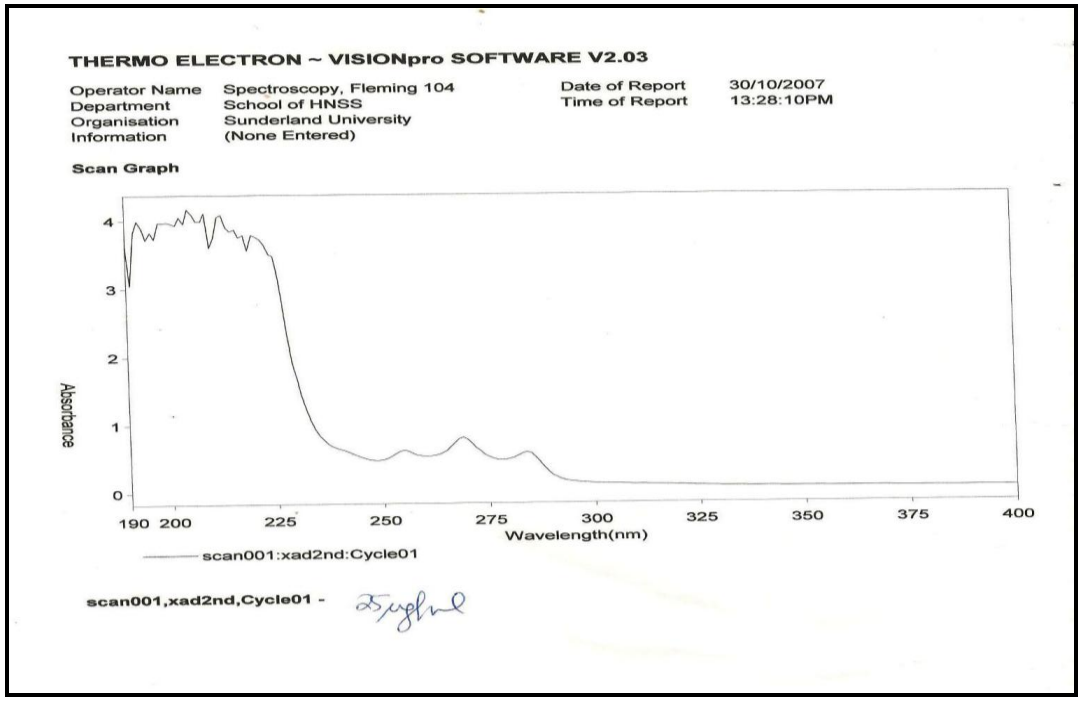
7.4.1 UV analysis of crude X. americana extract

The samples were subjected to UV spectrophotometry to obtain the UV profiles. The UV profiles showed prominent absorption peaks at 210, 230, 245, 265, 280 nm. However, UV spectrophotometry lack specificity and is prone to spectral interference so cannot be used for characteristic identification without comparison to known compounds or a UV library database. Subsequent experiments showed that extracts exhibited UV properties when visualised under UV light at 254 nm. In Figure 7.3 i the crude extract UV spectrum is shown by the red trace and three peaks obtained from preparative LC of hexane extract using 100 % DCM. From Figure 7.3 ii it can be seen that the UV spectrum of the dichloromethane extract is similar to the hexane extract spectrum. Subsequent LC analyses gave similar chromatograms for XAH and XAD and testing with TB revealed higher activity with XAD so further experiments were focused on XAD.

i)



ii)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Department School of HNSS
Organisation Sunderland University
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Date of Report 30/10/2007
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scan001:xad2nd:Cycle01
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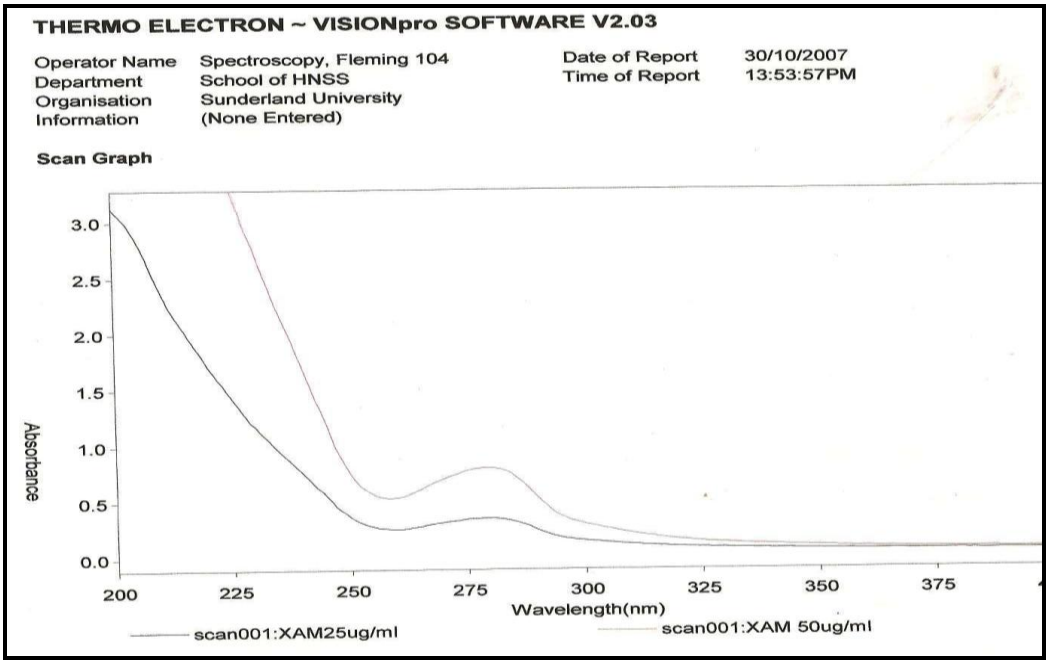
iii)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Operator Name Spectroscopy, Fleming 104
Department School of HNSS
Organisation Sunderland University
Information (None Entered)
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iv)

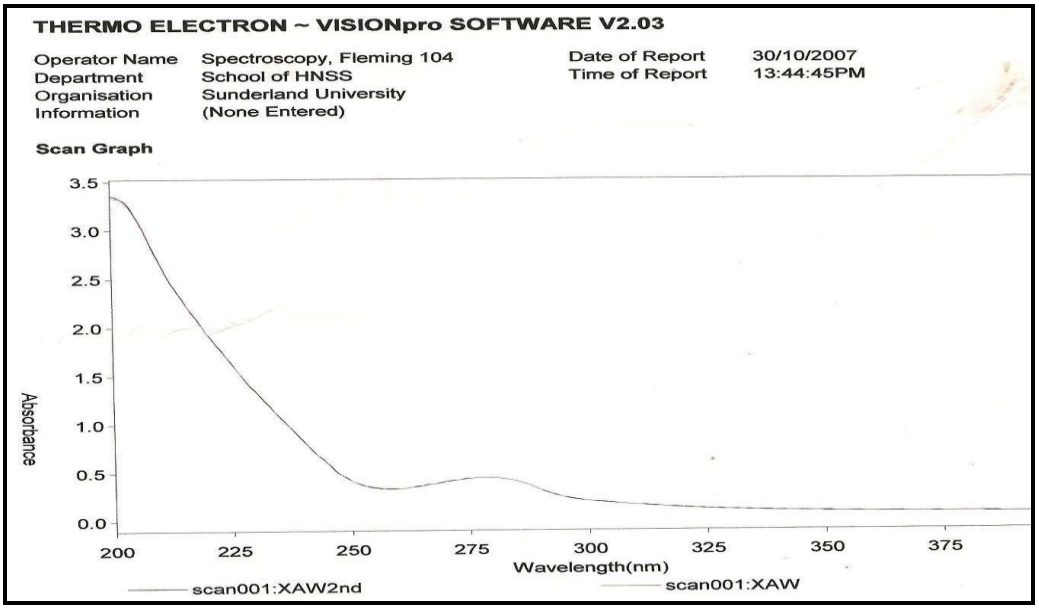
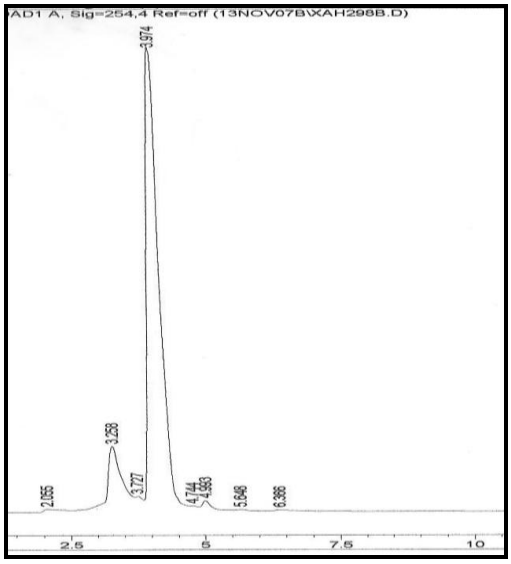


Fig. 7.3 i) UV spectrum of hexane extract and fractions obtained from elution with DCM on an ACE 5 CN semi-preparative column. ii) UV spectrum of dichloromethane extract. iii) UV spectrum of methanol extract. iv) UV spectrum of water extract.

7.4.2 Initial separation of X. americana extracts by HPLC

The plant fractions were subjected to LC to obtain LC fingerprints and to help develop conditions for preparative chromatography. Polar extracts (methanol and water) were analysed by reversed phase LC while hexane and dichloromethane fractions were analysed by normal phase LC. The methanol extracts of both plant materials were also subjected to analysis by analytical and preparative reversed phase HPLC on HP 1090 and Agilent 1200 preparative systems respectively. The chromatograms show the LC profiles obtained using different mobile phase systems (Figures 7.4 -7.5). Other LC chromatograms under different conditions are shown in Appendix 2. LC chromatograms of the most active fractions of *X. americana* against TB, *T. mentagrophytes*, *P. acnes* (hexane and DCM) and *S. aureus* (methanol fraction) are shown in Figures 7.4 and 7.5.

i) 

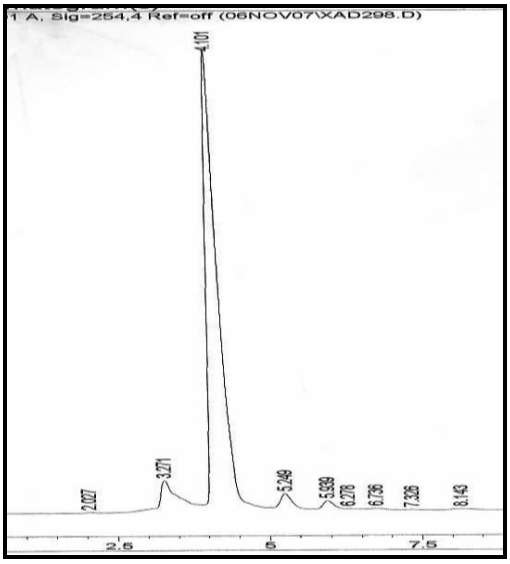
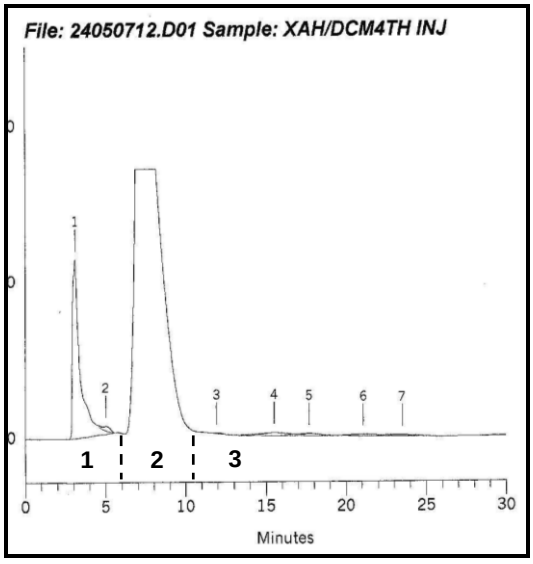
ii) 

Fig. 7.4 i and ii) Normal phase analytical LC profile of *X. americana* (hexane and dichloromethane) extracts on ACE 5 SILICA column, 250 x 4.6 mm, 5 µm at 254 nm using methanol - dichloromethane (2:98, v/v) mobile phase on HP 1090 LC system with PDA detector.

iii) 

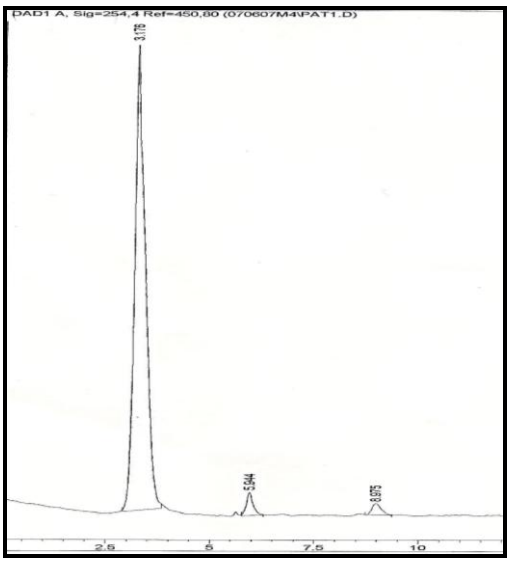
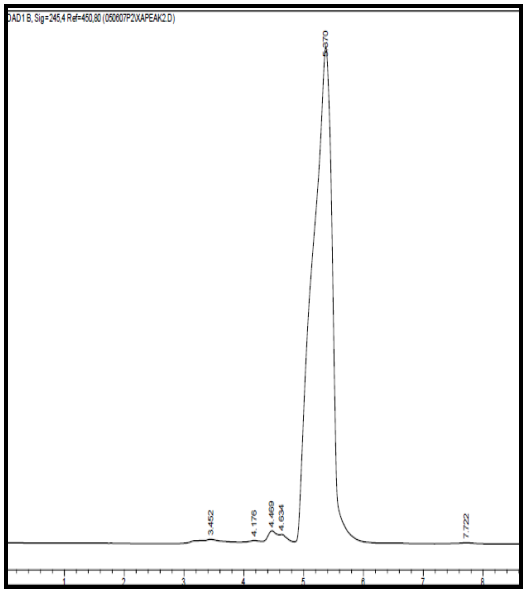
iv) 

Fig.7.4 iii) Normal phase semi-preparative LC profile of *X. americana* (hexane) extract separated with 100 % DCM on ACE 5 CN column (250 x 10 mm) at 254 nm using the LC-6 system with UV/vis detector collected in three fractions, 1,2,3. iv) Analytical LC profile of peak 1 from XAH preparative analysis on ACE 5

SILICA column, 250 x 4.6 mm using DCM - hexane (80:20, v/v) mobile phase system.

v. 

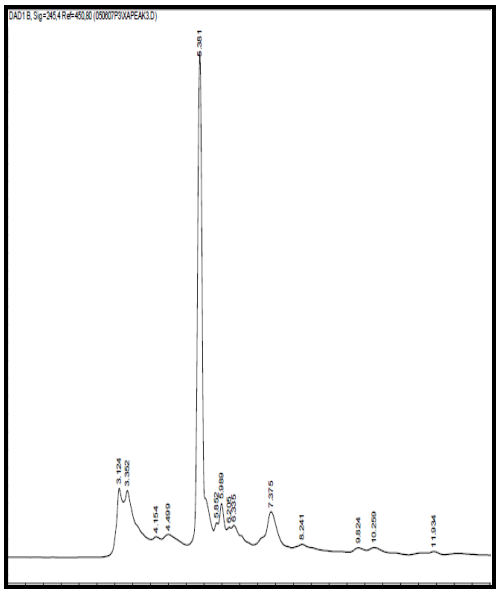
vi. 

Fig.7.4 v and vi) Analytical LC profile of peaks 2 and 3 from XAH preparative analysis on ACE 5 SILICA column, 250 x 4.6 mm separated with DCM: hexane (80:20, v/v) isocratic elution on HP 1090 LC system with PDA detector.

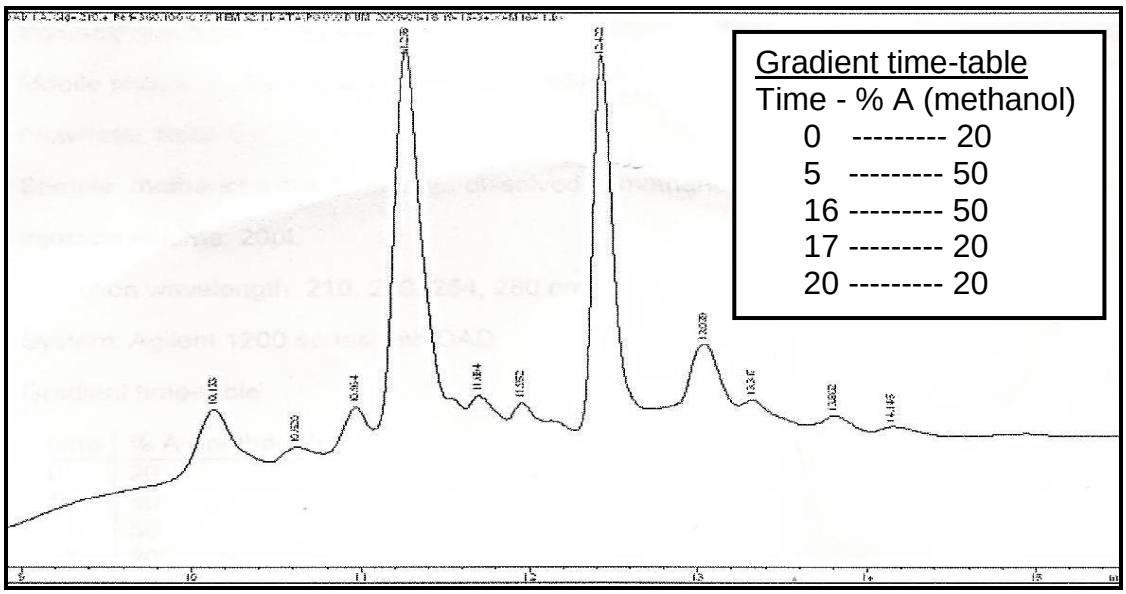


Fig.7.5. Reversed phase analytical profile of *X. americana* methanol extract (1 mg/mL) on ACE 5 C18, 250 x 4.6 mm column at 230 nm separated with methanol-water mobile phase gradient system.

7.4.3 Purity checks for crude hexane and dichloromethane fractions of X. americana and identification.

The NMR data for crude hexane and dichloromethane data are presented in Tables 7.2b and below

1. XAD: 500 MHz (Table 7.2b)
2. XAH 1: 300 MHz Proton NMR (Table 7.2b)

1. XAD

Brown viscous mass (50 mg): MS (m/z) (662.5 - 664.6, M+), 396, 255, 218,109. ^{13}C NMR (75 MHz,$CDCl_3$):13.82,14.12,19.34, 19.53,22.13,22.70,24.68, 28.23, 28.63, 28.73,28.78, 28.84, 28.92, 28.96,29.33, 29.72, 30.90, 31.93, 32.90, 33.82, 108.65, 128.28, 129.75, 129.78, 140.49, 143.41, 148.22, 173.83, 175.04, 178.29, 206.98.

2. XAH 1

White waxy solid (22 mg): MS (m/z): 426.4 (M+), 411.4 (M-15), 397.4 (M-29). ^{13}C NMR (75 MHz, $CDCl_3$): 11.87, 11.99, 14.11, 14.54, 15.22, 16.00, 16.18, 16.59, 18.02, 18.24, 18.79, 19.07, 19.33, 19.82, 21.06,22.69, 23.12, 24.31, 25.19, 26.17, 27.18, 27.24, 27.85, 27.99, 28.26, 29.12, 29.22, 29.27, 29.33, 29.37, 29.47, 29.53, 29.60, 29.71, 29.79, 29.88, 31.94, 33.99, 34.26, 34.87, 36.17, 36.63, 37.04, 37.13, 37.87, 38.10, 38.20, 39.77, 40.90, 42.35, 42.87, 43.02, 45.89, 48.03, 48.34, 50.09, 56.09, 56.73, 73.69, 80.63, 109.36, 118.85, 122.58, 129.78, 129.99, 139.76, 150.94, 173.26, 173.63.

Crude hexane and dichloromethane fractions sequentially obtained by maceration were initially analysed by NMR and mass spectrometry without chemical modification to prevent the formation of artefacts. Mass spectral data search did not match any compound in the NIST database and no molecular formulae were proposed (Figure 7.6g). However there were similarities in fragmentation patterns of steroid-like compounds with the base peak (m/z 396) matching stigmast-3, 5-diene, a dehydration product of sitosterol. Comparison of NMR spectra of standard compounds (β-sitosterol and stigmasterol) to crude extracts (XAD and XAH) spectra showed additional signals suggesting that the base peak (m/z 396) observed in the mass spectrum might be an aglycone fragment from a larger molecule in the extract. This is expected being that the

sample is a crude mixture (Figure 7.6a - d). The Proton NMR spectrum was crowded and overlapping especially at 1 - 2.5 ppm (aliphatic region) and not resolvable (Figure 7.6a, b). Very little information could be gleaned but signals observed between chemical shift values (0.6 - 2.5 ppm) are indicative of aliphatic chains (Silverstein, 2005).

According to Croasmun and Carlson (1994) the 1 - 2.5 ppm region in NMR spectra for steroids is crowded with only the C18 and C19 angular methyl singlets being shifted beyond the 1 – 2.5 ppm region (Table 7.2). Though the spectra showed similarities to steroids in the 1 - 2.5 ppm region, XAD exhibited signals for protons in the region between 5.52 – 6.5 ppm which were better resolved and located away from the crowded 1 - 2.5 ppm region suggesting a spin system or network (Figure 7.6 a, Appendix D2.iv). The carbon 13 spectrum of XAD run at 75 MHz was very noisy and DEPT spectrum did not provide a better picture. The sample was also run at 125 MHz but only signals in the olefinic region were resolved while the HSQC spectrum showed that carbons within 15 - 60 ppm were not well resolved as well. The region 0.7- 2.4 ppm integrated for 58 protons while the olefinic region integrated for 25 protons giving a total of 83 protons (Figure 7.6, Table 7.2b). Signals suggestive of acyl carbon (173.83 ppm), alkene carbon (108 -140 ppm) were observed in ^{13}C spectrum of XAD run at 75 MHz. Stigmasterol signals were assigned using NMRanalyst software and by comparison to literature values (Dunkel, 2011; Zhang *et al*. , 2005).

Efforts to purify the extracts by preparative LC were pursued by using cyano and silica columns for different retentivity and possibly alternate selectivity. Initial separation and isolation of XAH by LC on cyano column (250 x 10 mm, 5 µm using 100 % DCM resulted in three fractions which were checked for purity by analytical LC and NMR. (Table 7.1, Figure 7.4 iii - vi). Fraction 1 which looked fairly pure with one major peak, was analysed by NMR and compared to sitosterol and stigmasterol NMR spectra. XAH1 proton spectrum exhibited similar signals to sitosterol and β-amyrin in addition to other signals. The proton spectrum showed a total of 58 protons with most of the signals in the aliphatic region. There were two signals consistent with alkene protons at 4.52 and 5.52 ppm integrating for a proton each. The ^{13}C NMR spectrum had additional signals at 173 ppm suggestive of an acyl carbon and the 3 - OH signal of a

triterpene alcohol which appears at about 3 – 4 ppm signal was not observed, instead there was a deshielded signal at 4.52 ppm. On expansion of the ^{13}C spectrum, signals were observed at 150 and 208 ppm (suggestive of a C=O group in acids, esters, anhydrides and keto-compounds).

Mass analysis gave ion peaks at 426.4 (M)+, 411 (M – 15), 397 (M – 29) (Figure 7.6h). This mass resembles C-30 terpenoids such as β-amyrin, cycloartenol, lupeol, friedelin with molecular formula, $C_{30}H_{50}O$ (MW - 426.72). However, comparison of β-amyrin NMR predicted by NMR predictor (www.nmrdb.org) shows that XAH1 may actually be a conjugated terpene fatty acid ester with close similarity to β-amyrin palmitate, $C_{48}H_{80}O_2$ (MW - 664.62). The ion peak 665.6 was observed in the high molecular weight region of the mass spectrum (Figure 7.6). Compounds such as terpenes, fatty acids and phytosterols are reported to be common constituents of non-polar fractions so the sample was tested for the presence of a steroidal nucleus using Lieberman-Burchard reagent (Evans, 1996). The crude extract tested positive to the test.

Based on the results of anti-TB studies, steroidal compounds such as saringosterol have been demonstrated to have anti-TB activities at MIC's ranging from 0.125 – 1 µg/mL. It has also been reported that anti-TB activities of a majority of natural products tested are attributed to secondary metabolites of terpenoid origin (Copp, 2003). Saludes *et al.* (2002) identified the presence of E-phytol, cycloartenol, stigmasterol, sitosterol and other derivatives as the anti-TB constituents from hexane extract of *Morinda citrifolia* while Okunade *et al.* (2004) reported anti-TB activities of sitosterol and stigmasterol at MIC - 128 and 32 µg/mL respectively. A number of triterpenes investigated for *in vitro* anti-TB and structure - activity studies suggest that the presence of structural features such as C-3 keto and/or β-hydroxy, cyclopropane ring and epoxide groups are essential for activity (Cantrell *et al*., 2001).

Table 7.1. Preparative LC data of XAH

Initial weight of crude	Peak 1	Peak 2	Peak 3
200 mg hexane extract	38.5 mg	52.98 mg	34.61 mg

Table 7. 2a. ^{13}C and ^{1}H NMR assignments of stigmasterol at 75 and 300 MHz respectively

Atom	stigmasterol	
	^{13}C ppm	^{1}H ppm
1	37.28	1.48
2	31.67	1.59
3	71.78	3.51
4	42.32	2.28
5	140.76	-
6	121.66	5.10
7	31.92	1.93
8	31.90	1.48
9	50.20	1.01
10	36.52	-
11	21.08	1.48
12	39.70	1.77
13	42.23	-
14	56.88	1.01
15	24.36	1.01
16	28.89	1.48
17	55.99	1.01
18	12.22	0.66
19	21.06	1.01
20	40.45	2.02
21	21.22	1.01
22	138.28	5.10
23	129.30	5.35
24	51.24	2.02
25	31.87	1.48
26	19.39	1.01
27	18.99	1.01
28	25.39	1.01
29	12.05	1.01

Table 7.2b. ^{1}H assignments of XAD (500 MHz) and XAH1 (300 MHz)

XAD			XAH1		
Chemical shift , δ (ppm)	multiplicity	No of protons	Chemical shift , δ (ppm)	multiplicity	No of protons
0.7- 2.39	u	58	0.68 – 0.79	s	1.07
3.69	s	1	0.81 – 1.09	u	17.35
5.15	u	5.65	1.13 – 1.68	u	32.22
5.32 - 5.37	u	6.52	1.99 – 2.23	u	4.37
5.45 - 5.52	u	4.79	2.26 – 2.32	u	2.59
5.75 - 5.78	m	0.79			
6.05 - 6.10	m	2.19			
6.27 - 6.33	q or d,t	3.00			
6.47 - 6.52	d,d	1.00			

u-unresolved, s-singlet, m-multiplet, q-quintet, d-doublet, t-triplet

Though hexane extract exhibited greater inhibitory activity against *T. mentagrophytes* and *P. acnes* at 250 µg/mL and 62.5 µg/mL respectively, XAD showed stronger anti-TB activity at an MIC of 30.5 µg/mL against *M. tuberculosis* H37Rv (Tables 3.4 and 6.1). The anti-dermatophyte and anti-acne activities of hexane extract may be attributed to the presence of triterpenoidal compounds. However, more rigorous purification is required to aid structure determination by NMR. XAD was further subjected to semi-preparative LC using methanol - DCM (2:98 v/v) mobile phase system to obtain cleaner fractions for NMR and mass spectral analysis.

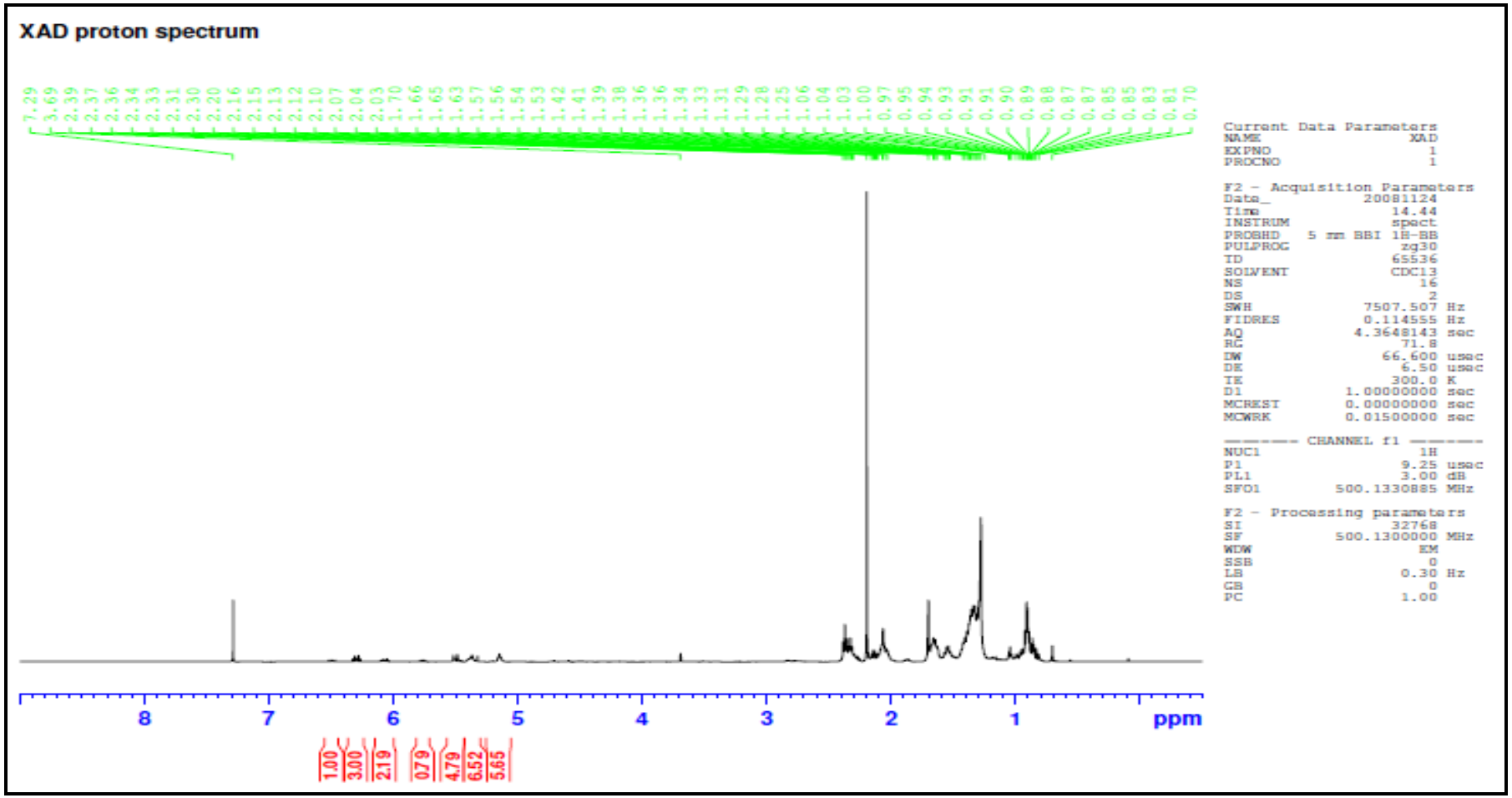


Fig.7.6a Proton NMR spectrum of dichloromethane extract of *X. americana* (XAD)

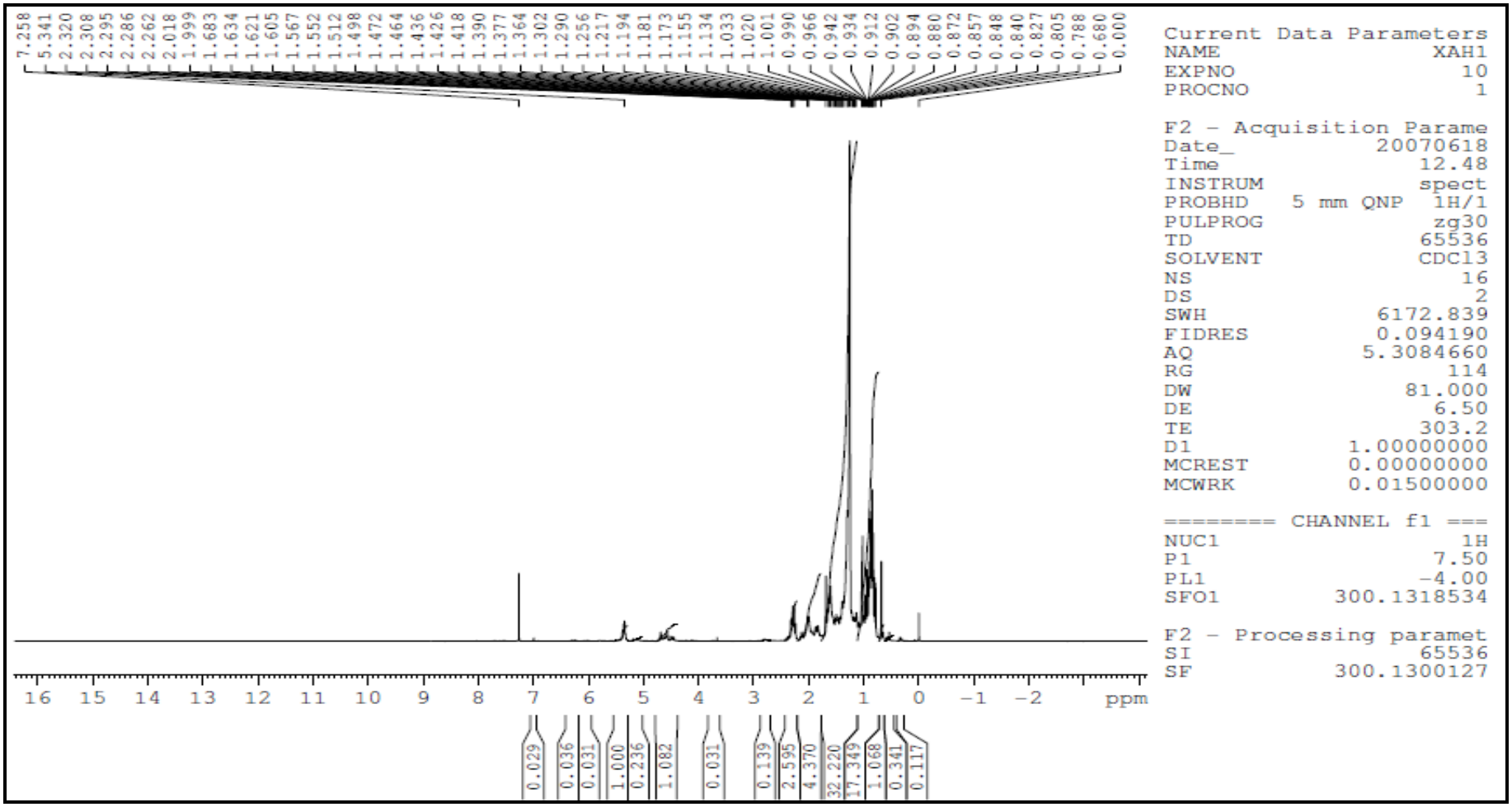


Fig. 7.6b Proton NMR spectrum of XAH peak 1 (*X. americana* hexane extract fraction 1).

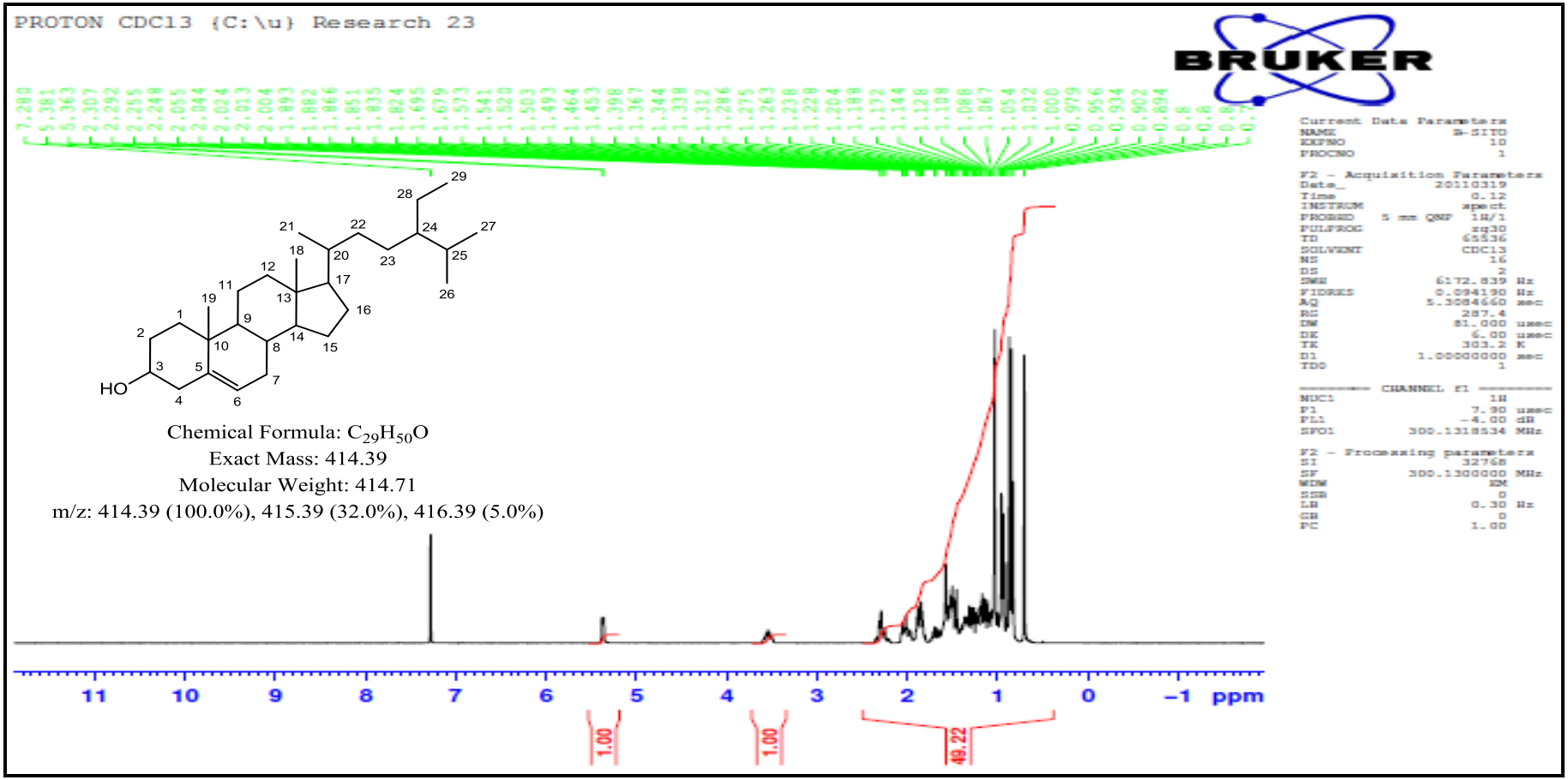


Fig. 7.6c Proton NMR spectrum of β-sitosterol (reference standard)

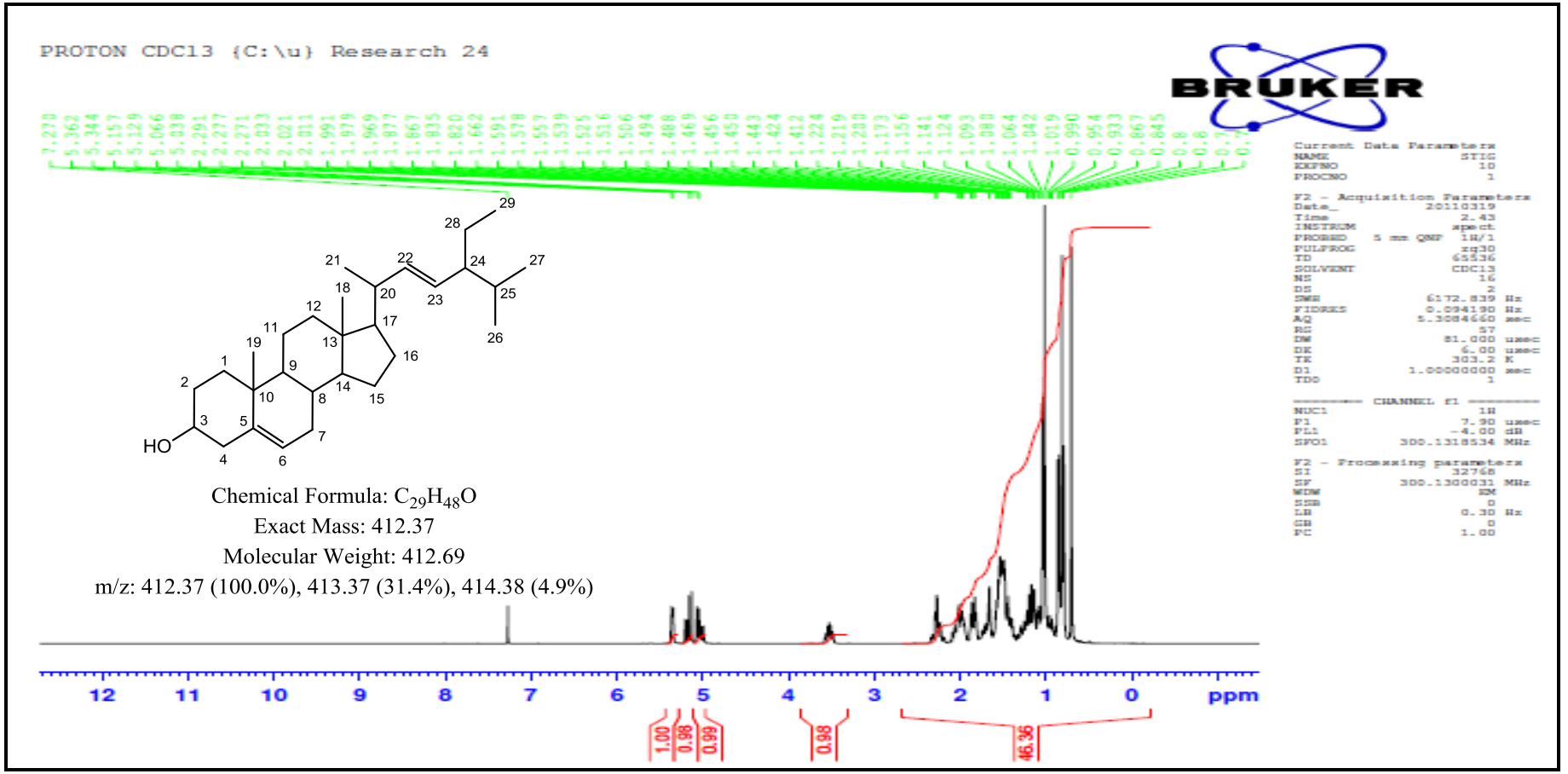


Fig.7.6d Proton NMR spectrum of stigmasterol (reference standard).

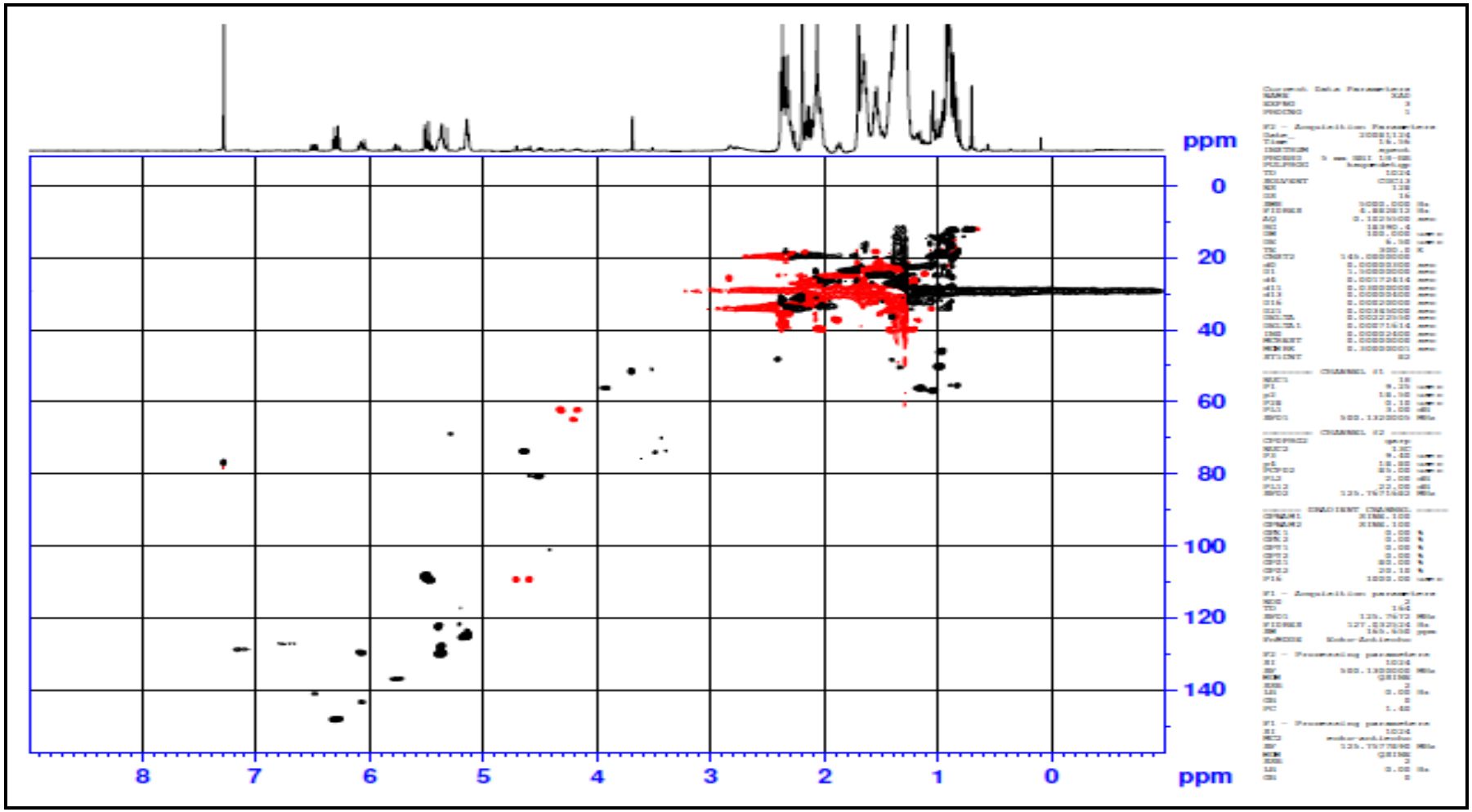


Fig.7.6e HSQC spectrum of *X. americana* dichloromethane fraction (XAD).

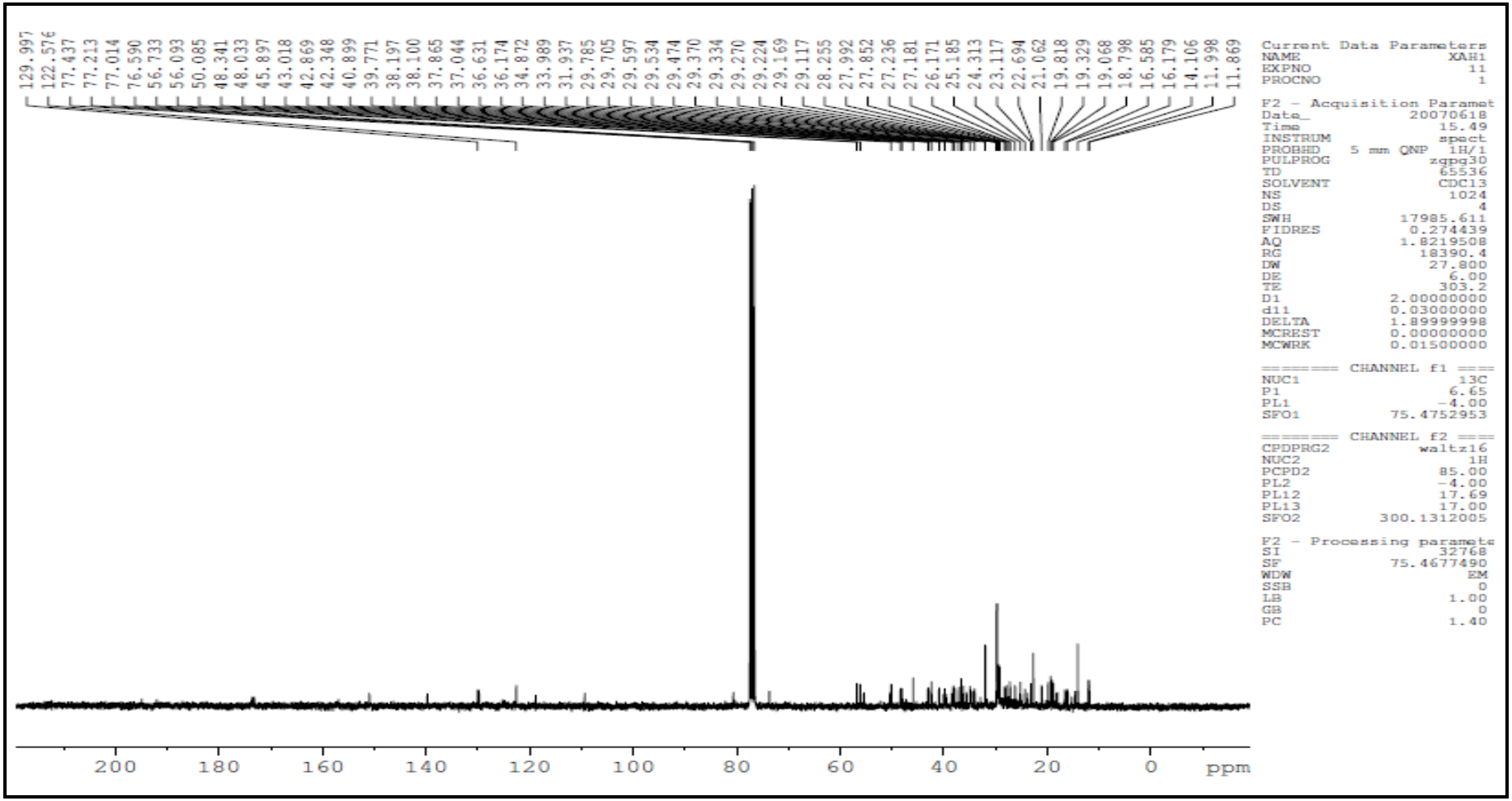


Fig.7.6f Carbon 13 NMR spectrum of XAH peak 1 (*X. americana* hexane fraction 1).

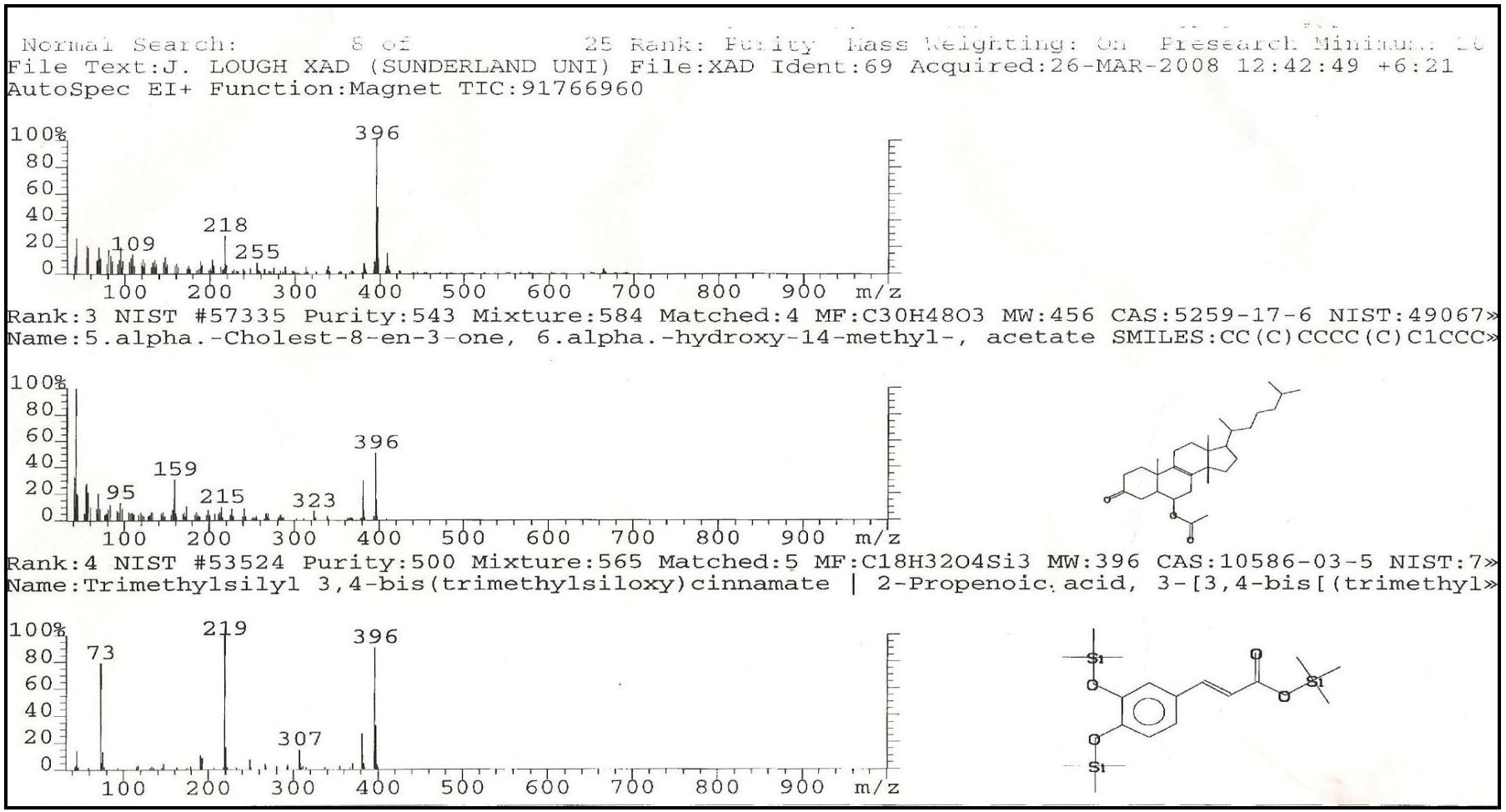


Fig. 7.6g EI Mass spectrum of crude dichloromethane extract (XAD).

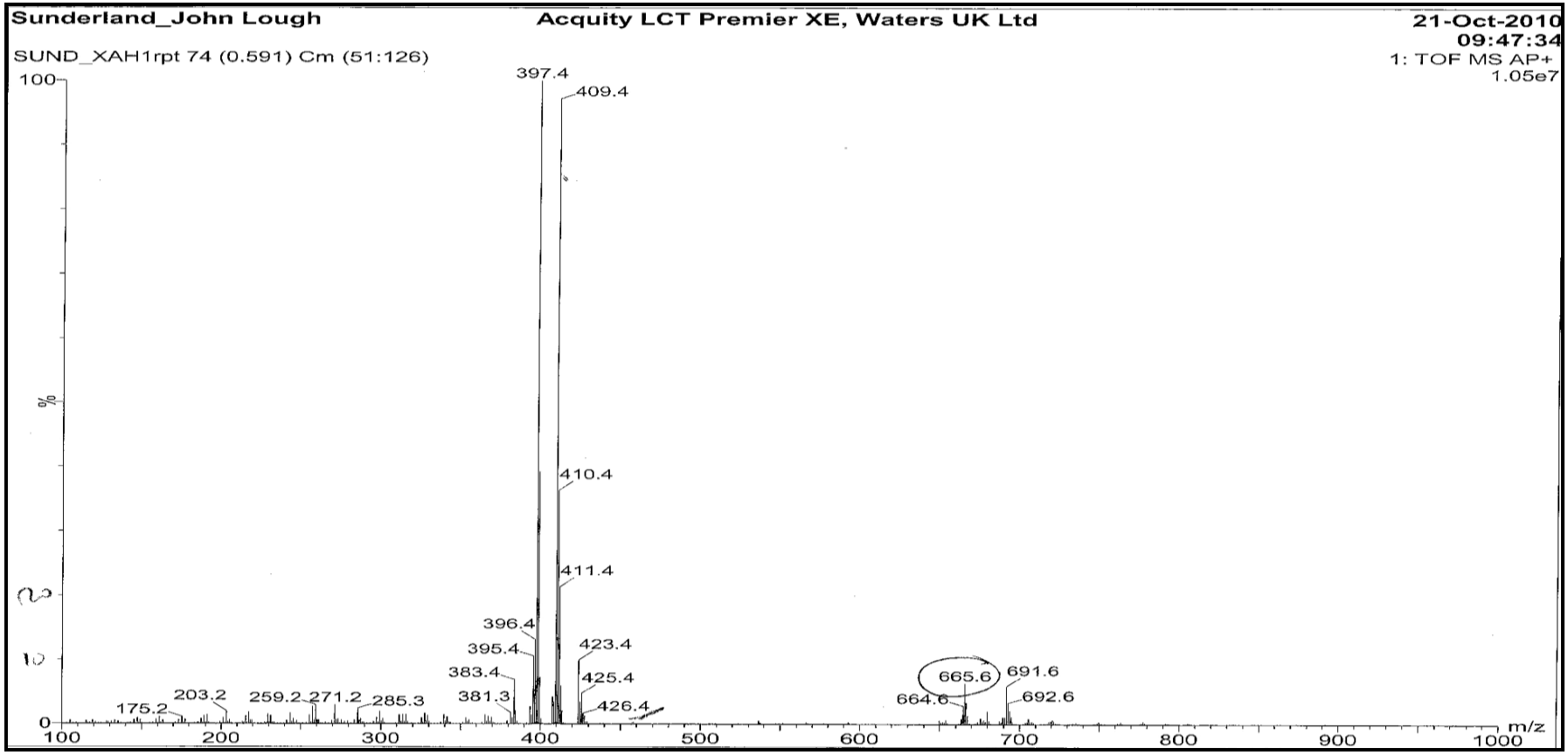


Fig. 7.6h Mass spectrum of *X. americana* hexane extract (peak 1 from preparative LC).

7.4.4 LC separation and mass spectral analysis of XAD fractions

Dichloromethane extract of *X. americana* was separated by methanol-DCM (2:98 v/v) to obtain four fractions labelled as XD1, XD2, XD3 and XD4. The data obtained is shown in Table 7.3 and Figure 7.7. The fractions were analysed by EI + MS and the fragmentation patterns showed similar ion peaks but due to the lack of molecular formulae and NMR data, it was difficult to predict structures based on the nominal masses obtained. XD1 showed base peaks at low frequency regions below 200 amu indicative of aliphatic or alicyclic chains. When compared to the data obtained by TOF MS EI, a consistent ion peak at 396 was observed which could possibly be due to an aglycone or genin (Appendix E4). Other ion peaks observed were 446 (XD1), 428 (XD2), 571 (XD3) and 280 (XDW). NIST data search matches showed some similarities to compounds such as ergosta-6, 22-diene-3-ol, 5, 8-epidioxy derivative ($C_{28}H_{44}O_3$, MW- 428), ergosta- 4, 6, 22-trien-3-α-ol ($C_{28}H_{44}O$, MW- 396). The ion peak at 662 was also consistent in previous and subsequent experiments. The crude extract of XAD tested positive to Lieberman-Burchard reagent and the ion peak at 662 – 664.6 is possibly a mixture of fatty acid esters of triterpenes such as β-amyrin palmitate ($C_{46}H_{80}O_2$ MW- 664.615). Further experiments were carried out to obtain accurate mass measurements to enable structure determination in conjunction with NMR analysis.

Table 7.3. LC semi-preparative data for XAD

Peak	Peak code	Volume of eluent	Dry weight (mg)
1	XD1	100 mL	12.4
2	XD2	60 mL	16.4
3	XD3	100 mL	2.3
4	XD4	1.2 L	34.6
Initial weight of crude – 265 mg (5 mg/mL solution in methanol-DCM, 2:98 v/v)			

The LC chromatograms looked fairly simple but the results showed that UV detection may not be picking up all the compounds in the mixture and there might have been several compounds under the peaks observed. The nominal masses alone could not be used for identification and library data matching search of fragmentation patterns did not yield positive results. Further purification procedures were continued to obtain cleaner samples for accurate mass measurements using a different ionisation technique and NMR analysis.

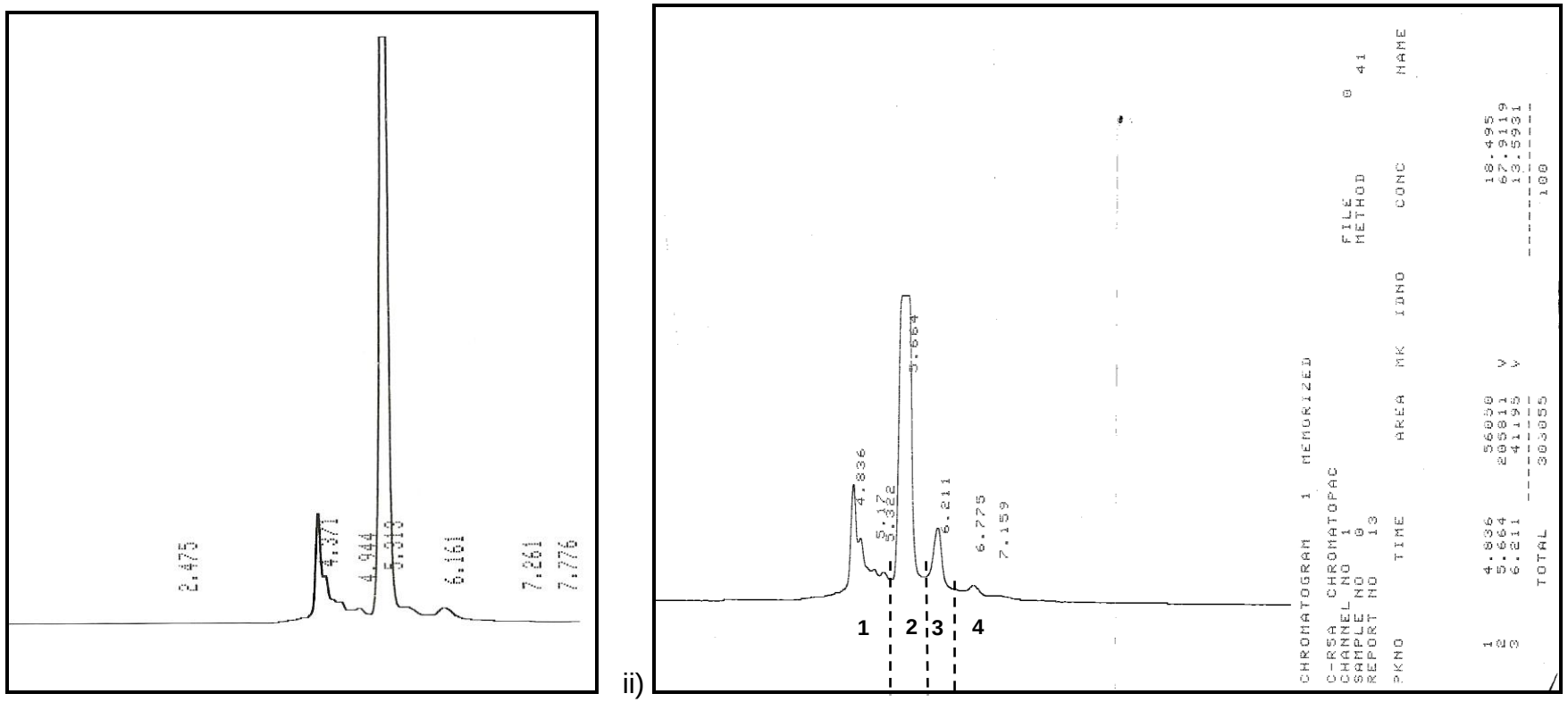


Fig. 7.7 i) Analytical and preparative LC profile of XAD on ACE 5 CN column (250 x 4.6 mm, 250 x 10 mm respectively) at 1 and 5 mL/min developed with methanol-DCM (2:98 v/v), injection volume of 1mL. Preparative fractions were collected and labelled as 1= XD1, 2= XD2, 3 = XD3, 4= XDW.

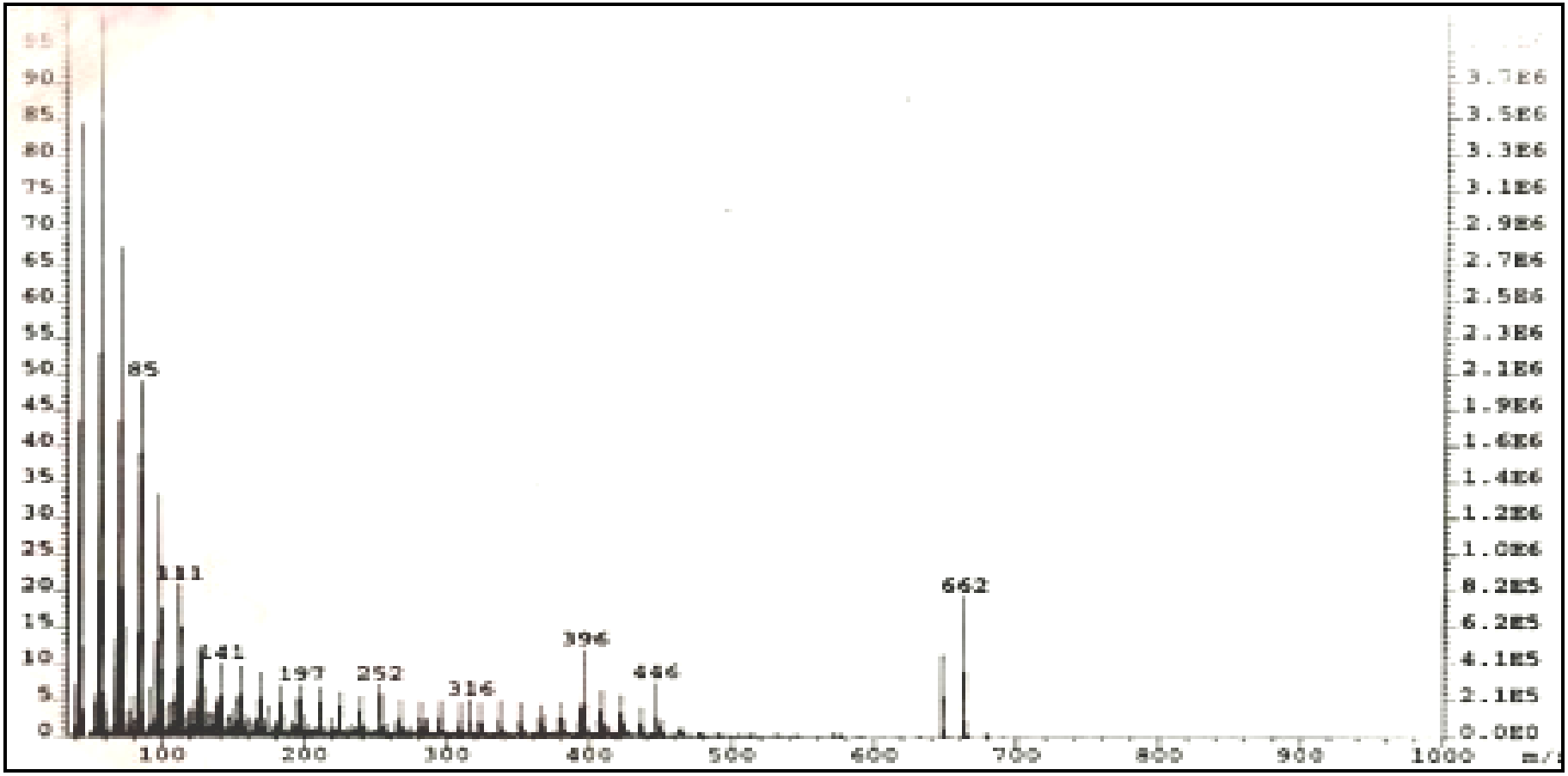


Fig. 7.7 ii) EI mass spectrum of *X. americana* dichloromethane extract (XD 1)

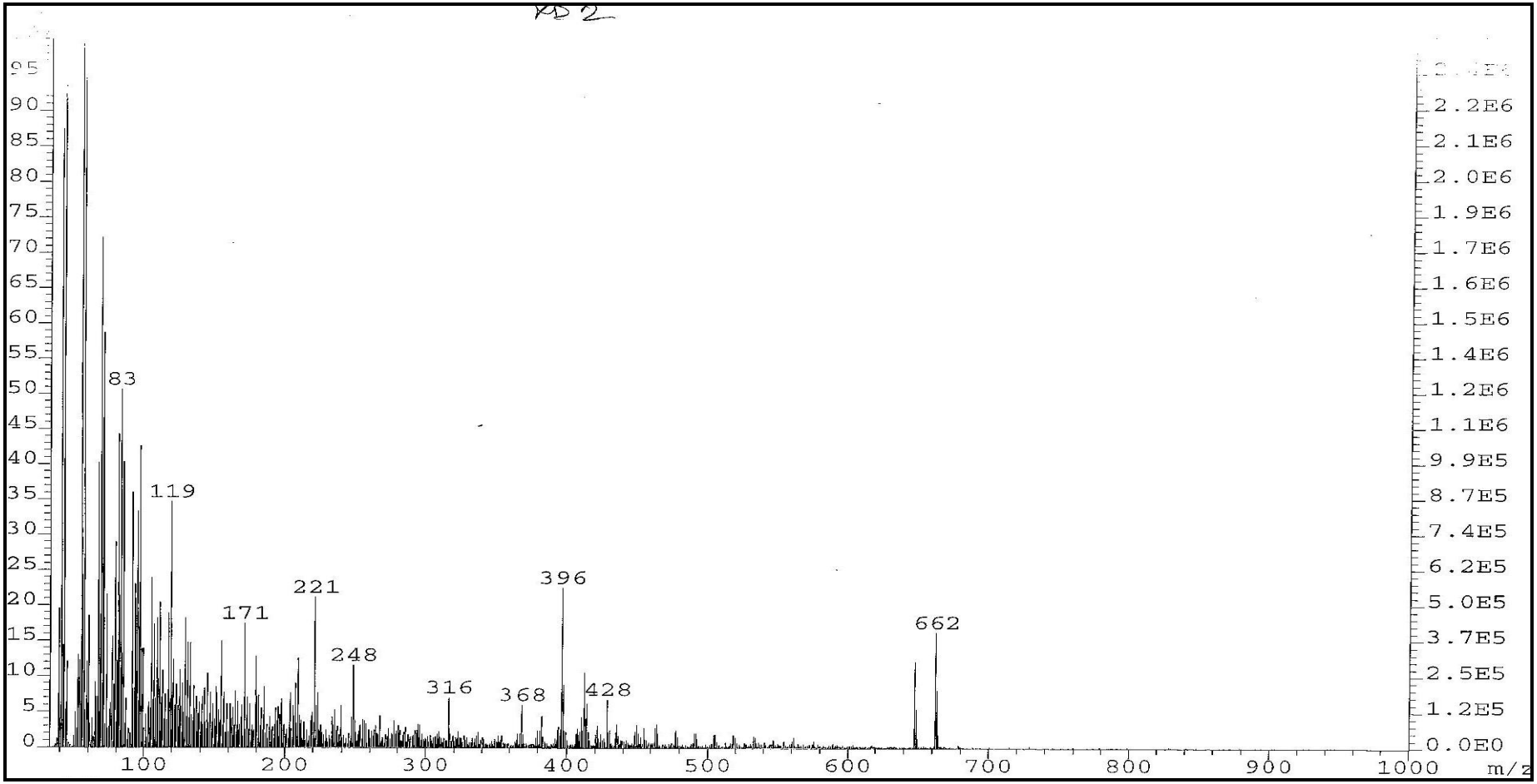


Fig. 7.7 iii) EI mass spectrum of *X. americana* dichloromethane extract (XD 2).

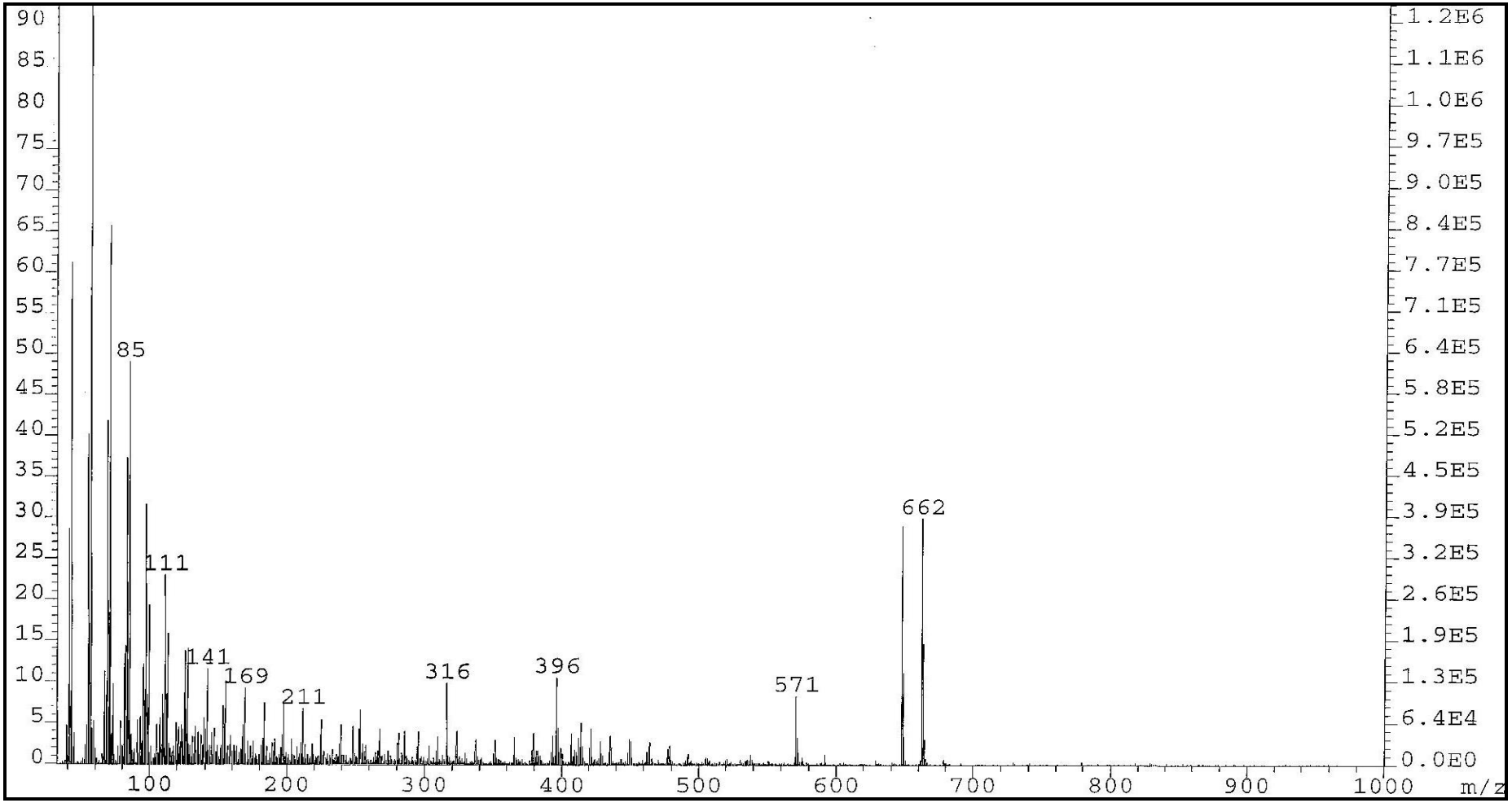


Fig. 7.7 iv) EI mass spectrum of *X. americana* dichloromethane extract (XD 3).

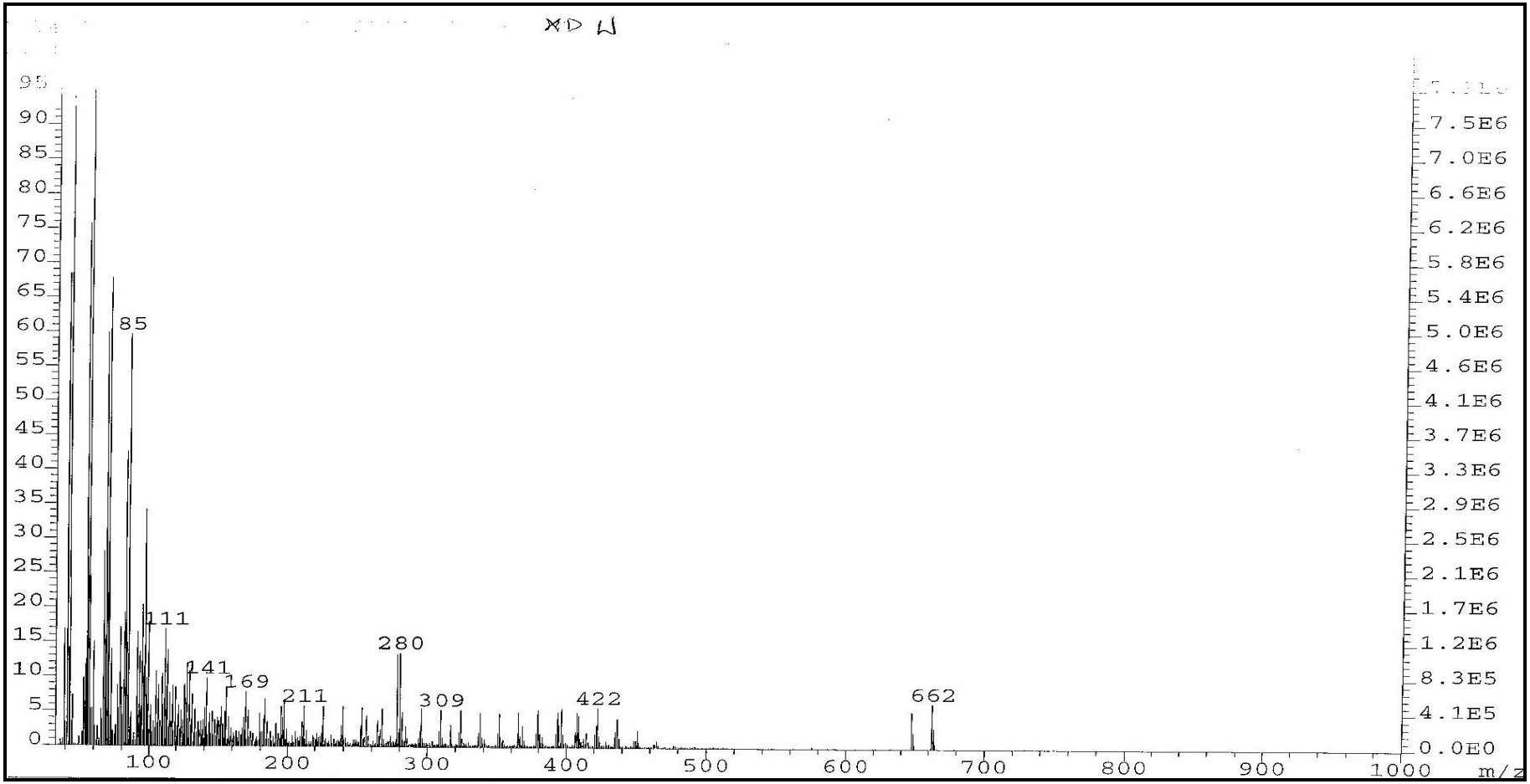


Fig. 7.7 v) EI mass spectrum of *X. americana* dichloromethane extract (XDW).

7.4.5 TLC analysis of LC, crude hexane and dichloromethane fractions

7.4.5.1 LC analysis

The crude fractions were separated using ACE 5 SILICA column, 250 x 7.75 mm in order to obtain cleaner samples for mass spectral analysis using a soft ionisation source (Time of Flight – Atmospheric Pressure Solids Analysis Probe, TOF- ASAP MS). It was expected that ASAP which is a modification of the atmospheric pressure ion (API) source would give molecular ions as radical cations $M^{+\bullet}$ or protonated molecules MH+ to aid identification unlike the EI instrument which has the capacity for extensive fragmentation and is not likely to give a molecular ion (Ray *et al*., 2010).

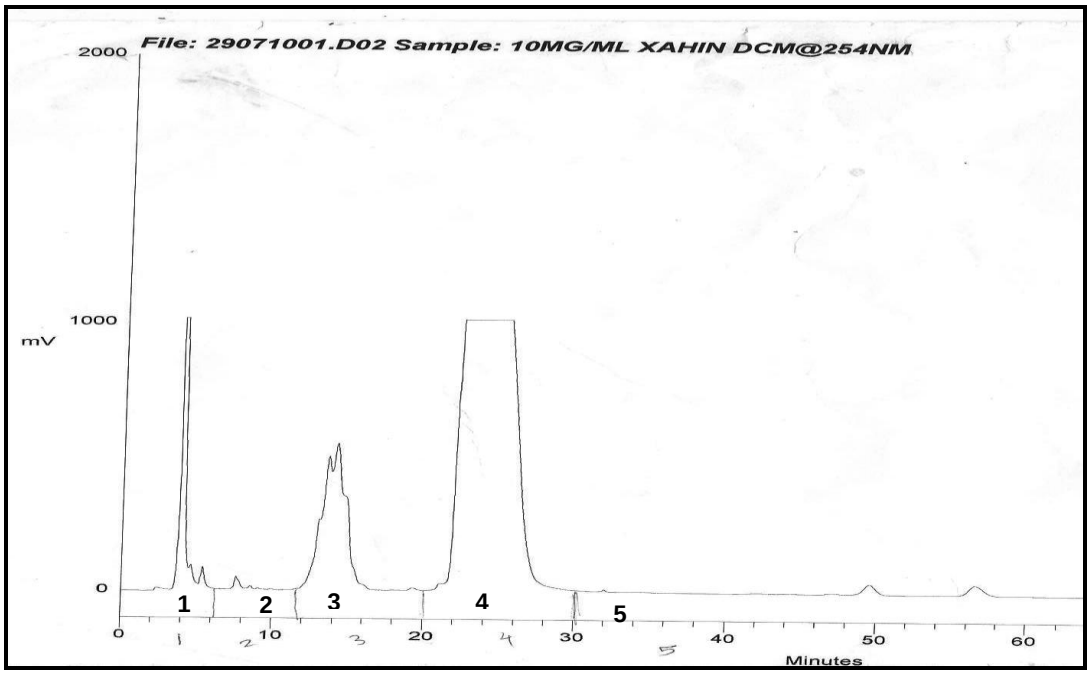


Fig.7.8 LC chromatogram of XAH on ACE 5 SILICA column using DCM

The crude extracts were separated with 100 % DCM and collected in five fractions (Figure 7.8). The fractions were tested against *M. aurum* by bioautography to localise the activity so as to focus on the bioactive component (Table 3.4).

The mass spectral data of fractions showed many fragment peaks which when compared to fractions obtained from blank solvent runs showed similar peaks.

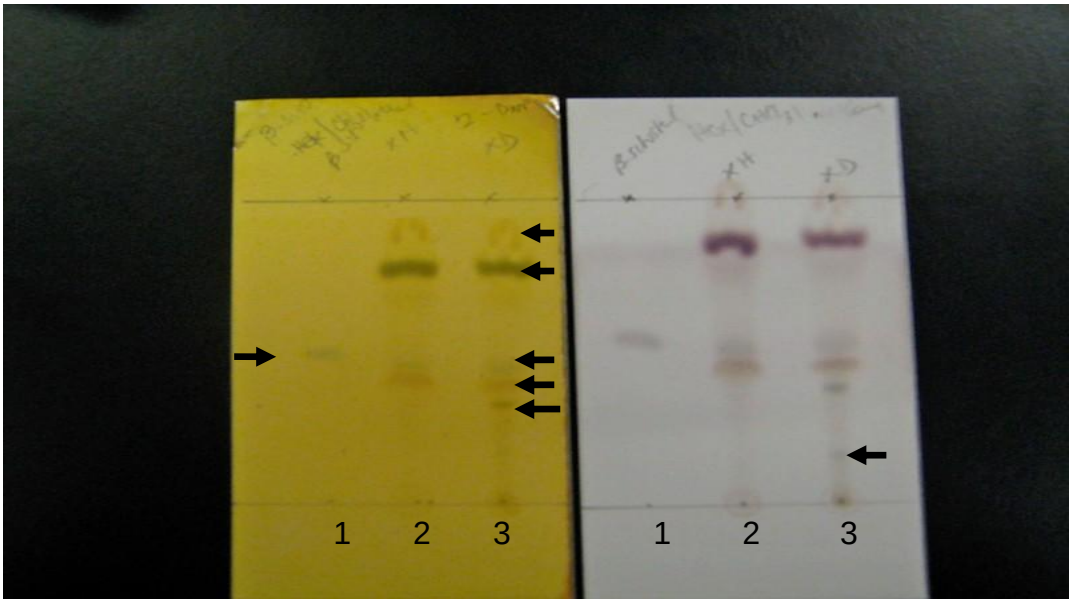
The peaks in the blank may have been due to a carry-over effect from previous analyses or contaminants. The mass peaks observed were similar to mass fragments seen with EI - MS (Figures 7.1-7.7). Detailed ASAP mass spectra are shown in Appendix E5/6. The mass spectra for the various fractions showed that the fractions required required further purification. TLC analyses of LC fractions were carried out to aid localisation of activity by bioautography and to possibly separate the components further such that better mass data could be obtained for easier interpretation.

7.4.5.2 TLC analysis of fractions (LC and crude samples)

Due to ambiguity in the data observed with EI - MS and ASAP- MS, isolation and purification by TLC was then considered. Thin layer chromatography was performed on analytical silica gel 60 F_{254} plates. Sample extracts and β-sitosterol (reference standard) were dissolved in chloroform to give 1 mg/mL solutions and spotted in 10 - 20 µL volumes. Plates were developed to a distance of 7 cm for analytical plates and 15 cm for preparative plates (Figure 7.9). Various solvent systems were used to develop the samples as shown in Figures 7.9 i - iii. The plates were visualised with iodine (Figure 4.4i) 2- di-nitrophenylhydrazine and sulphuric acid sprays as well as under UV lamp at 254 and 366 nm. Hexane – chloroform - methanol (4:4:1, v/v/v) solvent system gave better separation and sharper zones compared to hexane: IPA (95:5 v/v). The plates showed about seven bands (Figure 7.9.i).

The standard, β-sitosterol, R_f 0.65 (lane 1) migrated to about the same position as one of the spots in XAH and XAD extracts, Rf 0.55 (Figures 7.9. i and ii). The LC fractions showed no spots on spraying with 2-DNP and sulphuric acid reagents (Figure 7.9 ii). The LC and TLC fractions were all subjected to testing with *M. aurum* and then re-subjected to TLC analysis. A hexane: chloroform: methanol solvent system was then used for preparative TLC of XAD. The spots were scraped off and re-constituted in chloroform for spots 1 and 2 while spots 3 and 4 which were insoluble in chloroform were re-constituted in methanol (Figure 7.9. iii). Attempts to recrystallize the sample with acetone or ethylacetate did not yield crystals. The samples were then filtered, dried and subjected to MS and NMR analyses.

i.



ii.

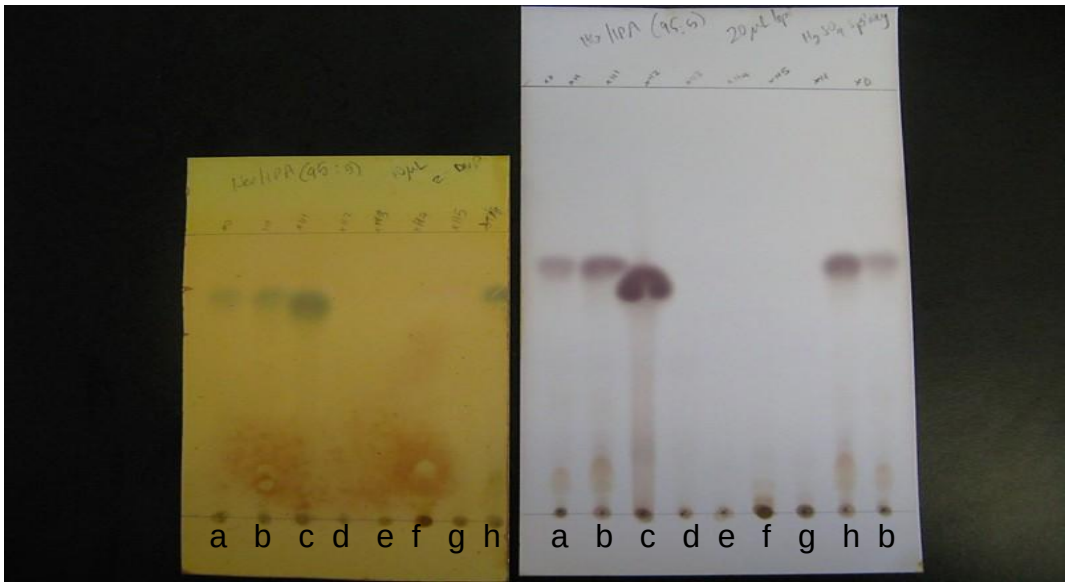


Fig.7.9 i) TLC plate sprayed with 2-DNP (left) and H_2SO_4 (right) with β-sitosterol in Lane1, XH in Lane 2 (hexane extract of *X. americana*), XD (dichloromethane extract of *X. americana*) in lane 3 developed with hexane-chloroform-methanol (4:4:1, v/v/v) solvent system.

ii) H_2SO_4 sprayed TLC plate with crude and LC fractions from XH and XD in IPA - hexane (5:95 v/v) solvent system. (a = XH, b = XD, c =XH1, d =XH2, e =XH3, f =XH4, g =XH5, h = XH)

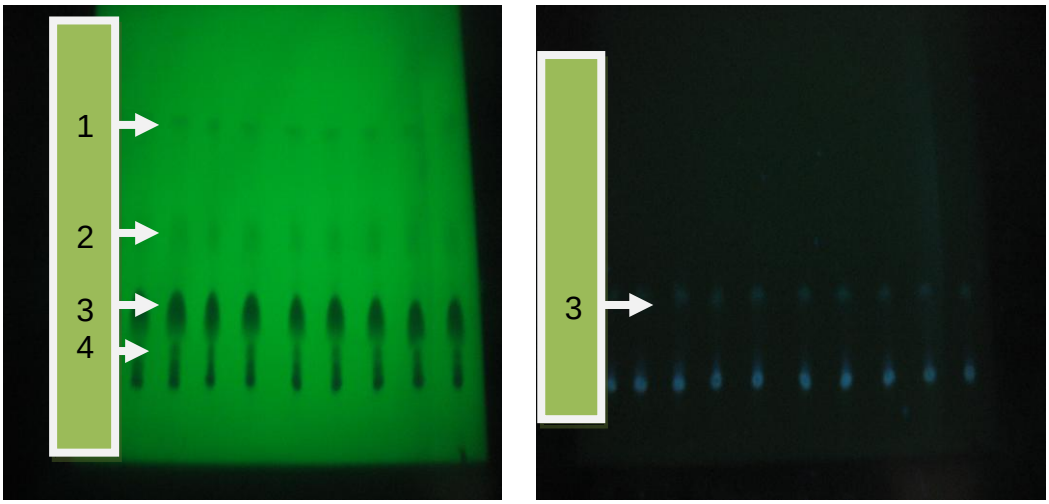
iii) 

Fig.7.9 iii) Preparative TLC plate (20 x 20 cm) spotted with XAD and developed with hexane - $CHCl_3$ - methanol (4:4:1, v/v/v) solvent system visualised with UV lamp at 254 and 365 nm. Four spots were observed and numbered as 1, 2, 3, and 4. Spot 3 fluoresced at 365 nm.

7.4.5.3. Identification of compounds from crude dichloromethane X. americana root extract by HR TOF-MS EI +

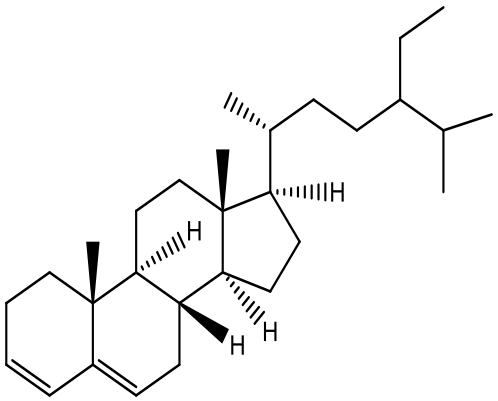
The results obtained from accurate mass measurements predicted three compounds at < 5 ppm error from TLC spots 1 and 2 as stigmasten- 3,5-diene [**1**] (MW – 396.3751 calculated 396.3751 for $C_{29}H_{48}$), Stigmast-5-en-3-ol oleate [**2**] (MW - 678.6329, calculated 678.6315 for $C_{47}H_{82}O_2$) and β-sitosterol [**3**] (MW – 414.3861, calculated 414.3862 for $C_{29}H_{50}O$) [Figure 7.10, 11a,b].

Other compounds predicted from TLC spot 2 and 3 having an error margin greater than 5 ppm are shown in Table 7.4a.

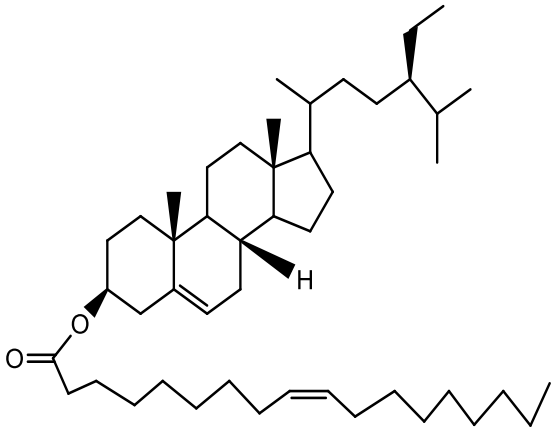
[**1**] Mass data (m/z): 41, 57, 69, 81, 147, 105,147, 283, 396

[**2**] Mass data (m/z): 41, 69, 95, 105, 147, 161, 203, 283, 339, 678

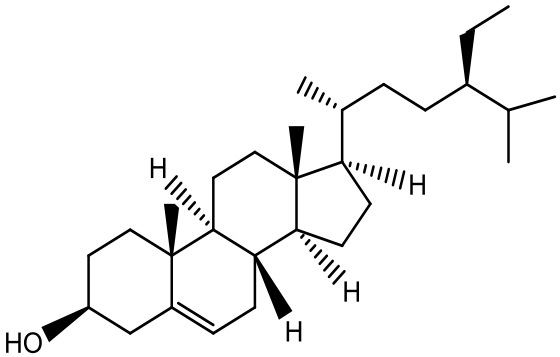
[**3**] Mass data (m/z): 41, 57, 81,145, 213, 255, 329, 414



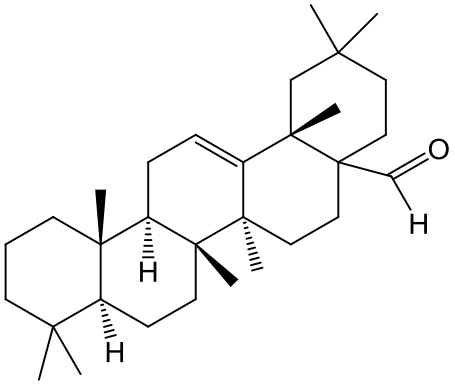
[1]



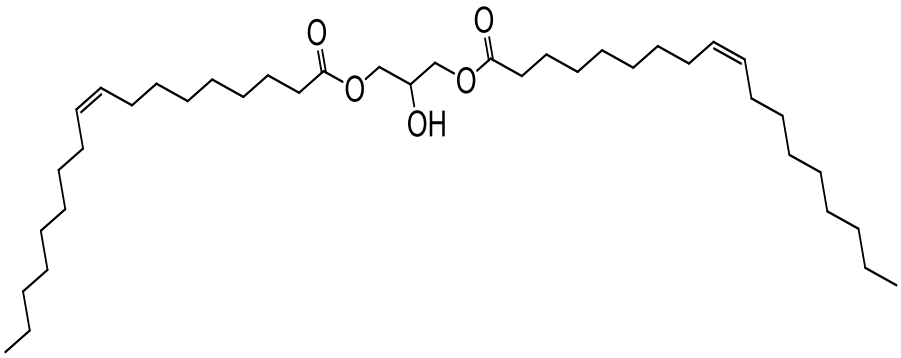
[2]



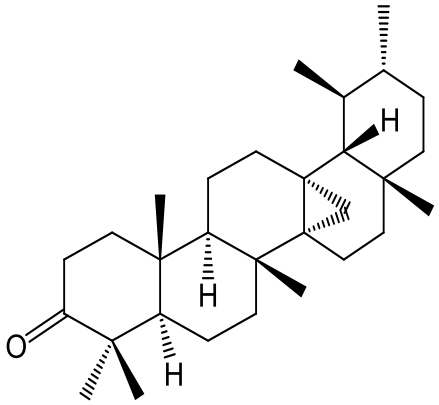
[3]



[4]



[5]



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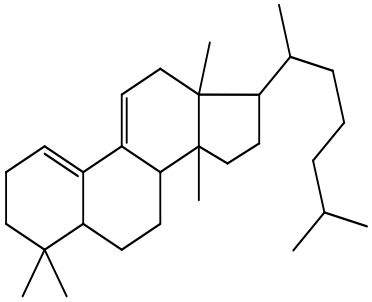

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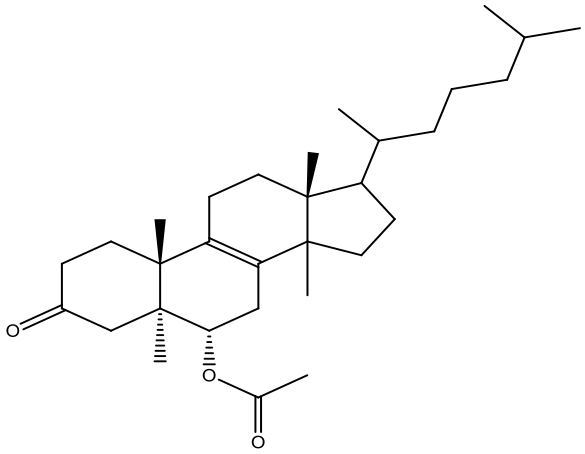


[8]

Fig.7.10 Chemical structures of suggested compounds

Table 7.4. Properties of some of the predicted compounds

TLC spot	Description	Dry Weight mg	Exptal. mass	Calculated mass	Error margin ppm	Predicted compound match	Molecular formula	Molecular weight
2	Brown mass	4.4	424.3733 620.5483 424.3680	424.3705 620.5380 424.3705	6.6 16.6 - 5.9	[**4**] Olean-12-en-28-al [**5**] 2-hydroxy-3-[(Z)-octadec-9-enoyl]oxypropyl](Z)-octadec-9-enoate [**6**] 13,27-cycloursan-3-one	$C_{30}H_{48}O$ $C_{39}H_{72}O_5$ $C_{30}H_{48}O$	424 620 424
3	Yellow oily mass	2.9	410.3852 456.3650	410.3913 456.3603	- 14.9 10.3	[**7**]4,4,14-trimethyl-9,19-cyclo-9,10-secocholesta-1(10),9(11)-diene [**8**] 5-α-cholest-8-en-3-one, 6-α-hydroxy-14-methyl-acetate	$C_{30}H_{50}$ $C_{30}H_{48}O_3$	410 456

Initial weight of crude used in TLC isolation - 223.1 mg

The NMR proton and carbon spectra obtained for TLC spot 1 was overlapping and crowded at the aliphatic region but attempts to account for the structures of the first three compounds from the data proved challenging with the spectra obtained. However, the proton NMR spectrum is presented in Figure 7.11d. Difficulties were also encountered in obtaining NMR data for TLC spots 2 and 3 due to the amounts obtained which had to be subjected to mass, NMR and biological testings. Only proton and COSY spectra could be obtained for TLC spots 2 and 3 with insufficient material for ^{13}C and other 2D NMR spectra.

Based on accurate mass measurements, three compounds were proposed at < 5 ppm as stigmasten-3, 5-diene, stigmast-5-en-3-ol oleate and β-sitosterol. The results of accurate mass measurements indicated that the TLC spots were a mixture of compounds. Probably as a result of this, the inadequacy of the NMR data obtained made the task of proper identification of the structures challenging.

Hyphenated techniques involving LC/NMR and SFC-UV/ELSD/MS were then considered for further exploration of the fractions but LC/NMR involving NP solvents was not practicable without a significant amount of initial development work and the fact that earlier separations and isolation using UV detector did not give good results. GC/FID/MS was considered as an alternative technique when the Faculty of Applied Sciences in Sunderland University acquired new equipment such as the triple quadruple mass spectrometers linked to GC with a library database. Subsequent experiments were then carried out with GC/FID in the hope that the structures of some of the predicted compounds could be correctly identified.

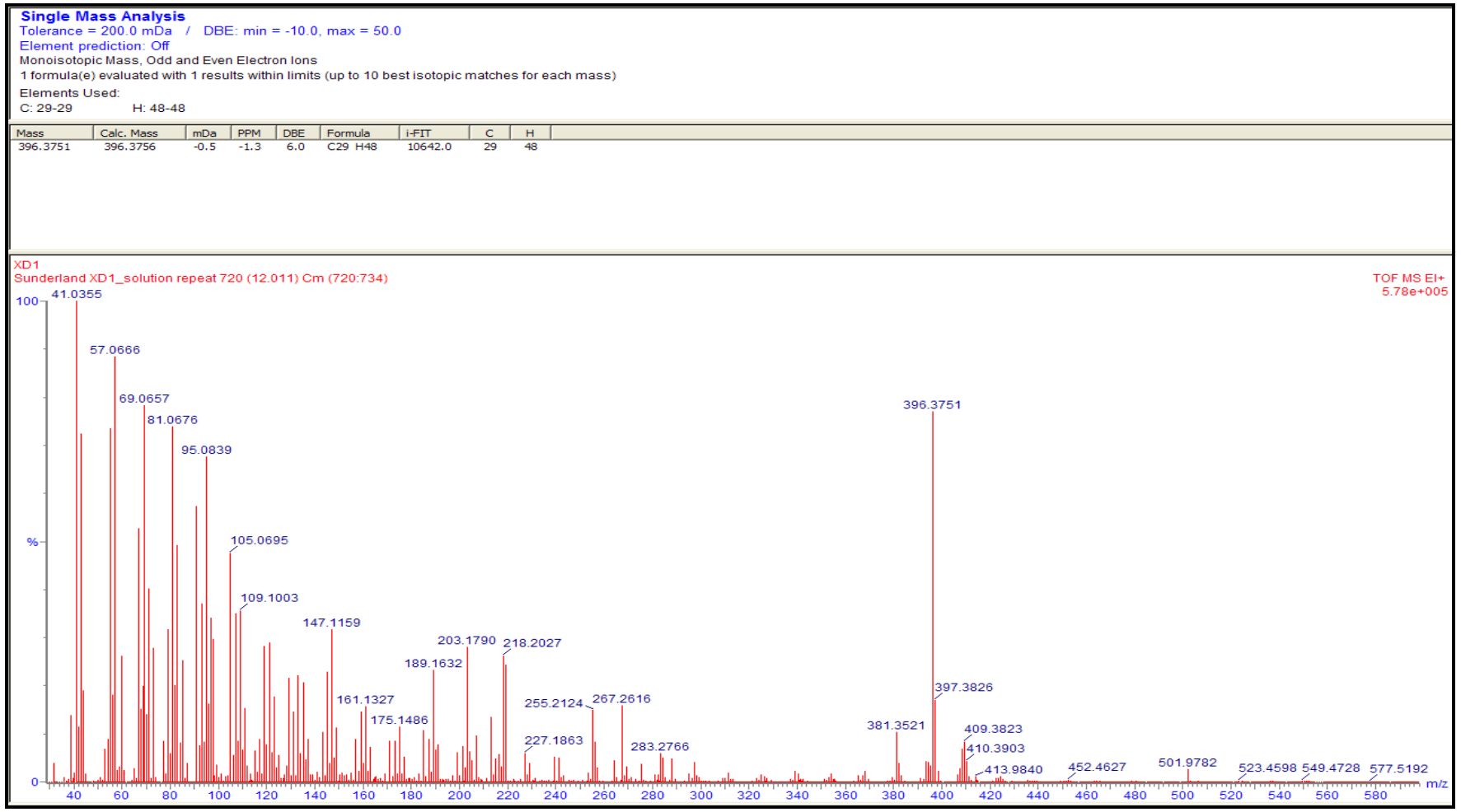


Fig. 7.11a i) Mass spectrum of XD1 compound 1 (TOF MS EI +)

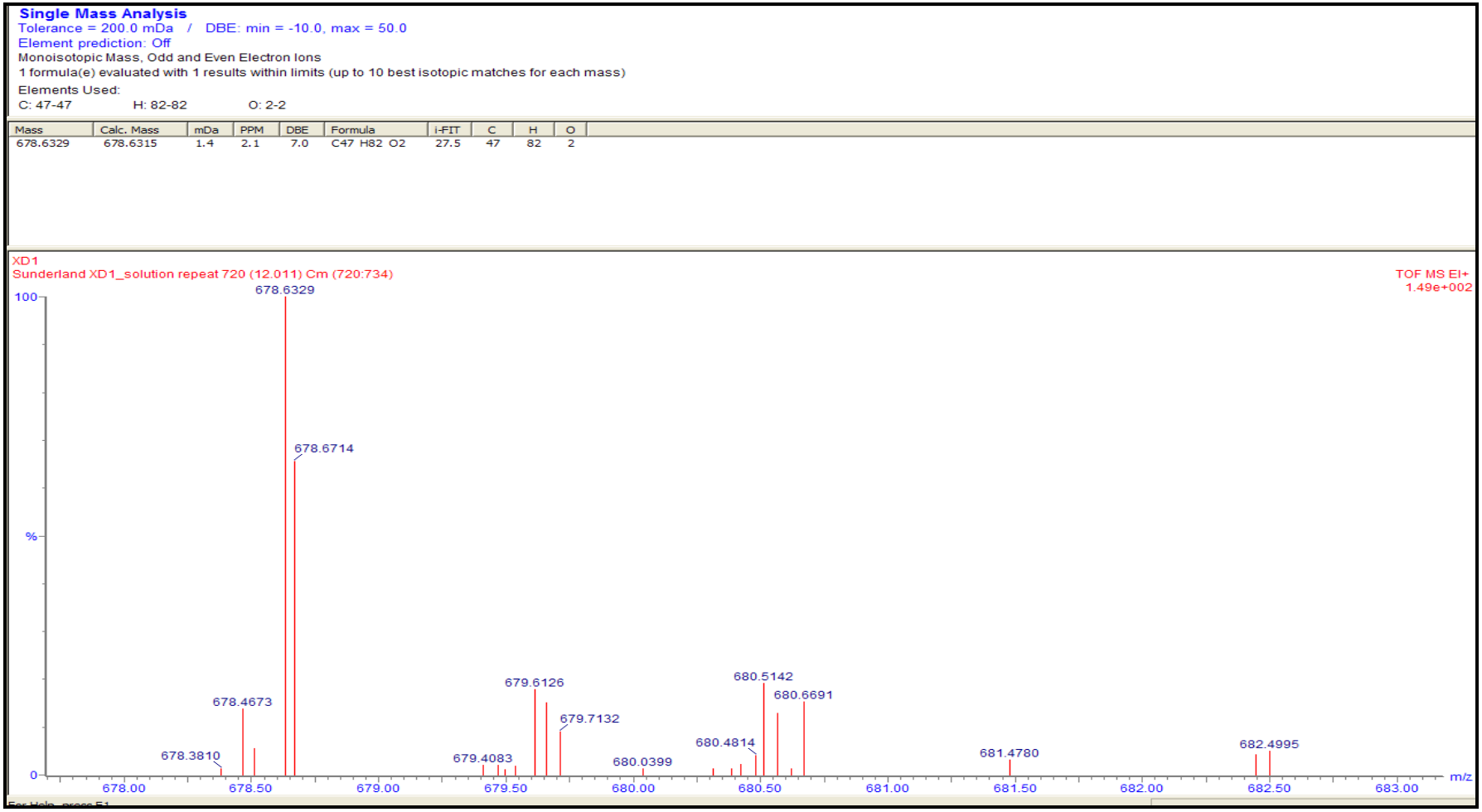


Fig. 7.11a ii) Mass spectrum of XD1 compound 2

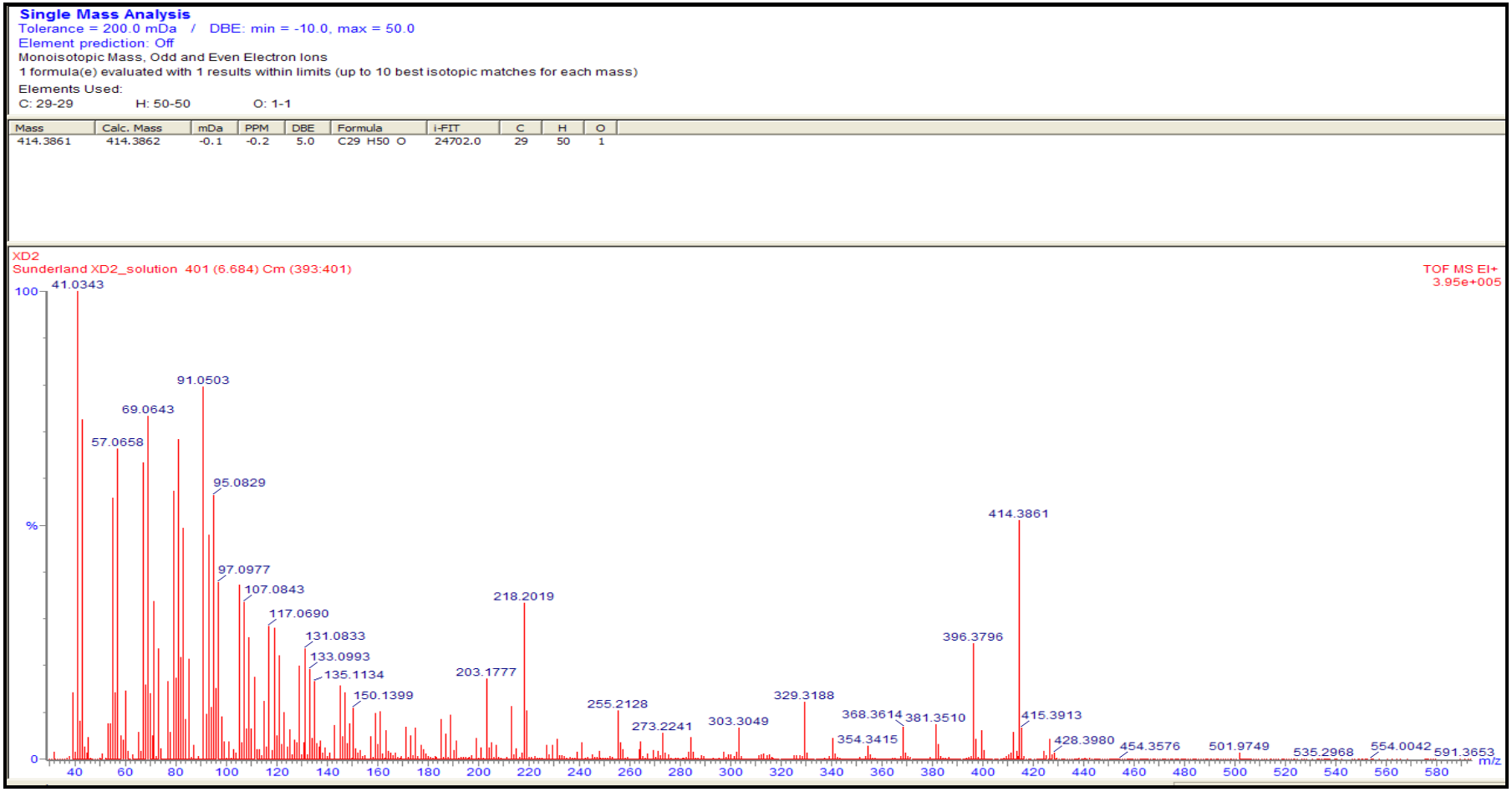


Fig.7.11b i) Mass spectrum of XD2 compound 1

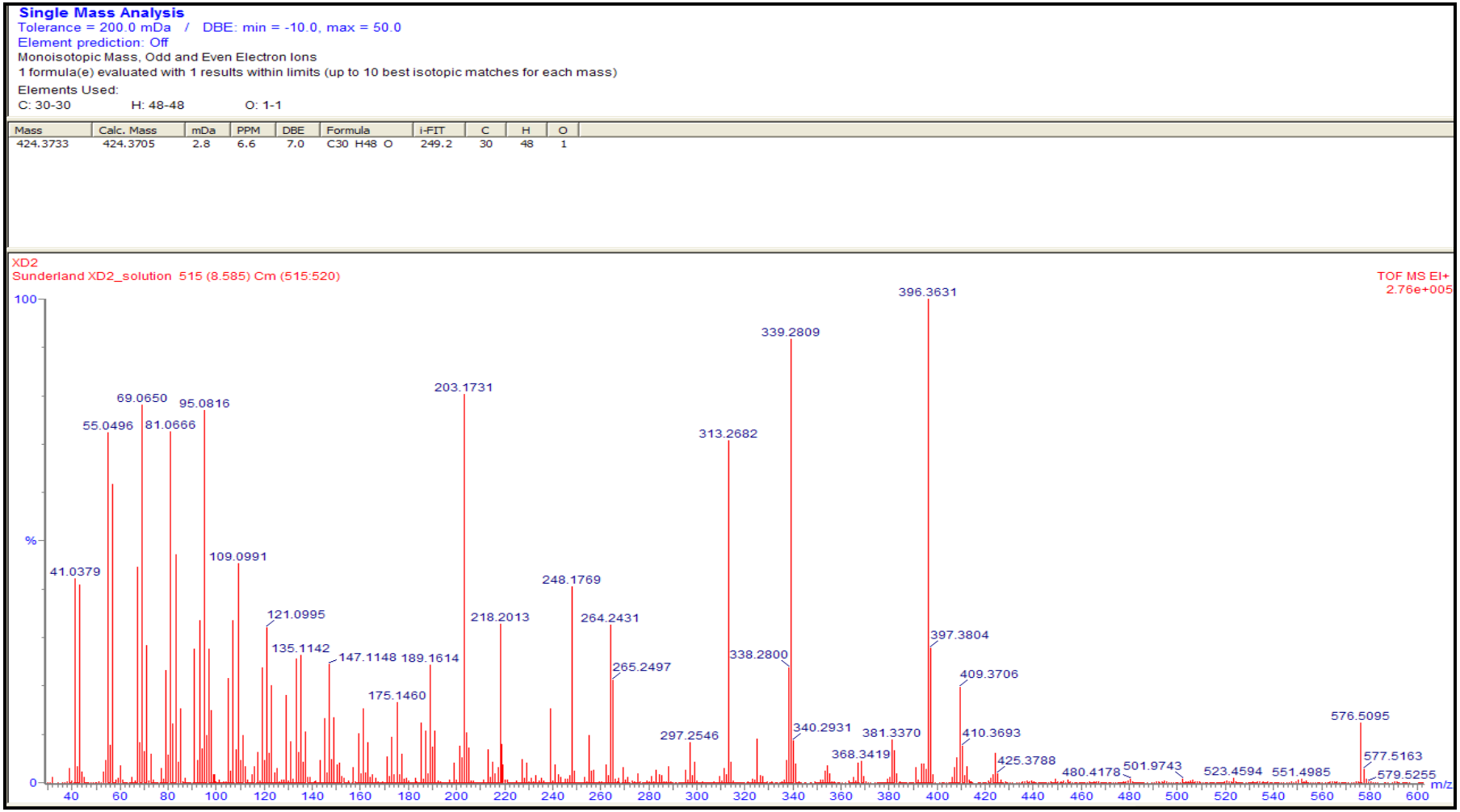


Fig. 7.11b ii) Mass spectrum of XD2 compound 2

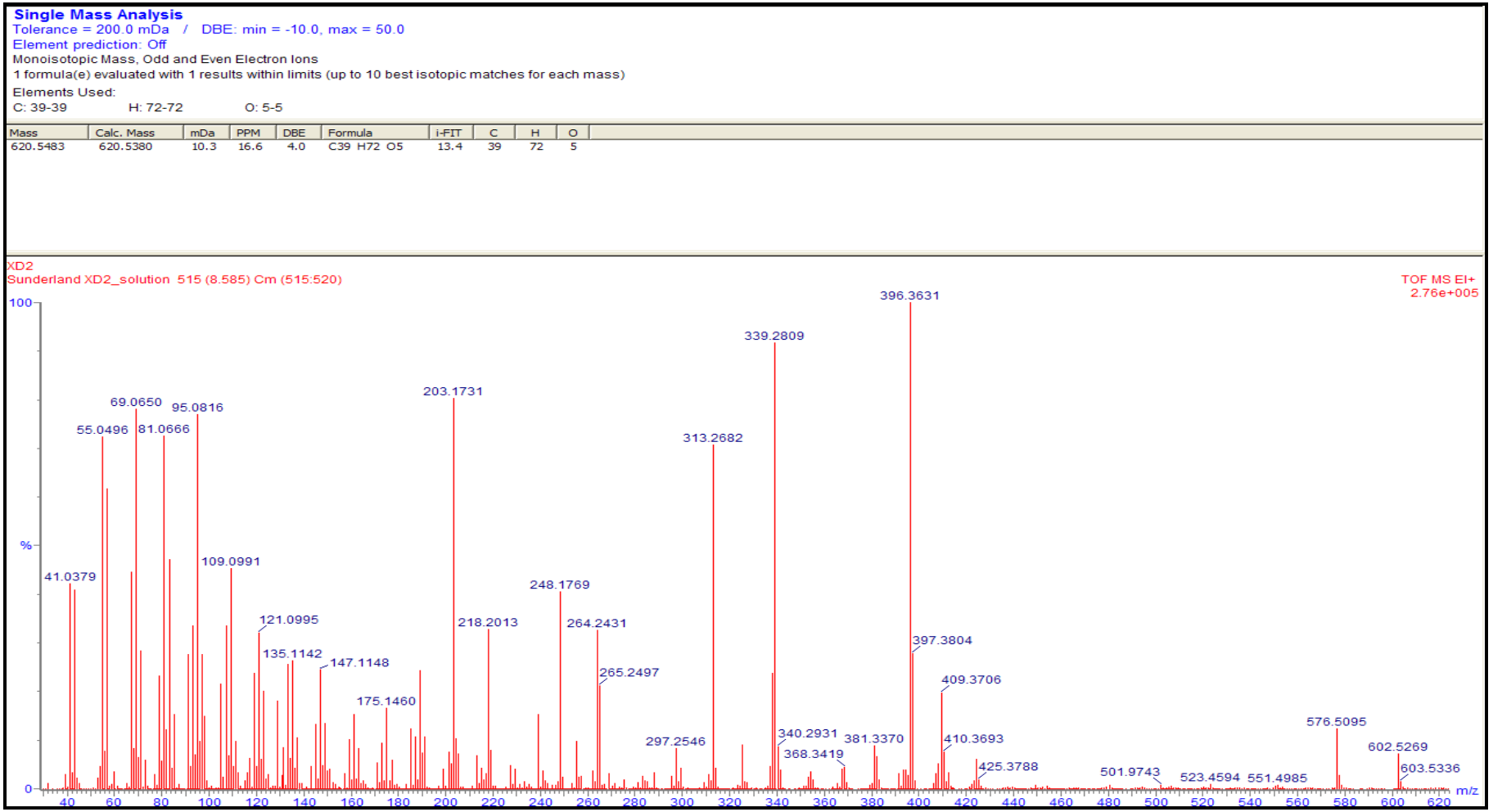


Fig. 7.11b iii) Mass spectrum of XD2 compound 3

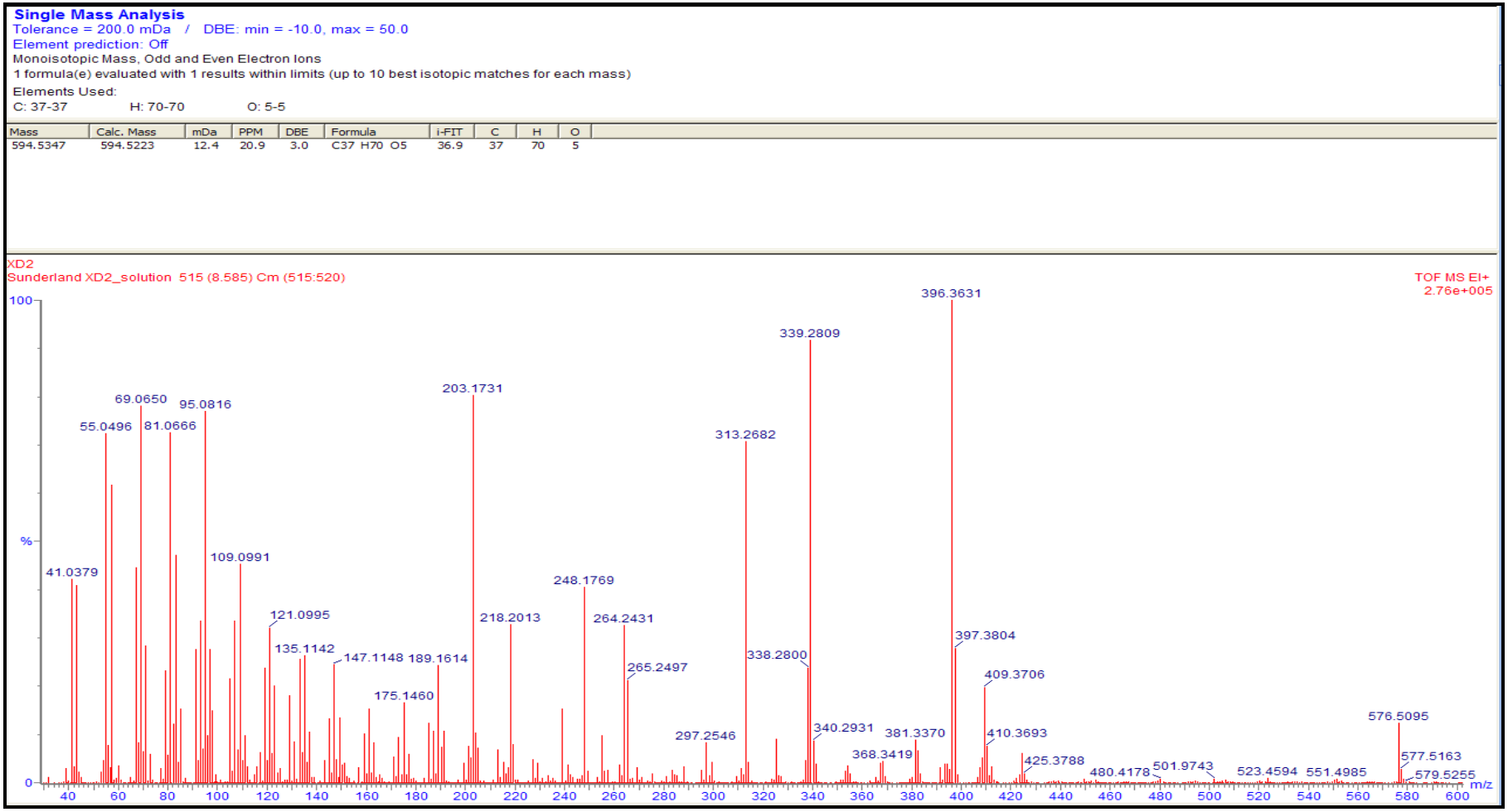


Fig.7.11b iv) Mass spectrum of XD2 compound 4

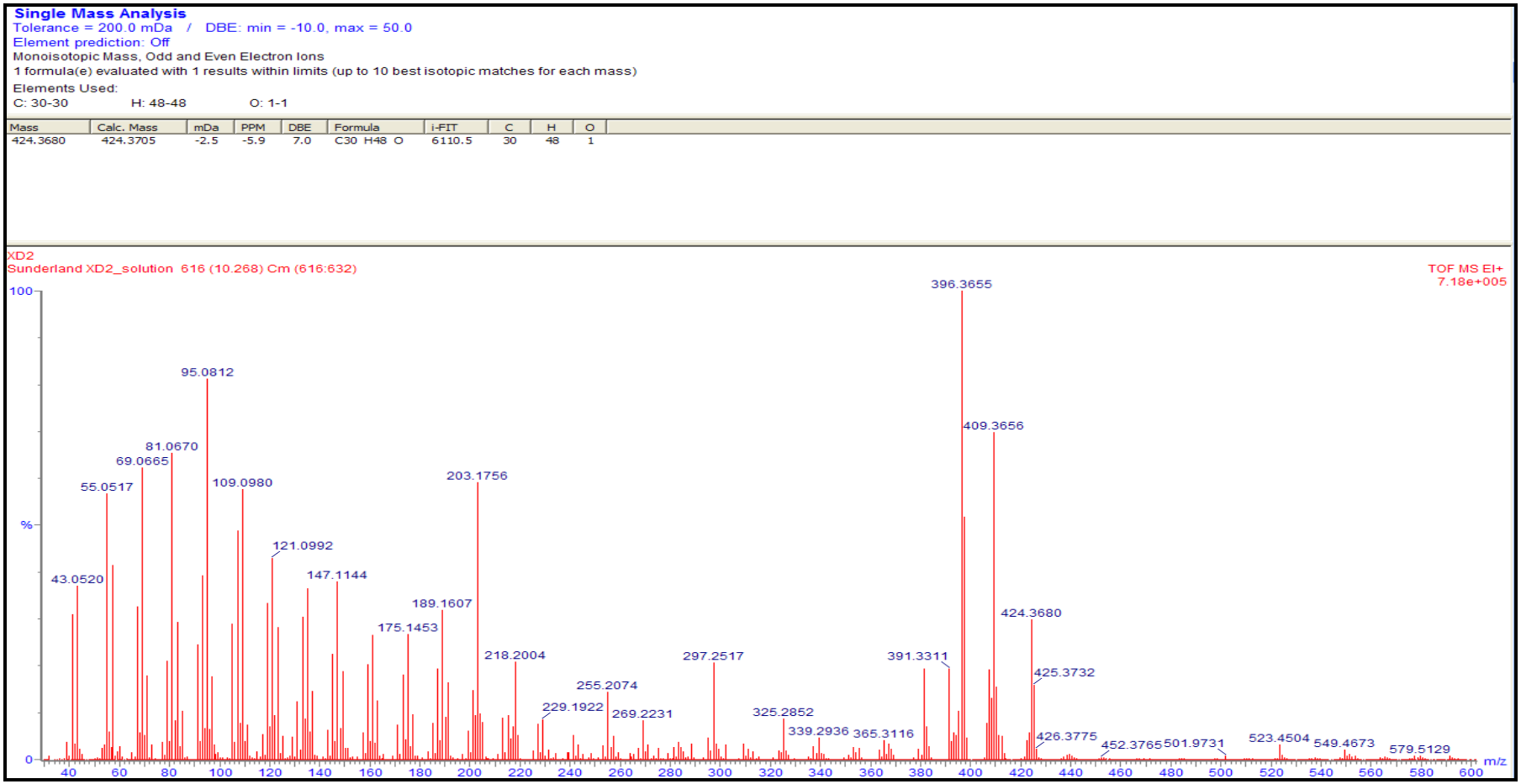


Fig.7.11b v) Mass spectrum of XD2 compound 5

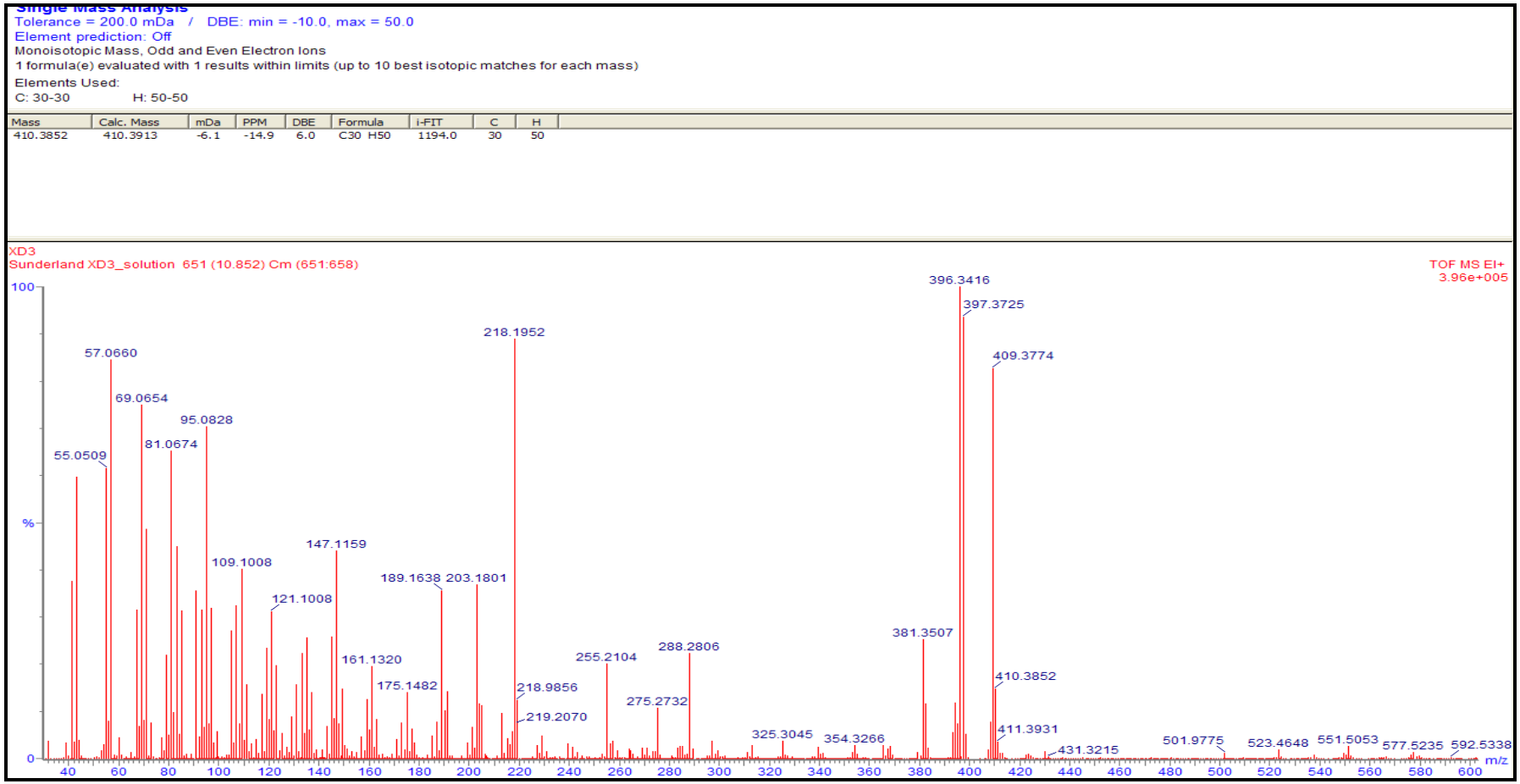


Fig.7.11c i) Mass spectrum of XD3 compound 1

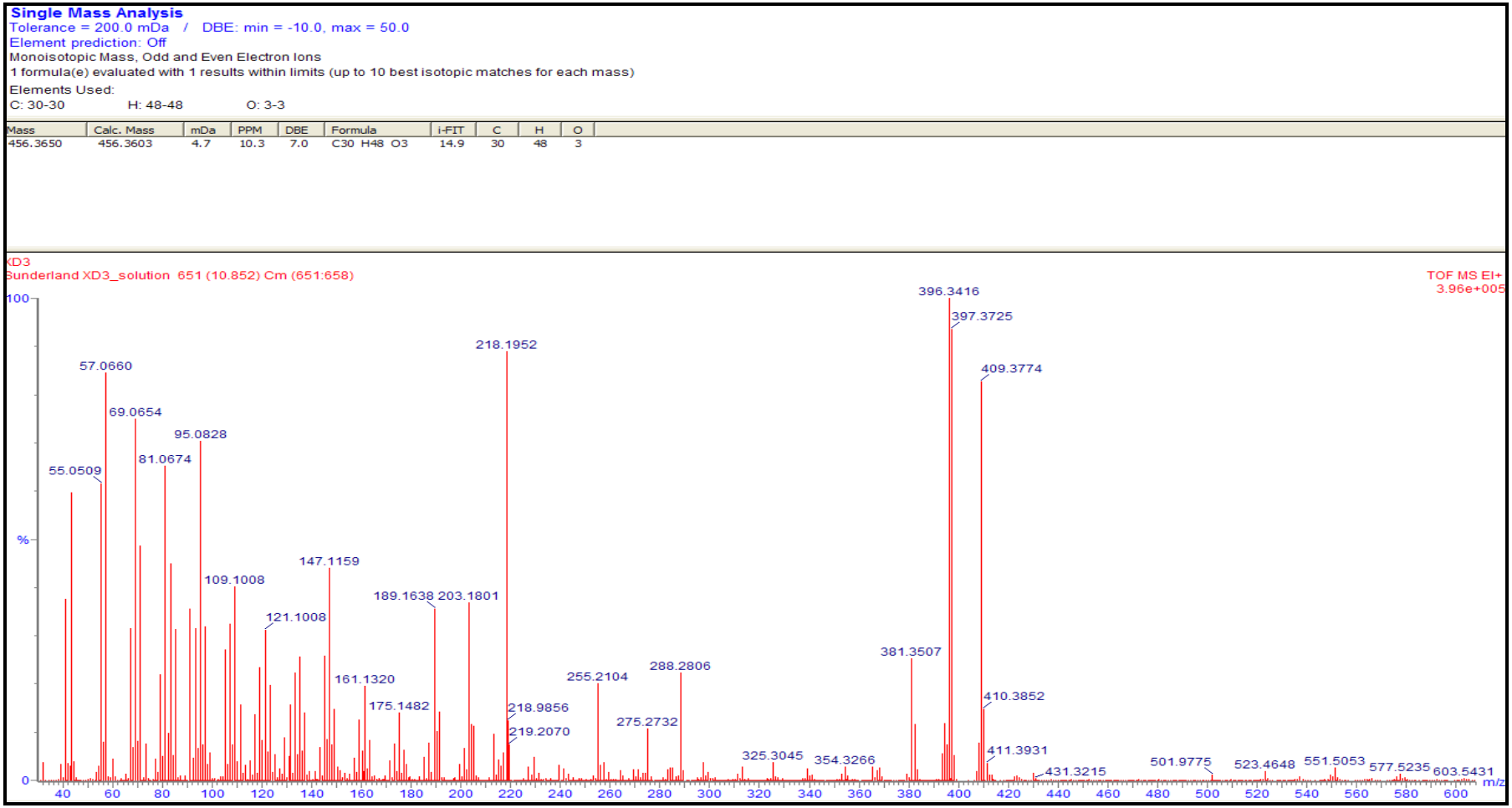


Fig. 7.11c ii) Mass spectrum of XD3 compound 2

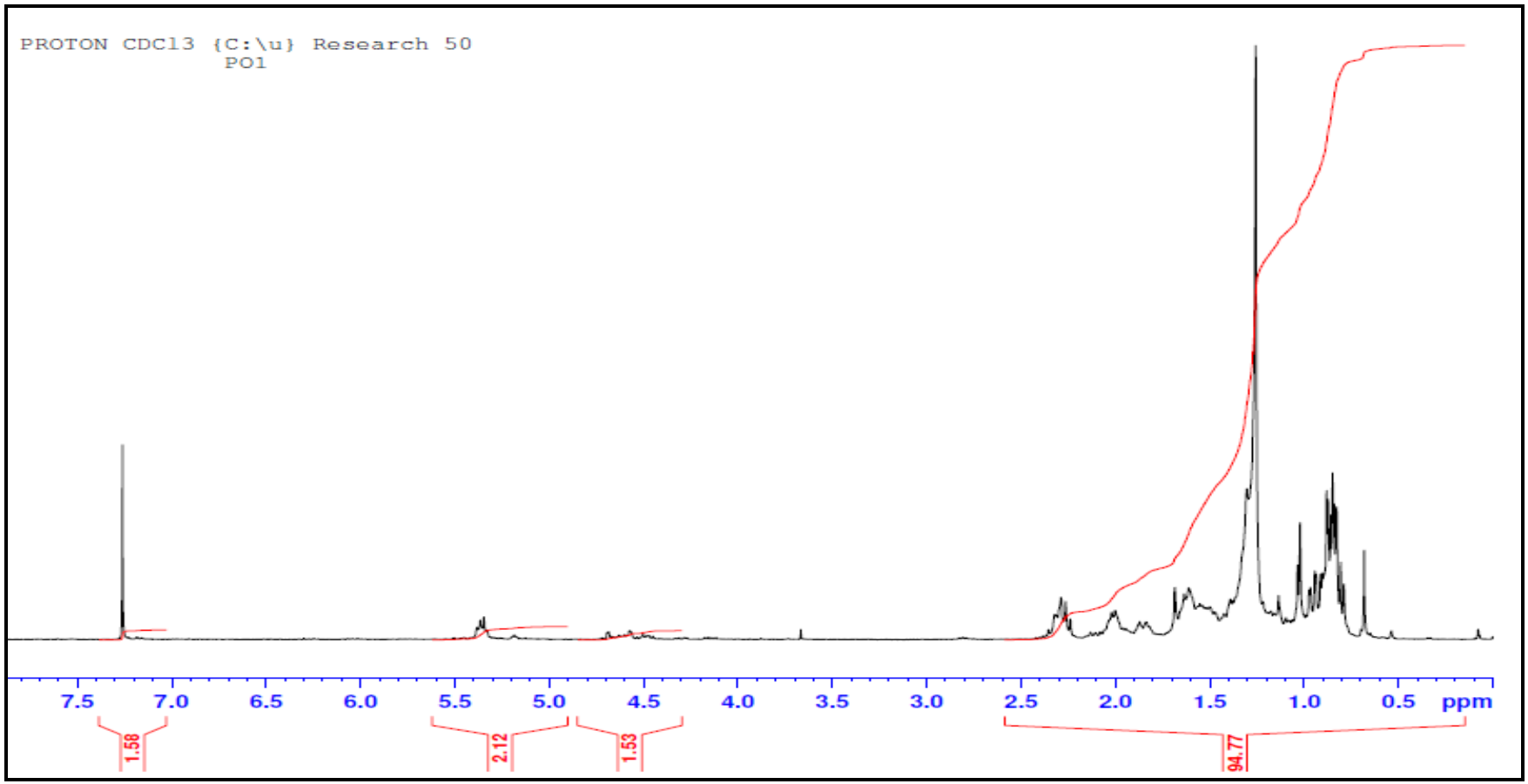


Fig.7. 11d i) Proton NMR spectrum (300 MHz) of TLC spot 1 – XD1

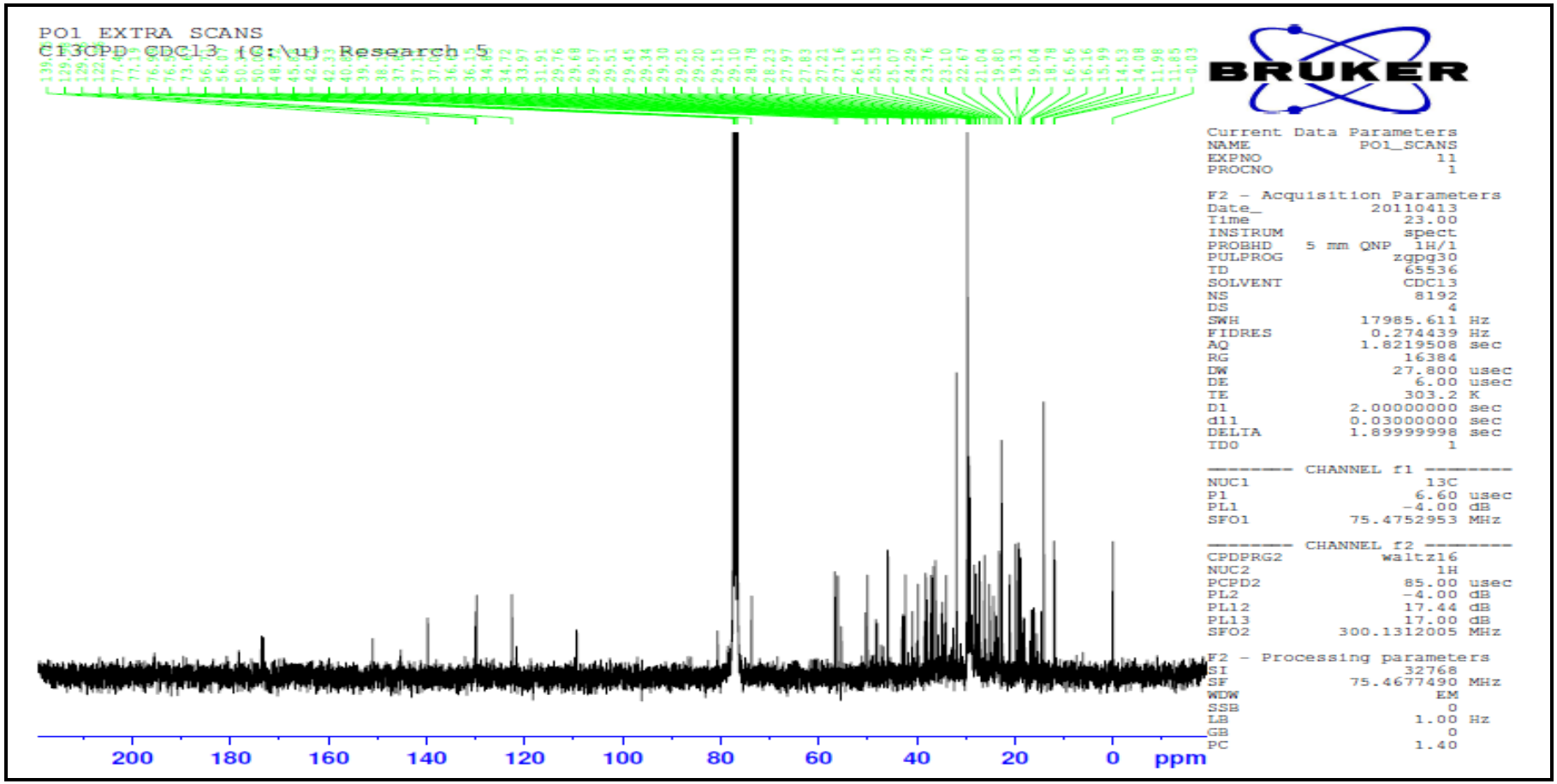


Fig.7.11 d ii) ^{13}C NMR (75 MHz) spectrum of TLC spot 1 (XD 1)

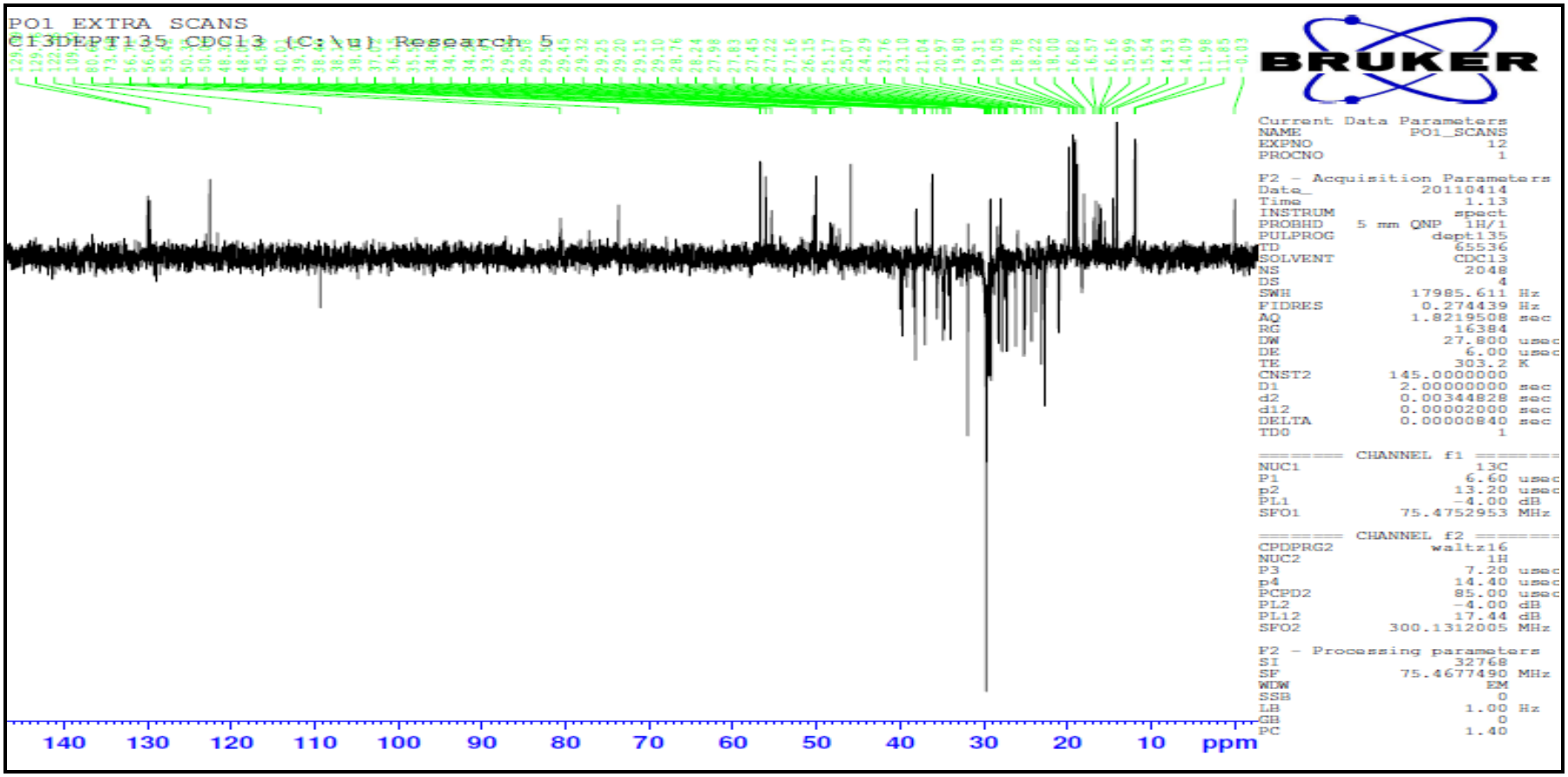


Fig. 7.11d iii) ^{13}C DEPT NMR (75 MHz) spectrum of XD1

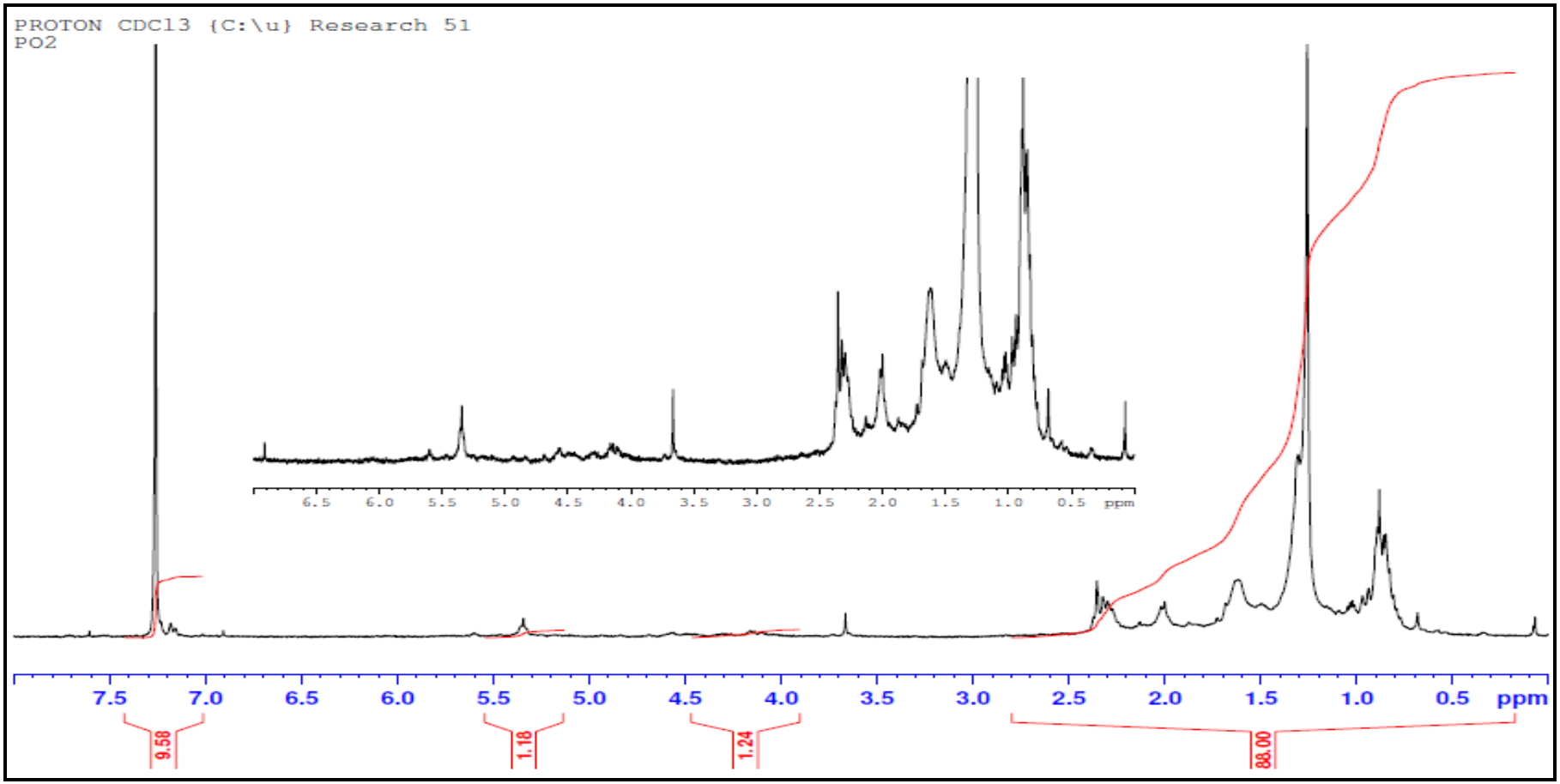


Fig. 7.11d iv) Proton NMR spectrum (300 MHz) of TLC spot 2 – XD2

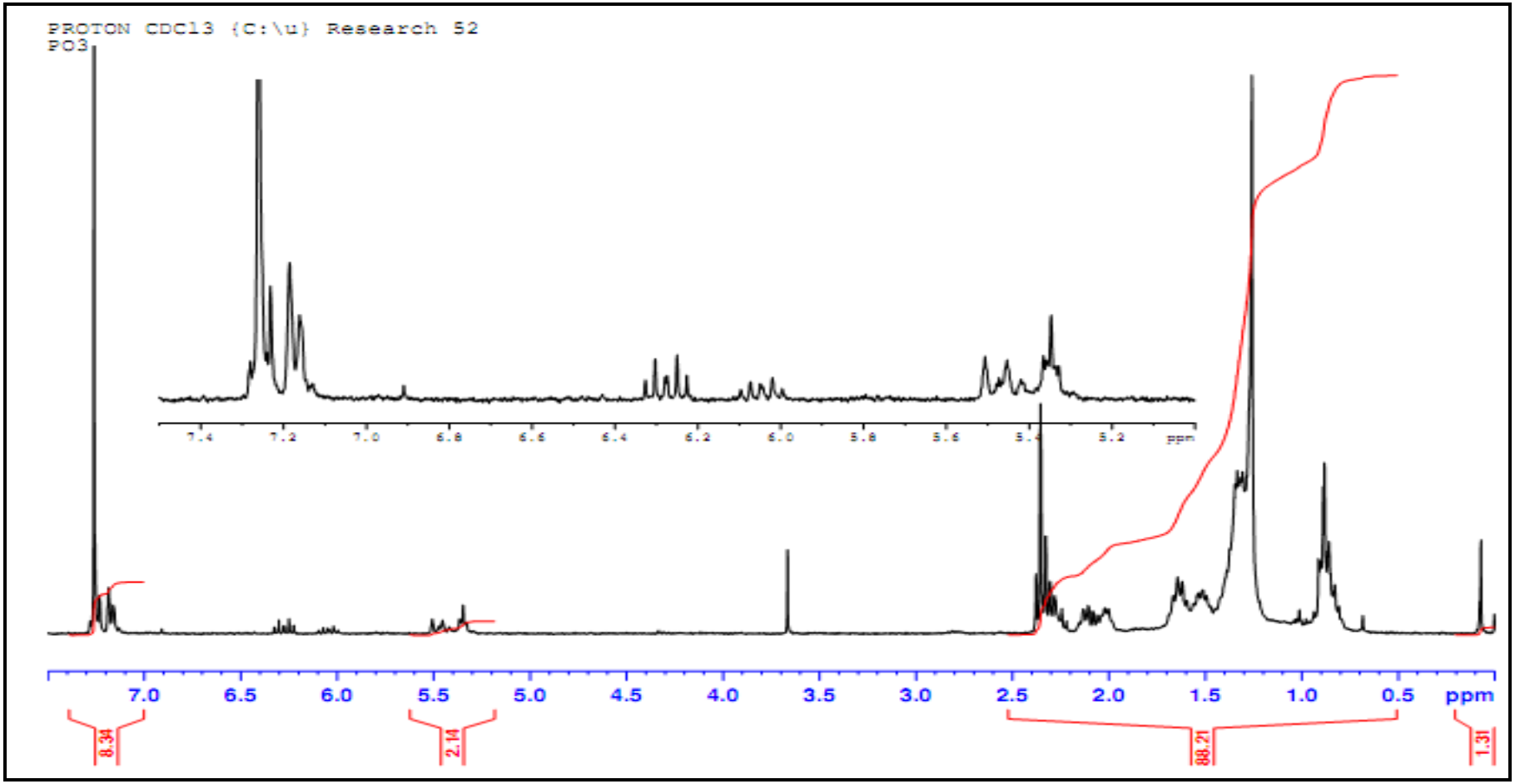


Fig. 7.11d v) Proton NMR (300 MHz) spectrum of TLC spot 3 (XD 3)

7.4.5.4 Gas chromatographic analysis of crude hexane, dichloromethane and column fractions of *X. americana*

GC analysis was considered as an alternative method since resolution of the active fractions by LC was difficult. Acetylenic fatty acids and oleanolic acid saponin have been reported in the chloroform extract of *X. americana* root by other investigators (Fatope *et al*., 2000; D'Agostino *et al*., 1994). Crude samples were analysed by GC and the retention times of available standard compounds, stigmasterol, β-sitosterol, cholesterol and stearic acid were compared to the retention times of the plant extracts. Crude hexane and DCM extracts (500 mg each) were individually subjected to dry column flash chromatography using n-hexane/ethylacetate (90/10v/v) and hexane/DCM/ethanol (25/25/50 v/v/v) to obtain two fractions which would be enriched in fatty acids and phytosterols respectively (Verleyen *et al*., 2002).

The results show the individual retention times of the standards and extracts (Figure 7.12, Appendix F). The standard compounds were tested individually and as a mixture. Following the experiment, extracts were also tested alone and spiked with 10 µL volumes of the reference compounds. The initial temperature programme was for 51 minutes (initial temperature, 100 ˚C (hold 1 min.), ramped at 5 °C/min to 300 °C (hold for 10 min) which was shortened to 31 minutes in the final experiments (Figure 7.12a). The retention times of stearic acid, cholesterol, stigmasterol and sitosterol are as shown in Figure 7.12a. The crude extract fractions showed intense peaks (retention time, 1.546 -7.371) which eluted within the temperature range 100 – 140 °C and could be attributed to the fatty acid and low molecular weight components. The standard compounds (stearic acid, cholesterol, stigmasterol and sitosterol) eluted at 4.568, 15.960, 17.732 and 18.434 minutes while crude XH and XD showed less intense peaks at 17.753 (XH) and 17.074, 17.788, 19.445 for XD (Figure 7.12bi and ii). The retention time of the most intense peaks at 17.184, 17.753 (XH) and 17.299, 17.788 (XD) were close to the retention time for an unknown peak and stigmasterol (17.227, 17.732 min) in standard mixture as shown in Figure 7.12aii.

The intensity of the peaks observed with 10 mg/mL solutions of the crude extracts show that the phytosterol content is small compared to the low

molecular weight components while the enriched fractions show intense peaks at 17.922 and 18.055 for 10 mg/mL XH2 and XD2 respectively (Figure 7.12b.v and vi). The enriched fractions were each spiked with a 10 µL volume of the standard mixture and an increase in the intensity of peak at 17.1 min was observed however the intensity of the low molecular weight enriched fractions decreased when compared to the crude fractions (Figure 7.12bi- iv). The order of elution of the phytosterol standards followed a similar pattern to that obtained by INA using Rtx-5 column (The Institute for Nutraceutical Advancement; http://www.restekcorp.com). The hyphenated GC/MS was unavailable to continue further work so subsequent arrangements were made to analyse the samples by SFC-UV-ELSD in collaboration with Waters Corporation. While this would not give such high efficiency, volatility would not be an issue, as with FID all eluting compounds would be detected with ELSD and there would be a possibility of subsequently transferring methods to SFC-MS.

a)

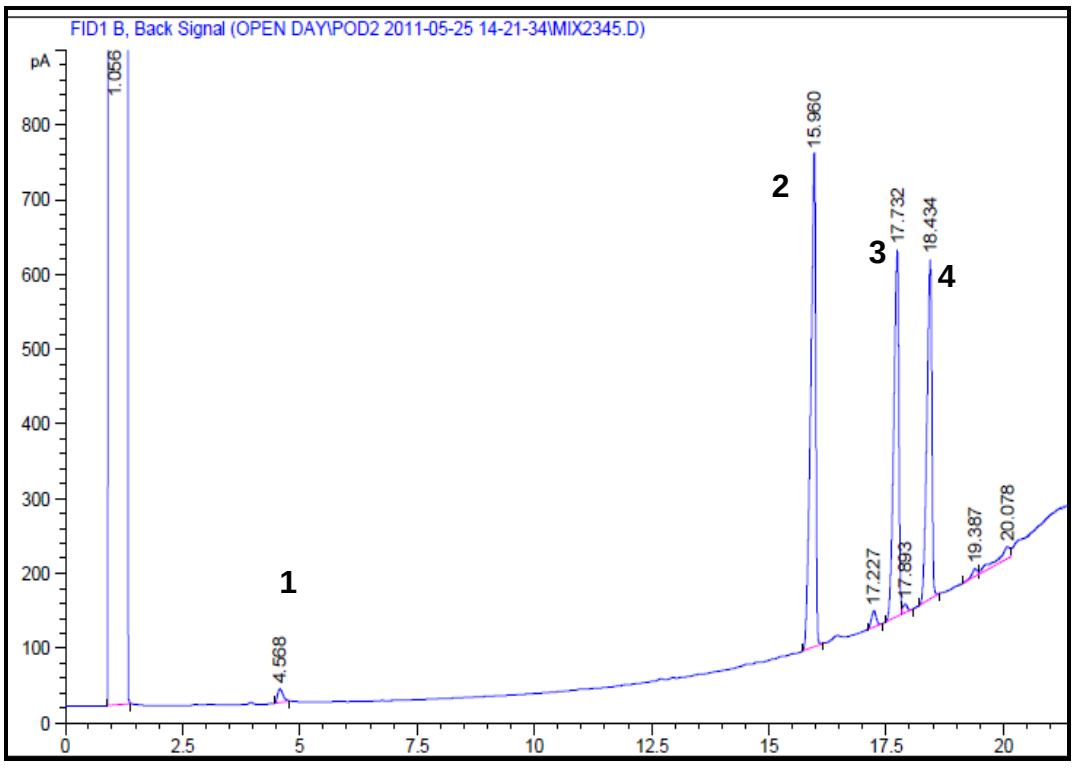
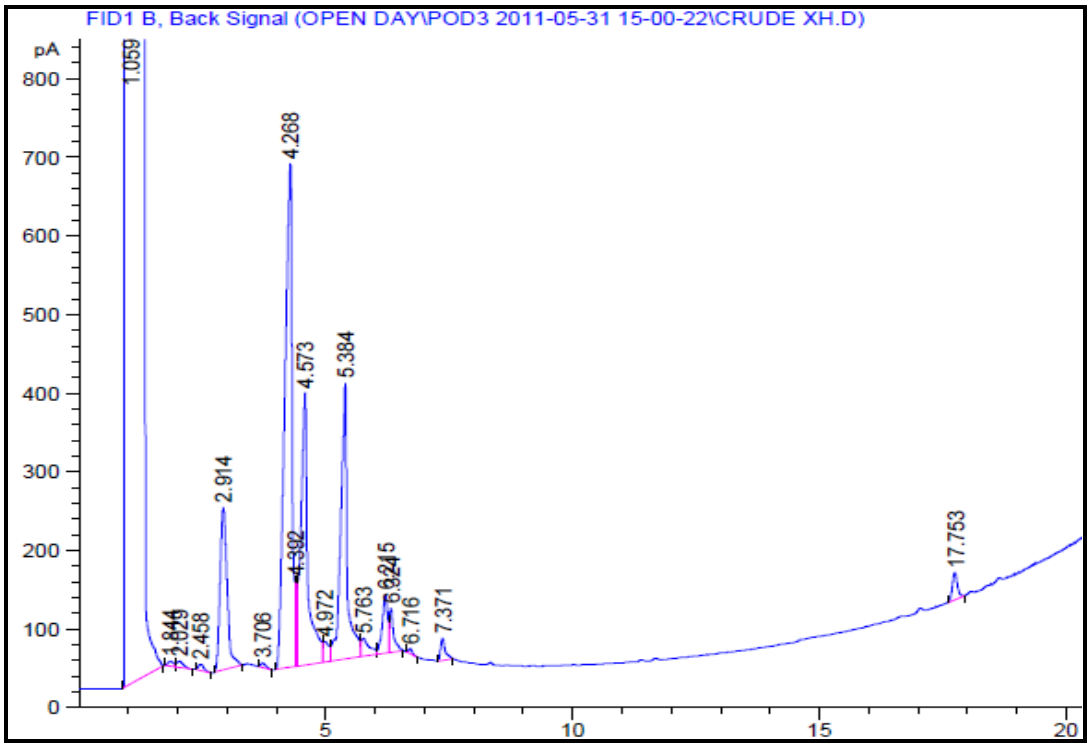


Fig. 7.12.a GC analysis of a mix of **1** - stearic acid, **2** - cholesterol, **3** - stigmasterol, **4** – sitosterol. Temperature programme: Initial 200 °C (hold for 1min), rate 5 °C/min to 300 °C final temperature (hold for 10 mins).

i.



ii.

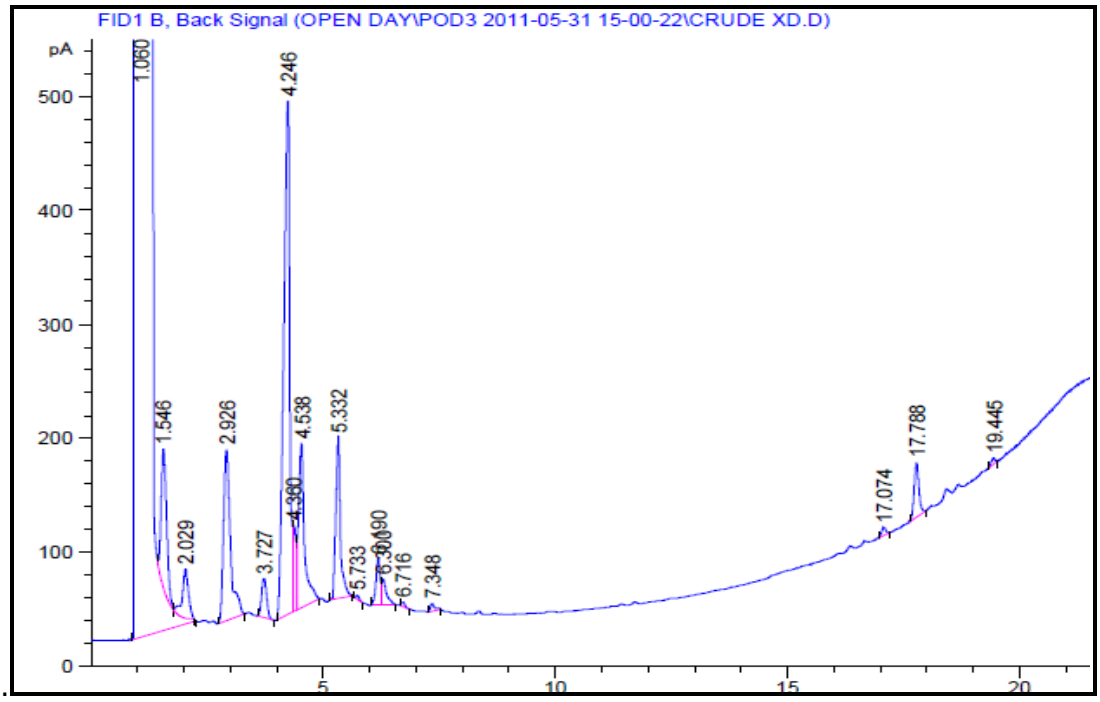
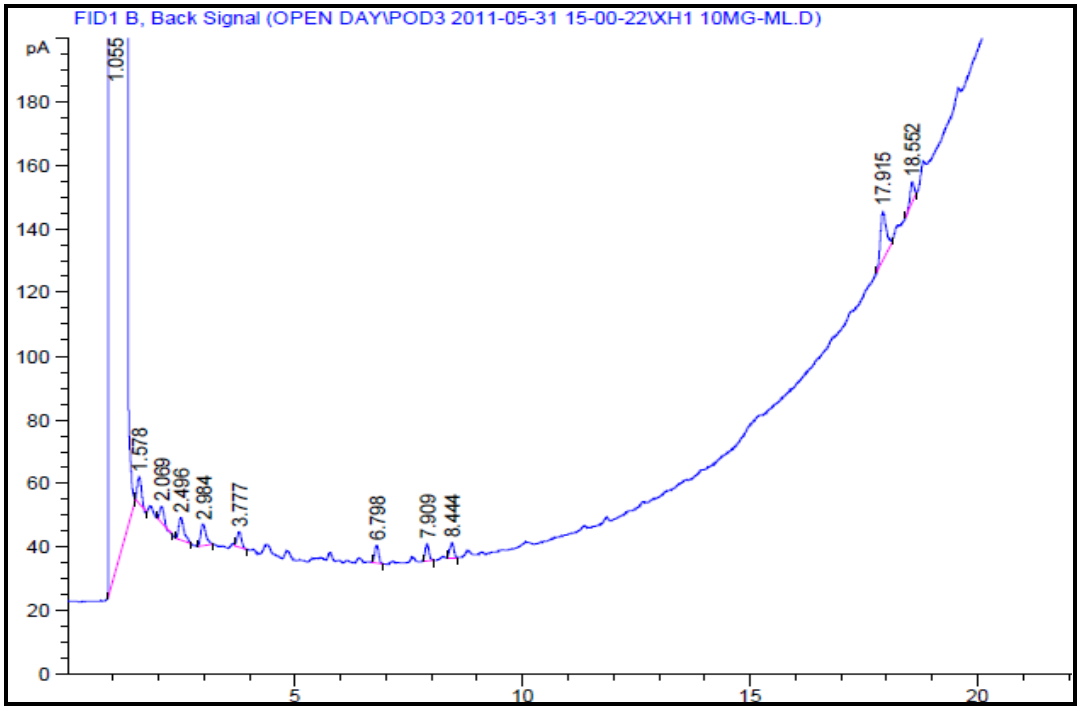


Fig.7.12bi and ii) GC chromatographic profiles for crude XH (i), XD (ii)

iii.



iv.

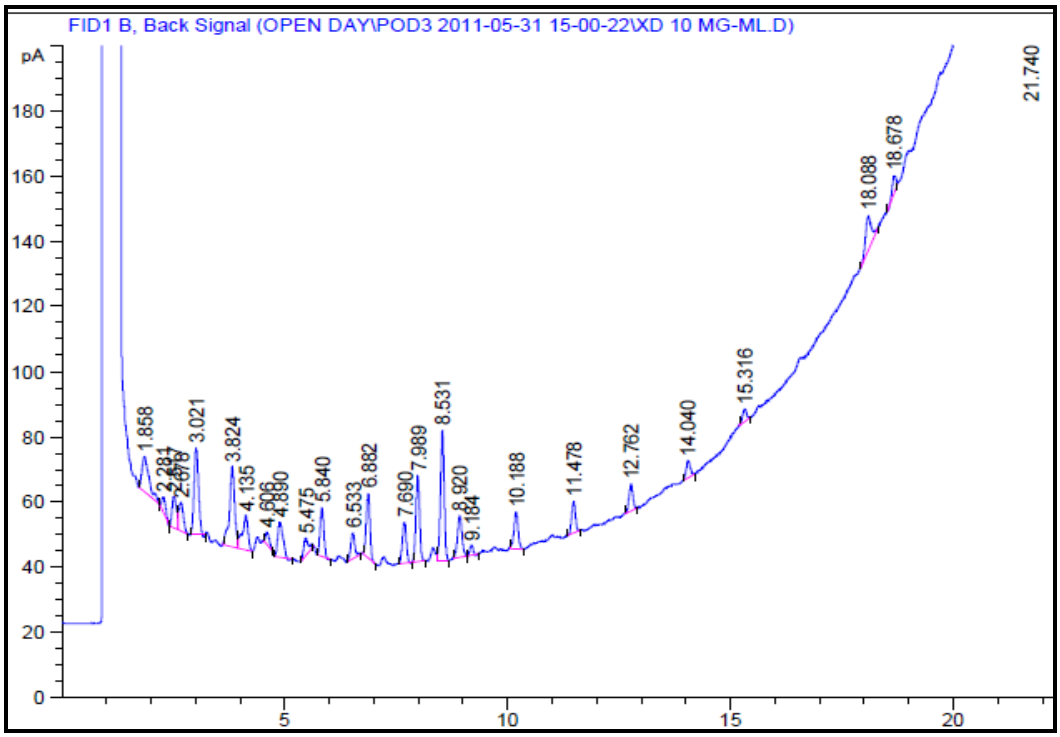
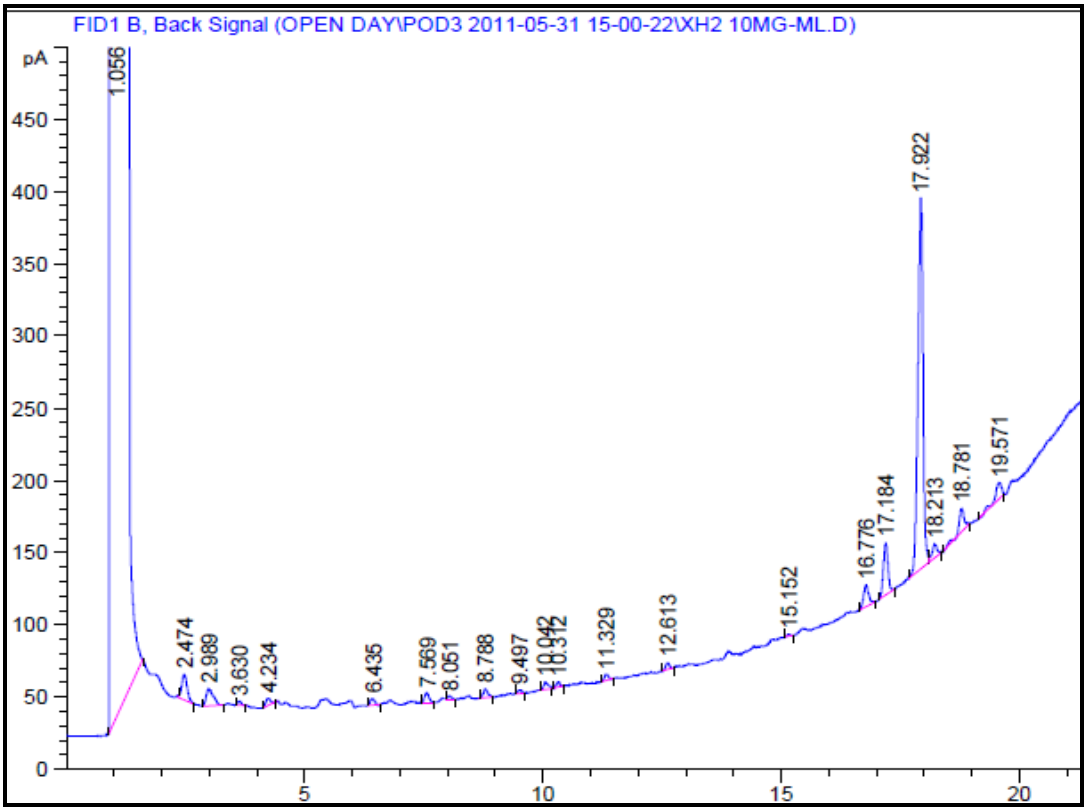


Fig.7.12b.iii and iv) GC chromatographic profiles for enriched fractions XH1 (iii), XD1(iv)

v.



vi.

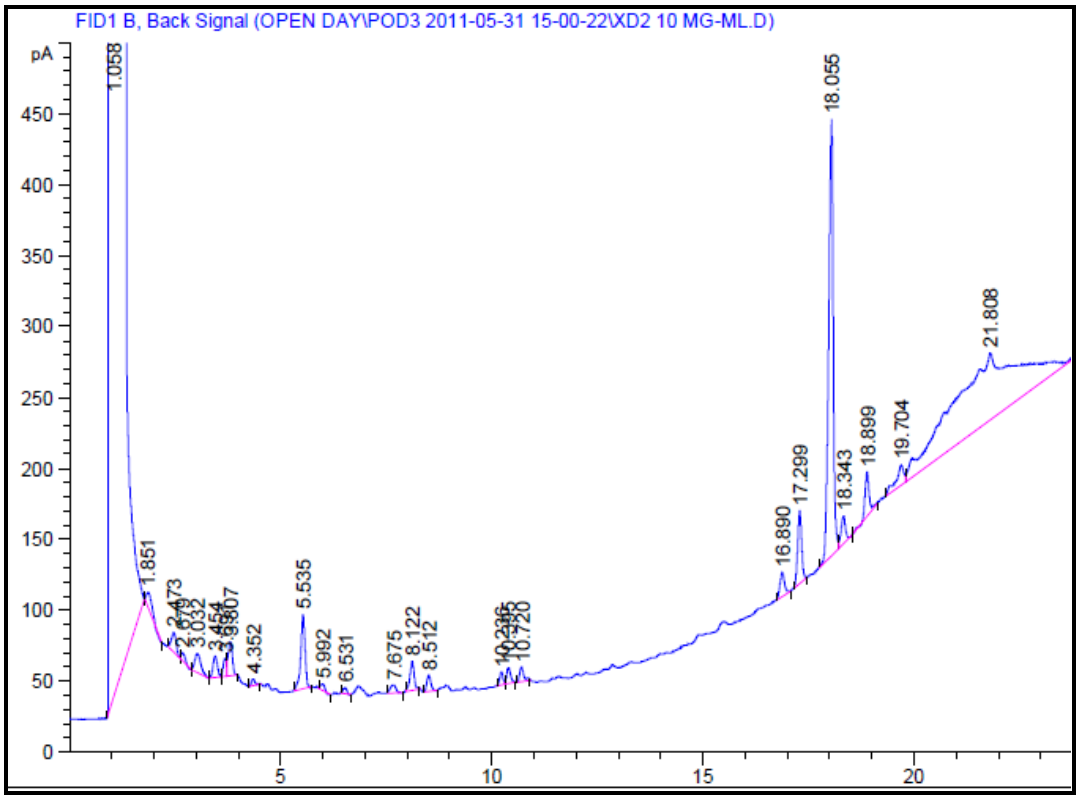
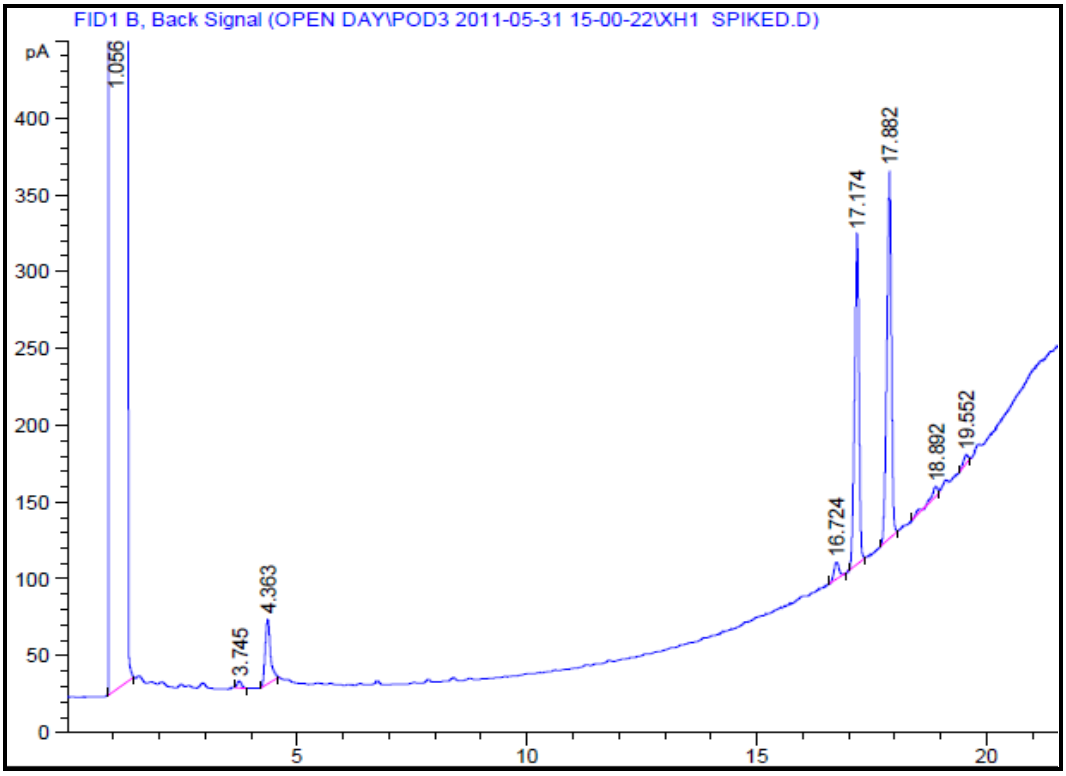


Fig.7.12b. v and iv) GC chromatographic profiles for fractions XH2 (v) and XD2 (vi)

vii.



viii.

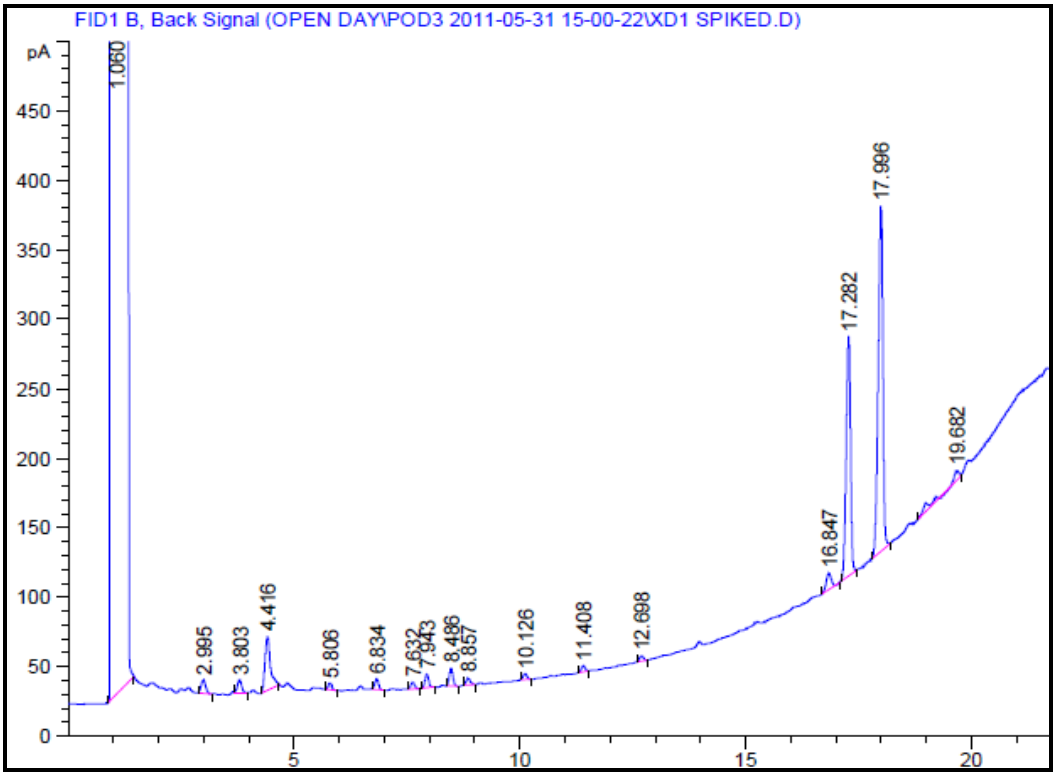
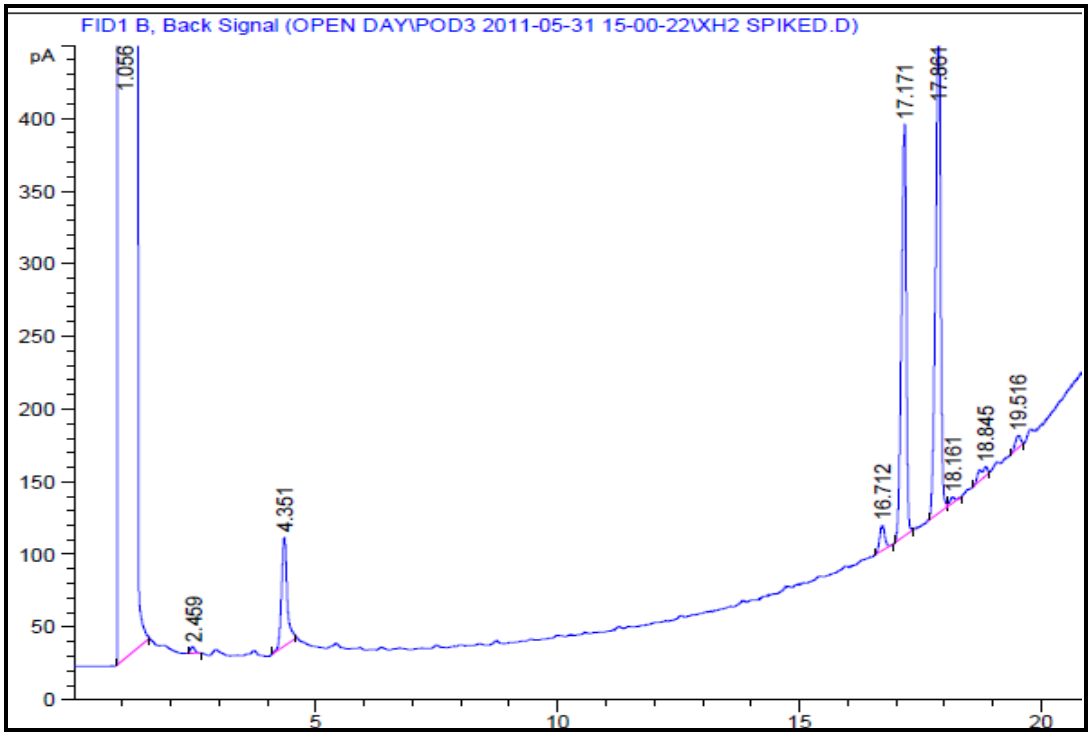


Fig.7.12b. vii and viii) GC chromatographic profiles for spiked fractions XH1 (vii), XD1 (viii),

ix.



x.

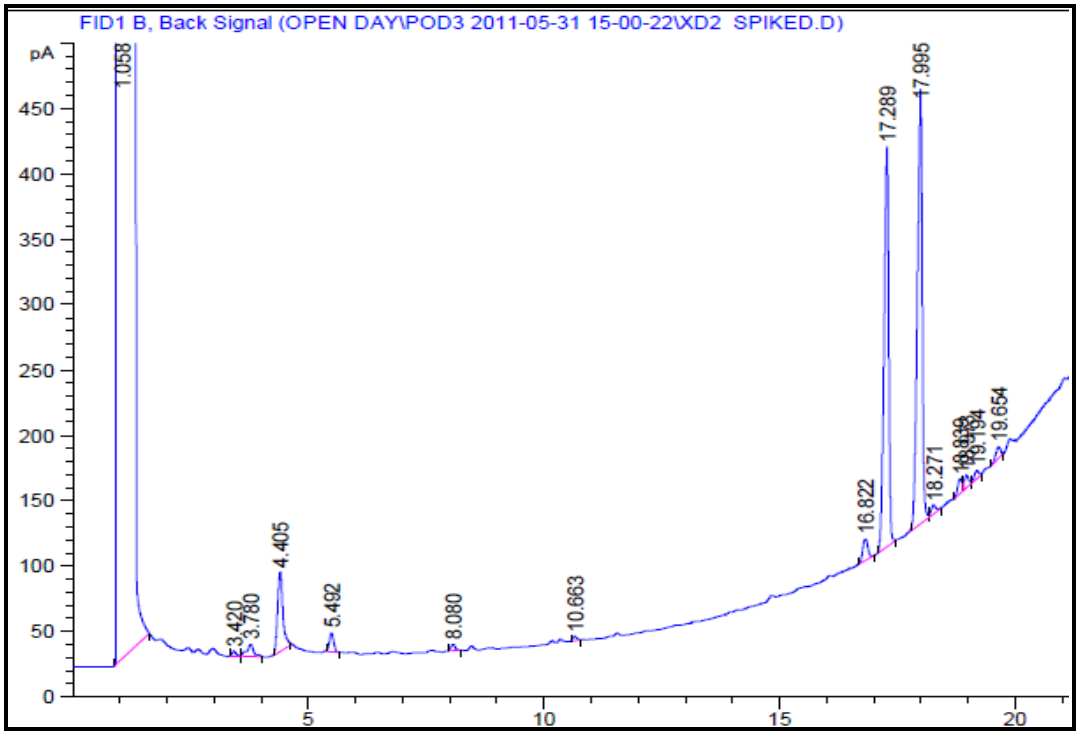


Fig.7.12b ix, x) GC chromatographic profiles for spiked fractions XH2 (ix), XD2 (x)

While the GC work revealed that the crude extract contained some quite volatile components it was not possible to identify these because of the non-availability

of GC-MS at the time. There was clearly very little in the way of phytosterols present. While it might have been possible to study some of the more polar constituents in the materials by using more polar columns, there was insufficient time to carry out the method development needed or to wait for the repair of the GC-MS.

7.4.5.5 LC analysis of XAH, XAD, XAT, XD3 and XD4 (active sub-fractions) using refractive index detector and photodiode array detector.

Following the series of analytical approaches described in earlier sections, it seemed that the fractions of the non-polar extracts of *X. americana* responsible for anti-TB activity contained mixtures of hydrophobic compounds, probably diacylglycerols, dehydrated sterols, phytosterol conjugates or pentacyclic triterpenoids which were not readily resolvable into single compounds by TLC using UV detection.

On further exploration of the literature on analysis of phytosterols, it was then necessary to consider NPLC of dichloromethane and XD3/4 extracts (TB active fractions) as well as RP LC of the total water extract which was used in the initial testing TB with RI or ELSD detection. Discussions with industry experts led to suggestions that NPLC-ELSD or SFC- FID-MS would give much better sensitivity compared to LC-UV methods previously used (Personal communication with G. Cox, August, 2011). The chromatographic profiles of the TB-active fractions and standard phytosterols were carried out using a universal detector prior to analysis by SFC-FID-MS.

The extracts showed the presence of compounds with poor UV absorbing properties and this was corroborated by the presence of NMR signals within the aliphatic regions from earlier experiments. The first experiment was conducted using silica column (ACE 5 SILICA, 250 x 7.75 mm) on Agilent 1290 Infinity series with refractive index andvariable wavelength detectors (G1362A and G1314E respectively). The chromatographic profiles and conditions are shown in Figure 7.13a. A major peak at 8.295 minutes was observed with RID for the crude samples (XAH, XAD). IPA-hexane (2:98, v/v) mobile system was used to analyse samples XAT, XAH and XAD on ACE 5 CN column, 150 x 4.6 mm on the same system in order to compare the profiles.

The results for XAT are presented in Figure 7.13a iv and v. The peaks observed with UV eluted close to the solvent peak while an additional peak at 18.5 minutes was observed with RID suggesting the presence of a carbohydrate molecule in the total water extract. Further experiments using NP on hypercarb showed that XAT contained some peaks with the same retention time as XAD suggesting that the total water extract contained peaks that were observed in the TB-active dichloromethane fraction (Figure 7.13 v and vi).

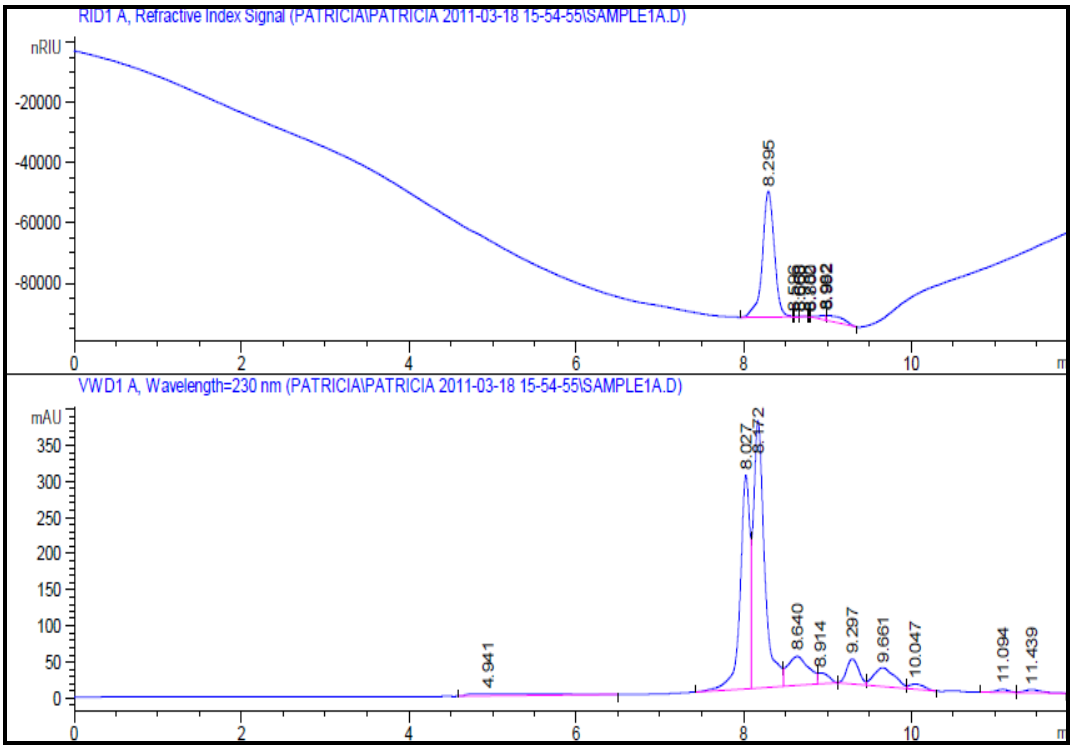
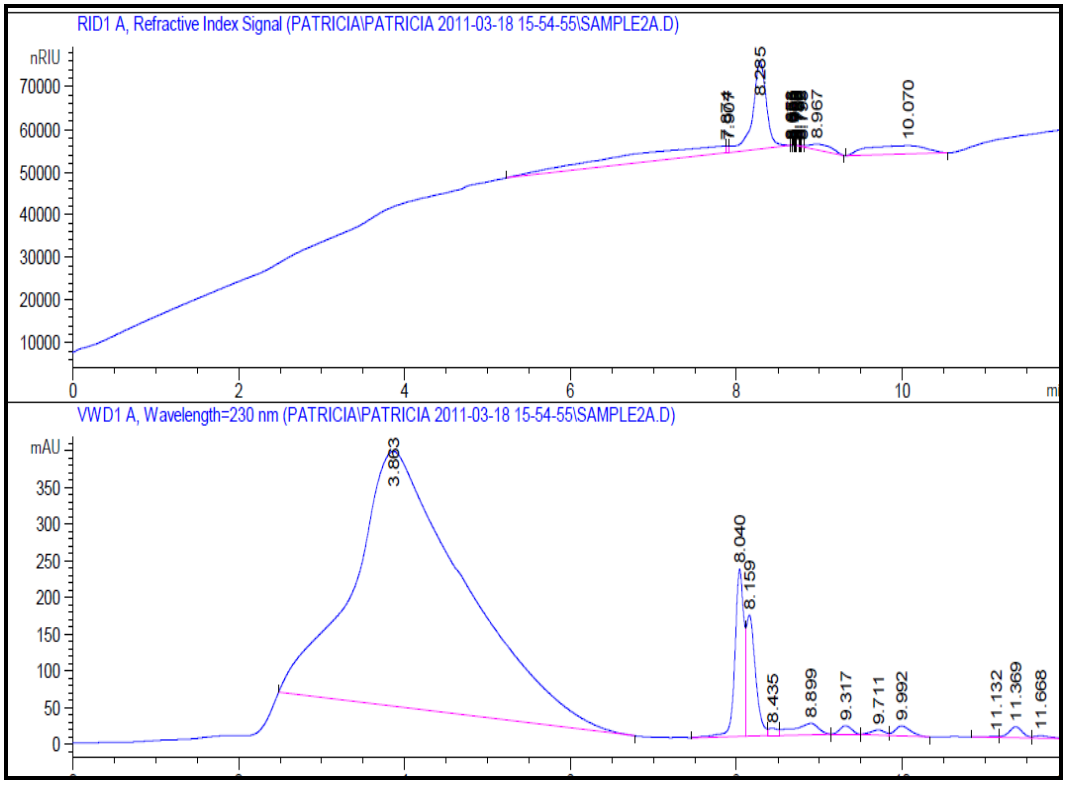


Fig.7.13a i) Chromatographic profiles of samples analysed on Agilent 1290 Infinity system with RID detector and on ACE 5 SILICA column at 230 nm. Sample: XH (1 mg/mL), Conditions: column length - ACE 5 SILICA, 250 x 7.75 mm i.d; Mobile phase - methanol: DCM (2:98, v/v); Flow rate - 1mL/min; column temperature - 21.06 °C; VWD - 230 nm; RID - 40 °C; Run-time: 60 min

ii.



iii.

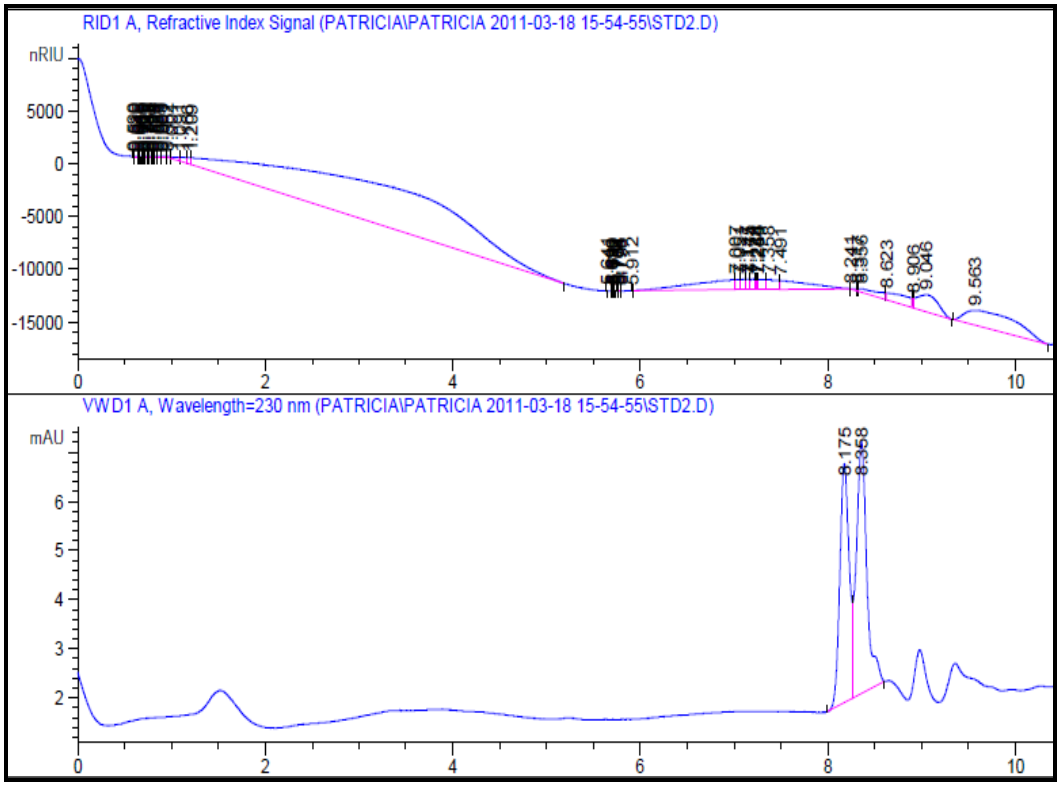


Fig.7.13a ii and iii) Sample containing 1 mg/mL XD (ii) sample containing 1 mg/mL sitosterol (iii) conditions: same as Fig.7.13a i.

iv .

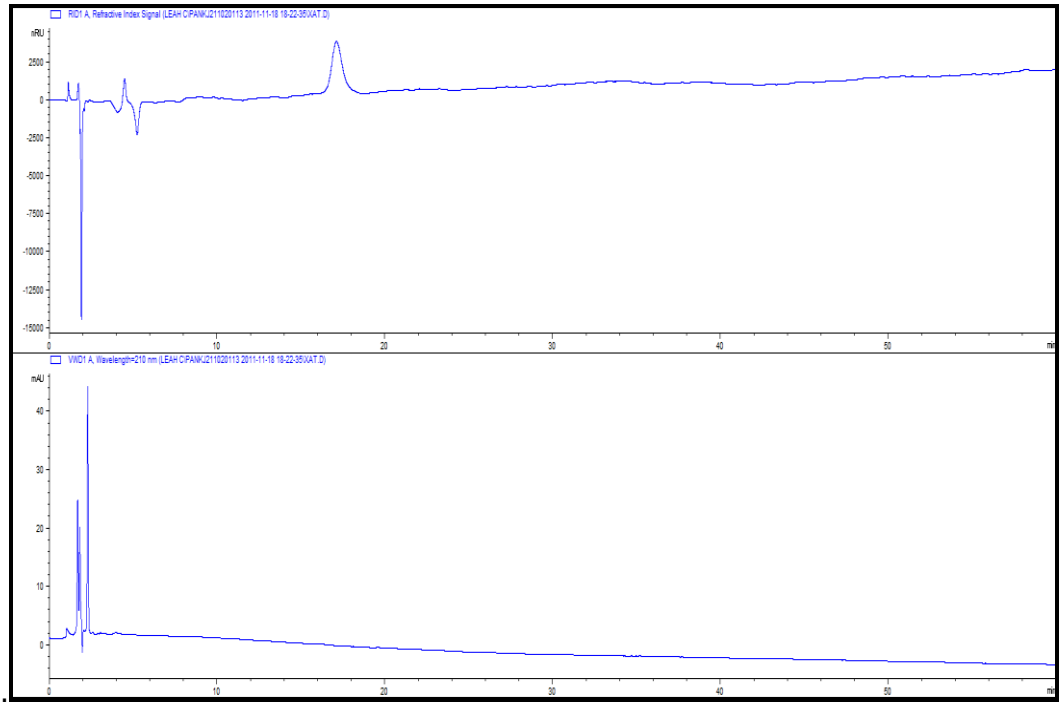


Fig.7.13a iv) Chromatographic profiles of XAT (1 mg/mL) on ACE 5 SILICA column at 210 nm. Conditions: same as above

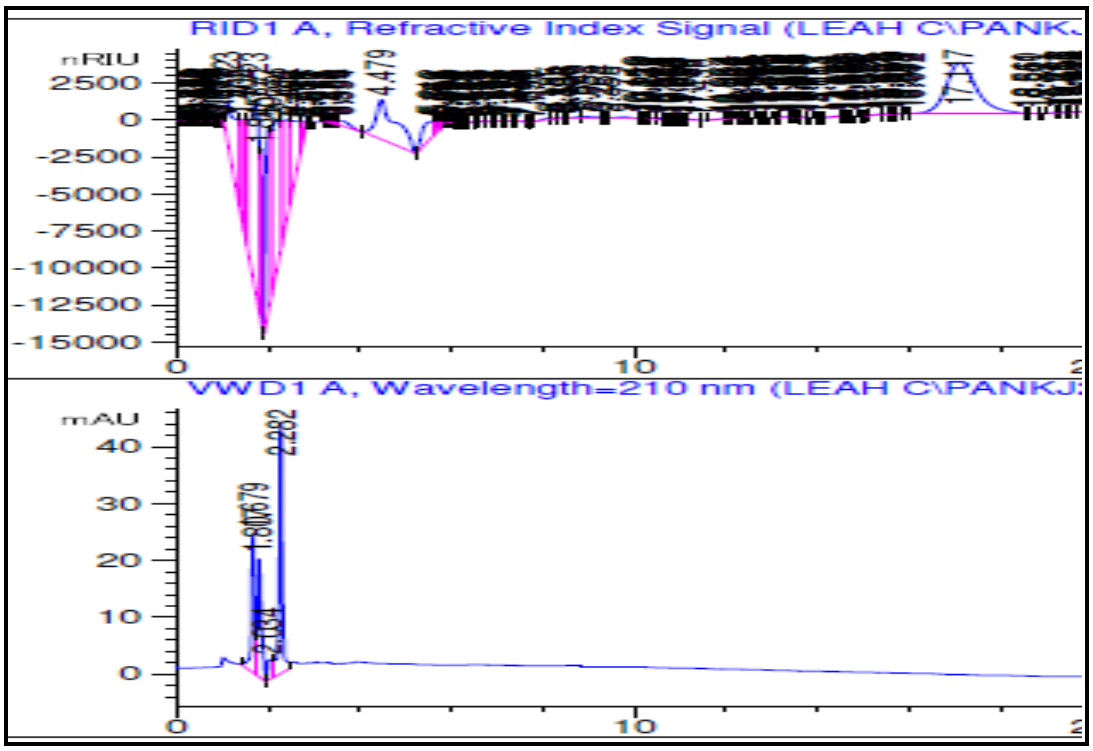


Fig. 7.13a v) Chromatographic profile of XAT (1 mg/mL) on HYPERCARB column run with DCM at 210 nm.

v

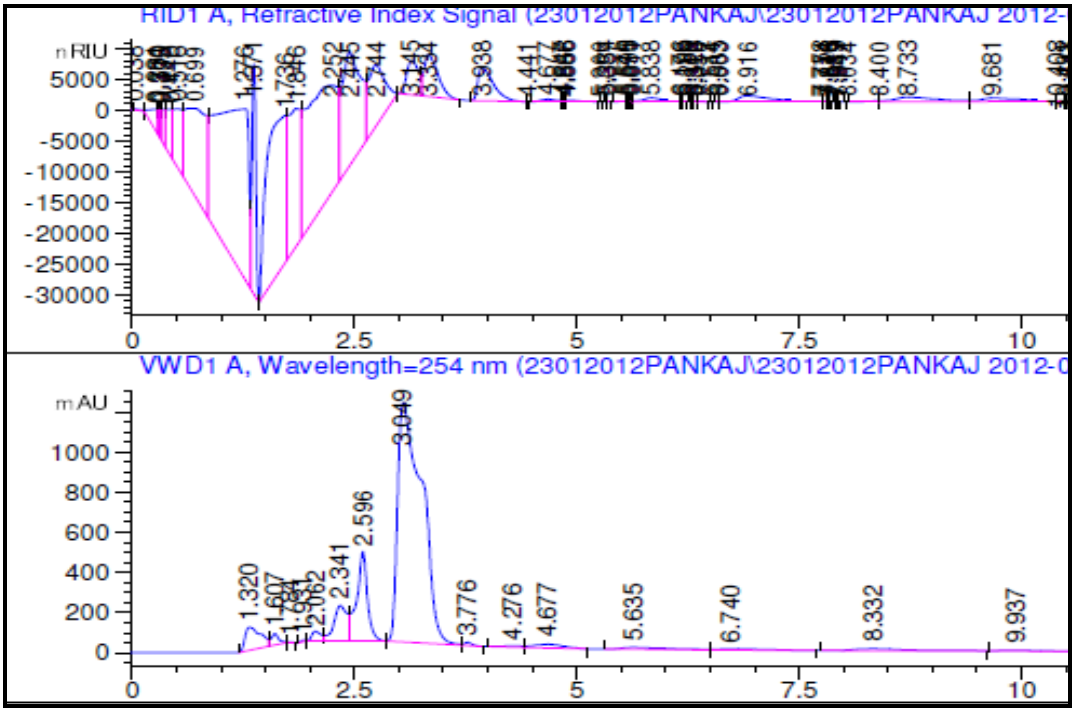


Fig. 7.13a vi) XAD 10 mg/mL run on HYPERCARB column with DCM at 254 nm.

Attempts were made to conduct some RPLC separations on the methanol soluble fraction of *X. americana* active fraction (XD3) obtained from TLC of XAD.

The methanol-soluble fractions of the dichloromethane extract (XAD) were also analysed by isocratic and gradient RPLC on HP 1090 LC with PDA detector to get an overview of the number of compounds present in the fractions. Mass spectral analysis of TLC spot 3 (XD3) had given signals for two distinct compounds with an error margin > 5 ppm. The anti-TB activity was shown to reside in this fraction by autobiography so efforts were aimed at finding out the active constituents by a more sophisticated and unambiguous method than those previously used.

LC analysis of XD3 by methanol-water (70:30, v/v) mobile phase showed a single peak at 210 and 230 nm while three peaks were observed at 254 nm (Figure 7.13b). Further experiments on the fraction were conducted by SFC in collaboration with Waters Corporation.

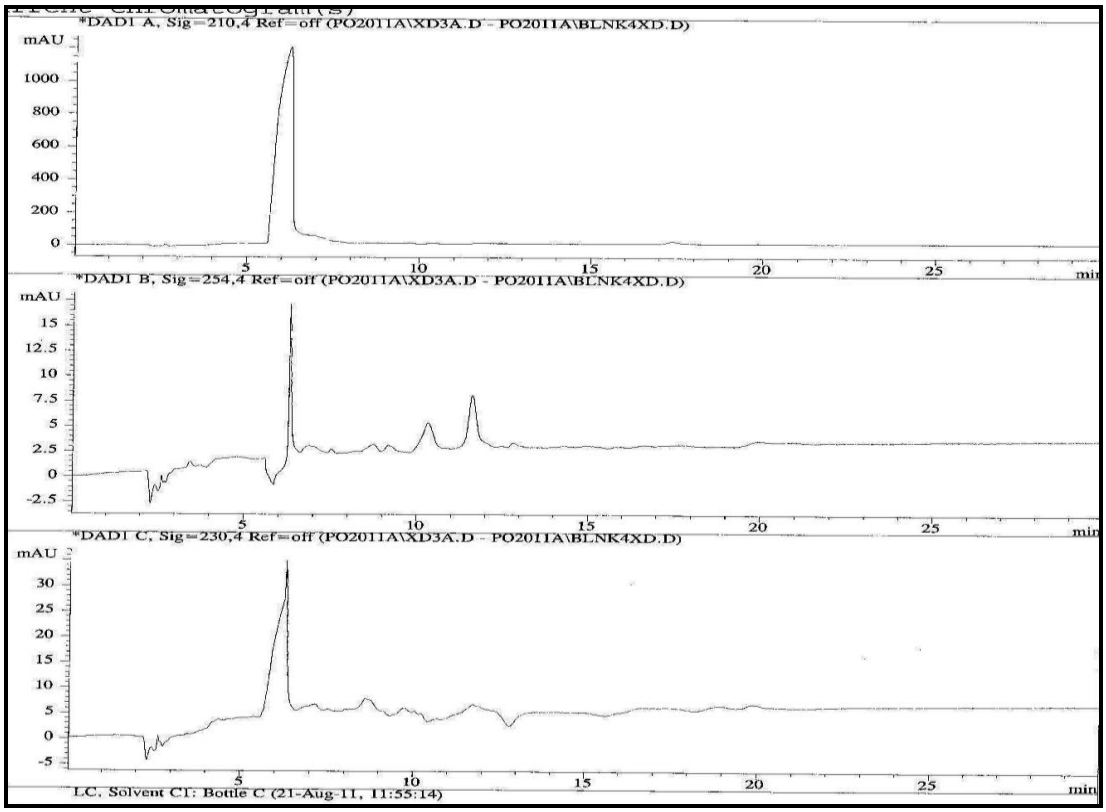


Fig. 7.13b. Chromatographic profile of XD3 on ACE 5 C18 column, 250 x 4.6 mm i.d.

The methanol - soluble fraction of XAD may be amenable to structure determination by LC-MS upon further optimisation of LC in conjunction with detectors other than UV-visible.

7.5 Supercritical fluid chromatography

SFC-MS provides an efficient and alternative route to analysing components of non-polar complex mixtures due to incompatibility issues arising from coupling NPLC to MS because of the solvents utilised in normal phase. It has also been reported to have the advantage of reducing work-up time for samples before analysis, faster run-times and equilibration, simultaneous chiral and achiral separation, decreased solvent consumption and combined detection capabilities of MS (www.waters.com). The mobile phase properties of carbon dioxide (e.g. diffusivity, density, viscosity, etc.) are intermediate between those of gases and

liquids and can be varied and controlled by small changes in pressure or temperature. These features allow for high mass transfer of lipid compounds with little consumption of solvents unlike NPLC (Wright and DePhillipo, 2010).
SFC can be carried out with open tubular and packed columns, with differences in selectivity, detection and need of modifier addition to the carbon dioxide. Both types have been employed in the separation of sterols in a wide variety of samples. Supercritical carbon dioxide has an adequate solvating power for sterol separation with both column types without the need of modifier addition. It is therefore possible to separate sterols in complex samples at lower temperatures than gas chromatography and in shorter times than liquid chromatography (Senorans and Markides, 2000). The equipment used in the analysis is shown in Figure 7.14.

7.6 Experimental conditions

Flow: 5 mL/min
column: 2 ethylpyridine, 15 cm x 4.6 mm
co-solvent: EtOH
Pressure: 125 bar
Temperature: 40 ° C
Injection volume: 5 µL (1 mg/mL of drug- lanosterol, stigmasterol and sitosterol)
Gradient:

Time (min)	% co-solvent	% CO_2	Flow(ml/min)
0	5	95	5
1	5	95	5
8	40	60	5
10	40	60	5

UV detector: 220 nm
ELSD detection
Make-up MS: 0.6 mL/min, MeOH

7.7 Results and Discussion

The standard samples of lanosterol, stigmasterol and sitosterol were analysed initially by SFC-UV-ELSD in order to establish detectability and to explore likely mobile phase conditions that might be needed for subsequent extract samples (TLC having shown the important TLC 3 and TLC 4 fractions to have been more polar than phytosterols. They showed good separation but ELSD gave sharper peaks with the retention time falling within three minutes. The separation of a mixture of the three compounds on fluorophenyl column gave better detectability when compared to 2-ethyl pyridine and silica columns (Figure 7.14b).

The main properties of supercritical fluid chromatography which affect sterol separations are related to the high solvating power of supercritical fluids and a low viscosity, which yields a high resolving power and rapid throughput. In addition to its other advantages, the ability of SFC to resolve complex mixtures of low volatility compounds allows the direct injection of samples that contain sterols with no or little pre-treatment. Some sterols can be degraded or lost during exposure to light, heat or extreme values of pH. In the SFC of sterols, all these factors can be avoided, providing a separation under mild conditions that preserves the integrity of the sample (Senorans and Markides, 2000).

i.

- Fluid delivery module
- Make-up pump
- Sample manager 2767
- Analytical 2 prep Oven
- PDA 2998
- ABPR
- Software MassLynx
- CO_2
- EtOH: Fisher LC-MS grade

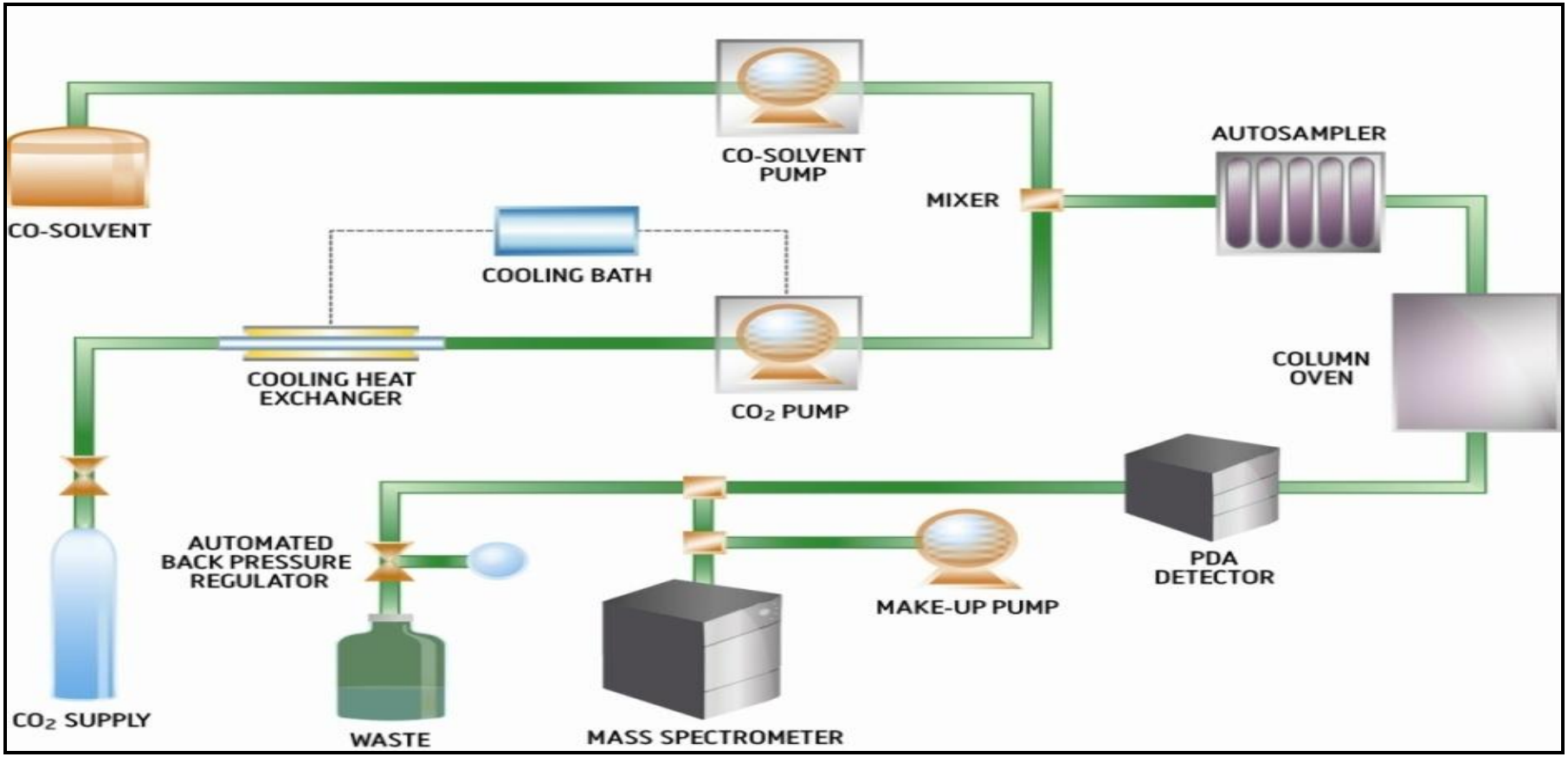
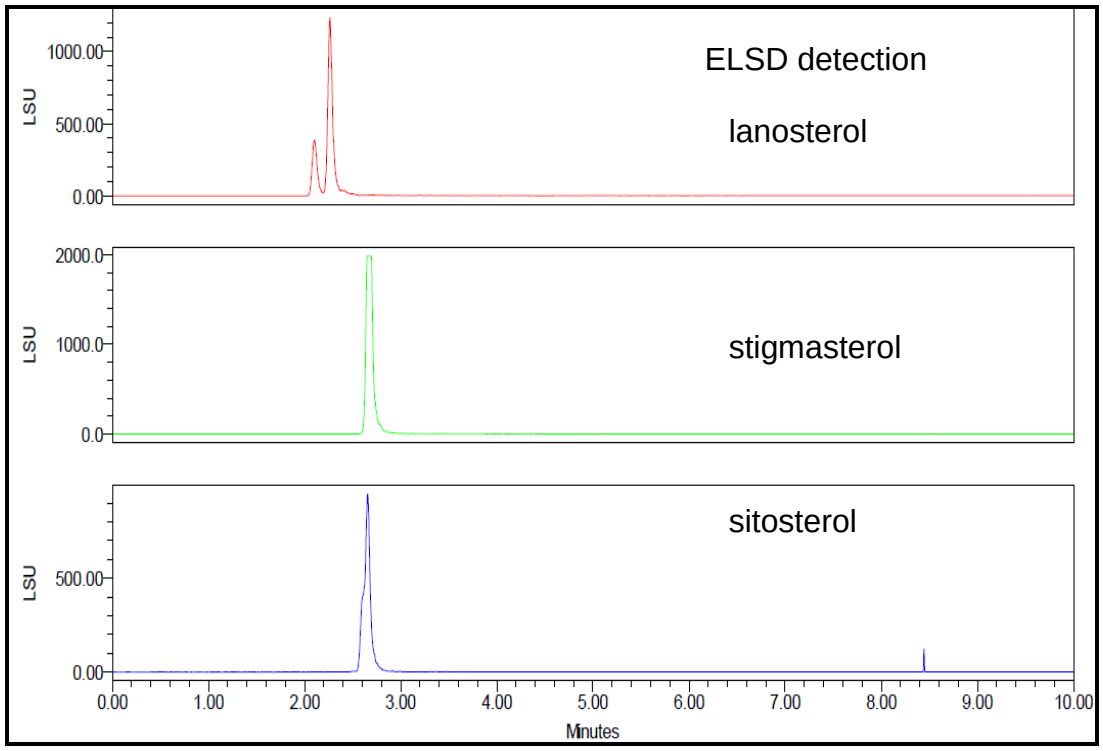


Fig.7.14a. i) Waters Analytical SFC system, Method Station II equipment. ii) schematic overview of the components of SFC coupled to PDA detector and mass spectrometer (Waters Corporation, 2011).

i.



ii.

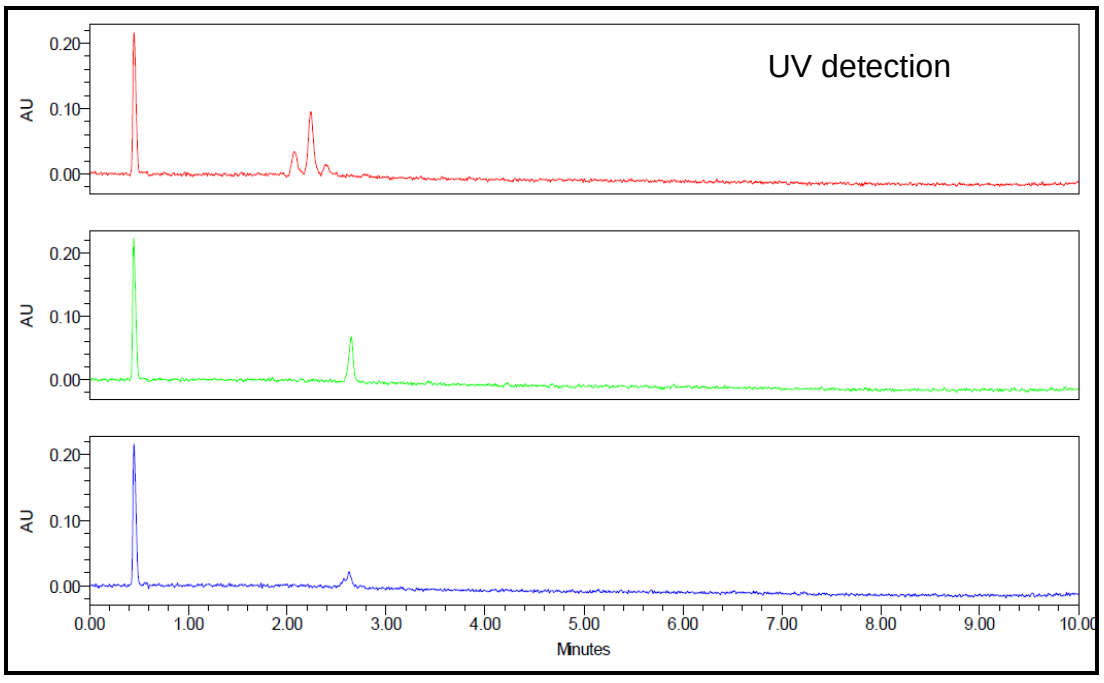


Fig. 7.14b i and ii) SFC profiles of standards; lanosterol, stigmasterol and sitosterol on 2-ethylpyridine column with ELSD and UV detection.

iii.

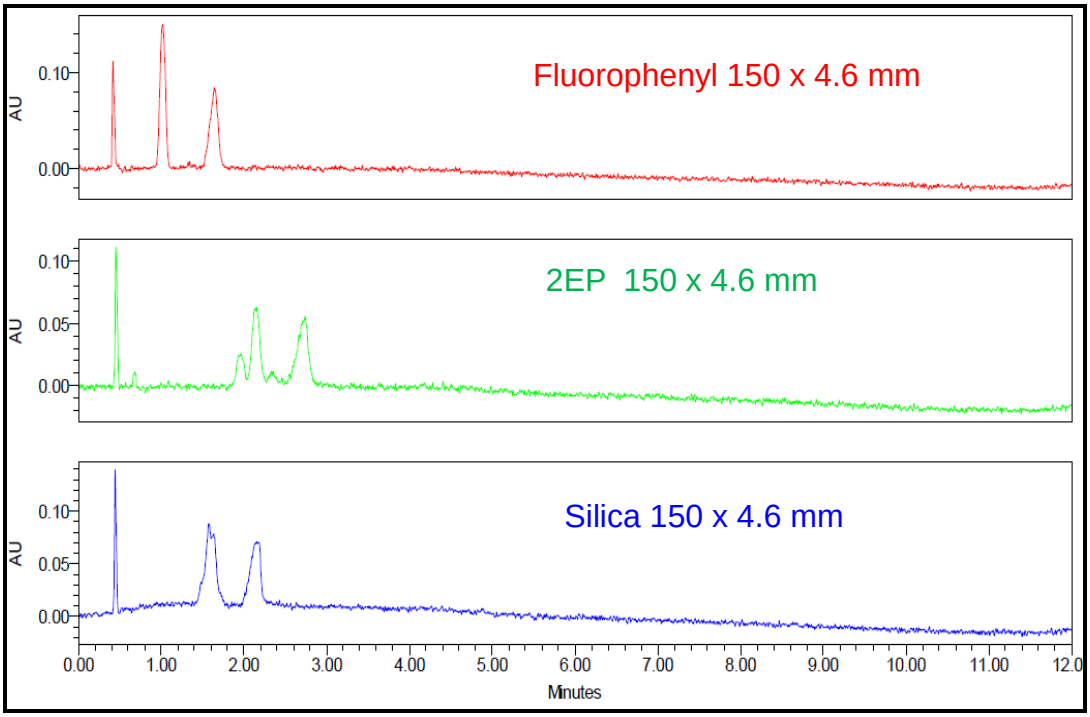


Fig.7.14b iii) Chromatographic profiles of a mixture of standard compounds, lanosterol, stigmasterol and sitosterol on different columns using UV detection.

The standard compounds were separated by SFC and detection with ELSD gave better detectability compared to UV. Further experiments on the TB active fraction, XAD showed that detectability improved with ELSD under gradient conditions compared to isocratic analysis using UV detection (Figures 7.14e i-iii). The separation was not enhanced with pressure or temperature modification (Figure 7.14c iv). SFC analysis of XD3 showed similar profiles for the extract with the use of methanol and ethanol as co-solvent (Figures 7.14d i-iii). Column screening of XD4 (TB active fraction of XAD) showed weak retention of the compounds (Figures 7.14e 1-iv).

The presence of poor UV absorbing constituents might provide a possible explanation for the inability to separate the constituents by earlier methods involving LC/UV.

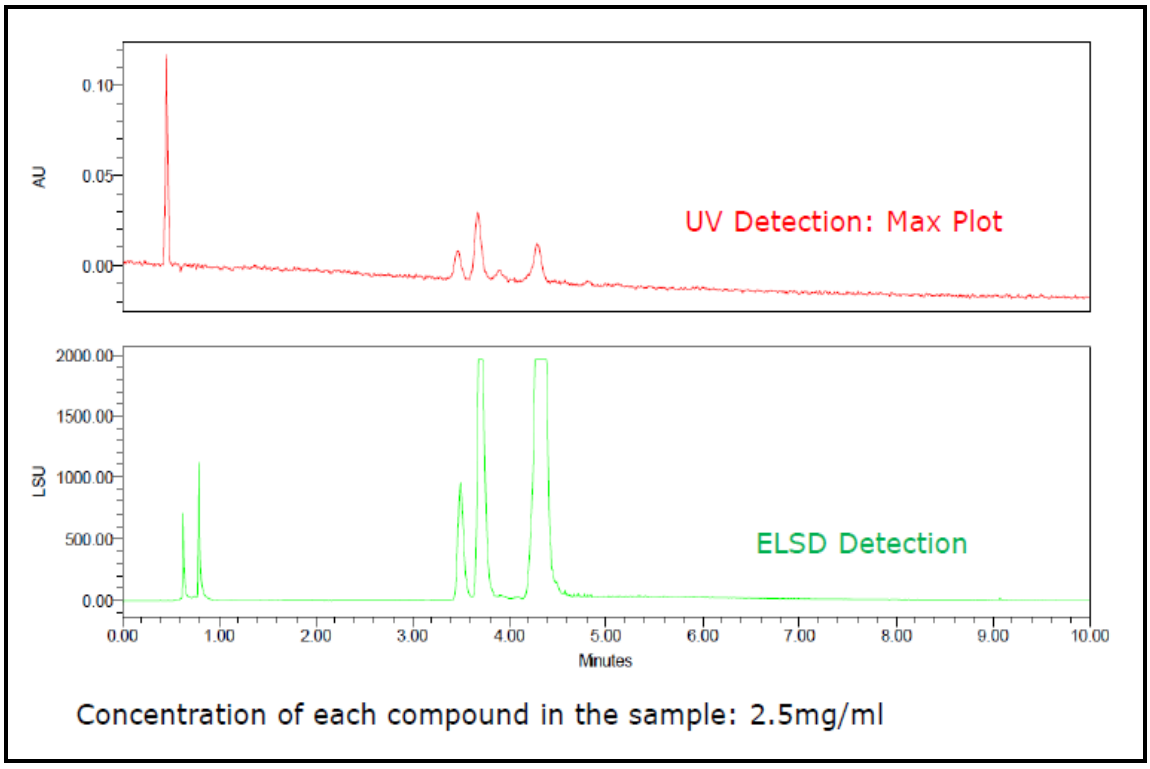


Fig. 7.14c i) SFC profile of a mixture of lanosterol, stigmasterol and sitosterol on 2-ehylpyridine column. Conditions are described in Section 7.6

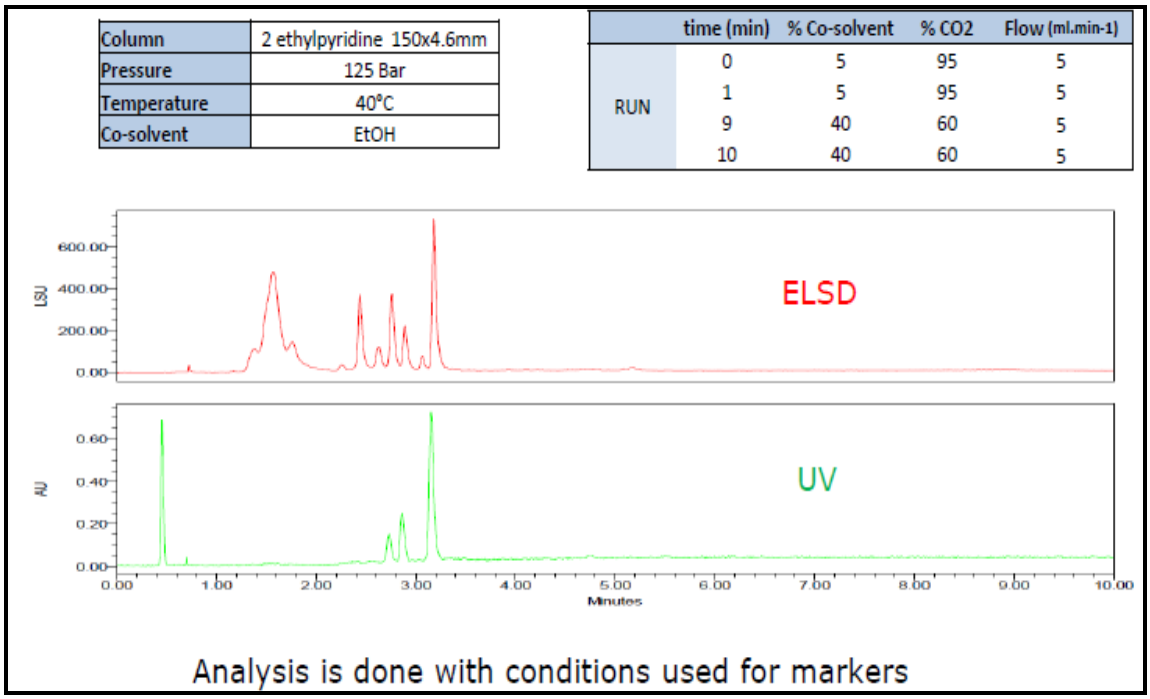


Fig. 7.14c ii) SFC profile of XAD using the same gradient conditions as the standards.

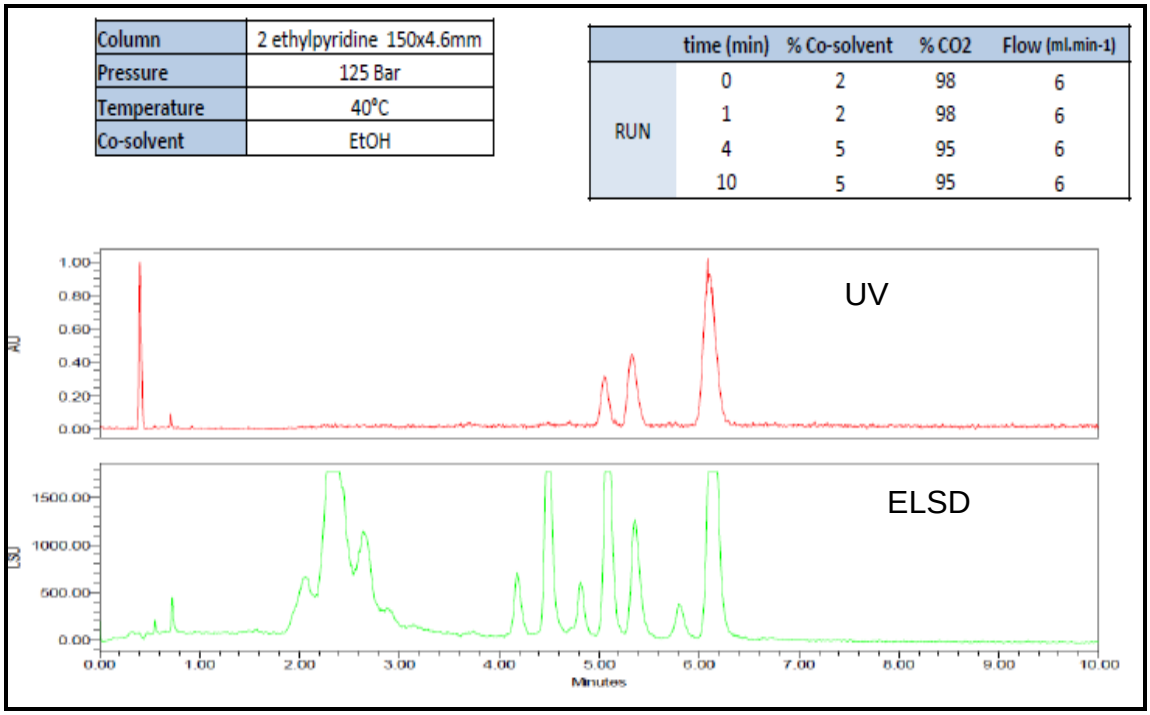


Fig. 7.14c iii) SFC profile of XAD with modified gradient condition as described above.

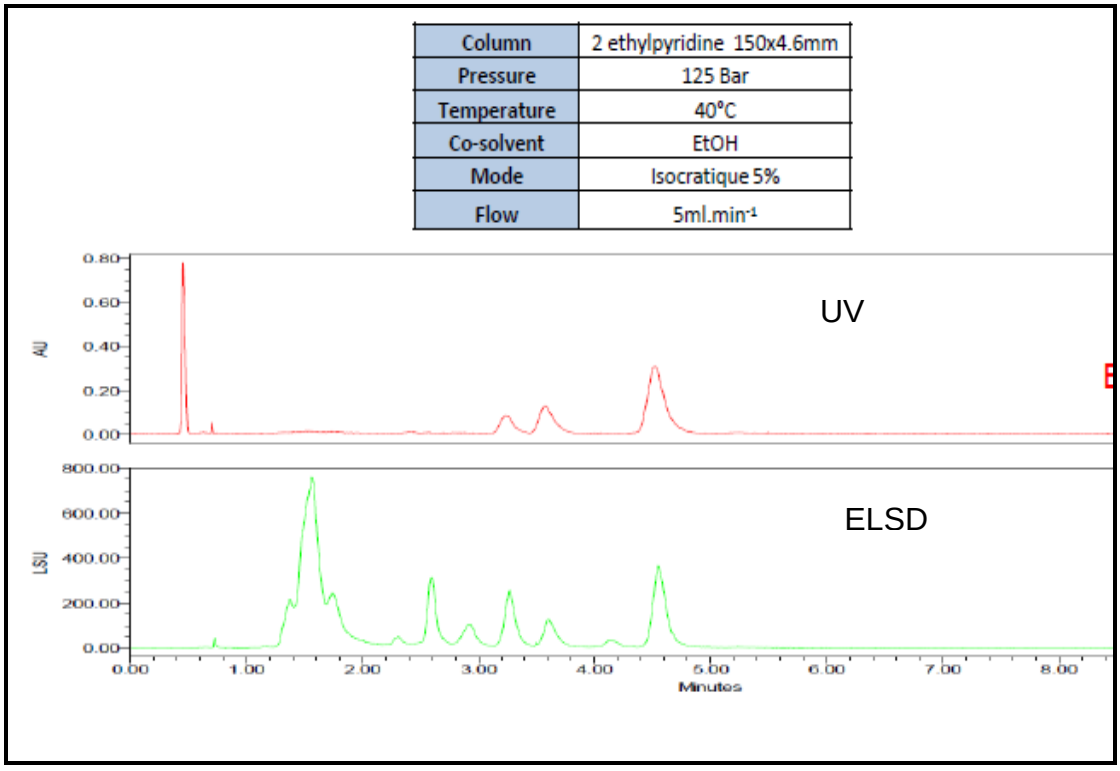


Fig. 7.14c iv) SFC profile of XAD on 2- ethylpyridine column using isocratic conditions as described above.

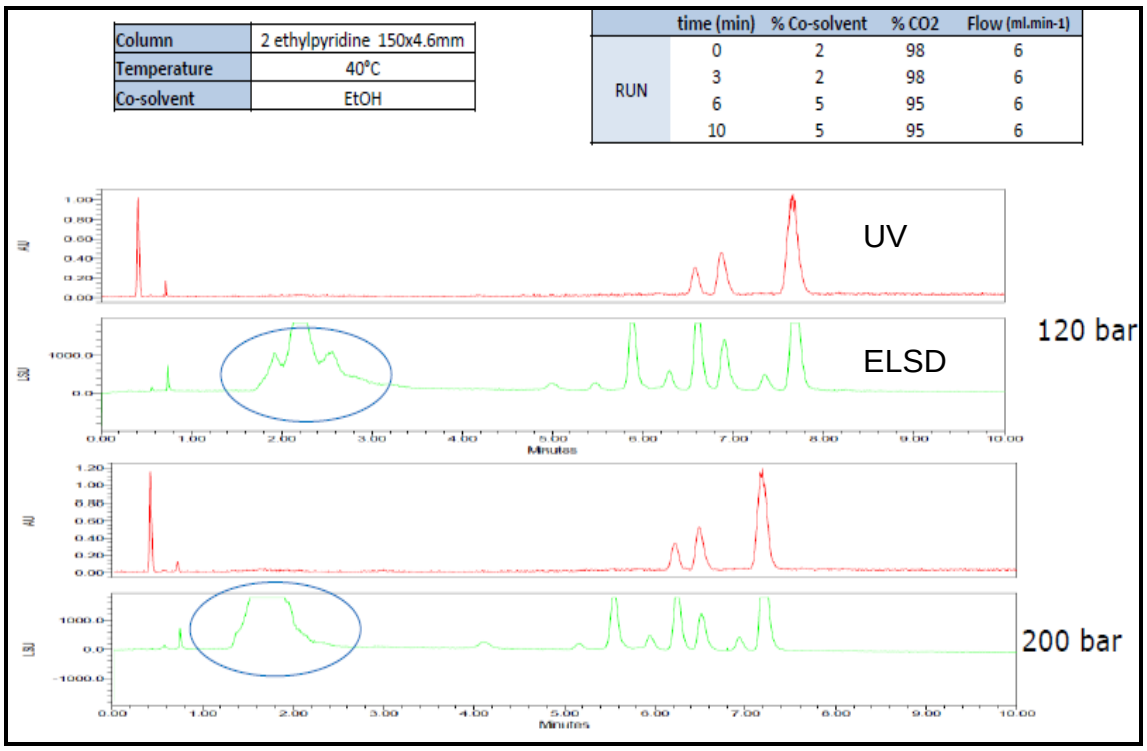


Fig. 7.14c v) SFC profile of XAD on 2- ethylpyridine column with pressure modification.

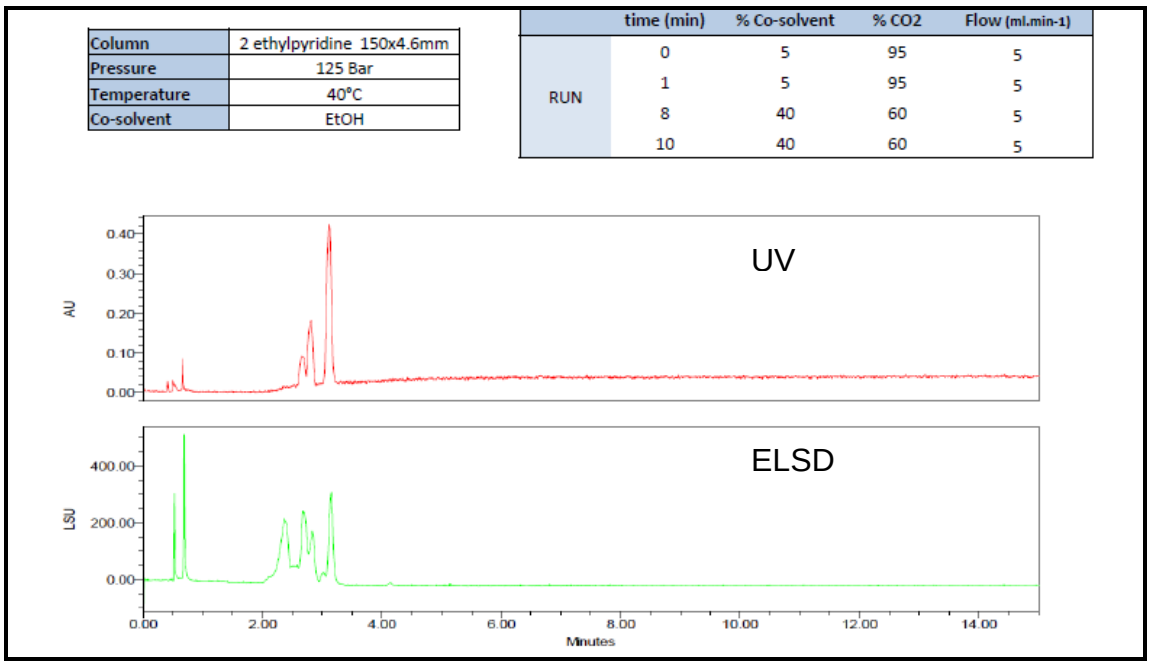


Fig. 7.14d i) SFC profile of XD3 on 2-ethylpyridine column using gradient conditions with EtOH as co-solvent.

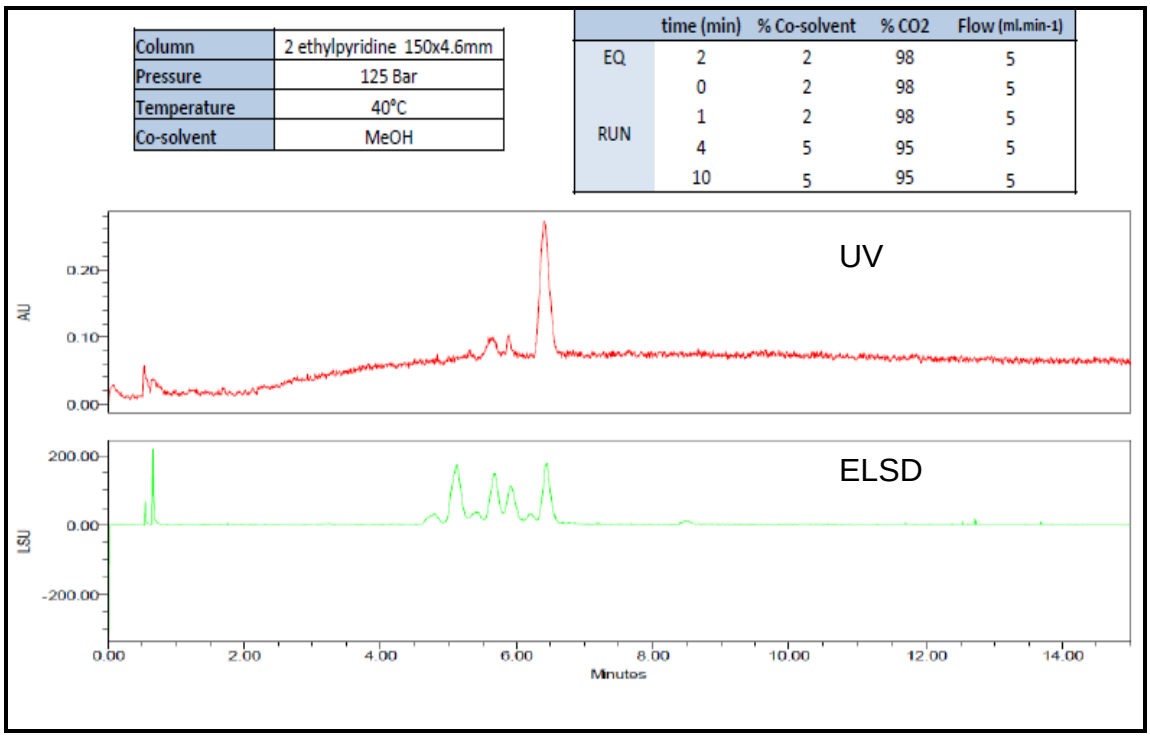


Fig. 7.14d ii) SFC profile of XD3 on 2-ethylpyridine column using a modified gradient condition with MeOH as co-solvent.

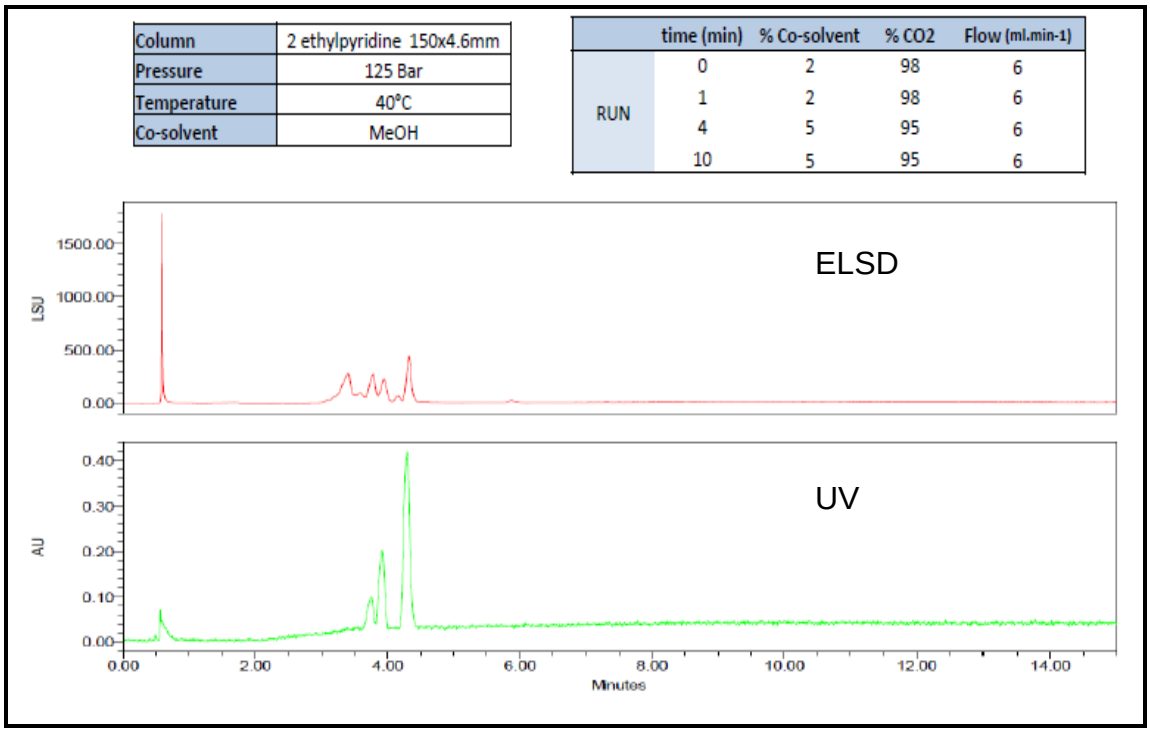


Fig. 7.14d iii) SFC profile of XD3 on 2-ethylpyridine column using a modified flow rate with MeOH as co-solvent.

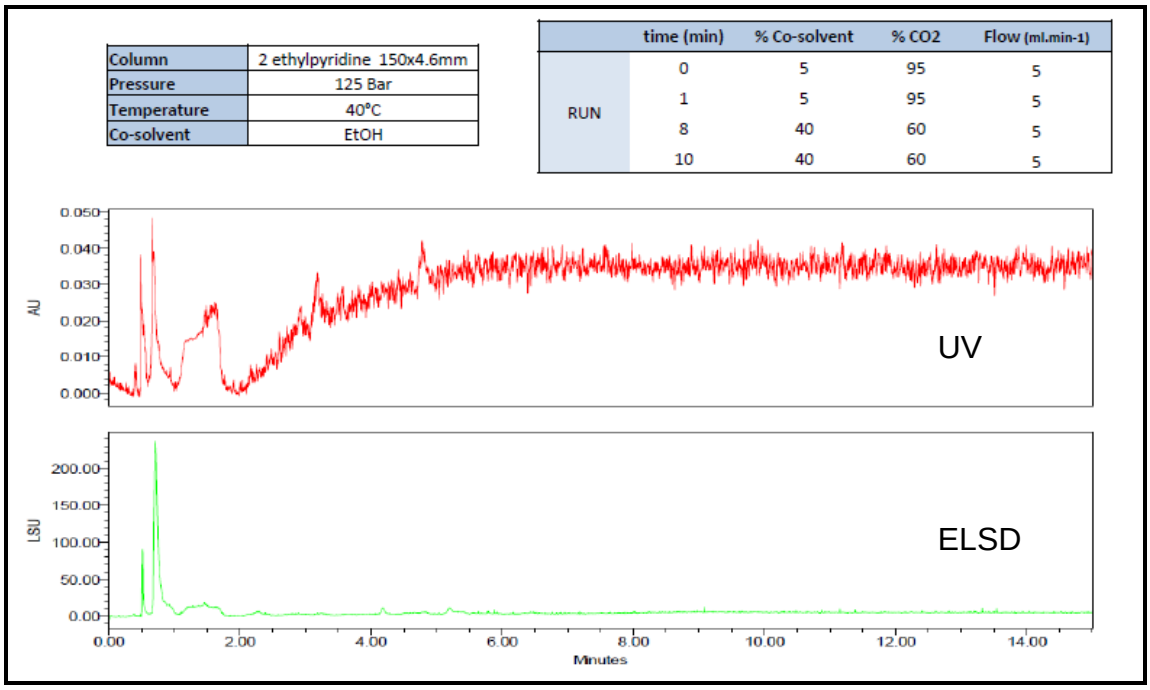


Fig. 7.14e i) SFC profile of XD4 on 2-ethylpyridine column using gradient conditions with EtOH as co-solvent

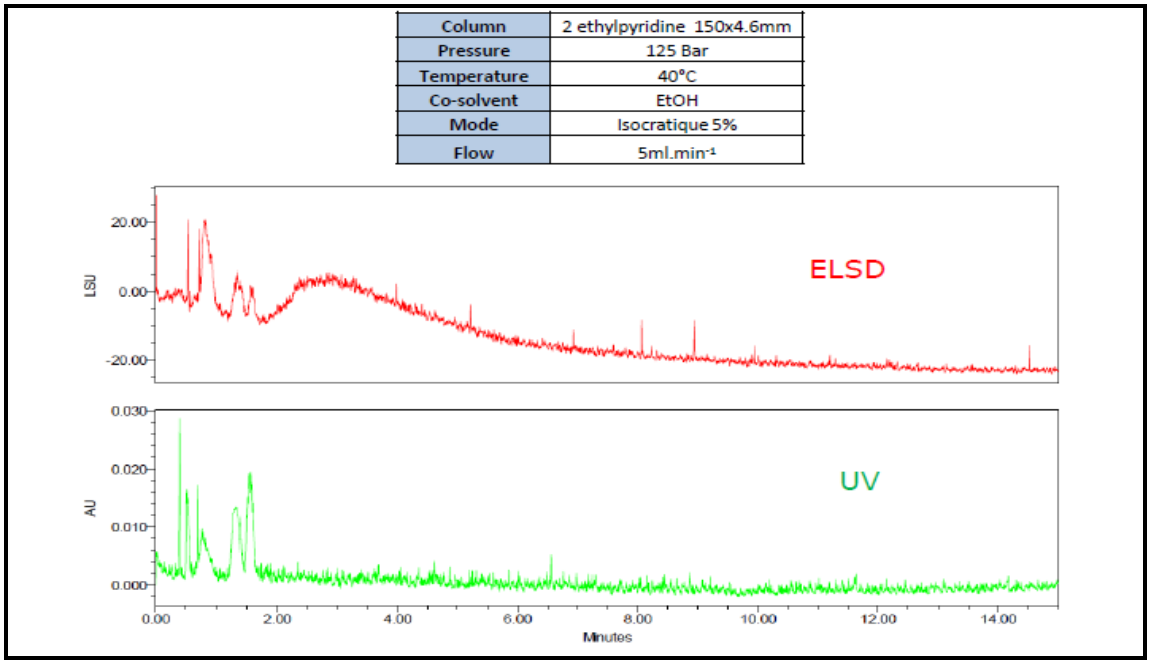


Fig. 7.14e ii) SFC profile of XD4 on 2-ethylpyridine column using isocratic conditions with EtOH as co-solvent**.**

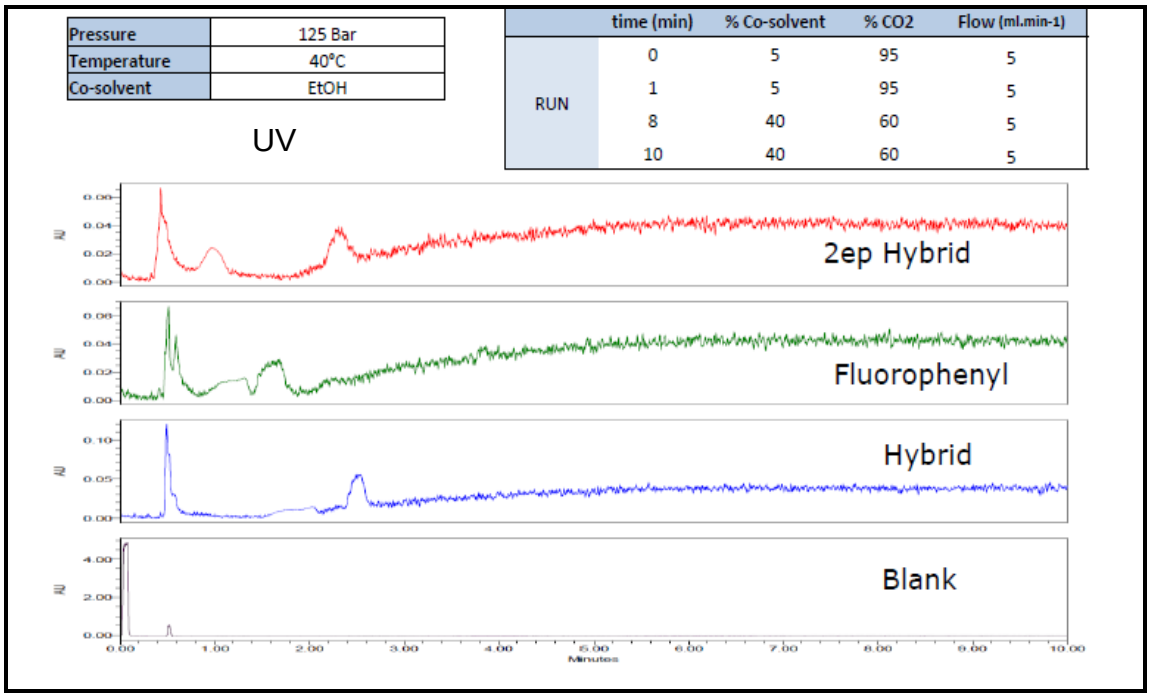


Fig. 7.14e iii) SFC profile of XD4 screened with different columns using UV detection and gradient conditions with EtOH as co-solvent**.**

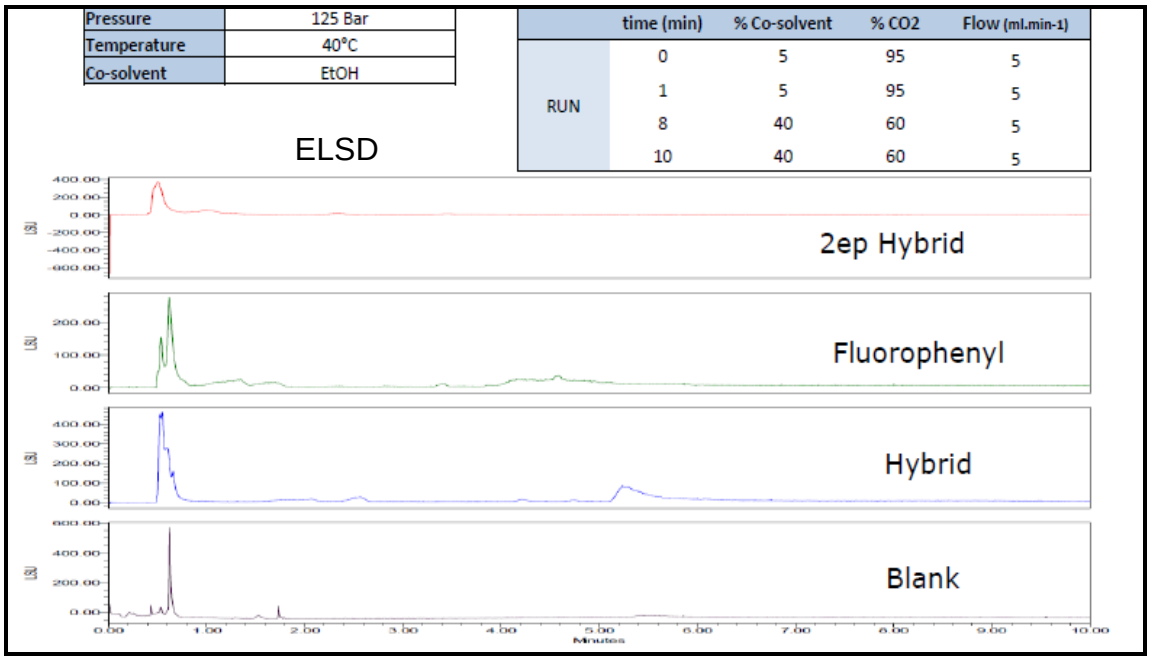


Fig. 7.14e iv) SFC profile of XD4 screened with different columns using ELSD detection and gradient conditions with EtOH as co-solvent**.**

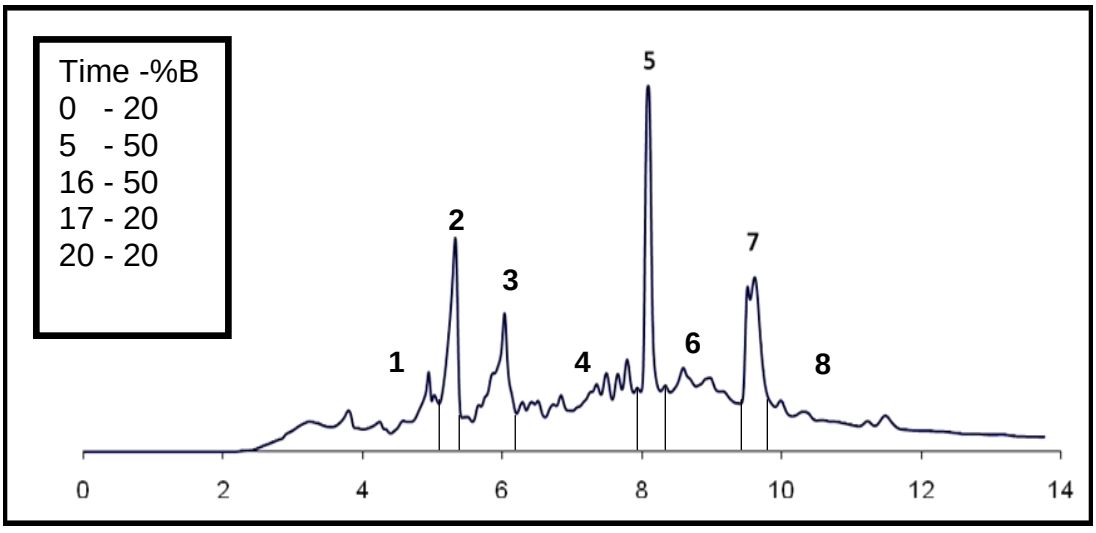
7.8 Identification of compounds from *P. crassipes* leaf.

Initial screening studies with the methanol extract of *P. crassipes* showed some inhibitory activity against *M. aurum* so attempts were made to separate and identify the constituents of the extract using LC, MS and NMR.

Reversed phase LC analysis of the methanol fraction resulted in eight fractions, two of which were found to be of high enough purity for analysis by NMR and mass spectrometry measurements. The crude methanol extract tested positive with $FeCl_3$ solution and NaOH suggesting that tannins and flavonoids were present (Evans, 2009). The compounds, 5-caffeoyl quinic acid methyl ester and quercetin-3-rutinoside were identified by accurate mass measurements and confirmed by NMR data and literature. The results obtained by mass spectrometry are presented in Figures 7.15d and e while assignments of the signals are presented in Tables 7.5b and c respectively. Compounds 5 and 7 were identified by accurate mass measurements as 5-O-caffeoyl quinic acid methyl ester and rutin respectively after separation and isolation by reversed phase semi-preparative LC (Figure 7.15a).

The structures were confirmed by comparison of 1H and ^{13}C NMR data of the isolated compound to a standard sample of rutin (Sigma-Aldrich, S2424, Lot # 129K2568V) and with literature values for 5-O-caffeoyl quinic acid methy ester (Chan *et al*., 2009; Clifford *et al*., 2003).

i)



Retention time (minutes)

Fig.7.15a. Semi-preparative LC profiles of *P. crassipes* (methanol) extract (10mg/mL) on ACE 5 C18 column, 250 x 10 mm with gradient elution using methanol: water (B – methanol).

Compound 5 (Me 5-CQA) has a molecular formula $C_{17}H_{19}O_9$ established by HR-ESI-MS (m/z: 367.10339 $[M-H]^-$, calculated as 367.10346, δ - 0.18 ppm (Figure 7.15d). Compound 7 (rutin) was compared to NMR data of an authentic sample and both compounds showed similar signals (Table 7.5c). The molecular formula was established by HR-ESI-MS as $C_{27}H_{29}O_{16}$ (m/z: 609.14603 $[M-H]^-$, calculated as 609.14611, δ – 0.13 ppm (Table 1).

Table 7.5a. Preparative LC data of *P. crassipes* methanol extract

Fraction	**compound**
5	5-caffeoyl-quinic acid methyl ester
7	Quercetin-3-rutinoside (rutin)
1	unknown
2	unknown
3	unknown
4	unknown
6	unknown
8	unknown

Table 7.5b. ^{1}H and ^{13}C NMR spectral assignment of compound 5 from *P. crassipes* leaf dissolved in CD_3OD

Atom	**5 - Me 5-CQA**			
	^{13}C	^{1}H	^{13}C HSQC	^{13}C HMBC
1	72.14	-		

2		2.04 - 2.05 (m)	~36.9	
3		4.16 (m)	~69	
4		3.72 (m)	~71.7	
5		5.29 (m)	~71	
6		2.22 (m)	~37	
COO-	175.43			
OCH_3	52.96	3.76 (s)		
1'	122.98	6.96 (dd, 2.06 Hz, 8.25 Hz)		
2'	116.54	6.79 (d, 8.25 Hz)		
3'	146.91	-		
4'		-		~149
5'	115.09	7.06 (d, 2.06 Hz)		
6'	127.61	-		
7'	147.22	7.53 (d, 15.81 Hz)		
8'	115.01	6.22 (d, 15.81 Hz)		
9'	168.27	-		

~ estimated chemical shift values in ppm from HSQC and HMBC spectra

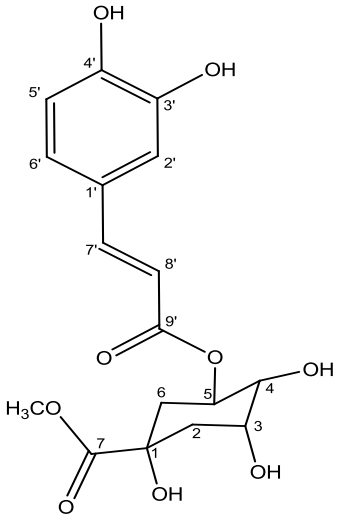
Table 7.5c. ^{1}H and ^{13}C NMR spectral assignment of compound 7 from *P. crassipes* leaf dissolved in CD_3OD

Atom	**7- Rutin**		**Rutin reference standard**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	179.38	-	179.41	-

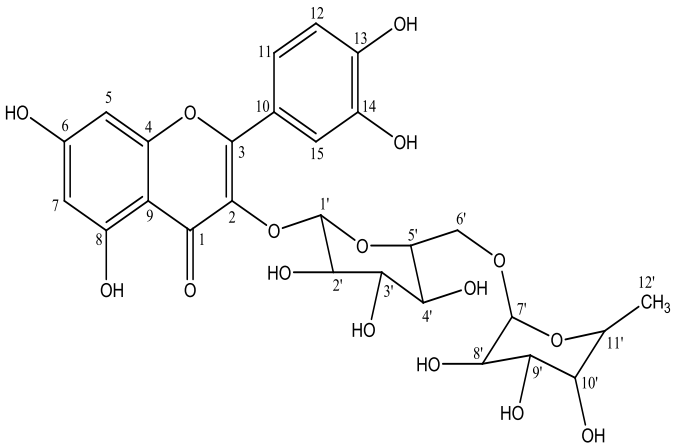
2	135.6	-	135.64	-
3	159.29	-	159.33	-
4	158.56	-	158.50	-
5	94.95	6.39 (d, 1.83 Hz)	94.87	6.42 (d, 2.07 Hz)
6	166.48	-	165.98	-
7	100.09	6.20 (d, 1.83 Hz)	99.95	6.24 (d, 2.07 Hz)
8	162.98	-	162.96	-
9	105.51	-	105.65	-
10	123.54	-	123.57	-
11	123.12	7.62 (dd, 1.83 Hz, 8.25 Hz)	123.15	7.64 (dd, 2.17, 8.48 Hz)
12	116.06	6.87 (d, 8.48 Hz)	116.07	6.92 (d, 8.48 Hz)
13	145.86	-	145.82	-
14	149.84	-	149.78	-
15	117.66	7.66 (d, 1.83 Hz)	117.71	7.69 (d, 2.17 Hz)
1'	104.75	5.10 (d, 7.56 Hz)	104.74	5.15 (d, 7.44 Hz)
2'	78.20	3.40 (m)	78.20	3.40 (m)
3'	75.73	3.46 (m)	75.73	3.47 (m)
4'	69.71	3.44 (m)	69.70	3.45 (m)
5'	73.93	3.27 (m)	73.95	3.27 (m)
6'	68.55	3.36 (m), 3.81 (dd, 1.15,11 Hz)	68.57	3.35, 3.82 (d, 10.27 Hz)
7'	102.43	4.51 (s)	102.41	4.55 (d, 1.41 Hz)
8'	77.23	3.32 (m)	77.23	3.32 (m)

9'	72.24 Hz)	3.52 (dd, 3.44, 9.39	72.26 9.42 Hz)	3.53 (dd, 3.49,
10'	71.4	3.25 (m)	71.41	3.26 (m)
11'	72.11	3.62 (m)	72.10	3.66 (dd, 1.60 Hz)
12'	17.88	1.12 (d, 6.19 Hz)	17.87	1.16 (d, 6.22 Hz)

Values of ^{1}H and ^{13}C chemical shifts are in ppm (δ), and those of coupling constants, J in Hz



a)



b)

Fig.7.15b Structures of 5-O-caffeoyl quinic acid methyl ester (a) and quercetin-3-rutinoside (rutin) (b).

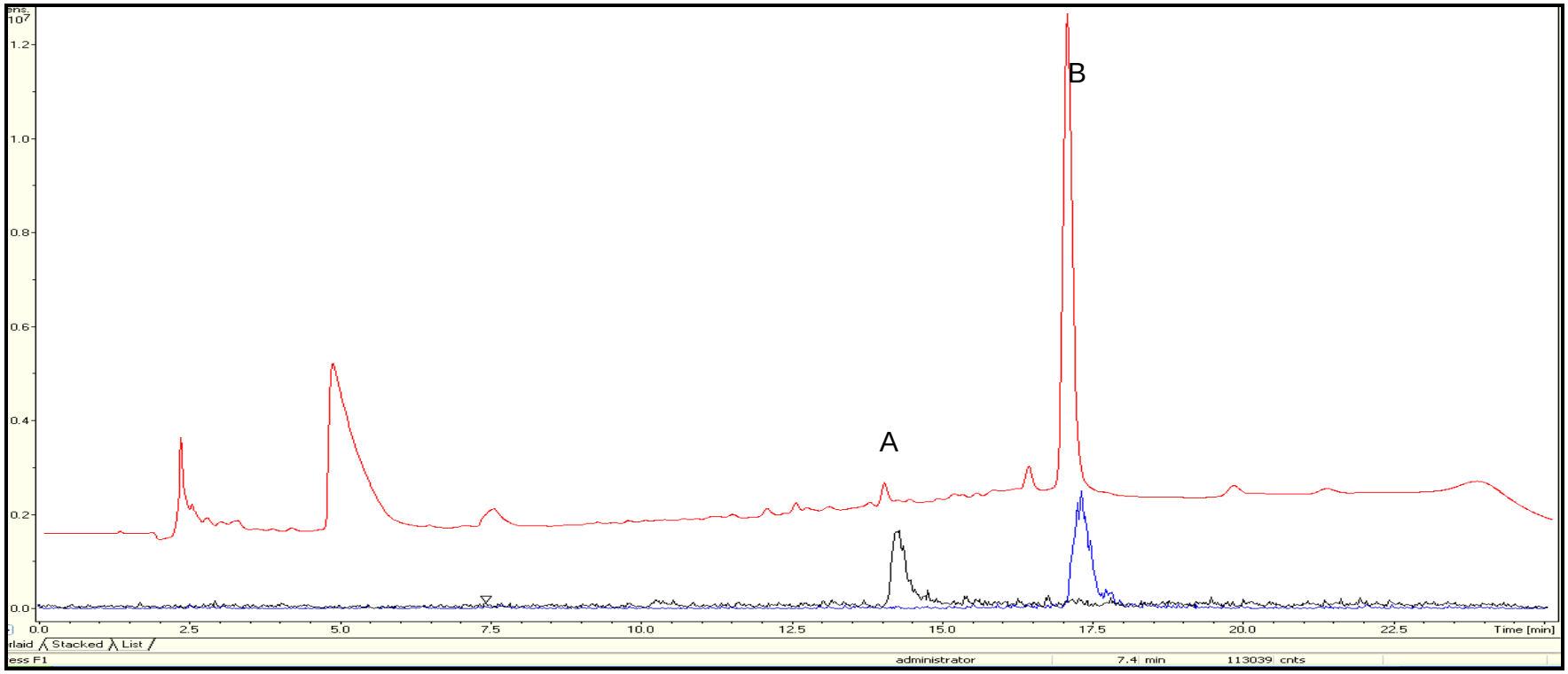


Fig. 7.15c i. LC-MS UV trace of *P. crassipes* methanol extract obtained on Esquire 3000+ mass spectrometer at 225 nm (top trace). Bottom trace: Extract ion chromatogram for 369 ion - PCM 5 (trace A) extract ion chromatogram for 611 ion - PCM 7 (trace B) acquired in positive ion mode.

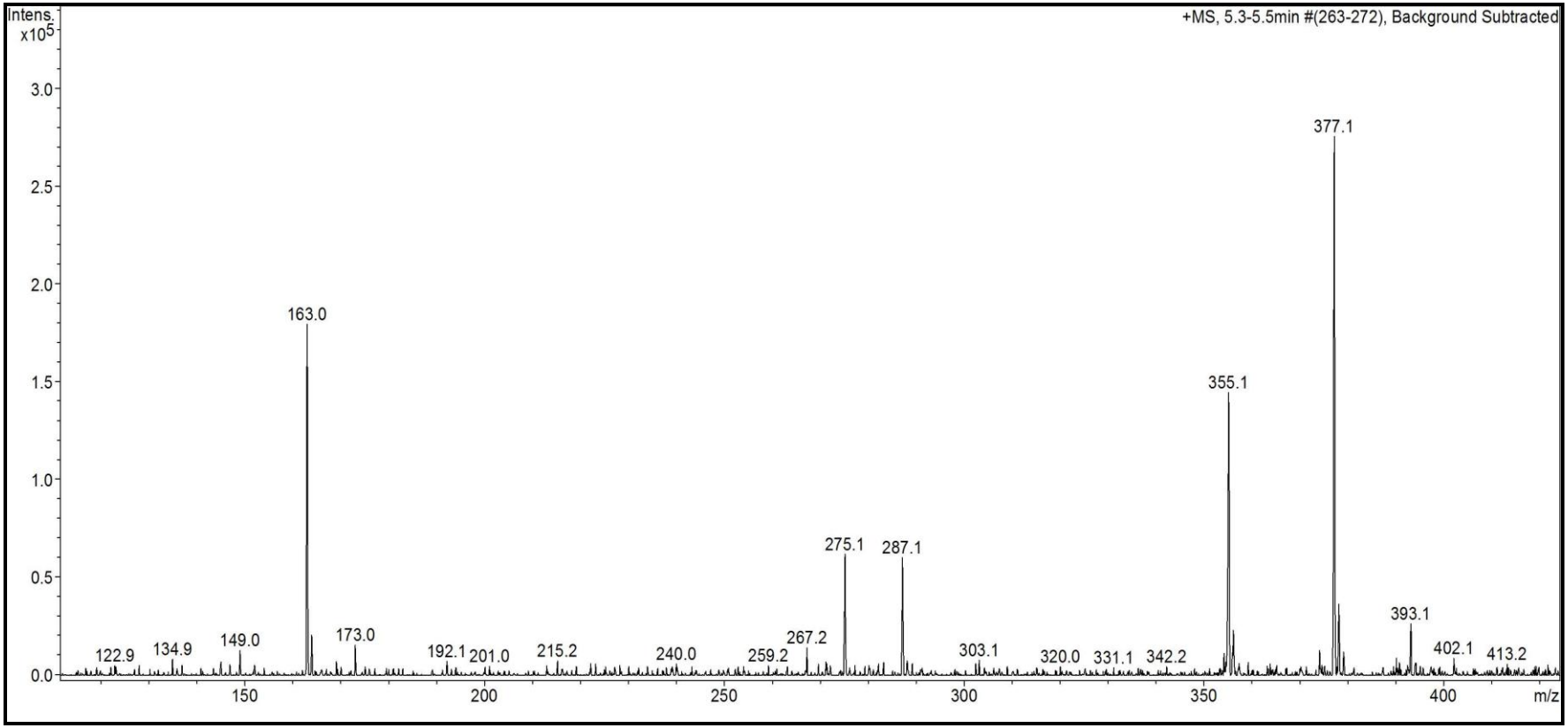


Fig. 7.15 c ii. Mass spectrum of *P. crassipes* methanol extract in positive ion mode (Esquire 3000 + spectrometer).

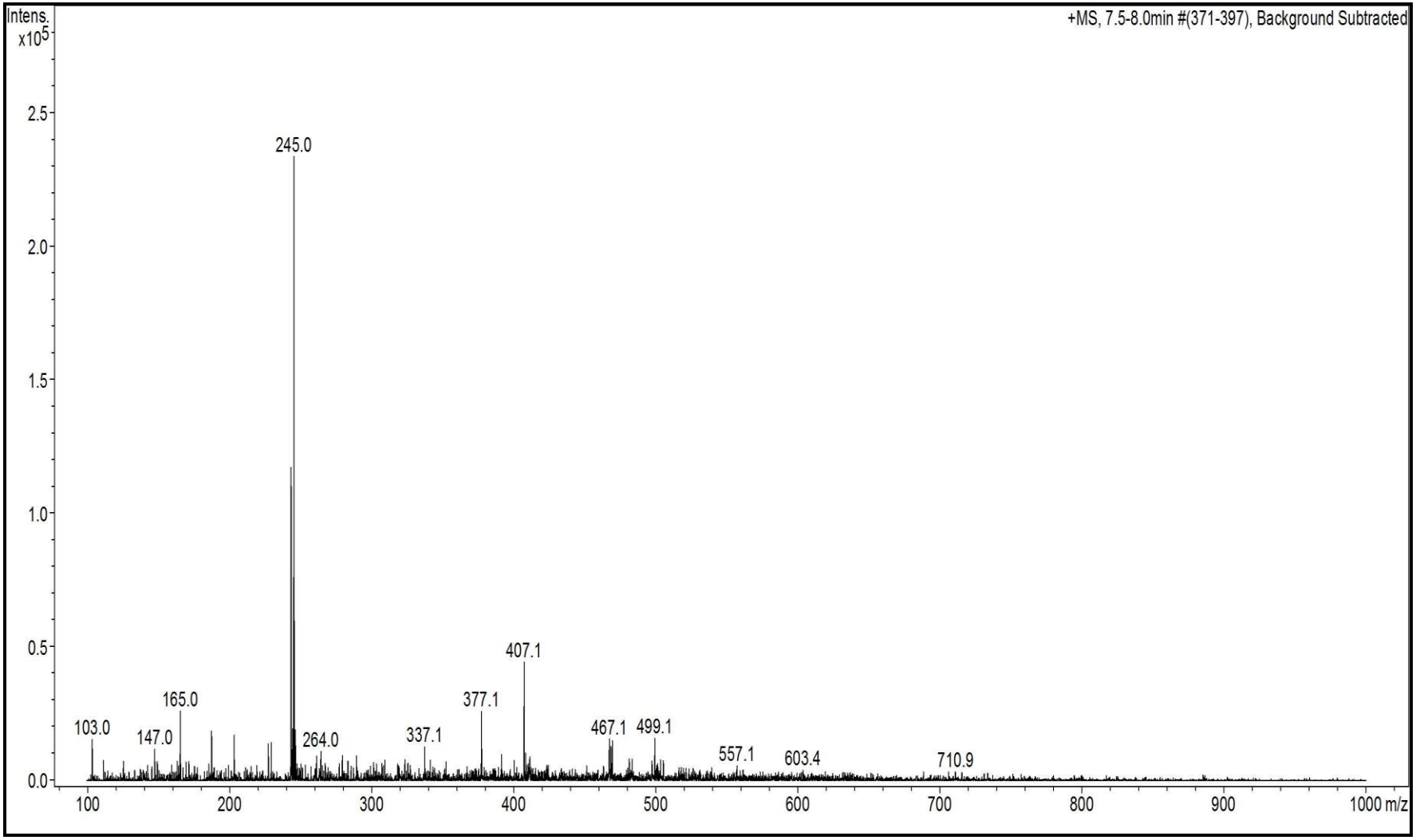


Fig.7.15 c iii. Mass spectrum of *P. crassipes* methanol extract in positive ion mode (Esquire 3000 + spectrometer).

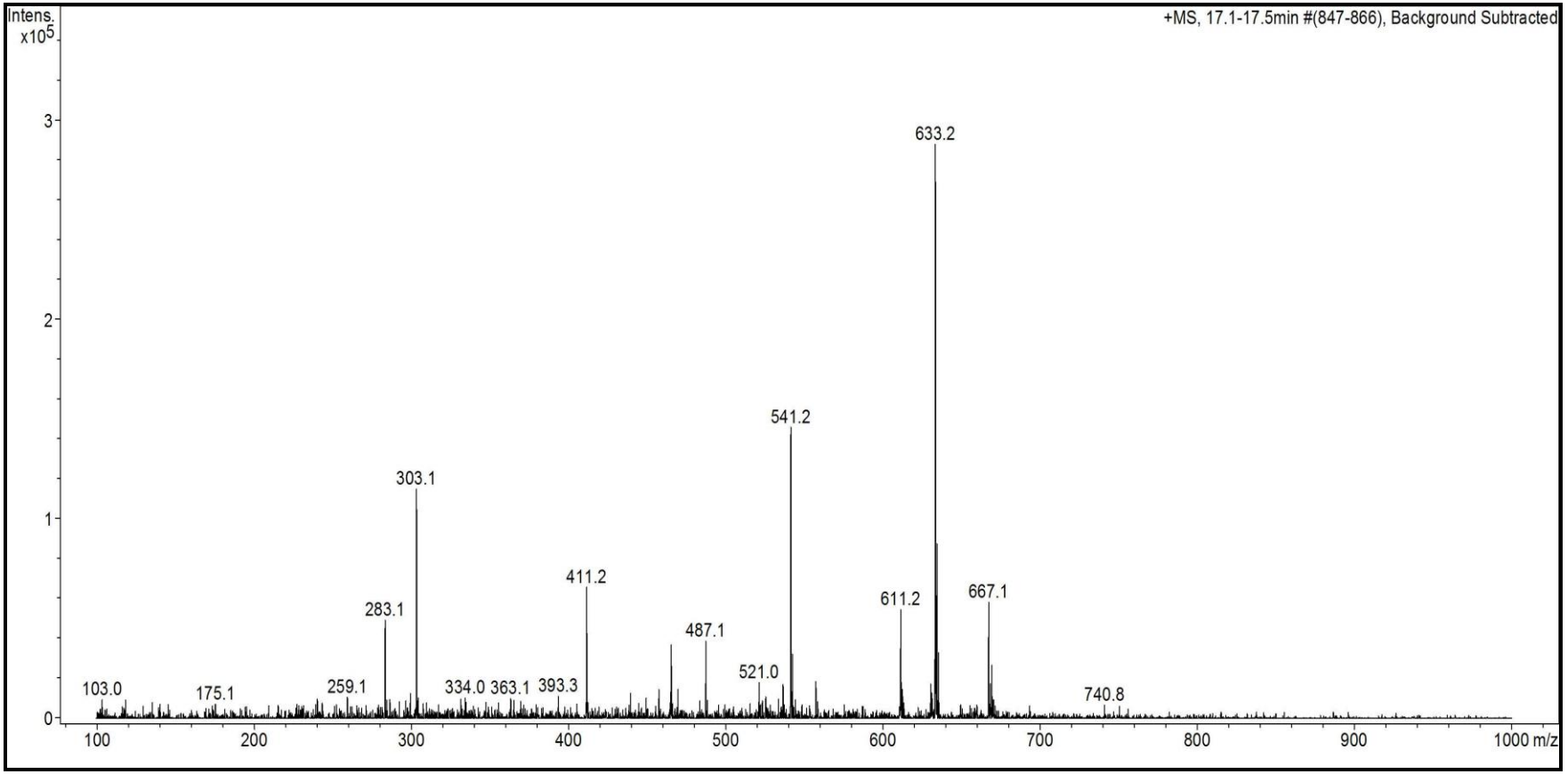


Fig.7.15 c iv. Mass spectrum of *P. crassipes* methanol extract in positive ion mode (Esquire 3000 + spectrometer).

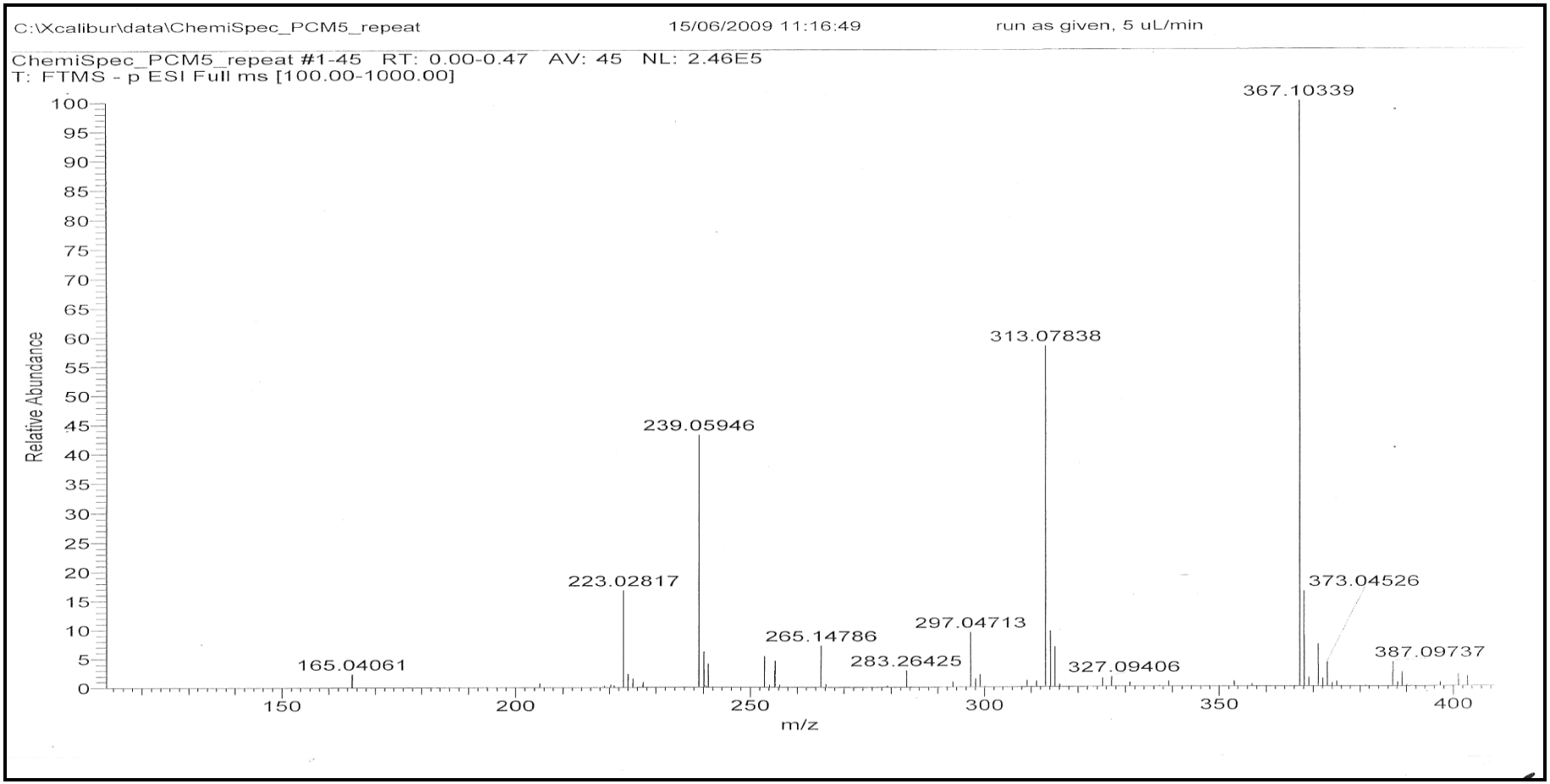


Fig.7.15 d i. Accurate mass spectrum of PCM 5 from *P. crassipes* leaf by ESI-MS (negative ion mode)

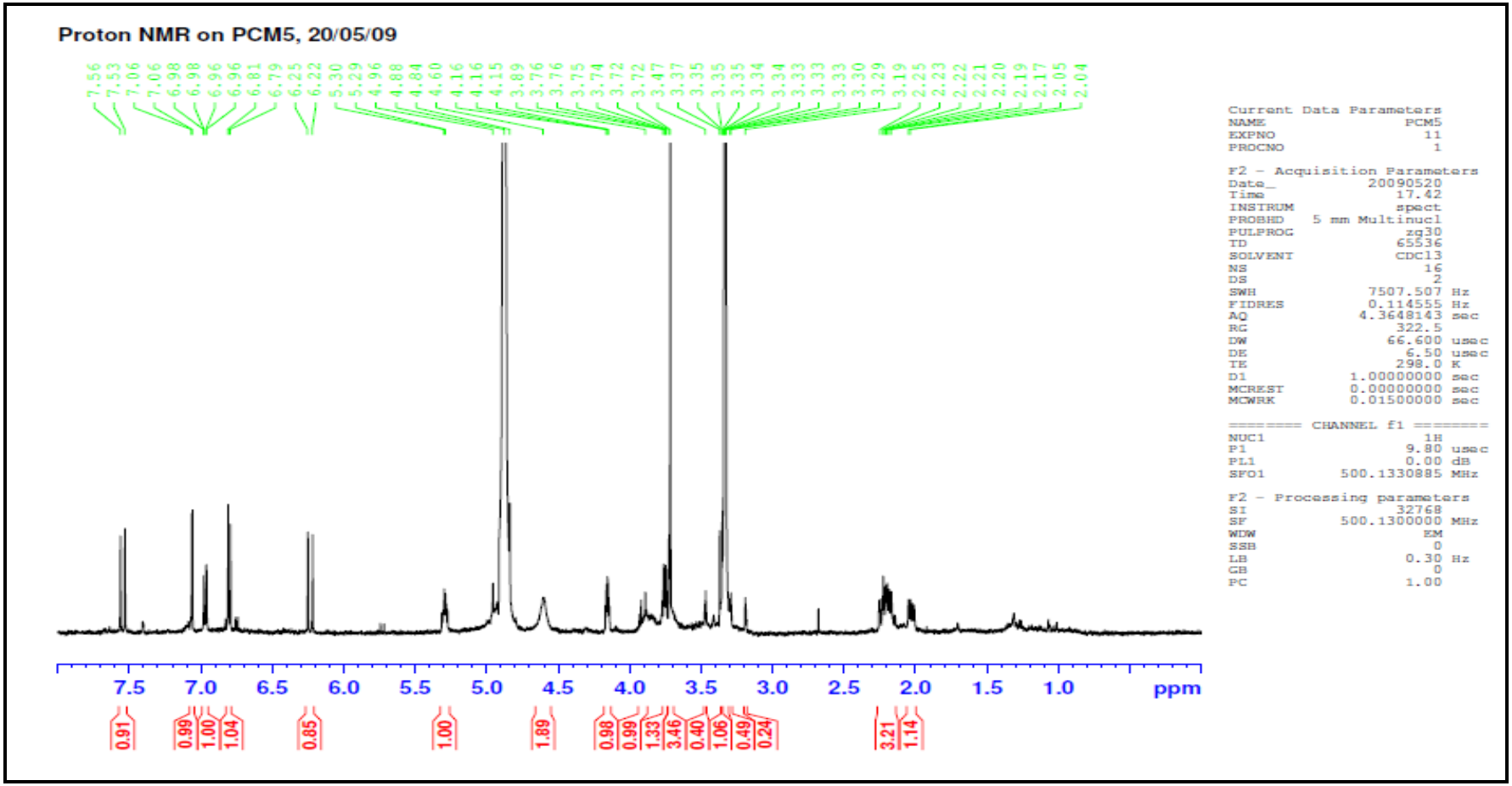


Fig.7.15 d ii. Proton NMR spectrum (500 MHz) of PCM 5 from *P. crassipes* leaf.

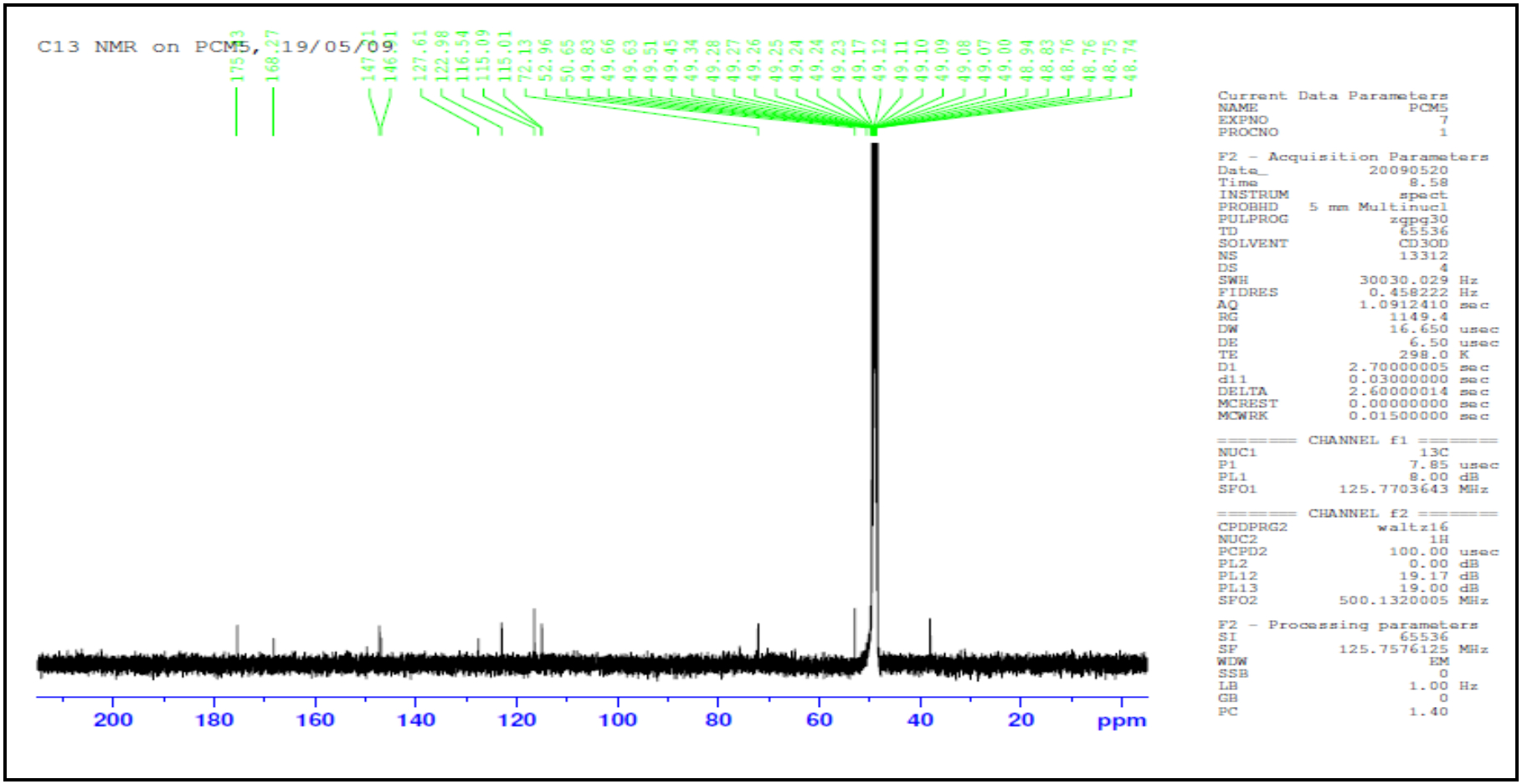


Fig.7.15d iii. Carbon 13 NMR (125 MHz) spectrum for PCM 5 from *P. crassipes* leaf.

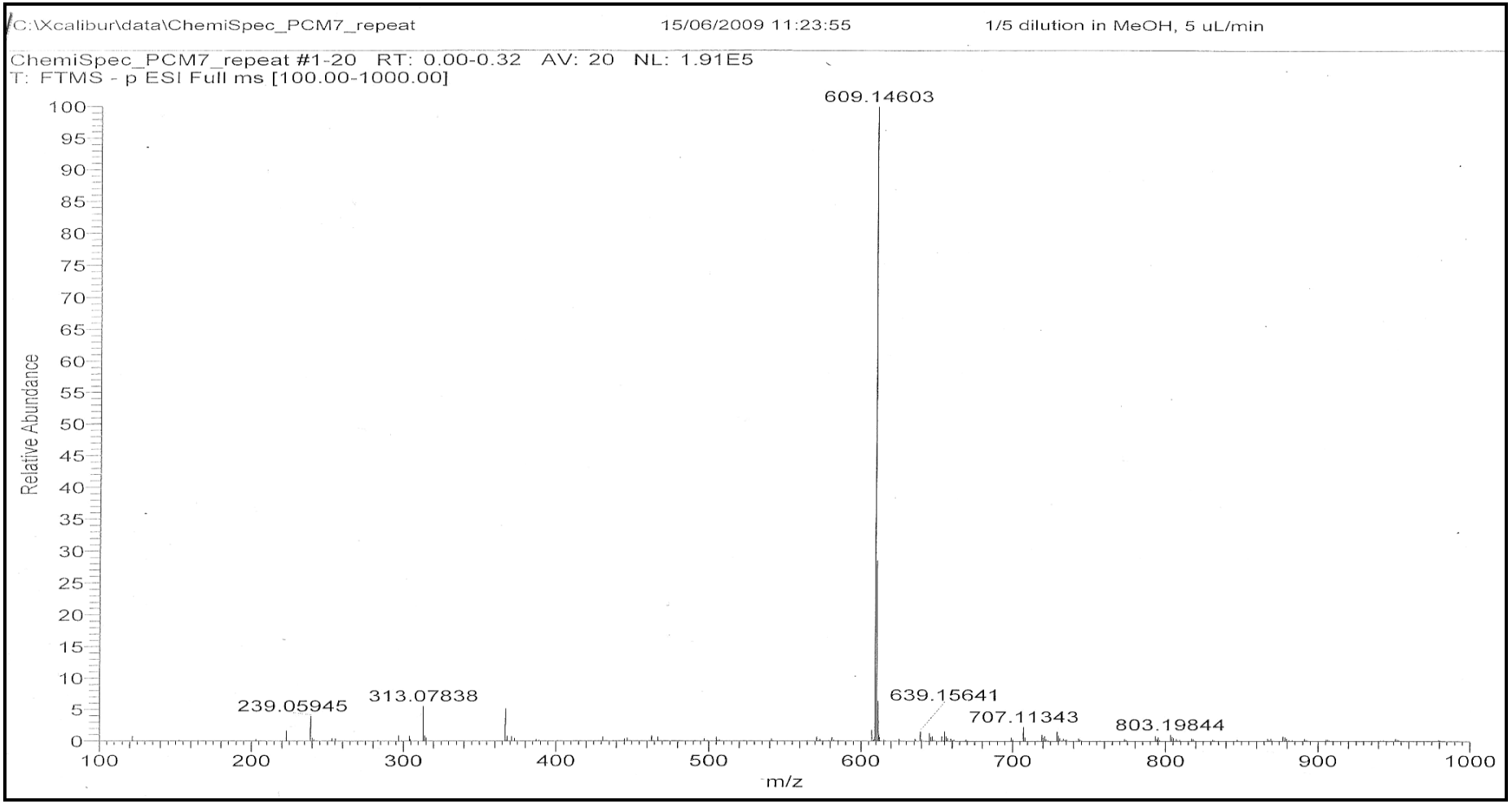


Fig.7.15e i. Accurate mass measurement for PCM 7 from *P. crassipes* leaf by ESI- MS (negative ion mode)

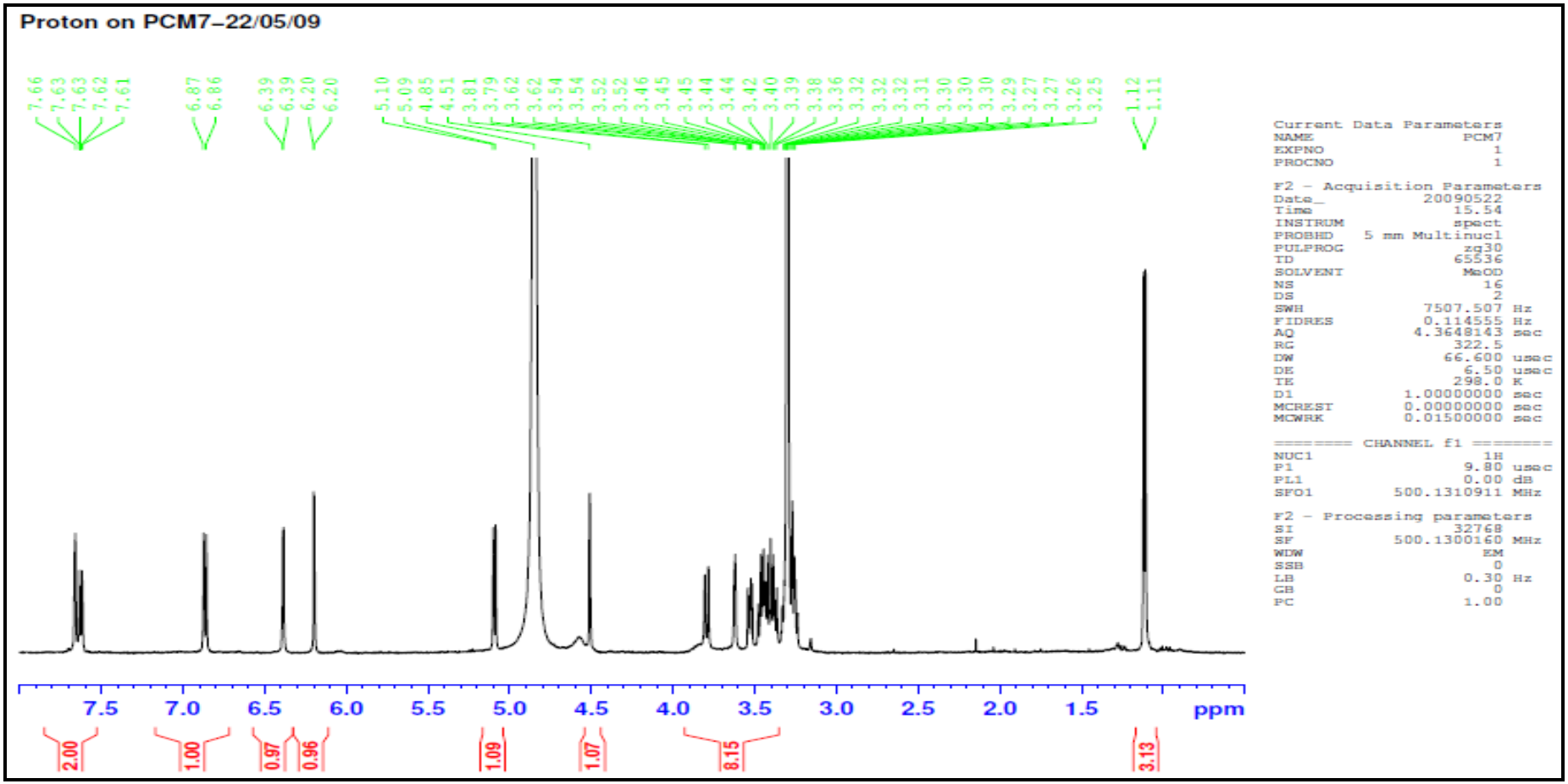


Fig.7.15e ii. Proton NMR spectrum (500 MHz) of PCM 7 from *P. crassipes* leaf

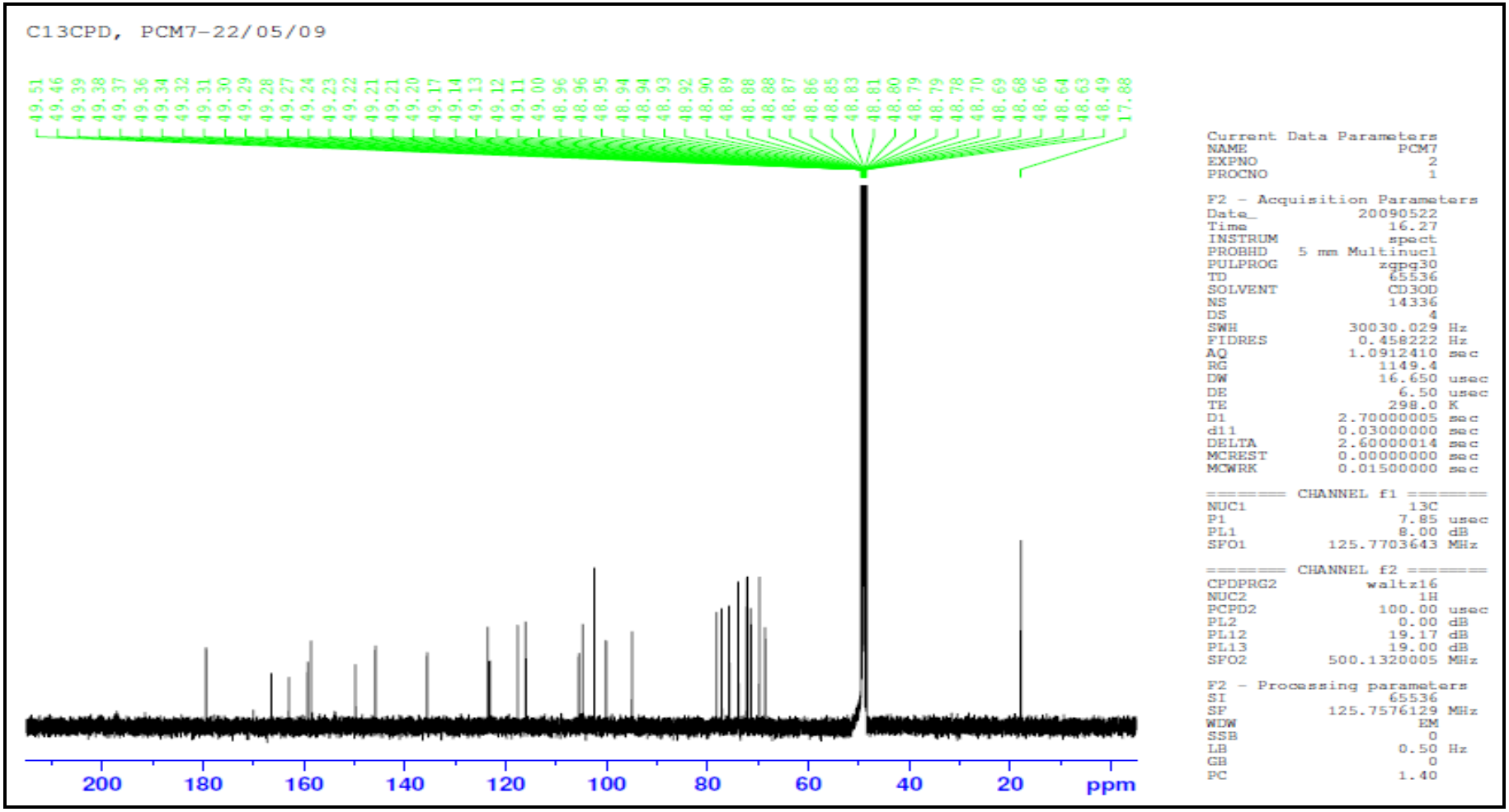


Fig.7.15e iii. Carbon 13 NMR (125 MHz) spectrum of PCM 7 from *P. crassipes* leaf

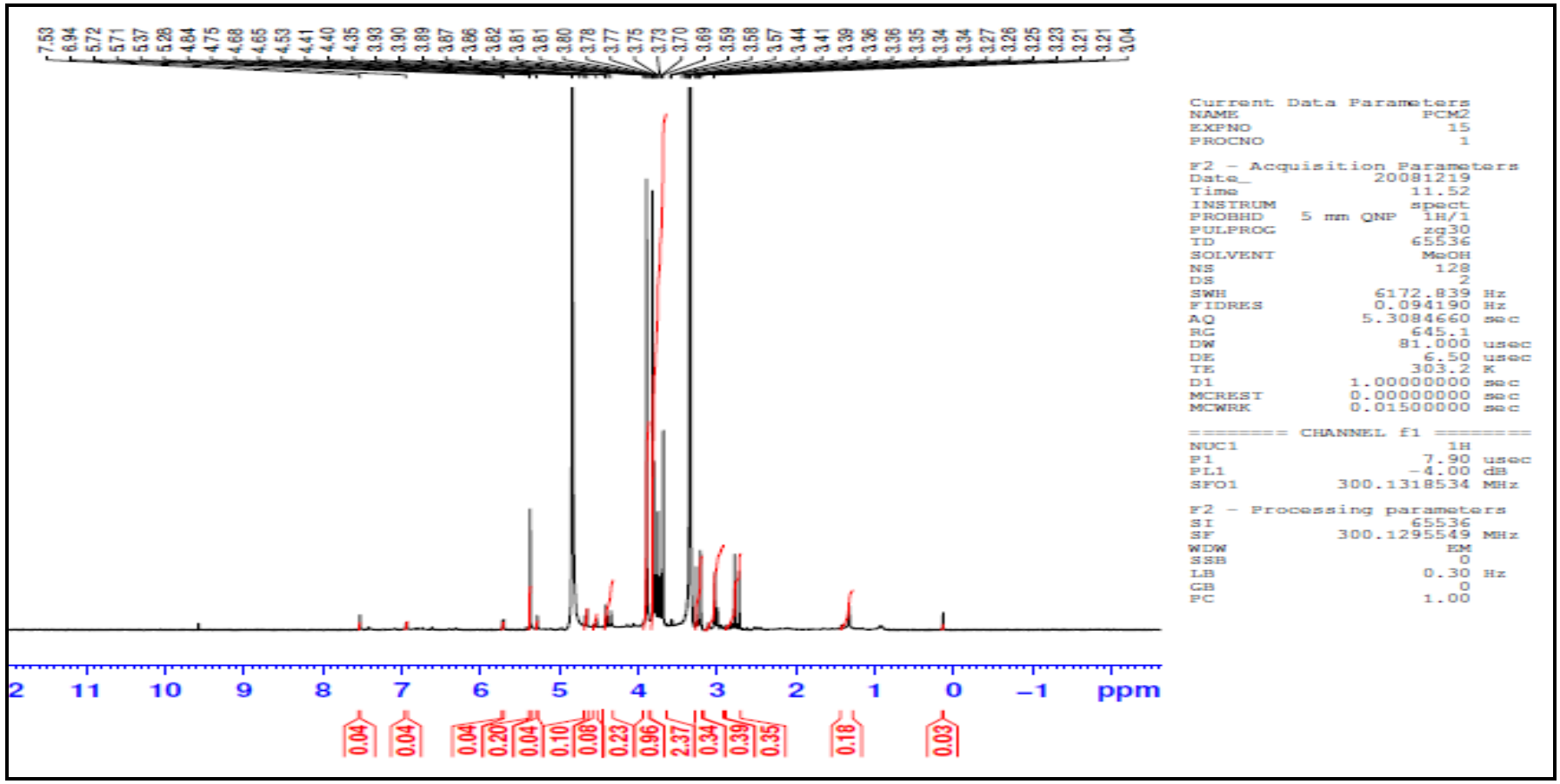


Fig.7.16.i. Proton NMR spectrum of PCM 2 from *P. crassipes* leaf

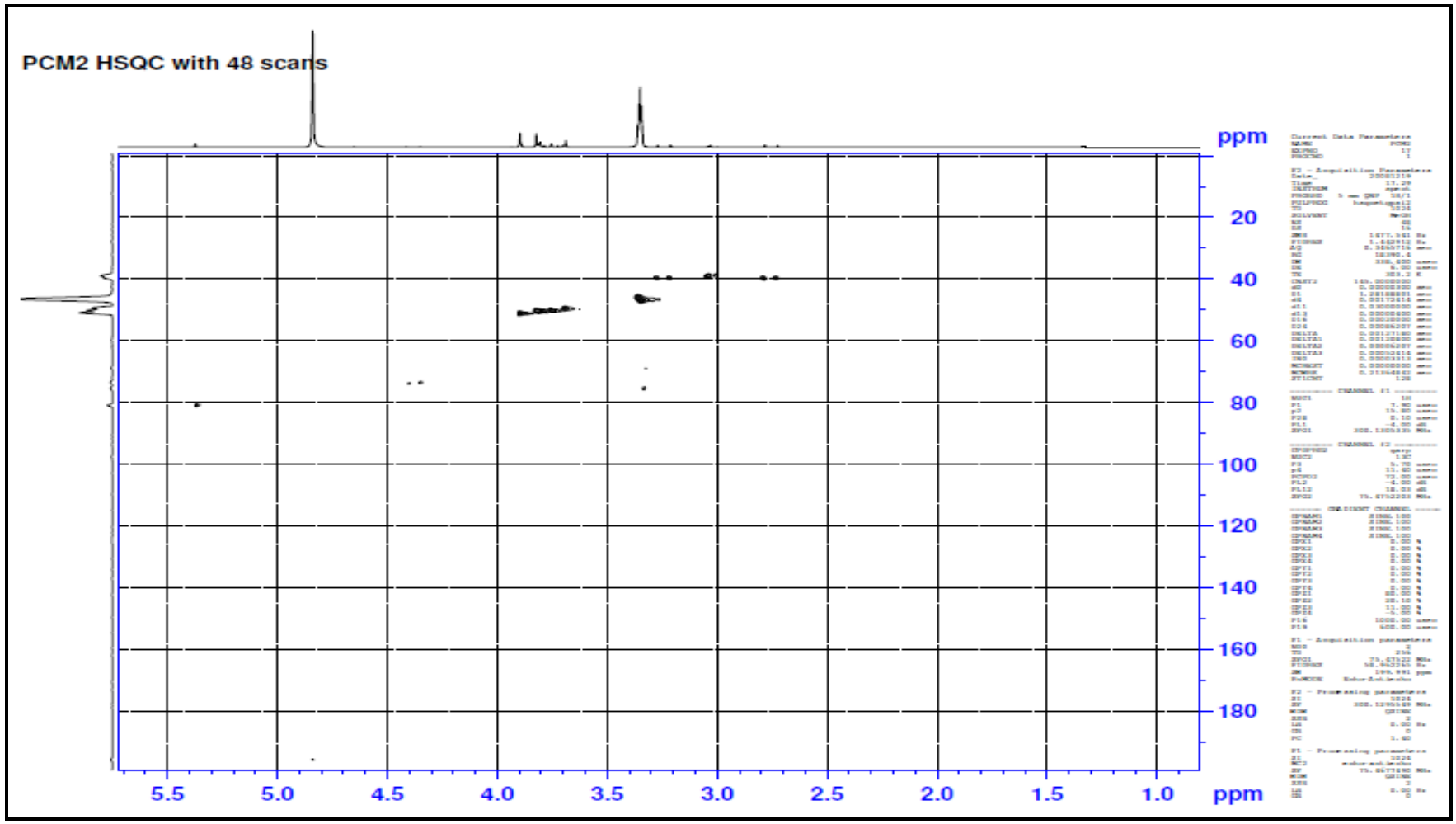


Fig.7.16.ii. HSQC spectrum of PCM 2 from *P. crassipes* leaf

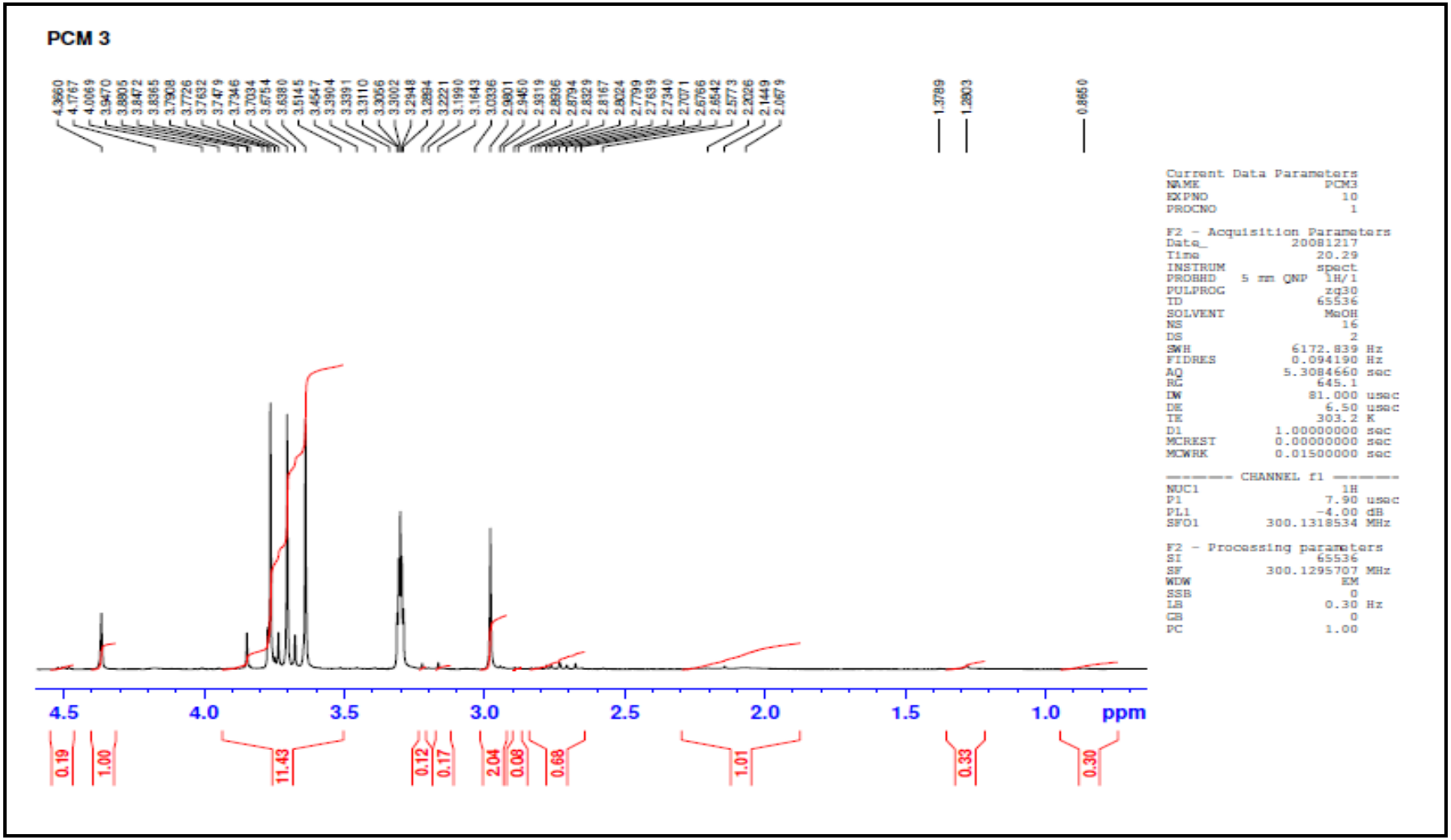


Fig.7.17.i. Proton NMR spectrum (300MHz) of PCM 3 from *P. crassipes* leaf

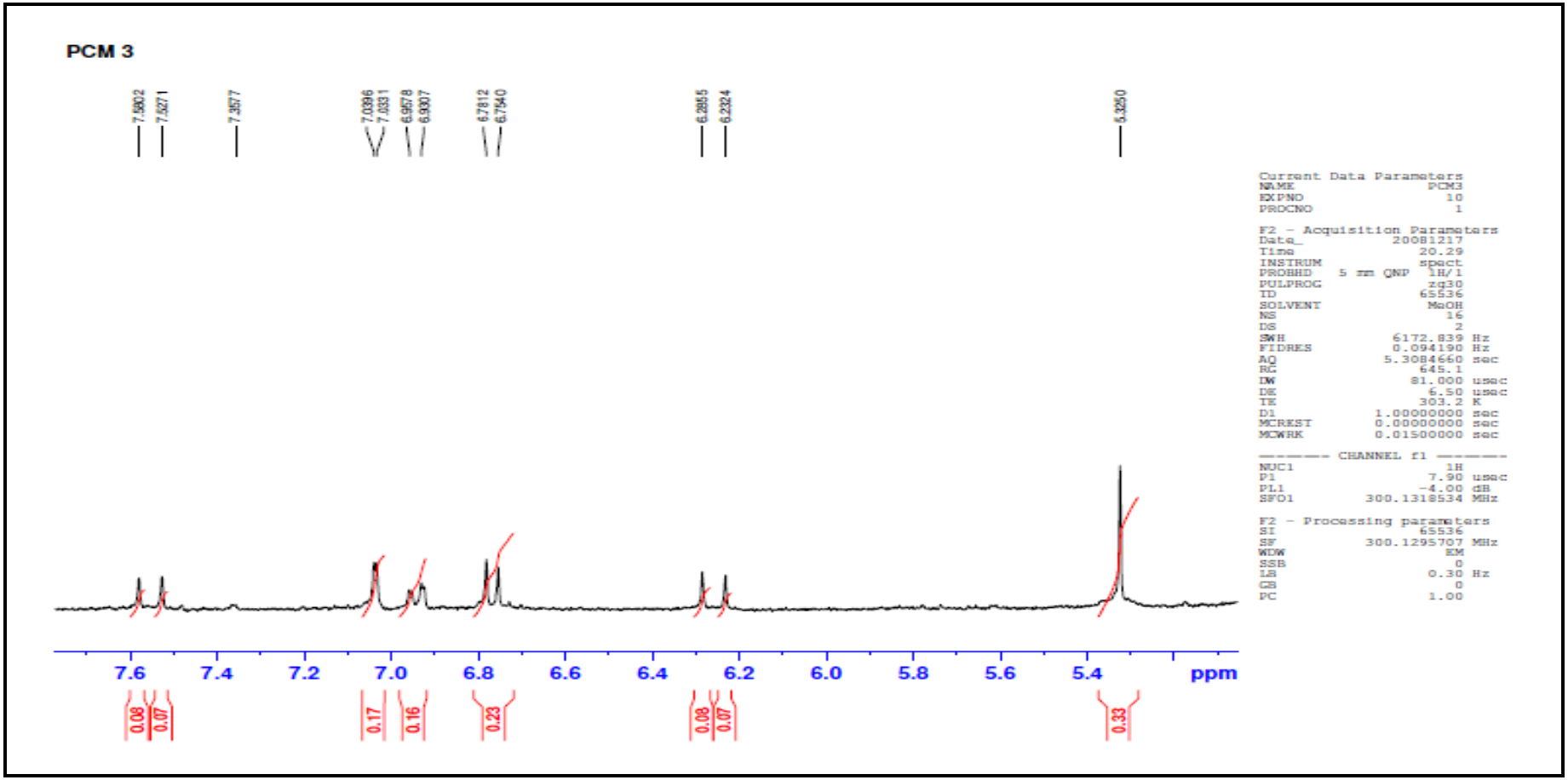


Fig.7.17.ii. Proton NMR spectrum (300 MHz) of PCM 3 from *P. crassipes* leaf

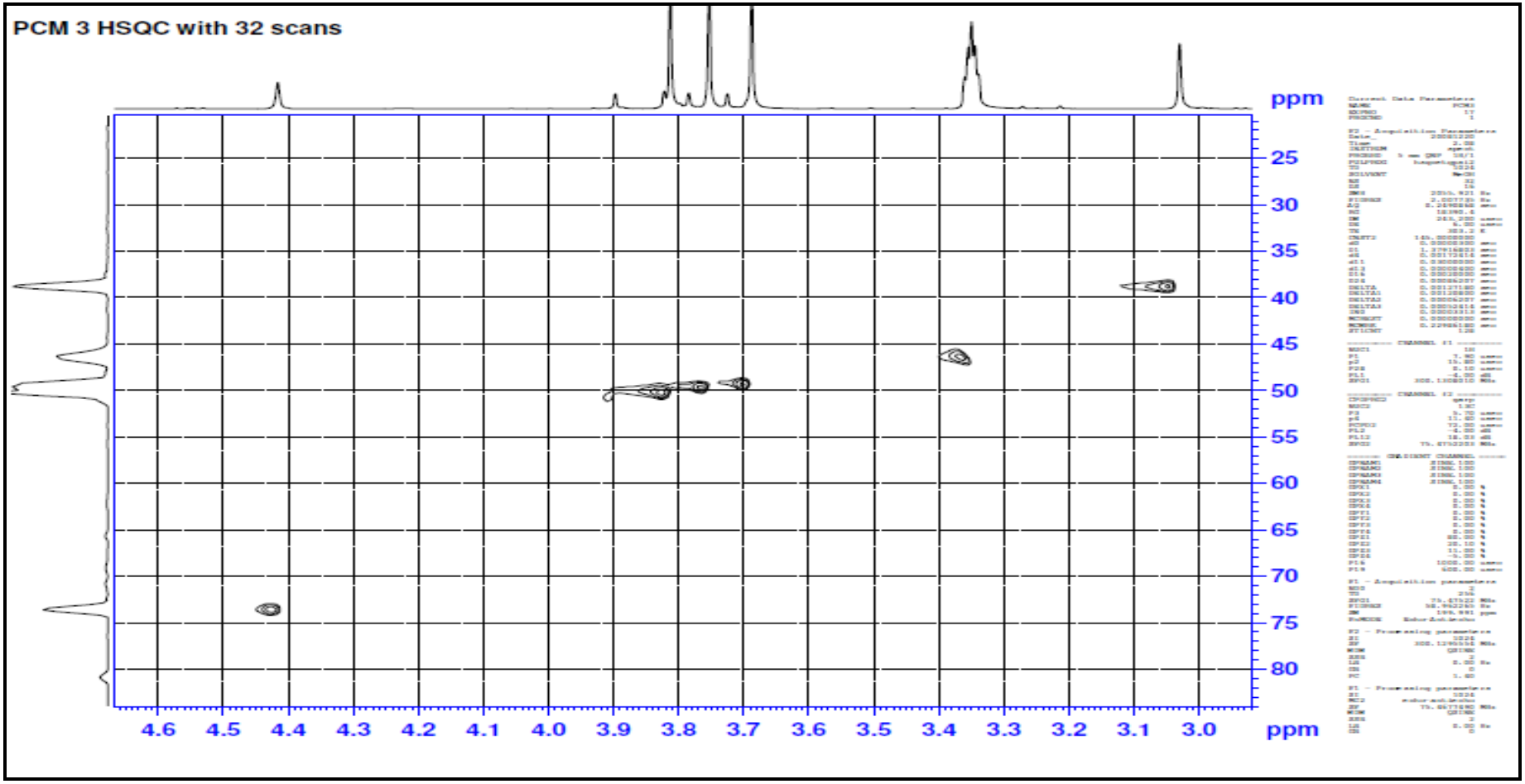


Fig.7.17.iii. HSQC spectrum of PCM 3 from *P. crassipes* leaf

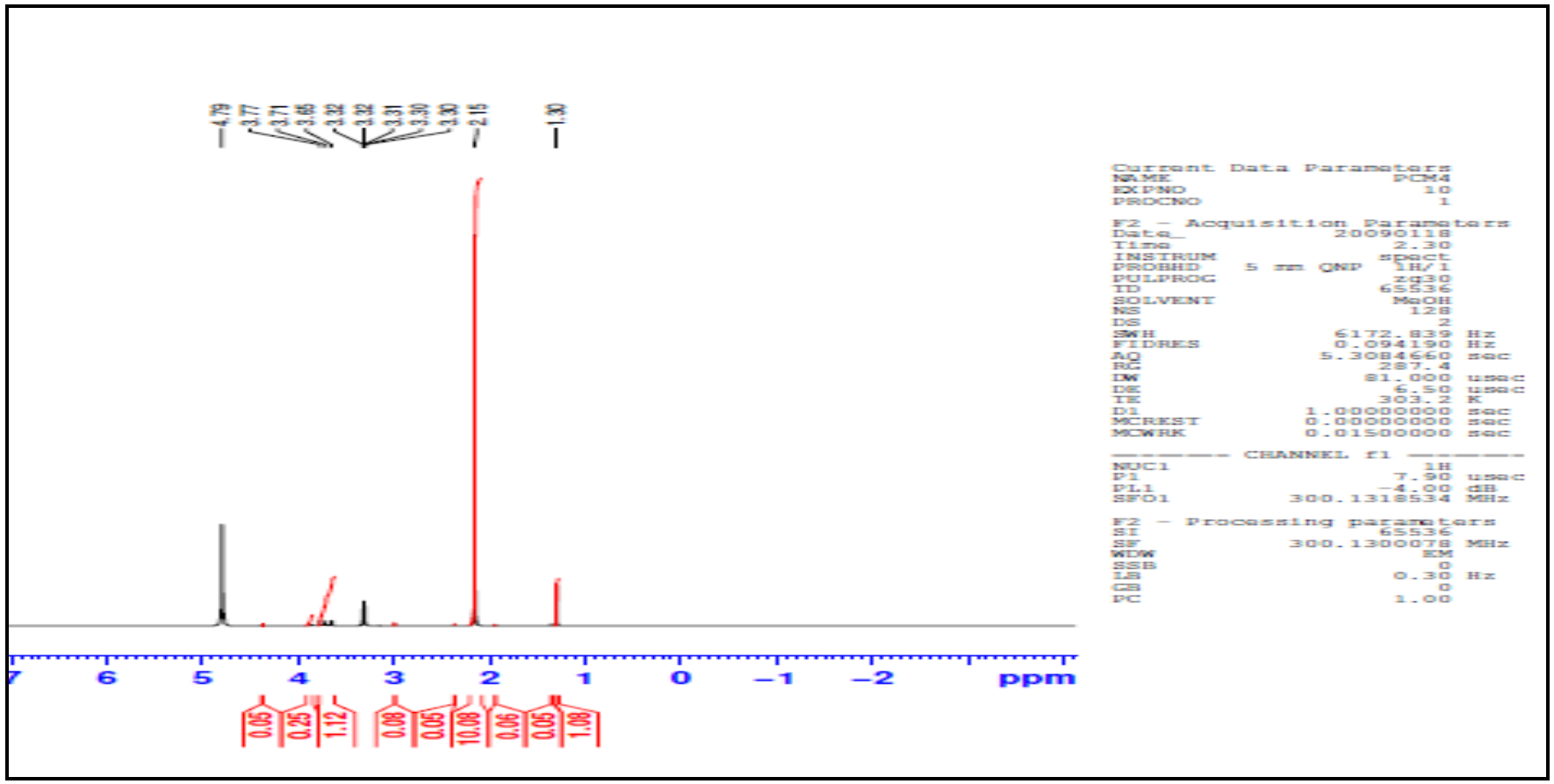


Fig.7.18.i. Proton NMR (300 MHz) spectrum of PCM 4 from *P. crassipes* leaf

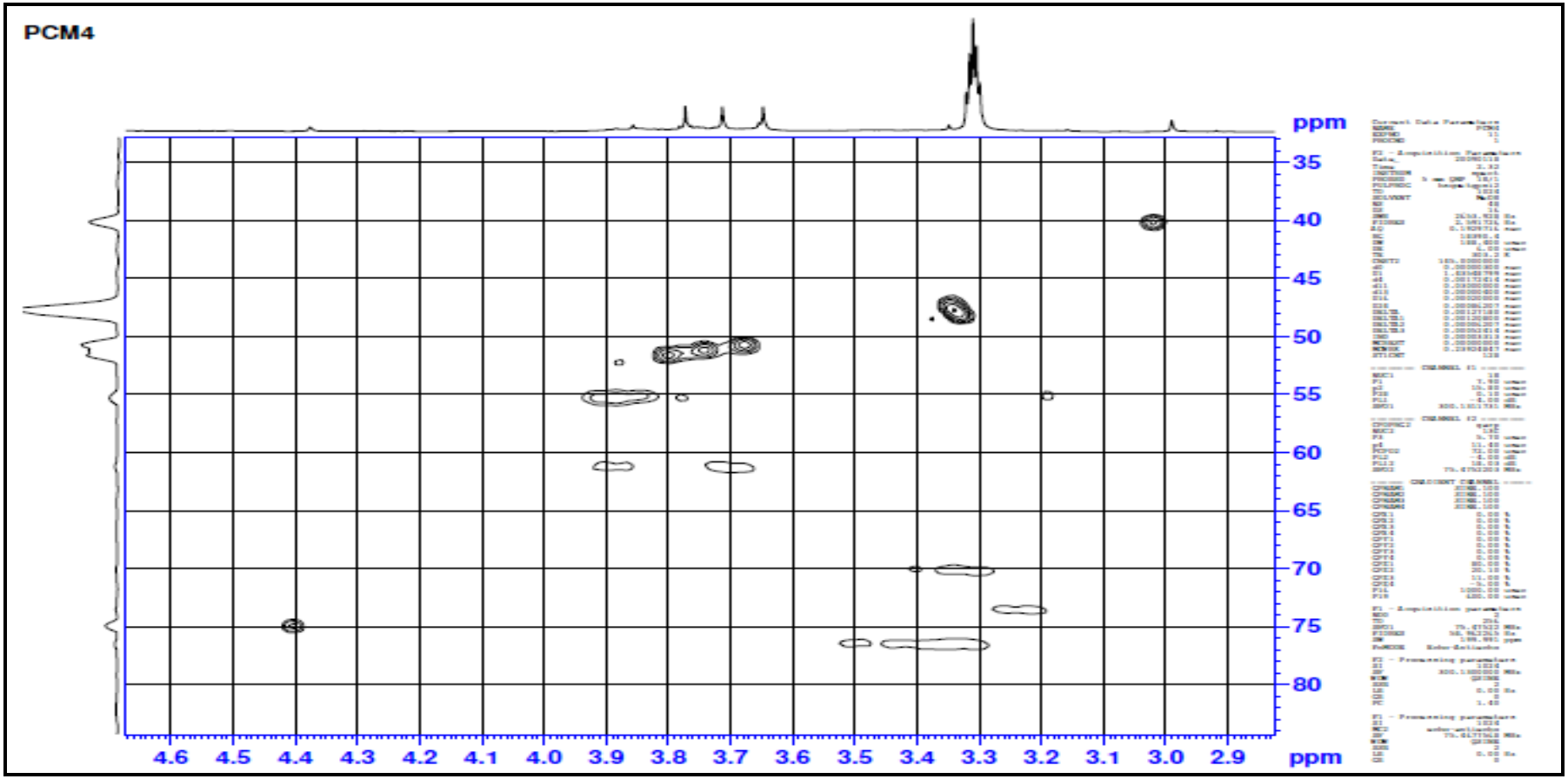


Fig.7.18.ii. HSQC spectrum of PCM 4 from *P. crassipes* leaf

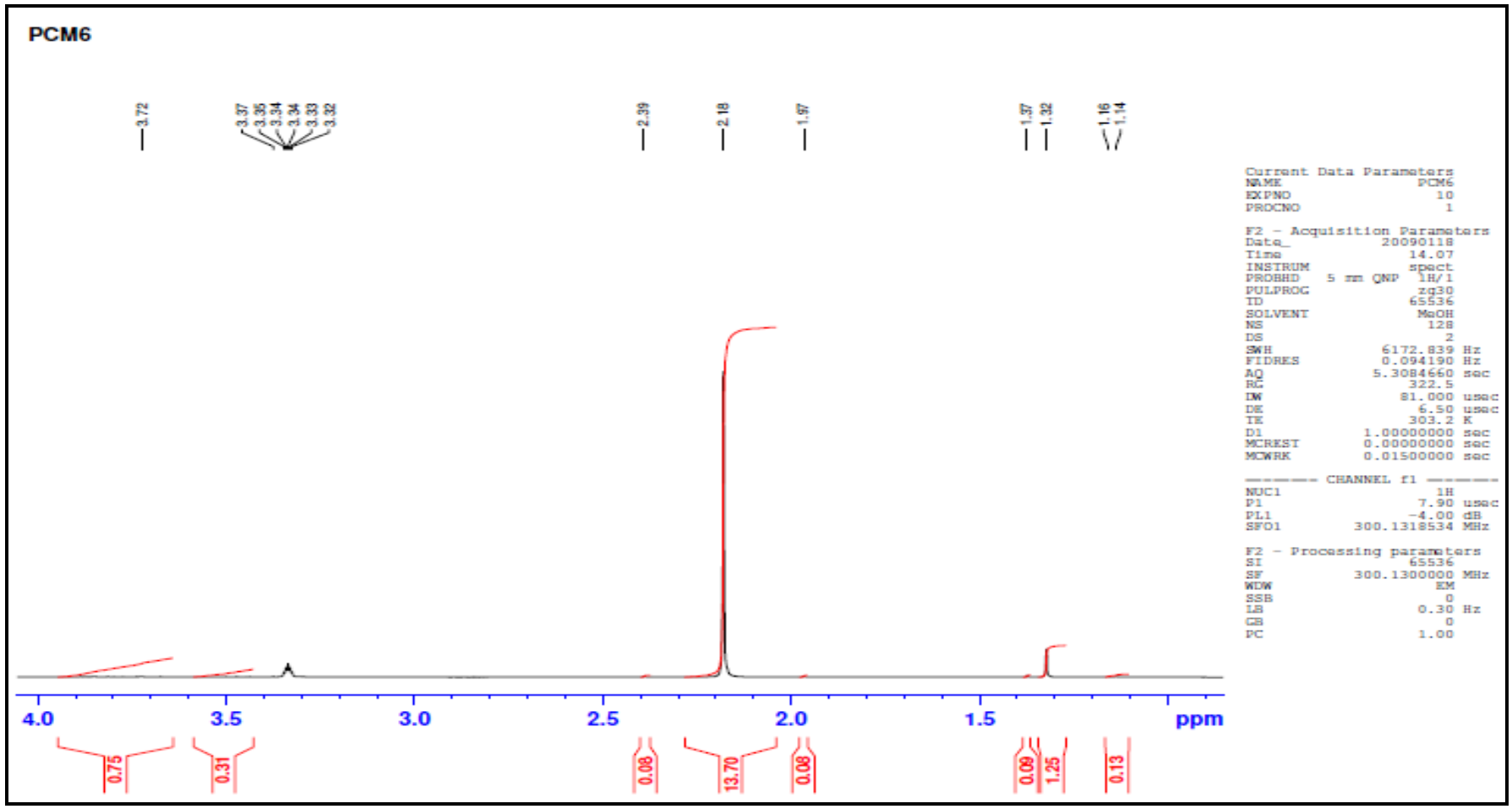


Fig.7.19.i. Proton NMR (300 MHz) spectrum of PCM 6 from *P. crassipes* leaf

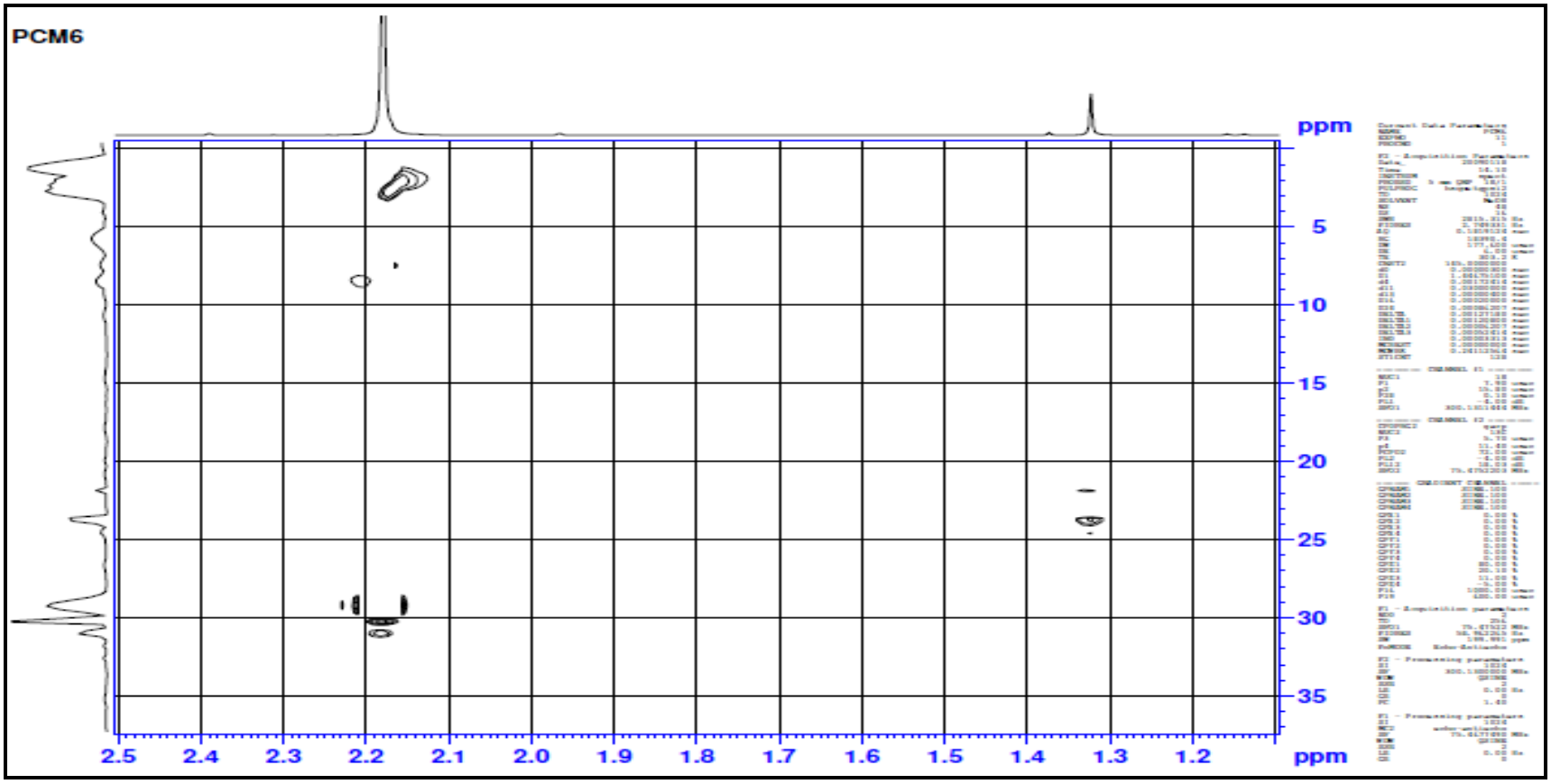


Fig.7.19.ii. HSQC spectrum of PCM 6 from *P. crassipes* leaf

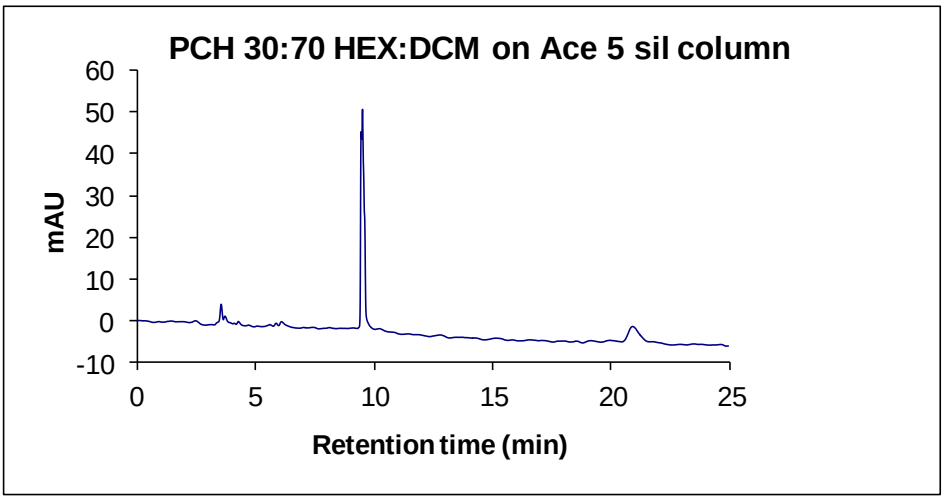


Fig.7.20 Chromatographic profile of *P. crassipes* hexane extract on ACE 5 SILICA column, 250 x 4.6 mm using hexane:DCM (30:70, v/v) mobile phase.

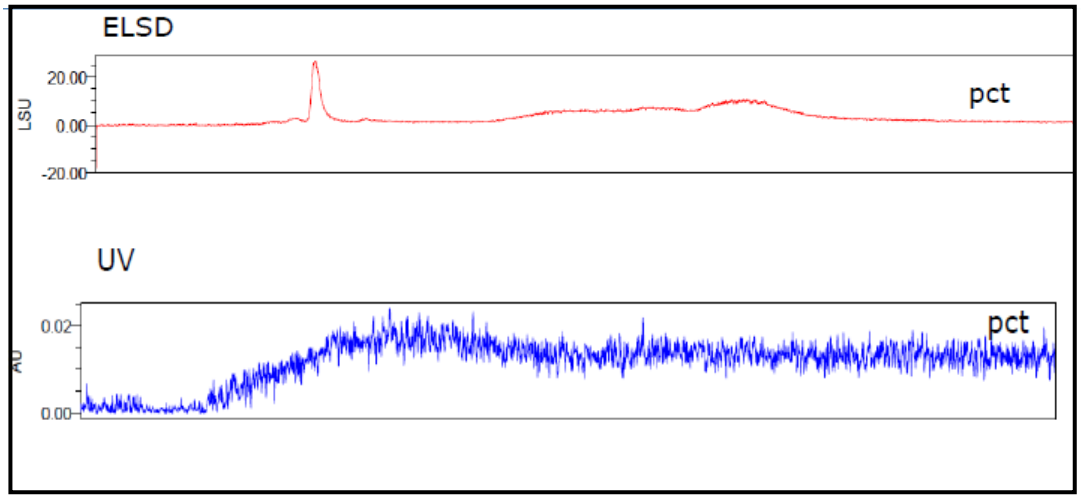


Fig. 7.21 SFC profile of *P. crassipes* total water extract solvent screening on fluorophenyl column, 150 x 4.6 mm i.d. using methanol-water (98:2).

LC/UV and SFC profiles of *P. crassipes* hexane and total water extracts are shown in Figures 7.20 and 7.21 respectively. The Preparative RPLC of *P. crassipes* methanol extract was successfully used to identify two compounds in conjunction with accurate mass measurement and NMR analyses without rigorous isolation or purification processes. Further work is required in order to identify compounds in the other fractions.

Chapter 8 Conclusions and Suggestions for Further Work

The plants selected for the study were the basis for one of several recipes used in North Central Nigeria for the traditional treatment of coughs and respiratory infections of bacterial origin (Harira Adamu, 2006; Personal communication). This particular remedy had been used by the indigenous people of North Central Nigeria for many years but there was no documented evidence for the bioactive constituents in relation to the use in traditional medicine.

The main objective of the study had been to evaluate the anti-tubercular activities of the plant extracts with a view to isolating the active constituents. The process of structural identification proved very challenging due to the lack of resources at the initial stage of the research and testing of extracts against *M. tuberculosis* virulent strain was performed off-site. However, the results obtained from this study show that the plants had potential anti-TB activities but the most active TB fraction was found to be complex in nature and not amenable to structure determination by the methods used. To help clarify the extensive analytical processes followed in attempting to evaluate the plant materials, these are shown in Figures 8.1 and 8.2.

From systematic classification, families such as Olacaceae, Santalaceae and Loranthaceae which belong to the order Santales are characterised by acetylenic acids, mono- and sesquiterpene alcohols, glycoproteins, lignans, flavonoids *etc* (Evans, 2009). Compounds such as oleanolic acid saponin, C18 acetylenic fatty acids and ximonicane (sesquiterpene) have been reported to be present in *X. americana,* Family Olacaceae (D'Agostino *et al*., 1994; Fatope *et*

al., 2000; Araujo *et al*., 2009). The extracts exhibited anti-bacterial, anti-acne and anti-TB activities at MIC levels of 31.25 µg/mL, 62.5 µg/mL and 30.5 µg/mL. Anti-TB activities of natural products have been attributed to terpenes in a majority of cases while oleanolic acid derivatives have been used in cosmetic products (Copp, 2003; Gordien, 2009). Steroidal constituents have also been demonstrated to have anti-TB activities (Saludes, 2002). There were suggestions that stigmast-3, 5 -diene, stigmasterol oleate and β-sitosterol derivatives were possible constituents of the TB - active fraction. The anti -TB activities of the plant extracts may therefore be attributed to the presence of a mixture of fatty acids, triterpenoid and phytosterol- related compounds.

Comparatively, *P. crassipes* which belongs to family Rubiaceae is characterised by a wide variety of classes of natural products e.g. alkaloids of indole, oxindole, quinolone and purine types, anthocyanins, iridoids as well as anthraquinones (Heinrich *et al.,* 2004). In this study, *P. crassipes* extracts exhibited less inhibition against most of the gram positive bacteria tested but the activity against gram negative bacteria was better compared to *X. americana* extracts. Caffeoylquinic acid methyl ester and rutin were isolated and characterised from *P. crassipes*. Rutin has been reported to have anti-inflammatory actions and anti-oxidant properties by other investigators (Guardia *et al*., 2001; Amos *et al*., 1998). Based on the philosophy of synergy in alternative medicine, it may thus be acting in concert with *X. americana* in an anti-inflammatory or anti-oxidant capacity to enhance the overall pharmacological effect.

SFC results showed that ELSD was better adapted for detection of constituents from the TB active fractions than UV detection. Pressure and temperature modifications in the operating conditions did not result in better separations while gradient analysis gave better separations compared to isocratic conditions. Importantly, there was clear evidence that complexity of the TLC and LC fractions had been the reason for the difficulties in identifying individual constituents. In future work, preparative SFC or SFC-MS with the aid of the NIST database would clearly be useful in further studies to identify constituents. LC-NMR with $CDCl_3$ as mobile phase would be another option.

Overall though, the results obtained from this study contributed to current knowledge about the plants and provide a platform for prioritizing further

studies. The information could also be of great benefit in the quality control and toxicity testing of the herbal remedy which would ultimately aid the provision of safer ethnomedicines for the indigenous users.

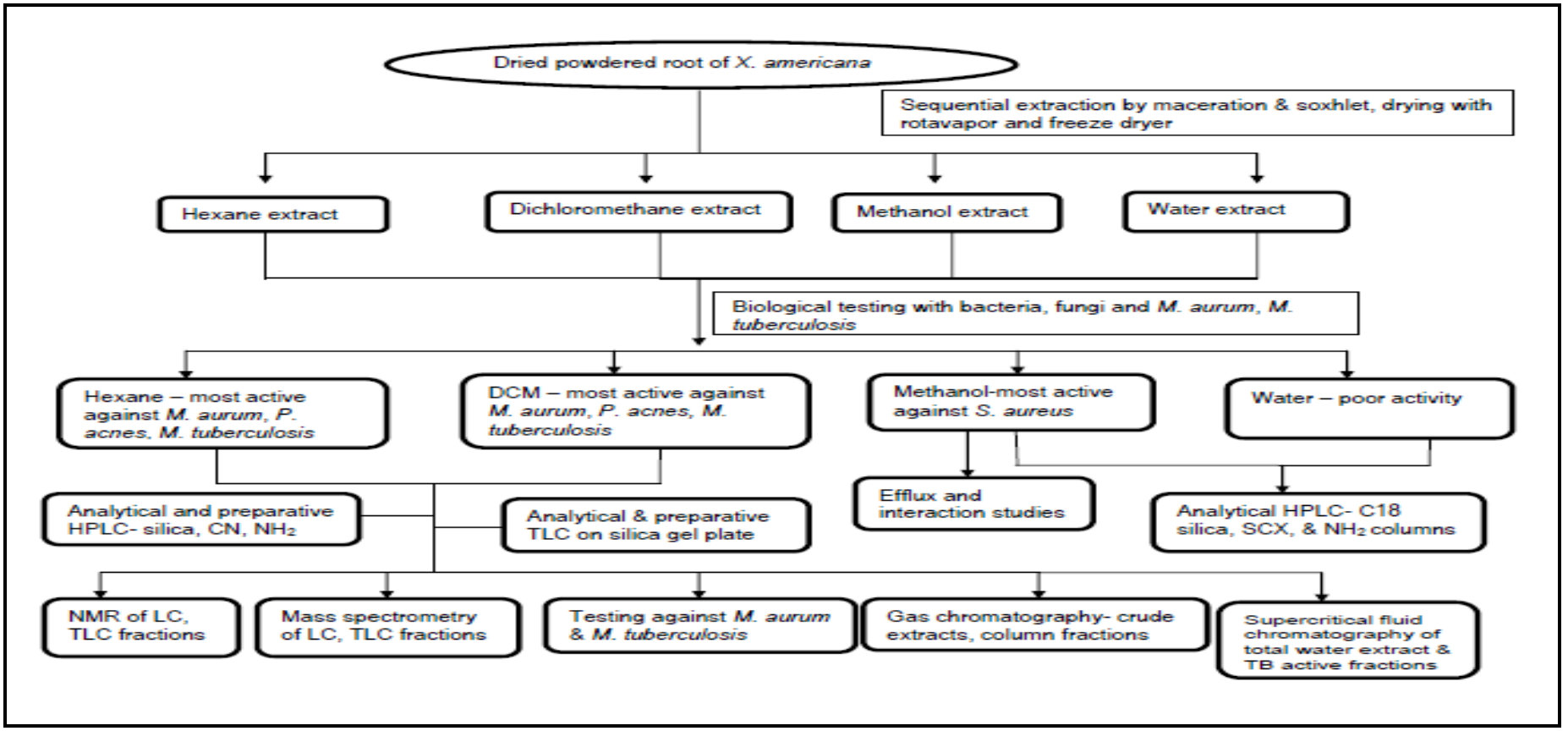


Fig. 8.1. Flow chart showing the analytical steps taken with *X. americana* extract.

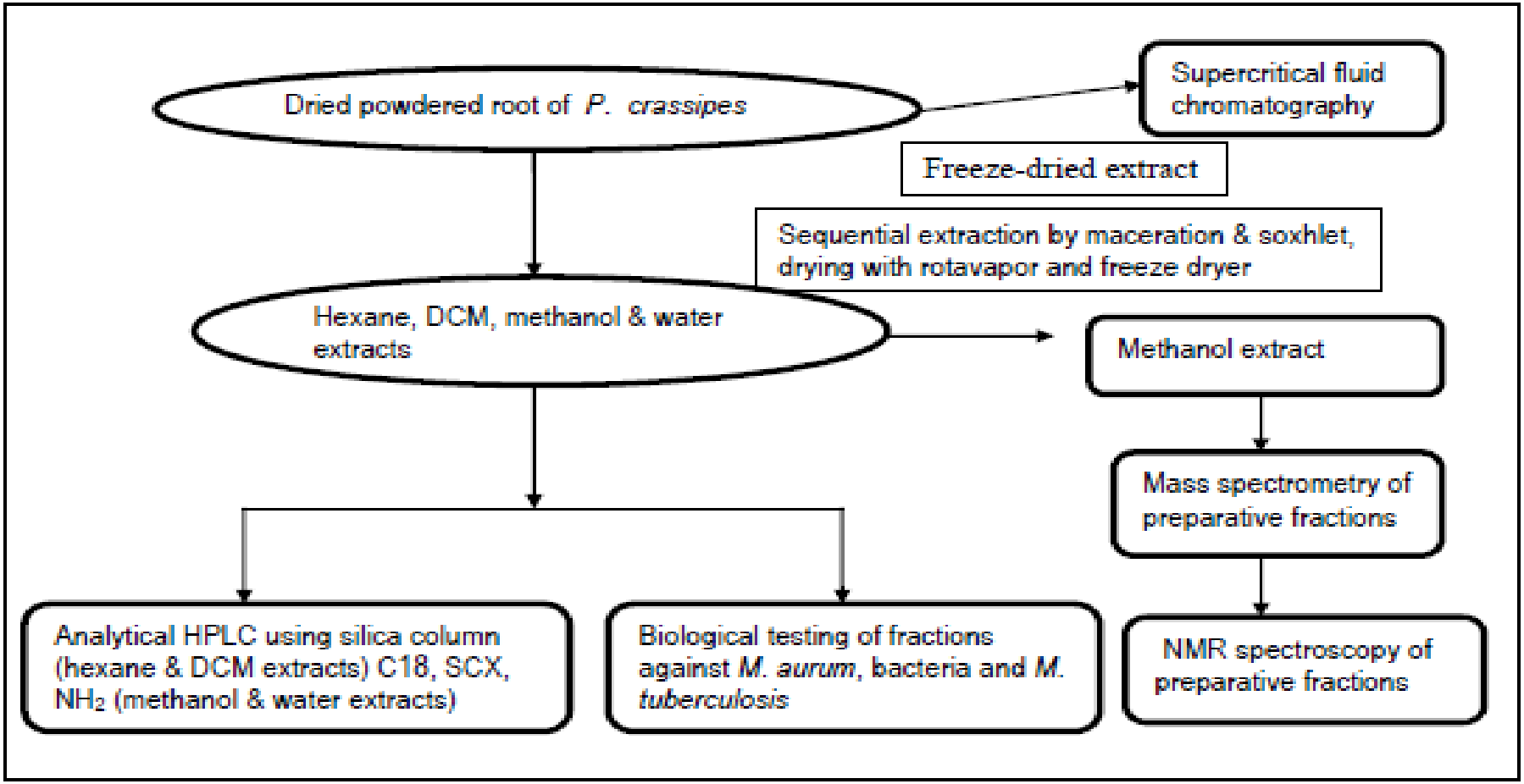


Fig. 8.2. Flow chart showing analytical steps taken with *P. crassipes*

8.1 Further Work

The full elucidation of the ambiguous structures from *X. americana* and *P. crassipes* could be continued by using SFC-UV/ELSD-MS as a tool of green technology. LC-NMR is another useful technique which could aid the elucidation of structures from the extracts after further purification of the fractions. In particular, the use of deuterochloroform and/or deuteromethanol on a microbore LC set-up with mobile phase delivered by a large volume loop set up between the pump and the LC column which might contain hypercarb to give more suitable retentivity and better selectivity than ACE CN or ACE silica.

Also SFC separations obtained would be transferrable to USFC-MS (Capillary SFC not being readily available commercially nowadays), for identification (EI; NIST) or prep SFC to isolate individual compounds. Characterised pure compounds could be subjected to further TB and cytotoxic testing, potentially leading to a lead optimisation semi-synthetic programme.

Furthermore, enzyme inhibition studies on the active drug molecule could be initiated to determine whether the extracts are enzyme inducers and could affect anti-TB activity in the probability of co-administration with HIV drugs. Current TB drug regimens have shown some incompatibility with HIV/AIDS drugs due to cytochrome P3A4 enzyme induction (CYP3A4) by rifampicin (Lilienkampf *et al*., 2009). The ability of drugs to act as inducers, inhibitors, or substrates for CYP3A is predictive of whether concurrent administration of these compounds with a known CYP3A substrate might lead to altered drug disposition, efficacy or toxicity (Zhou, 2008). Several herbal and dietary components have been implicated as CYP3A4 inhibitors suggesting that this may be a productive line of investigation.

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Appendix A: Tables showing methods used to prepare antibiotics and to determine MIC values.

1). Preparation and storage of antibiotic solutions (≥ 1000 mg/L)

Antibiotic	solvent	diluent	4°C	-20°C	-70°C
a. Ampicillin(trihydrate) (prepare fresh)	sat.$NaHCO_3$ solution	water	7days	unstable	30 days
b. Gentamicin SO_4	water	water	6 mths	NR	NR
c. Strept. SO_4	water	water	-	-	-
d. Sulphamethoxazole (free acid)	Water+0.1M NaOH to dissolve	water	1 mth	6 mths	2 yrs
e. Trimethoprim base	1 mL water+ 10 µL Acetic acid	water	1 mth	6 mths	2 yrs
f. Tetracycline HCl (prepare fresh)	water	Water	-	NR^i	NR^i

NR- Not recommended, NR^i – precipitates on freezing

2). MIC determination (µg / mL) by macrodilution method (BSAC).

	S. aureus	*E. coli*	*P. aeruginosa*
Ampicillin (etest strips)	8	>256	>256
Streptomycin	0.5	0.25	2
Gentamicin	0.03	0.25	0.13
Tetracycline	0.03	0.06	2
Sulphamethoxazole	>128	<2	>128
Trimethoprim	0.13	<0.03	32

3). Data for synergy testing by checkerboard analysis

DYNEX REVELATION 3.04

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 26/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 22:33:53
PLATE	: stm vs xm1	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.112	0.141	0.216	0.268	0.373	0.580	0.595	0.411	0.034	0.074	0.033	0.036
B	0.120	0.135	0.185	0.312	0.509	0.690	0.586	0.422	0.034	0.080	0.033	0.036
C	0.116	0.155	0.206	0.371	0.551	0.589	0.613	0.426	0.038	0.089	0.033	0.034
D	0.119	0.143	0.208	0.259	0.465	0.546	0.578	0.425	0.034	0.091	0.033	0.034
E	0.114	0.160	0.257	0.290	0.498	0.575	0.584	0.445	0.033	0.096	0.032	0.036
F	0.114	0.164	0.208	0.307	0.532	0.653	0.629	0.455	0.034	0.109	0.035	0.035
G	0.120	0.148	0.204	0.293	0.521	0.701	0.626	0.501	0.033	0.109	0.035	0.035
H	0.121	0.155	0.203	0.403	0.538	0.710	0.635	0.455	0.033	0.113	0.033	0.034

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

16

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 14:54:48
PLATE	: stm-xm1final	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.073	0.083	0.227	0.333	0.653	0.784	0.835	0.504	0.033	0.562	0.033	0.035
B	0.085	0.074	0.190	0.406	0.735	0.922	0.768	0.534	0.033	0.396	0.033	0.036
C	0.083	0.171	0.210	0.413	0.755	0.946	0.876	0.555	0.034	0.421	0.033	0.035
D	0.145	0.360	0.352	0.340	0.636	0.792	0.846	0.542	0.033	0.450	0.032	0.034
E	0.227	0.734	0.686	0.425	0.632	0.848	0.787	0.574	0.033	0.464	0.032	0.037
F	0.213	0.739	0.494	0.402	0.691	0.900	0.810	0.587	0.034	0.457	0.034	0.035
G	0.564	0.793	0.344	0.489	0.676	0.803	0.827	0.655	0.033	0.491	0.035	0.035
H	0.390	0.722	0.758	0.477	0.640	0.984	0.846	0.573	0.033	0.463	0.033	0.034

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

1c

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 17:06:33
PLATE	: xm-stm,mtt 1	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.264	0.128	0.362	0.384	0.804	1.233	1.805	1.898	0.033	0.724	0.033	0.035
B	0.095	0.112	0.293	0.483	1.254	1.241	1.770	1.714	0.033	0.475	0.033	0.035
C	0.234	0.123	0.503	0.668	1.349	1.477	1.814	1.699	0.034	0.738	0.033	0.035
D	0.331	0.215	0.209	0.649	0.808	1.337	1.609	1.876	0.033	0.978	0.032	0.034
E	0.200	0.901	0.616	0.644	1.023	1.244	1.602	2.108	0.033	0.710	0.033	0.037
F	0.860	0.446	0.277	0.479	0.960	1.570	1.651	1.977	0.035	0.677	0.034	0.036
G	0.349	1.289	0.194	0.418	1.098	1.438	2.149	1.749	0.035	0.808	0.034	0.034
H	0.692	1.000	0.788	0.656	0.942	1.412	1.806	1.928	0.033	0.754	0.033	0.034

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 26/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 22:36:20
PLATE	: stm vs xm2	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.120	0.124	0.127	0.244	0.363	0.702	0.624	0.429	0.034	0.075	0.033	0.034
B	0.108	0.137	0.198	0.275	0.334	0.555	0.586	0.430	0.033	0.079	0.033	0.034
C	0.120	0.166	0.233	0.263	0.471	0.575	0.602	0.458	0.033	0.080	0.033	0.035
D	0.111	0.147	0.229	0.295	0.525	0.661	0.594	0.437	0.034	0.086	0.033	0.034
E	0.105	0.174	0.212	0.220	0.545	0.661	0.570	0.459	0.034	0.089	0.033	0.034
F	0.127	0.132	0.217	0.311	0.526	0.806	0.635	0.469	0.033	0.102	0.033	0.035
G	0.117	0.141	0.232	0.387	0.534	0.639	0.626	0.467	0.033	0.104	0.033	0.036
H	0.118	0.190	0.228	0.346	0.561	0.693	0.658	0.475	0.033	0.102	0.033	0.035

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

26

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 14:56:50
PLATE	: stm-xm2 final	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.098	0.067	0.118	0.350	0.498	0.865	0.944	0.558	0.033	0.318	0.033	0.034
B	0.092	0.106	0.233	0.291	0.464	0.872	0.735	0.567	0.033	0.450	0.033	0.034
C	0.195	0.541	0.311	0.369	0.559	0.869	0.889	0.571	0.034	0.370	0.033	0.035
D	0.206	0.730	0.461	0.295	0.611	0.845	0.703	0.540	0.034	0.414	0.033	0.034
E	0.203	0.651	0.413	0.252	0.612	0.807	0.697	0.588	0.033	0.670	0.033	0.035
F	0.200	0.737	0.641	0.340	0.619	0.942	0.773	0.588	0.033	0.428	0.035	0.036
G	0.479	0.742	0.576	0.415	0.689	0.777	0.771	0.603	0.033	0.478	0.033	0.036
H	0.481	0.691	0.644	0.456	0.662	0.844	0.801	0.590	0.033	0.425	0.034	0.035

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

2c

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 17:08:14
PLATE	: xm-stm,mtt2	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.084	0.134	0.186	0.362	0.721	1.312	1.748	2.004	0.034	0.697	0.033	0.034
B	0.106	0.237	0.338	0.379	0.499	1.313	1.816	1.860	0.033	0.832	0.033	0.034
C	0.155	0.672	0.438	0.446	0.814	1.301	1.690	2.006	0.034	0.583	0.034	0.036
D	0.540	0.476	1.209	0.759	1.033	1.505	1.762	1.845	0.034	0.910	0.033	0.034
E	0.369	0.930	0.525	0.741	0.790	0.999	1.599	1.975	0.033	0.839	0.035	0.035
F	0.248	0.201	0.764	0.542	1.012	1.617	1.641	2.078	0.035	0.905	0.035	0.036
G	0.427	0.962	1.080	0.704	1.081	1.544	2.370	2.094	0.033	0.712	0.033	0.038
H	0.153	0.988	1.071	0.667	1.083	1.405	1.613	2.019	0.033	0.939	0.034	0.035

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

Name	: Spectroscopy Suite
Address	: 104 Fleming Building
	: School of HNSS
	: University of Sunderland
Phone	:
FAX	:

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 26/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 22:38:07
PLATE	: Neg contr, stm1	REF. FILTER	: *	OPERATOR	: PO

OVER Limit	: 4.000
Calculation mode	: Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.072	0.068	0.068	0.067	0.066	0.068	0.077	0.073	0.032	0.079	0.033	0.034
B	0.067	0.068	0.067	0.070	0.072	0.073	0.079	0.072	0.033	0.080	0.033	0.034
C	0.068	0.068	0.068	0.067	0.070	0.079	0.081	0.075	0.035	0.088	0.033	0.035
D	0.071	0.072	0.068	0.074	0.069	0.072	0.082	0.077	0.034	0.088	0.033	0.037
E	0.069	0.070	0.068	0.075	0.070	0.074	0.091	0.091	0.033	0.096	0.033	0.034
F	0.068	0.069	0.069	0.083	0.075	0.077	0.095	0.092	0.033	0.111	0.034	0.038
G	0.069	0.069	0.070	0.086	0.075	0.083	0.101	0.100	0.034	0.108	0.034	0.035
H	0.069	0.071	0.073	0.099	0.081	0.091	0.115	0.105	0.034	0.107	0.033	0.035

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

4b

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 15:04:30
PLATE	: nc,stm final 1	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

Empty Empty Empty

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.076	0.069	0.070	0.066	0.066	0.065	0.080	0.069	0.025	0.083	0.033	0.034
B	0.067	0.064	0.064	0.069	0.072	0.072	0.073	0.069	0.033	0.077	0.033	0.034
C	0.067	0.065	0.065	0.064	0.067	0.079	0.081	0.070	0.036	0.082	0.033	0.035
D	0.070	0.080	0.068	0.102	0.072	0.069	0.105	0.378	0.034	0.082	0.033	0.036
E	0.427	0.495	0.541	0.647	0.491	0.434	0.429	0.519	0.033	0.086	0.034	0.034
F	0.821	0.568	0.585	0.561	0.663	0.675	0.545	0.558	0.034	0.099	0.036	0.036
G	0.876	0.770	0.596	0.706	0.481	0.581	0.639	0.640	0.034	0.102	0.035	0.039
H	0.805	0.933	0.847	0.770	0.790	0.702	0.723	0.634	0.034	0.105	0.033	0.035

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 16:51:11
PLATE	: nc1stm-mtt	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.070	0.070	0.068	0.066	0.066	0.071	0.083	0.073	0.025	0.082	0.033	0.034
B	0.069	0.067	0.069	0.069	0.074	0.072	0.077	0.072	0.033	0.077	0.036	0.034
C	0.072	0.068	0.068	0.065	0.071	0.078	0.079	0.073	0.038	0.084	0.033	0.035
D	0.092	0.179	0.077	0.201	0.109	0.100	0.259	1.052	0.034	0.084	0.033	0.036
E	0.618	0.587	0.558	0.924	1.403	0.680	1.066	1.058	0.033	0.089	0.034	0.034
F	1.205	0.794	0.598	1.241	0.954	0.985	0.813	1.379	0.034	0.103	0.034	0.036
G	0.846	0.635	1.000	1.249	1.265	0.905	1.358	0.829	0.034	0.107	0.035	0.039
H	1.075	0.646	0.778	1.336	1.020	1.095	0.760	1.544	0.034	0.109	0.033	0.035

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

5a

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 26/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 22:39:42
PLATE	: Neg cont, stm 2	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.066	0.066	0.066	0.067	0.066	0.068	0.071	0.072	0.033	0.076	0.033	0.034
B	0.068	0.067	0.067	0.071	0.069	0.068	0.076	0.074	0.033	0.081	0.034	0.034
C	0.075	0.071	0.068	0.070	0.069	0.078	0.078	0.072	0.033	0.086	0.033	0.035
D	0.069	0.071	0.070	0.074	0.072	0.074	0.083	0.074	0.034	0.085	0.033	0.035
E	0.068	0.069	0.069	0.076	0.070	0.073	0.089	0.082	0.034	0.088	0.035	0.035
F	0.072	0.073	0.071	0.079	0.075	0.074	0.087	0.075	0.033	0.107	0.034	0.036
G	0.069	0.071	0.069	0.096	0.077	0.076	0.096	0.081	0.033	0.105	0.033	0.036
H	0.072	0.070	0.074	0.086	0.079	0.078	0.097	0.092	0.033	0.102	0.033	0.035

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

56

lame : Spectroscopy Suite
ddress : 104 Fleming Building
: School of HNSS
: University of Sunderland
hone :
AX :

EST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
EST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 15:07:35
LATE	: negc, stm2 final	REF. FILTER	: *	OPERATOR	: PO

VER Limit : 4.000
alculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.066	0.066	0.063	0.064	0.063	0.067	0.077	0.074	0.033	0.074	0.033	0.034
B	0.070	0.064	0.063	0.067	0.065	0.065	0.075	0.069	0.033	0.077	0.034	0.034
C	0.077	0.068	0.065	0.066	0.065	0.069	0.072	0.068	0.033	0.080	0.033	0.035
D	0.086	0.068	0.080	0.075	0.072	0.085	0.077	0.105	0.035	0.079	0.033	0.035
E	0.642	0.491	0.429	0.435	0.431	0.502	0.417	0.375	0.033	0.082	0.035	0.035
F	0.521	0.620	0.523	0.436	0.566	0.524	0.486	0.441	0.033	0.095	0.035	0.036
G	0.565	0.642	0.488	0.530	0.511	0.590	0.432	0.473	0.034	0.098	0.034	0.036
H	0.697	0.592	0.673	0.701	0.626	0.514	0.625	0.461	0.033	0.098	0.033	0.035

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

ame : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 16:52:45
PLATE	: nc2stm-mtt	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.068	0.067	0.069	0.065	0.069	0.067	0.080	0.073	0.033	0.079	0.033	0.034
B	0.068	0.067	0.070	0.068	0.070	0.068	0.074	0.071	0.033	0.077	0.034	0.034
C	0.074	0.080	0.068	0.070	0.068	0.073	0.072	0.067	0.034	0.086	0.033	0.035
D	0.272	0.128	0.094	0.083	0.101	0.217	0.081	0.126	0.035	0.084	0.035	0.034
E	0.609	0.886	0.887	1.141	0.611	0.562	0.594	0.464	0.034	0.086	0.035	0.035
F	0.818	1.182	1.438	1.235	1.006	0.854	0.595	0.656	0.034	0.102	0.034	0.036
G	1.141	1.263	1.075	0.667	1.147	1.226	0.499	0.567	0.034	0.102	0.033	0.036
H	0.693	1.213	1.056	0.895	0.832	0.753	0.643	0.631	0.033	0.105	0.033	0.038

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

3a

Name : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland
Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 26/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 22:43:11
PLATE	: Neg cont,xm, dmsoREF. FILTER		: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

Empty Empty

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.160	0.193	0.383	0.489	0.473	0.645	0.459	0.715	0.033	0.073	0.033	0.121
B	0.162	0.188	0.162	0.323	0.607	0.676	0.481	0.712	0.033	0.082	0.033	0.084
C	0.167	0.236	0.290	0.416	0.686	0.712	0.459	0.695	0.033	0.107	0.032	0.107
D	0.170	0.221	0.345	0.427	0.638	0.679	0.469	0.716	0.034	0.097	0.032	0.113
E	0.178	0.242	0.397	0.390	0.640	0.693	0.464	0.723	0.033	0.098	0.033	0.159
F	0.173	0.252	0.335	0.513	0.713	0.692	0.478	0.695	0.033	0.121	0.034	0.076
G	0.170	0.244	0.428	0.444	0.668	0.720	0.479	0.699	0.033	0.119	0.033	0.114
H	0.176	0.253	0.414	0.485	0.609	0.687	0.499	0.732	0.033	0.109	0.032	0.144

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

ne	: Spectroscopy Suite
Address	: 104 Fleming Building
	: School of HNSS
	: University of Sunderland
Phone	:
FAX	:

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 14:59:24
PLATE	: N C-xm+dmso	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.663	0.230	0.435	0.650	0.816	0.794	0.582	0.758	0.034	0.072	0.033	0.147
B	0.607	0.223	0.172	0.542	0.831	0.844	0.627	0.758	0.034	0.072	0.033	0.126
C	0.677	0.264	0.346	0.585	0.900	0.898	0.600	0.752	0.033	0.099	0.033	0.137
D	0.763	0.273	0.475	0.630	0.773	0.827	0.604	0.777	0.035	0.091	0.033	0.159
E	0.744	0.283	0.442	0.565	0.871	0.864	0.595	0.780	0.033	0.091	0.034	0.168
F	0.760	0.296	0.391	0.627	0.886	1.025	0.615	0.760	0.033	0.113	0.034	0.137
G	0.814	0.270	0.463	1.063	0.925	0.874	0.613	0.762	0.034	0.114	0.033	0.170
H	0.697	0.266	0.467	0.658	0.894	0.890	0.629	0.792	0.034	0.105	0.033	0.196

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

DYNEX REVELATION 3.04

3c

me : Spectroscopy Suite
Address : 104 Fleming Building
: School of HNSS
: University of Sunderland

Phone :
FAX :

TEST NO.	:	W/L MODE	: SINGLE	DATE	: 27/08/10
TEST NAME	: 595NOREF	TEST FILTER	: 595 nm	TIME	: 17:02:02
PLATE	: nc,xm,dm,mtt	REF. FILTER	: *	OPERATOR	: PO

OVER Limit : 4.000
Calculation mode : Endpoint

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.250	0.292	1.153	0.798	1.440	1.889	2.378	1.417	0.035	0.084	0.033	0.134
B	0.186	0.223	0.279	0.846	1.303	1.871	2.091	1.527	0.034	0.078	0.033	0.117
C	0.217	0.431	0.453	0.760	1.454	2.115	2.245	1.503	0.035	0.106	0.033	0.125
D	0.352	0.333	0.720	0.928	1.215	1.902	2.115	1.504	0.035	0.098	0.032	0.141
E	0.329	0.360	0.654	0.826	1.620	1.763	2.203	1.467	0.034	0.100	0.034	0.167
F	0.478	0.436	0.513	1.113	2.038	1.811	2.213	1.407	0.033	0.102	0.034	0.135
G	0.425	0.315	0.579	1.324	1.543	2.032	2.164	1.564	0.033	0.115	0.033	0.141
H	0.228	0.750	0.909	0.908	1.569	1.852	2.166	1.487	0.033	0.111	0.033	0.190

***** Indicates an unread well or value out of range

DYNEX TECHNOLOGIES

Appendix B: Efflux data obtained from Rotor-Gene 3000 instrument**.**

1) EtBr controls assay data

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Concentration Measurement Report

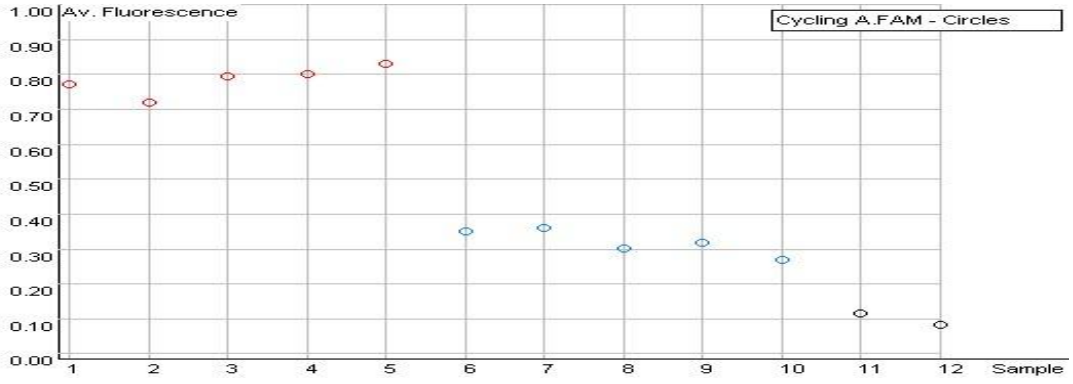
Experiment Information

Run Name	Run 2010-09-21 (2)
Run Start	21/09/2010 14:28:06
Run Finish	21/09/2010 15:46:08
Operator	Patricia
Notes	
Run On Software Version	Rotor-Gene 6.1.93
Run Signature	The Run Signature is valid.
Gain FAM	5.
Gain JOE	5.

Profile

Cycle	Cycle Point
Cycling (240 repeats)	Step 1 @ 37°c, hold 20 secs, acquiring to Cycling A(FAM,JOE)
	Step 2 @ 37°c, hold 20 secs
	Step 3 @ 37°c, hold 20 secs, acquiring to Cycling B(JOE)

Concentration data for Cycling A.FAM



No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
1		1 EtBr +glu +EC	0.78	[0.73 , 0.83]
2		1 EtBr +glu +EC		
3		1 EtBr +glu +EC		
4		1 EtBr +glu +EC		
5		1 EtBr +glu +EC		
6		1 EtBr + PBS+EC	0.32	[0.27 , 0.37]
7		1 EtBr + PBS+EC		
8		1 EtBr + PBS+EC		
9		1 EtBr + PBS+EC		
10		1 EtBr + PBS+EC		
11		1 EtBr +PBS+Glu	0.10	
12		1 EtBr +PBS+Glu		

Appendix B2: Controls used in EtBr assay

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Concentration Measurement Report

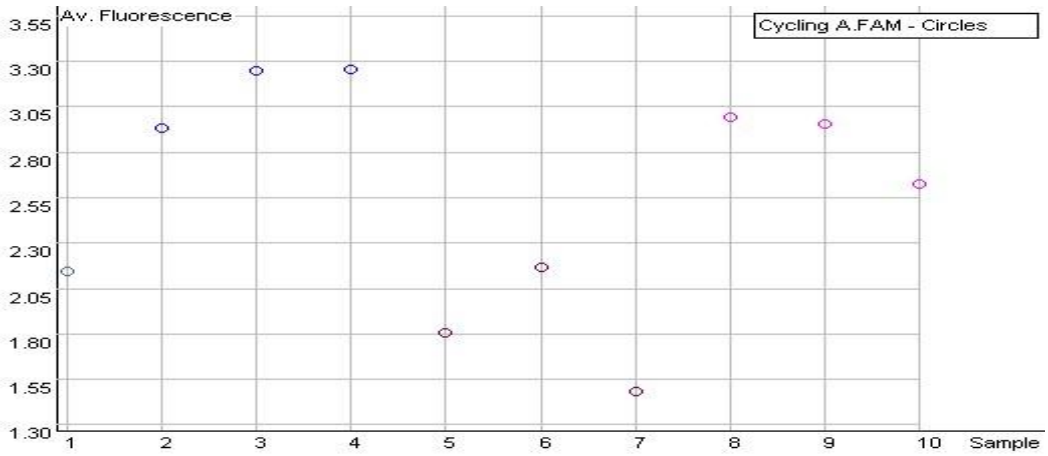
Experiment Information

Run Name	Run 2010-10-05 controls and EB assay (0.6GC)
Run Start	05/10/2010 16:49:31
Run Finish	05/10/2010 17:49:11
Operator	Patricia
Notes	
Run On Software Version	Rotor-Gene 6.1.93
Run Signature	The Run Signature is valid.
Gain FAM	10.
Gain JOE	5.

Profile

Cycle	Cycle Point
Cycling (30 repeats)	Step 1 @ 37°c, hold 60 secs, acquiring to Cycling A(FAM,JOE)
	Step 2 @ 37°c, hold 20 secs
	Step 3 @ 37°c, hold 20 secs, acquiring to Cycling B(JOE)

Concentration data for Cycling A.FAM



No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
1		PBS	2.14	
2		EC	3.14	[2.69 , 3.60]
3		EC		
4		EC		
5		EB 2.0	1.82	[0.97 , 2.67]
6		EB 2.0		
7		EB 2.0		
8		EB 2.0 + EC	2.86	[2.35 , 3.36]
9		EB 2.0 + EC		
10		EB 2.0 + EC		

Appendix B3: Chlorpromazine concentration assay and controls

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Concentration Measurement Report

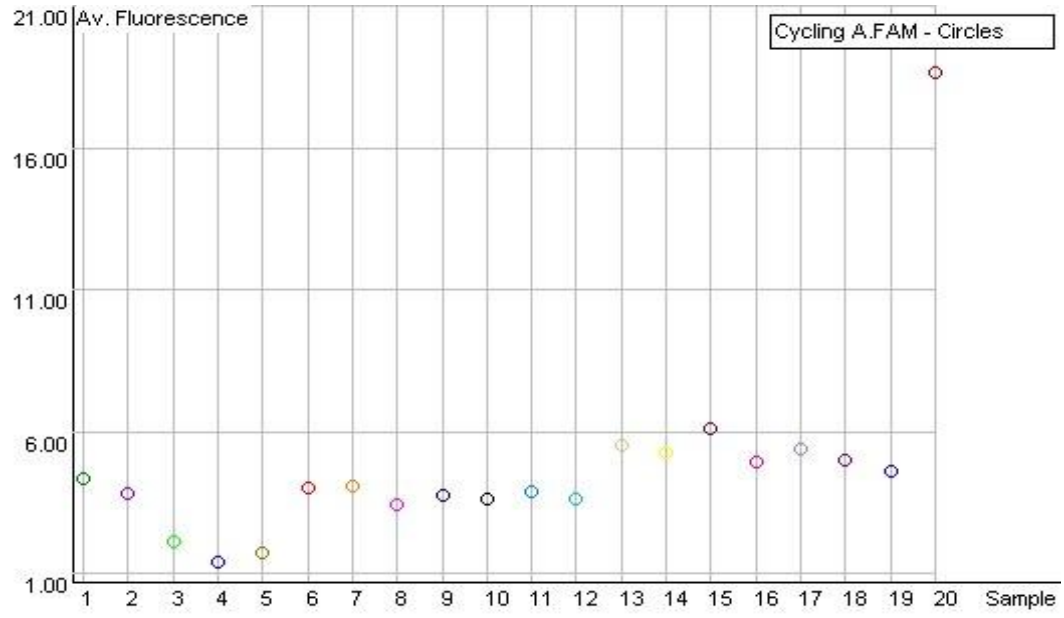
Experiment Information

Run Name	Run 2010-10-06 CPZ assay OVN B-C
Run Start	06/10/2010 14:47:48
Run Finish	06/10/2010 15:33:43
Operator	Patricia
Notes	
Run On Software Version	Rotor-Gene 6.1.93
Run Signature	The Run Signature is valid.
Gain FAM	10.
Gain JOE	10.

Profile

Cycle	Cycle Point
Cycling (30 repeats)	Step 1 @ 37°c, hold 60 secs, acquiring to Cycling A(FAM,JOE)
	Step 2 @ 37°c, hold 20 secs

Concentration data for Cycling A.FAM



No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
1		EC	4.39	
2		EC +EB 2	3.86	
3		EB 2	2.17	
4		CPZ 8	1.40	
5		EB 2.0 + CPZ 8	1.73	
6		EC + EB 2.0 + CPZ 1 in PBS	4.02	
7		EC + EB 2.0 + CPZ 2 in PBS	4.08	
8		EC + EB 2.0 + CPZ 3 in PBS	3.42	
9		EC + EB 2.0 + CPZ 5 in PBS	3.76	
10		EC + EB 2.0 + CPZ 4 in PBS	3.62	
11		EC + EB 2.0 + CPZ 6 in PBS	3.89	
12		EC + EB 2.0 + CPZ 8 in PBS	3.65	
13		EC + EB 2.0 + CPZ 1 in Glu	5.56	
14		EC + EB 2.0 + CPZ 2 in Glu	5.30	
15		EC + EB 2.0 + CPZ 3 in Glu	6.12	
16		EC + EB 2.0 + CPZ 4 in Glu	4.96	
17		EC + EB 2.0 + CPZ 5 in Glu	5.43	
18		EC + EB 2.0 + CPZ 6 in Glu	4.99	
19		EC + EB 2.0 + CPZ 8 in Glu	4.62	
20		EtBr + glu + EC	18.65	

Appendix B4: EtBr accumulation assay data in the presence of glucose and chlorpromazine

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Concentration Measurement Report

Experiment Information

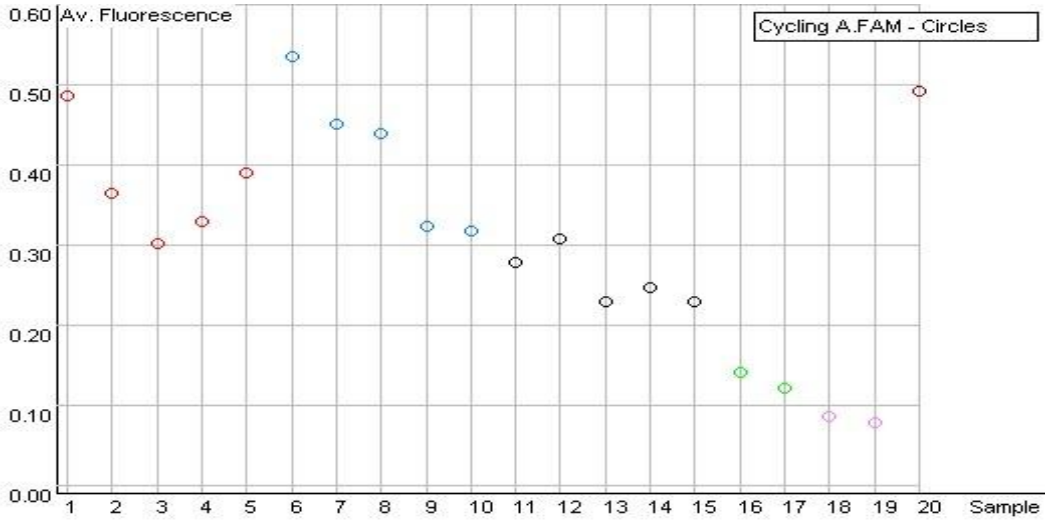
Run Name	Run 2010-09-23 (2)
Run Start	23/09/2010 16:58:53
Run Finish	23/09/2010 18:20:25
Operator	Patricia
Notes	
Run On Software Version	Rotor-Gene 6.1.93
Run Signature	The Run Signature is valid.

Gain FAM	5.
Gain JOE	5.

Profile

Cycle	Cycle Point
Cycling (240 repeats)	Step 1 @ 37°c, hold 20 secs, acquiring to Cycling A(FAM,JOE)
	Step 2 @ 37°c, hold 20 secs
	Step 3 @ 37°c, hold 20 secs, acquiring to Cycling B(JOE)

Concentration data for Cycling A.FAM



No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
1		1 EtBr + Ec in glu (CPZ)	0.49	
2		EtBr + Ec in glu (CPZ)	0.35	[0.28 , 0.41]
3		EtBr + Ec in glu (CPZ)		
4		EtBr + Ec in glu (CPZ)		
5		EtBr + Ec in glu (CPZ)		
6		1 EtBr + Ec in PBS ((CPZ)	0.54	
7		EtBr + Ec in PBS ((CPZ)	0.38	[0.27 , 0.50]
8		EtBr + Ec in PBS ((CPZ)		
9		EtBr + Ec in PBS ((CPZ)		
10		EtBr + Ec in PBS ((CPZ)		
11		1 EtBr + EC in Glu (CPZ)	0.26	[0.22 , 0.30]

No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
12		1 EtBr + EC in Glu (CPZ)		
13		1 EtBr + EC in Glu (CPZ)		
14		1 EtBr + EC in Glu (CPZ)		
15		1 EtBr + EC in Glu (CPZ)		
16		1 EtBr + Glu + CPZ	0.13	
17		1 EtBr + Glu + CPZ		
18		1 EtBr + PBS + CPZ	0.08	
19		1 EtBr + PBS + CPZ		
20		EtBr + glu + EC	0.49	

Appendix B5: *E. coli* efflux assay data

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Concentration Measurement Report

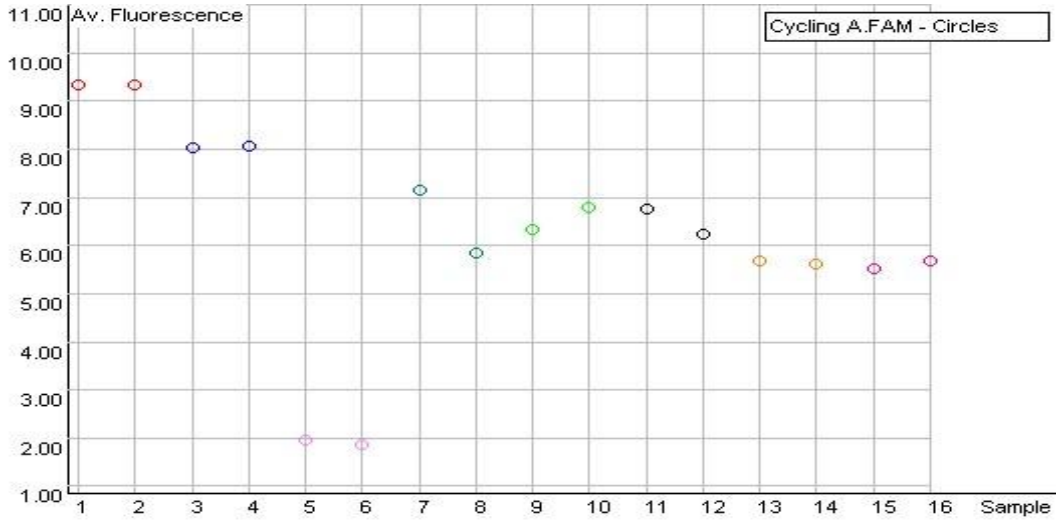
Experiment Information

Run Name	Run 2010-10-11 EC efflux assay
Run Start	11/10/2010 17:15:26
Run Finish	11/10/2010 18:13:52
Operator	Patricia
Notes	
Run On Software Version	Rotor-Gene 6.1.93
Run Signature	The Run Signature is valid.
Gain FAM	10.
Gain JOE	10.

Profile

Cycle	Cycle Point
Cycling (30 repeats)	Step 1 @ 37°c, hold 60 secs, acquiring to Cycling A(FAM,JOE)
	Step 2 @ 37°c, hold 20 secs
	Step 3 @ 37°c, hold 20 secs, acquiring to Cycling B(JOE)

Concentration data for Cycling A.FAM



No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
1		EC in PBS	9.33	
2		EC in PBS		
3		EC +EB 2.0 + CPZ 8.0	8.05	
4		EC +EB 2.0 + CPZ 8.0		
5		EB 2.0 + CPZ 8	1.91	
6		EB 2.0 + CPZ 8		
7		washed EC + CPZ 8.0- no glu	6.50	
8		washed EC + CPZ 8.0- no glu		
9		washed EC + CPZ 1.0 glu	6.56	
10		washed EC + CPZ 1.0 glu		
11		washed EC + CPZ 2.0 glu	6.50	
12		washed EC + CPZ 2.0 glu		
13		washed EC + CPZ 4.0 glu	5.64	
14		washed EC + CPZ 4.0 glu		
15		washed EC + CPZ 8.0 glu	5.60	
16		washed EC + CPZ 8.0 glu		

Appendix B6: EtBr and *M. aurum* initial concentration assay

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Concentration Measurement Report

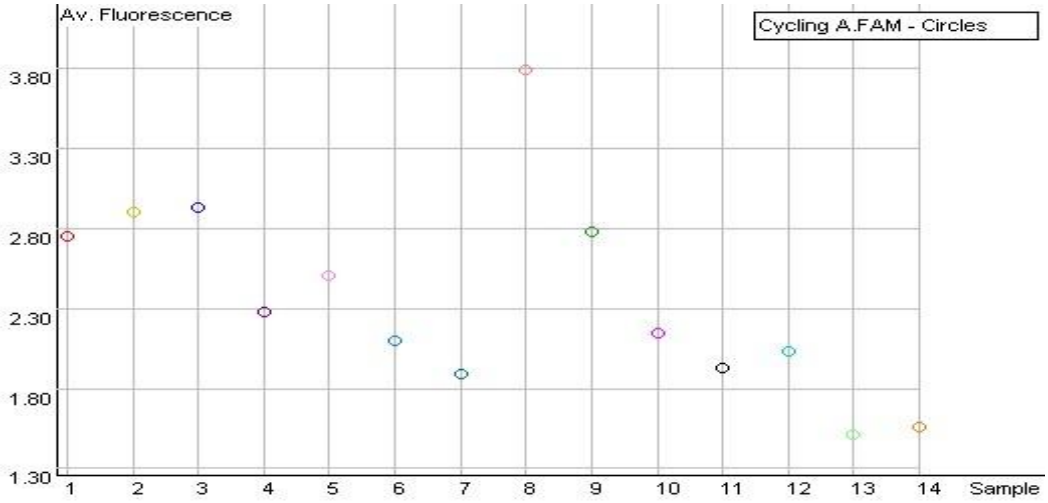
Experiment Information

Run Name	Run 2010-10-11 *M. aurum* EB initial assay
Run Start	11/10/2010 15:02:39
Run Finish	11/10/2010 16:01:07
Operator	Patricia
Notes	
Run On Software Version	Rotor-Gene 6.1.93
Run Signature	The Run Signature is valid.
Gain FAM	10.
Gain JOE	10.

Profile

Cycle	Cycle Point
Cycling (30 repeats)	Step 1 @ 37°c, hold 60 secs, acquiring to Cycling A(FAM,JOE)
	Step 2 @ 37°c, hold 20 secs
	Step 3 @ 37°c, hold 20 secs, acquiring to Cycling B(JOE)

Concentration data for Cycling A.FAM



No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
1		M0	2.75	
2		M + EB 1 pbs	2.91	
3		M + EB 2 pbs	2.93	
4		M + EB 3 pbs	2.28	
5		M + EB 4 pbs	2.50	
6		M + EB 5 pbs	2.10	
7		M + EB 6 pbs	1.89	

No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
8		M0 glu	3.79	
9		M + EB 1 glu	2.78	
10		M + EB 2 glu	2.15	
11		M + EB 3 glu	1.93	
12		M + EB 4 glu	2.03	
13		M + EB 5 glu	1.52	
14		M + EB 6 glu	1.56	

Appendix B7: EtBr assay with *M. aurum* in the presence of CPZ

Concentration Measurement Report

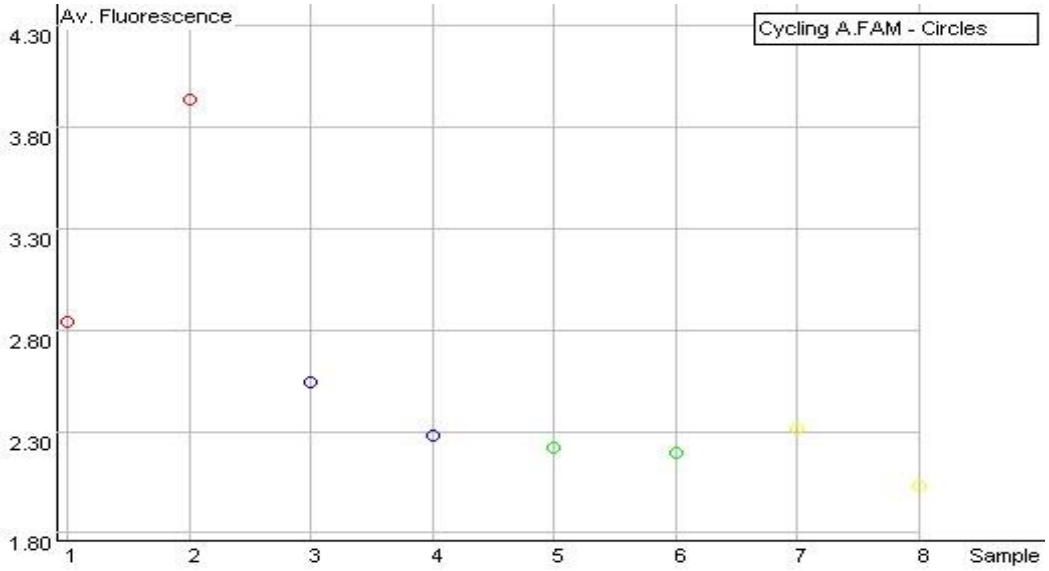
Experiment Information

Run Name	Run 2010-10-11 M aurum EB3, cpz 8 assay
Run Start	11/10/2010 16:22:57
Run Finish	11/10/2010 17:08:07
Operator	Patricia
Notes	
Run On Software Version	Rotor-Gene 6.1.93
Run Signature	The Run Signature is valid.
Gain FAM	10.
Gain JOE	10.

Profile

Cycle	Cycle Point
Cycling (30 repeats)	Step 1 @ 37°c, hold 60 secs, acquiring to Cycling A(FAM,JOE)
	Step 2 @ 37°c, hold 20 secs
	Step 3 @ 37°c, hold 20 secs, acquiring to Cycling B(JOE)

Concentration data for Cycling A.FAM

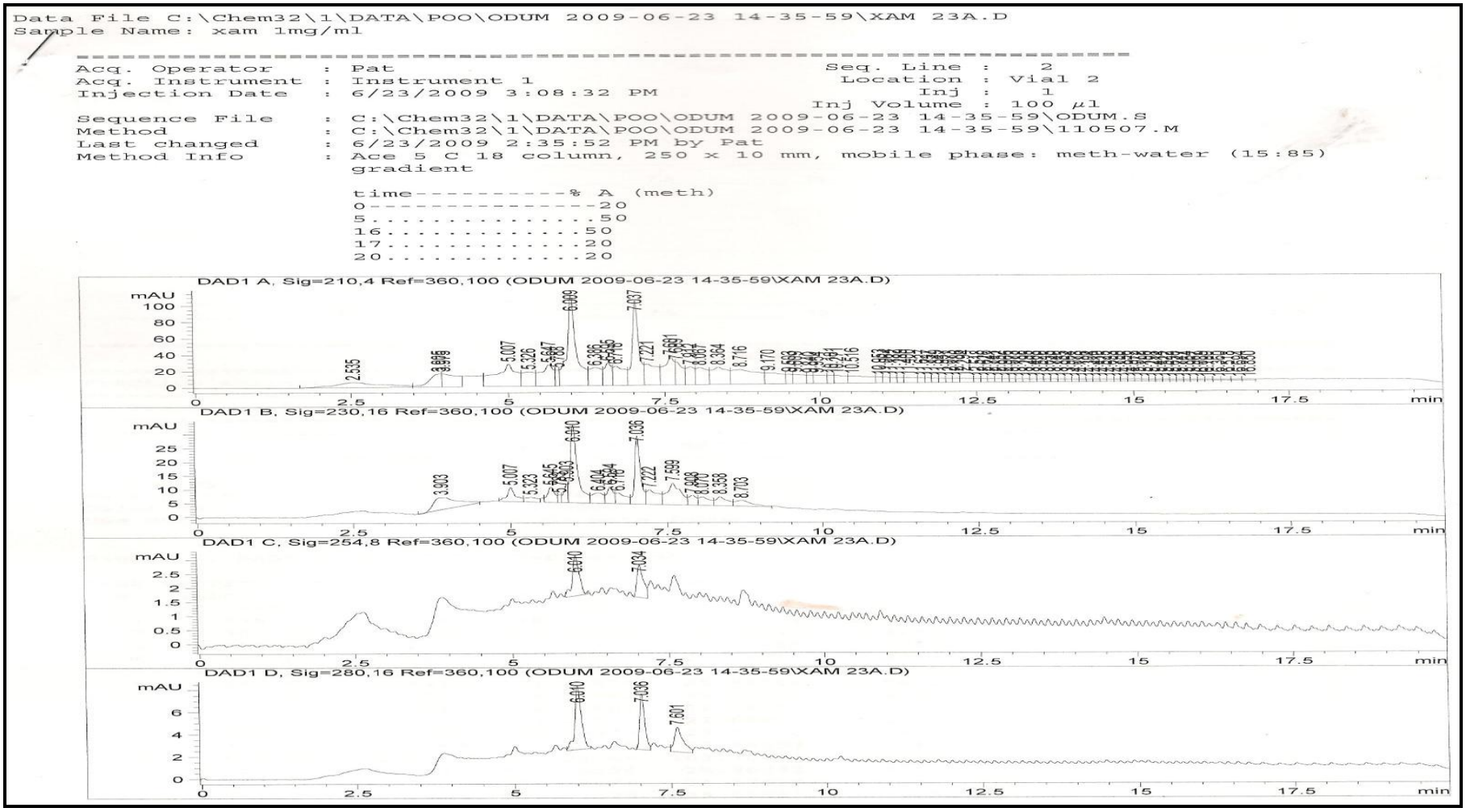


No.	Colour	Name	Rep. Fl.	Rep. Fl. (95% CI)
1		MA + EB 0 glu	3.39	
2		MA + EB 0 glu		
3		MA +EB3	2.41	
4		MA +EB3		
5		MA +EB3 + CPZ 8 pbs	2.21	
6		MA +EB3 + CPZ 8 pbs		
7		MA +EB3 + CPZ 8 glu	2.17	
8		MA +EB3 + CPZ 8 glu		

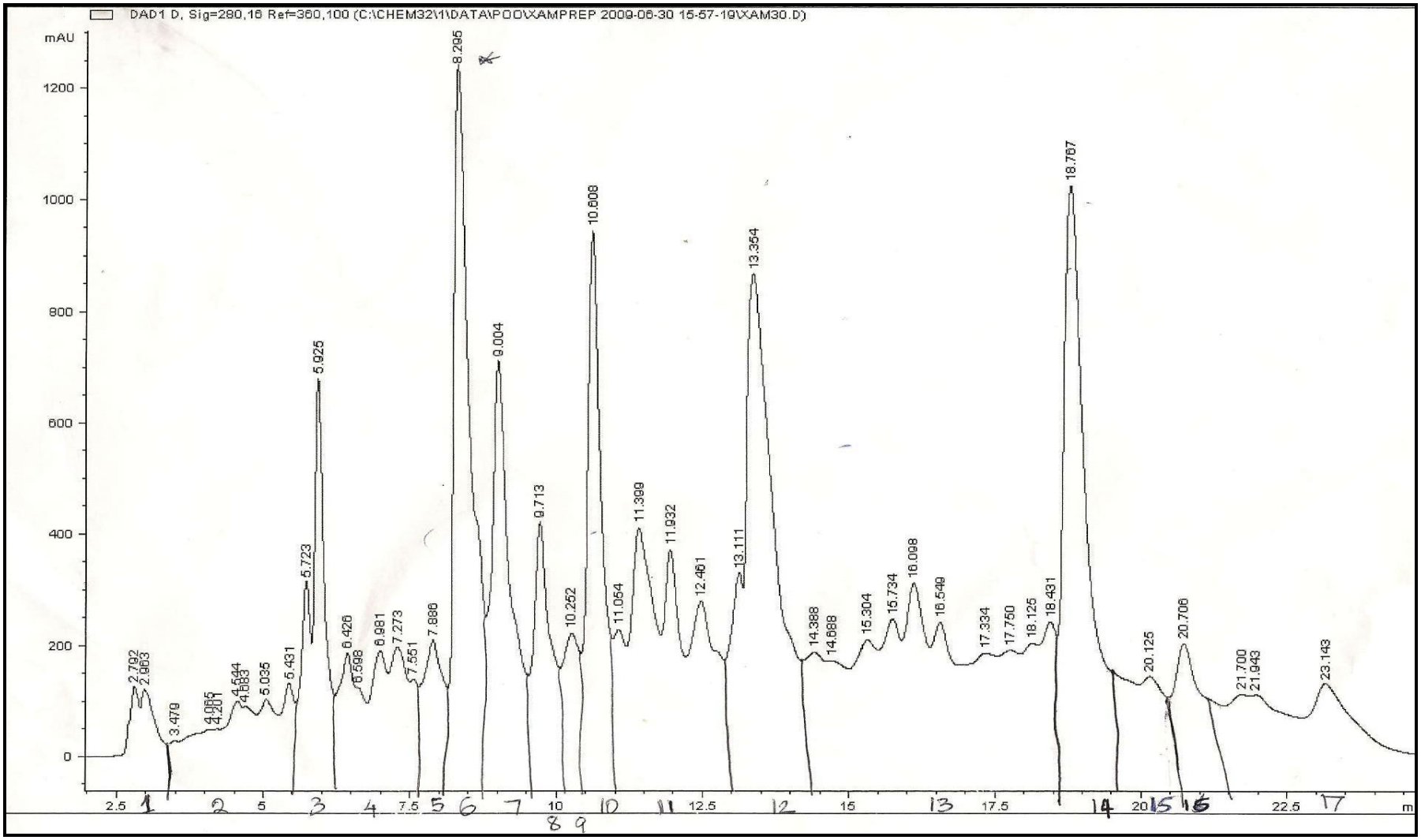
Appendix C : LC chromatograms for crude fractions and LC fractions.

a. LC chromatograms showing details of analytical and semi-preparative LC of *X. americana* methanol fraction.

i.

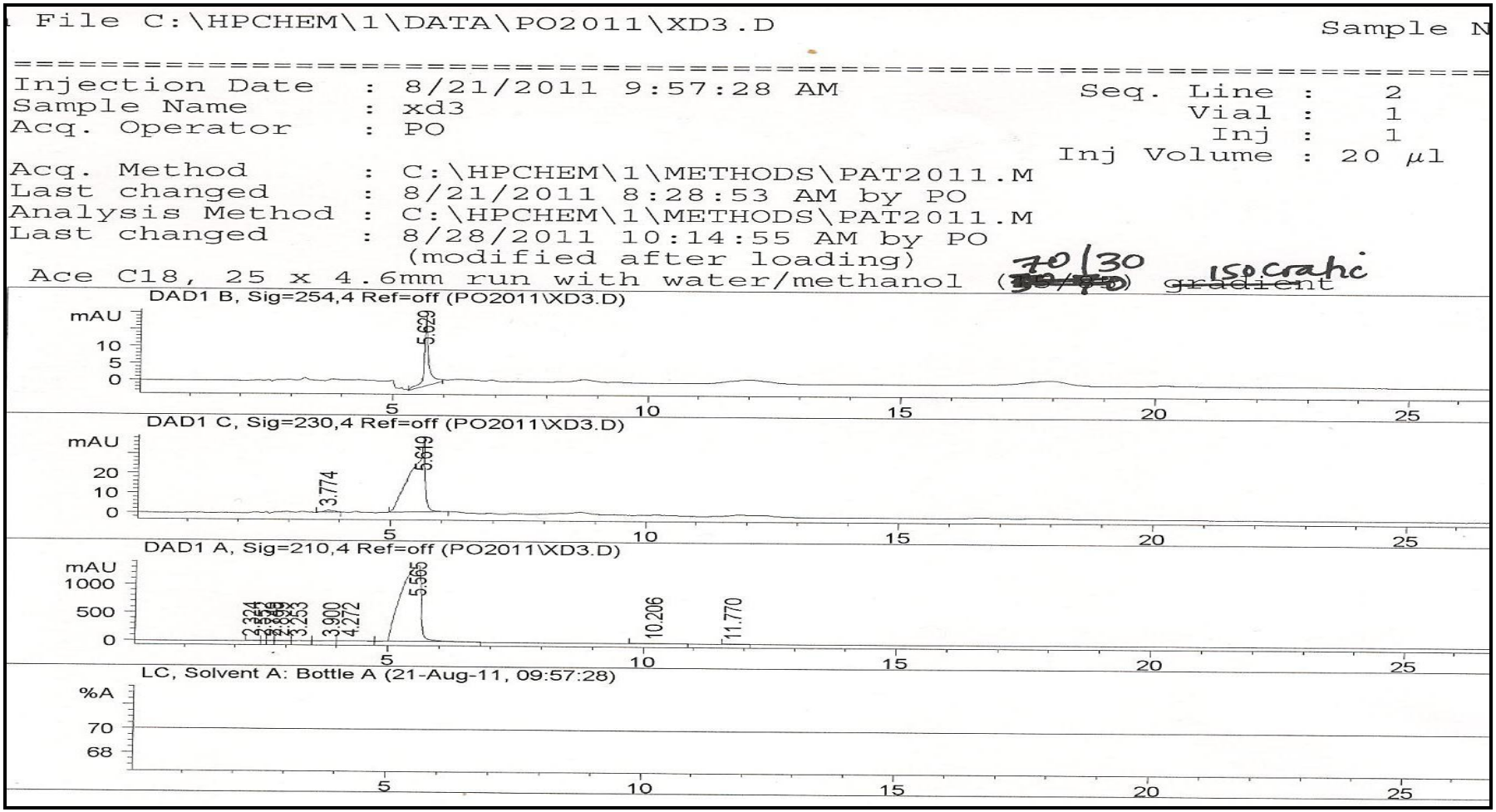


ii.


DAD1 D, Sig=280,16 Ref=360,100 (C:\CHEM32\1\DATA\POO\XAMPREP 2009-06-30 15-57-19\XAM30.D)
mAU
1200
1000
800
600
400
200
0
2.792
2.963
3.479
4.085
4.201
4.544
4.683
5.035
5.431
5.723
5.925
6.426
6.598
6.981
7.273
7.551
7.886
8.295
9.004
9.713
10.252
10.608
11.054
11.399
11.932
12.461
13.111
13.354
14.388
14.688
15.304
15.734
16.098
16.549
17.334
17.750
18.125
18.431
18.787
20.125
20.706
21.700
21.943
23.143
2.5
5
7.5
10
12.5
15
17.5
20
22.5
min

b. Reversed phase LC chromatograms of XD3 and XD4 active TB fractions on LC-DAD using (isocratic and gradient) methanol water (30:70) mobile phase on Ace 5 C18 column, 250 x 4.6 mm.

i.

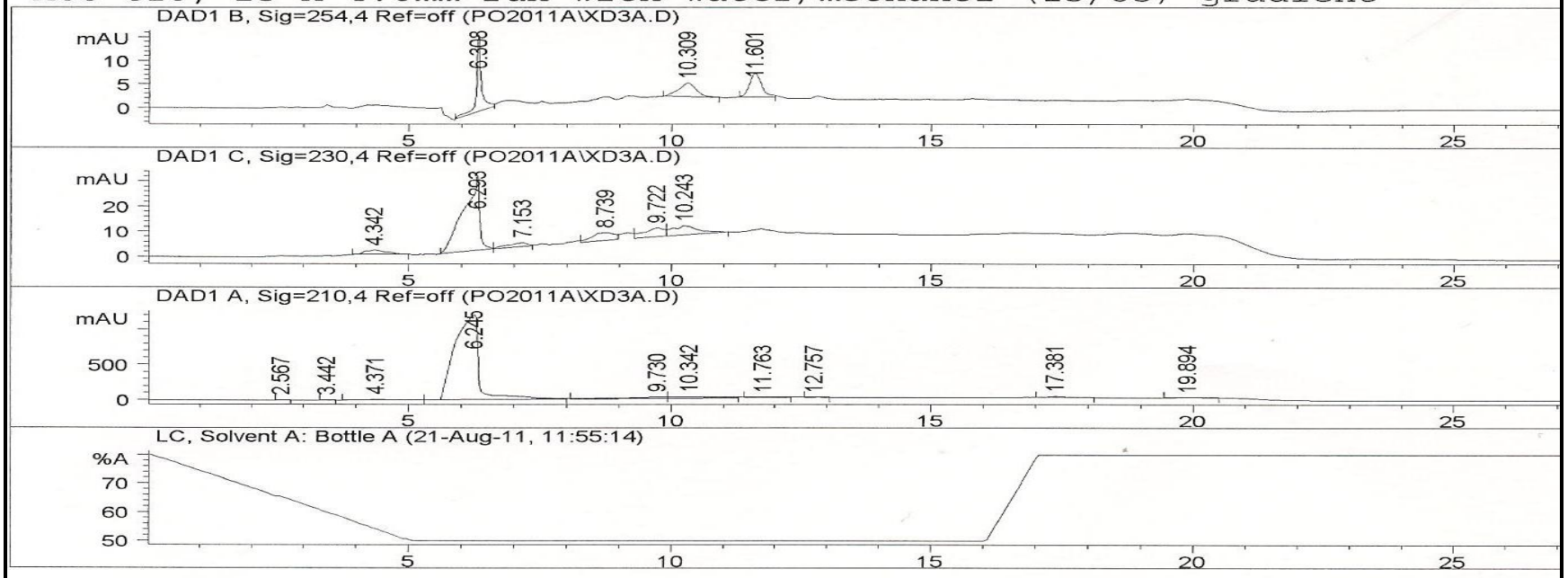


ii.

File C:\HPCHEM\1\DATA\PO2011A\XD3A.D Sample Na

Injection Date	: 8/21/2011 11:55:14 AM	Seq. Line	: 2
Sample Name	: xd3	Vial	: 1
Acq. Operator	: PO	Inj	: 1
		Inj Volume	: 20 µl
Acq. Method	: C:\HPCHEM\1\METHODS\PAT2011.M		
Last changed	: 8/21/2011 11:15:54 AM by PO		
Analysis Method	: C:\HPCHEM\1\METHODS\PAT2011.M		
Last changed	: 8/28/2011 10:14:55 AM by PO		
	(modified after loading)		

Ace C18, 25 x 4.6mm run with water/methanol (15/85) gradient

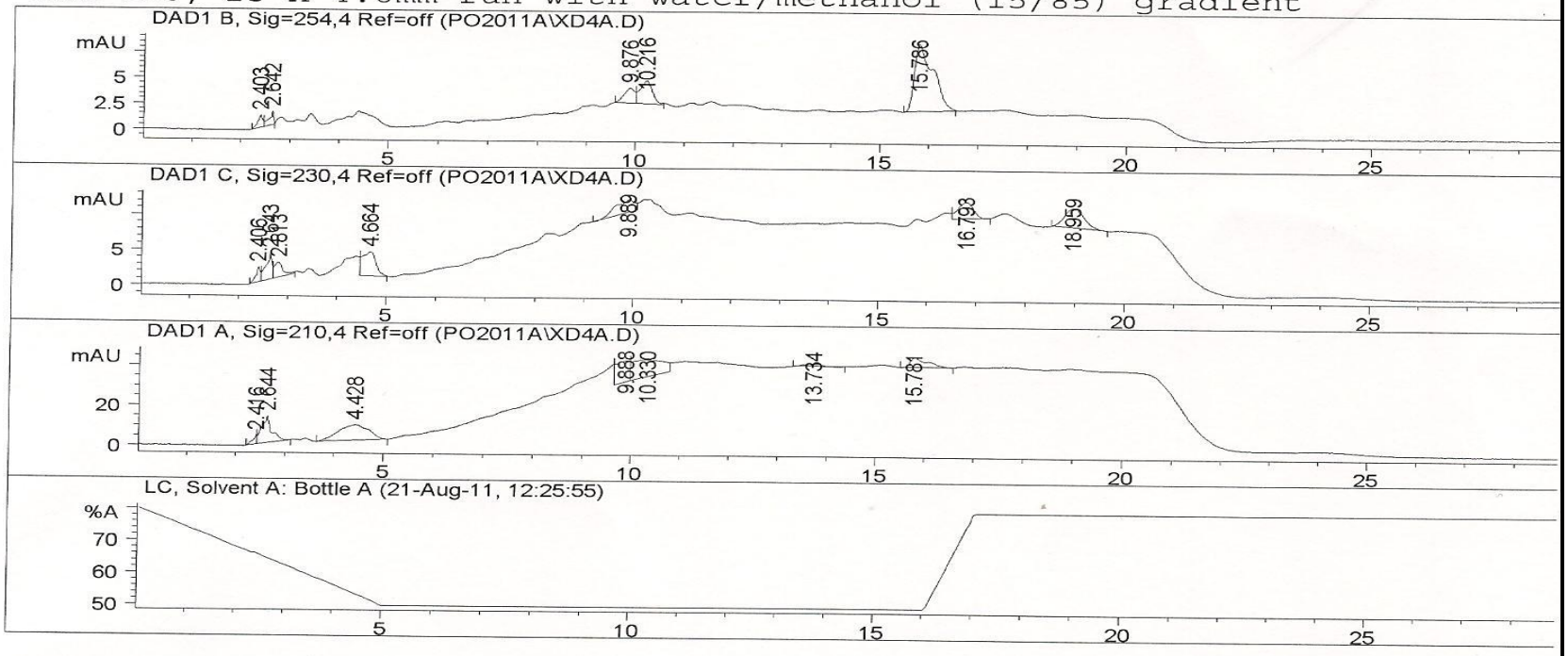


iii.

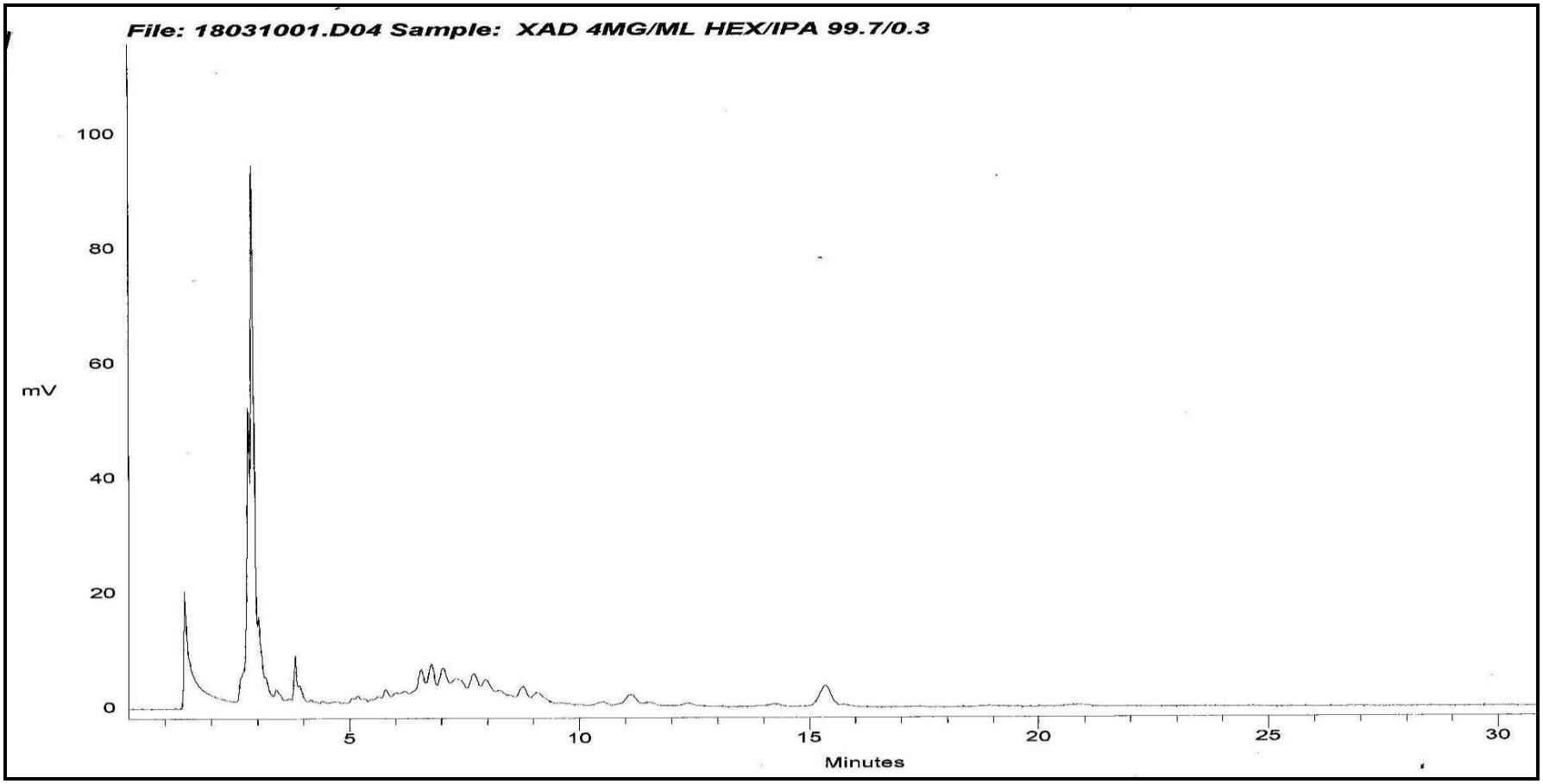
Data File C:\HPCHEM\1\DATA\PO2011A\XD4A.D Sample Name:

Injection Date	: 8/21/2011 12:25:55 PM	Seq. Line	: 3
Sample Name	: xd4	Vial	: 2
Acq. Operator	: PO	Inj	: 1
		Inj Volume	: 20 µl
Acq. Method	: C:\HPCHEM\1\METHODS\PAT2011.M		
Last changed	: 8/21/2011 11:15:54 AM by PO		
Analysis Method	: C:\HPCHEM\1\METHODS\PAT2011.M		
Last changed	: 8/28/2011 10:14:55 AM by PO (modified after loading)		

Ace C18, 25 x 4.6mm run with water/methanol (15/85) gradient



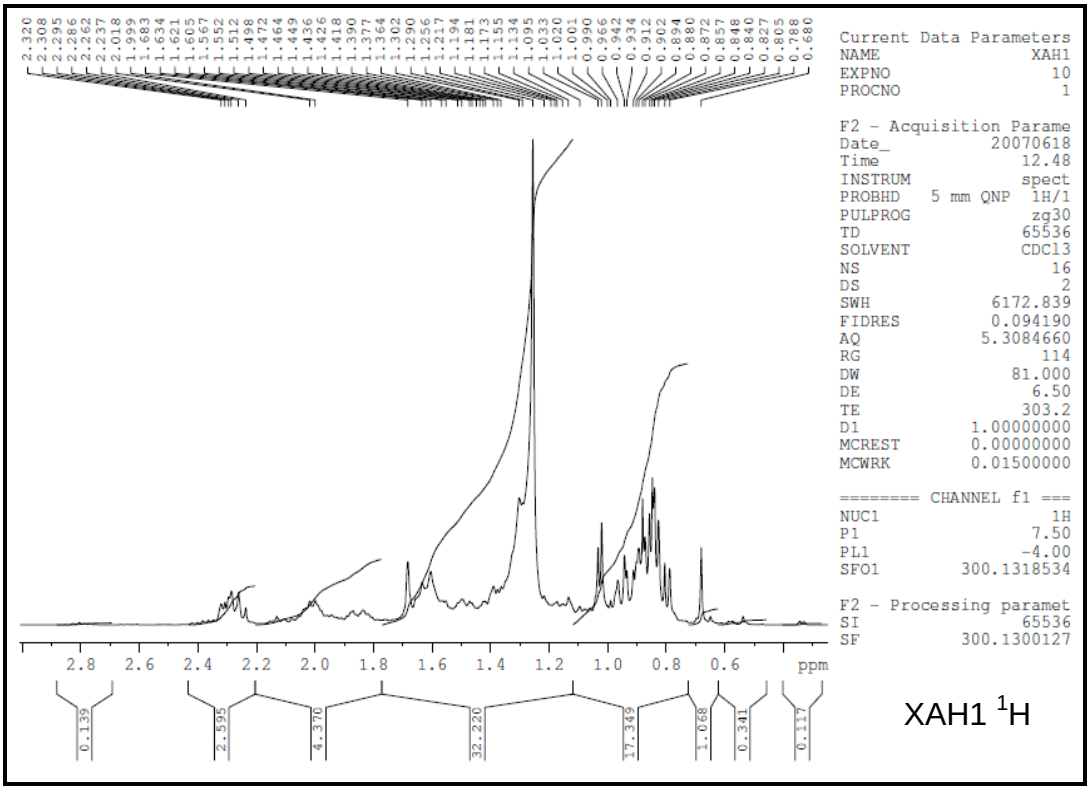
c. *X. americana* dichloromethane extract on Ace 5 silica , 250 x 7.7 mm column



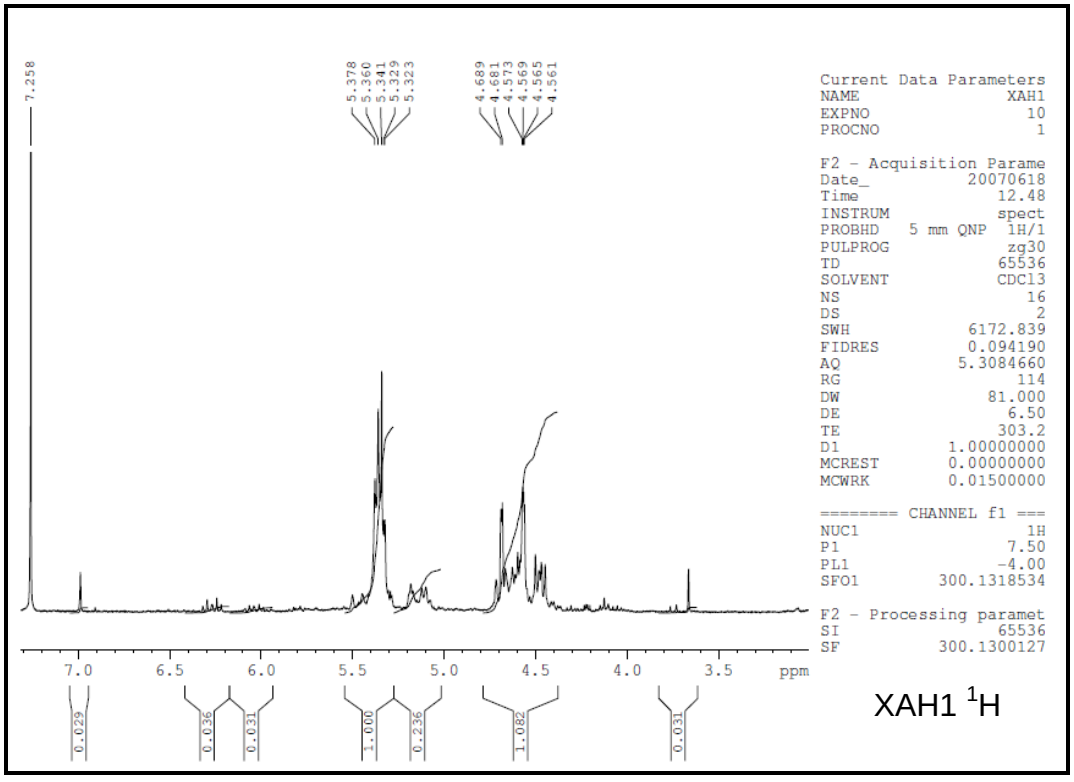
Appendix D: NMR spectra for fractions and reference compounds.

1. XAH (peak1) ^{1}H and ^{13}C , HMBC, HMQC spectra (expanded)

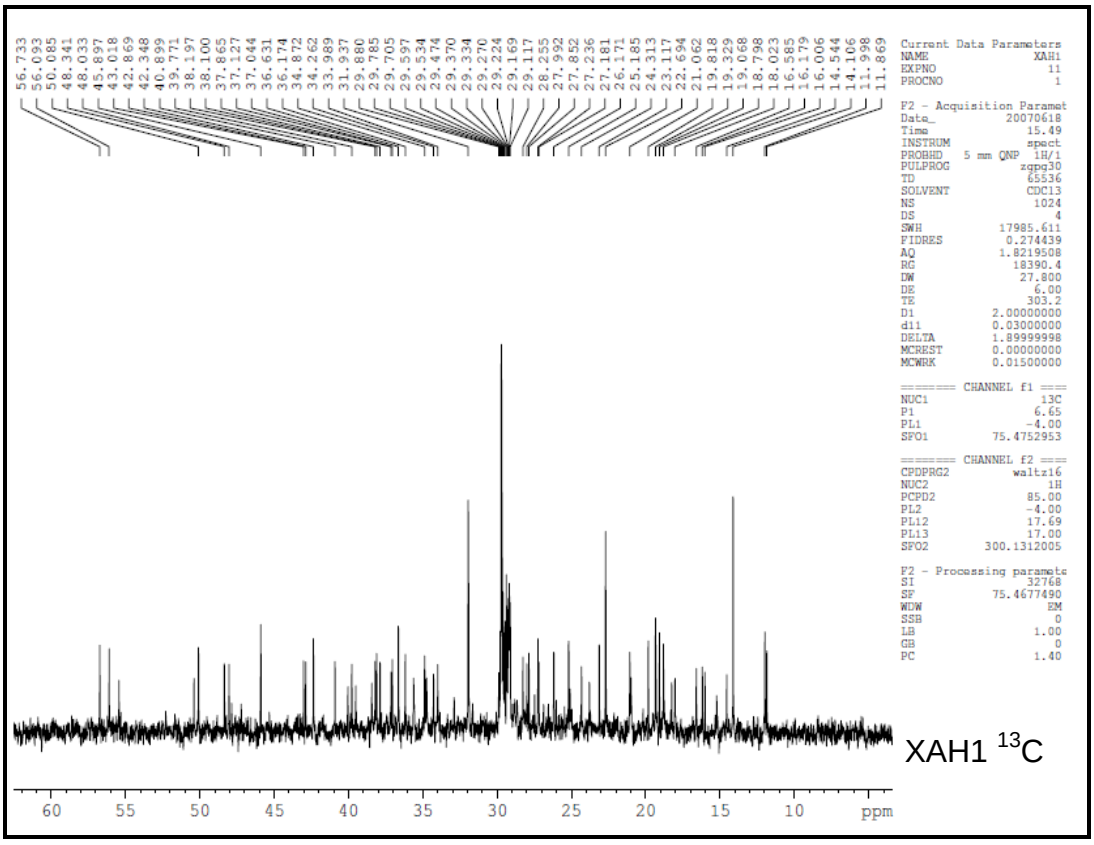
i.



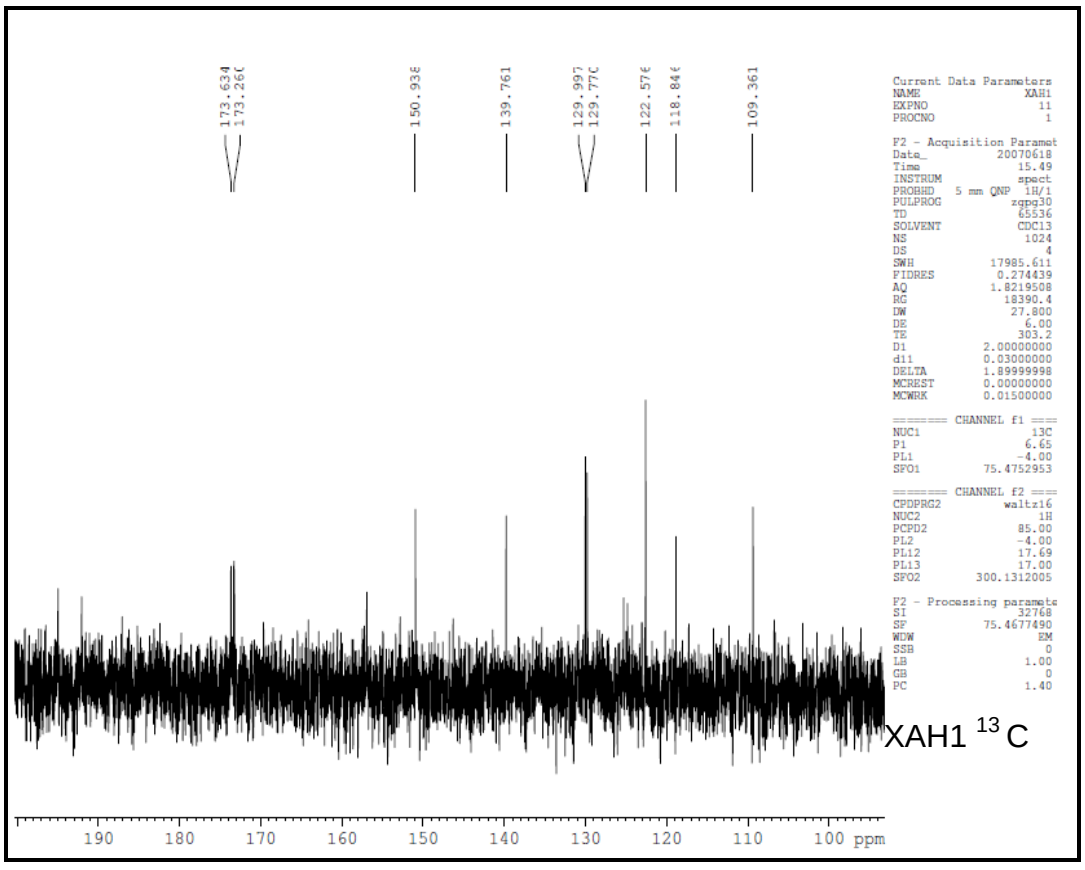
ii.



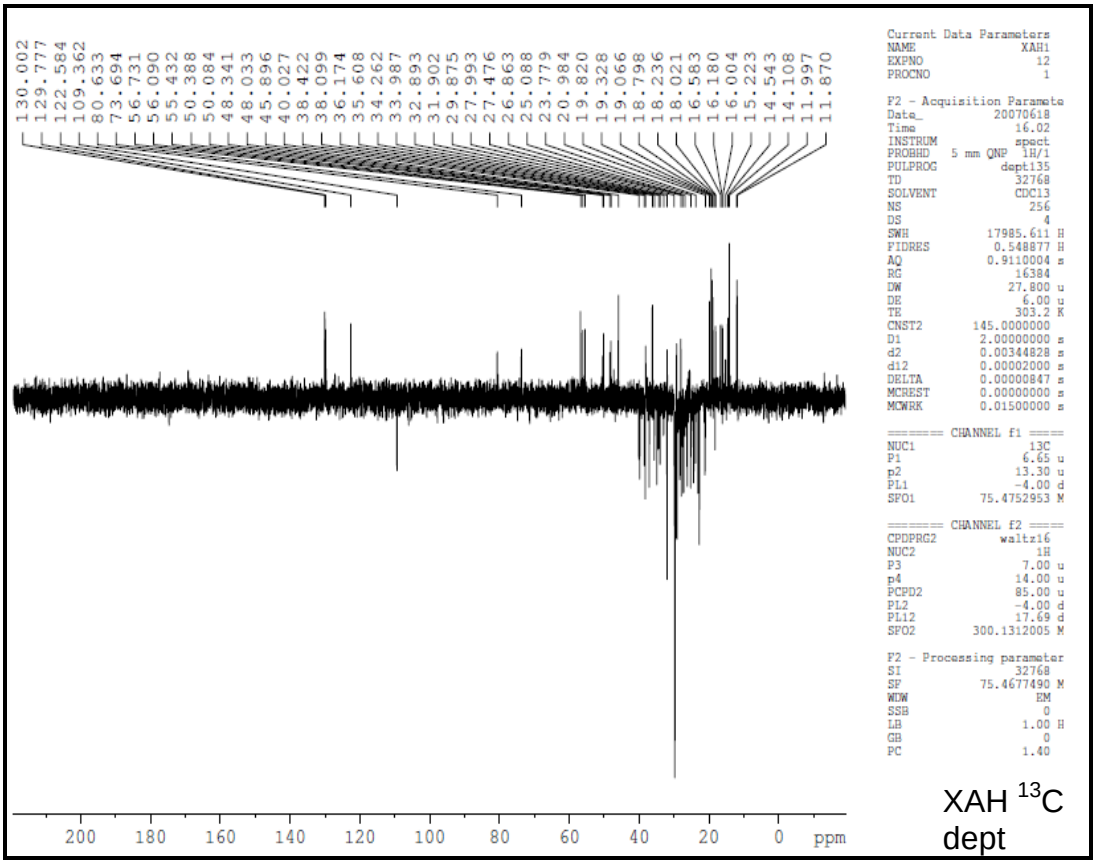
iii.


56.733
56.093
50.085
48.341
48.033
45.897
43.018
42.869
42.348
40.899
39.771
38.197
38.100
37.865
37.127
37.044
36.631
36.174
34.872
34.262
33.989
31.937
29.880
29.785
29.705
29.597
29.534
29.474
29.370
29.334
29.270
29.224
29.169
29.117
28.255
27.992
27.852
27.236
27.181
26.171
25.185
24.313
23.117
22.694
21.062
19.818
19.329
19.068
18.798
18.023
16.585
16.179
16.006
14.544
14.106
11.998
11.869
Current Data Parameters
NAME XAH1
EXPNO 11
PROCNO 1
F2 - Acquisition Paramet
Date_ 20070618
Time 15.49
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1024
DS 4
SWH 17985.611
FIDRES 0.274439
AQ 1.8219508
RG 18390.4
DW 27.800
DE 6.00
TE 303.2
D1 2.00000000
d11 0.03000000
DELTA 1.89999998
MCREST 0.00000000
MCWRK 0.01500000
CHANNEL f1
NUC1 13C
P1 6.65
PL1 -4.00
SFO1 75.4752953
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 85.00
PL2 -4.00
PL12 17.69
PL13 17.00
SFO2 300.1312005
F2 - Processing paramete
SI 32768
SF 75.4677490
WDW EM
SSB 0
LB 1.00
GB 0
PC 1.40
XAH1 13C
60 55 50 45 40 35 30 25 20 15 10 ppm

iv.

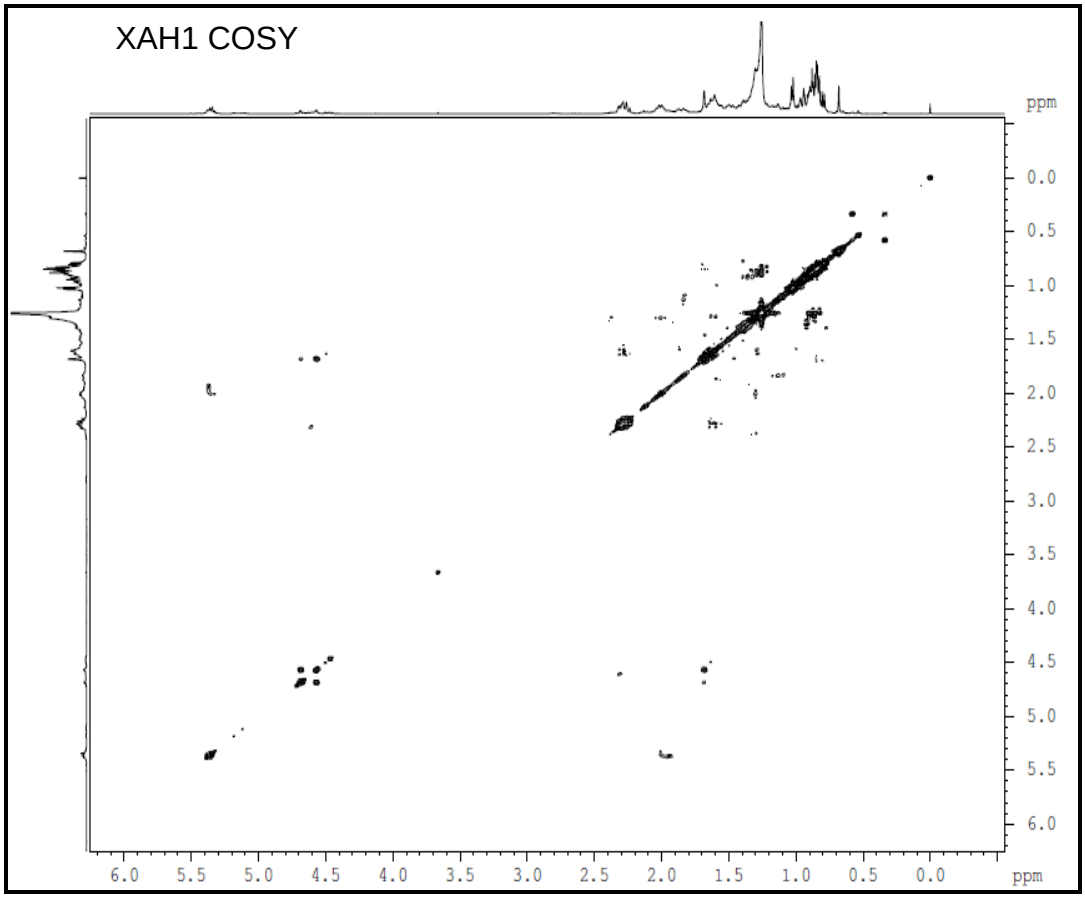

173.634
173.260
150.938
139.761
129.997
129.770
122.576
118.844
109.361
Current Data Parameters
NAME XAH1
EXPNO 11
PROCNO 1
F2 - Acquisition Paramet
Date_ 20070618
Time 15.49
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1024
DS 4
SWH 17985.611
FIDRES 0.274439
AQ 1.8219508
RG 18390.4
DW 27.800
DE 6.00
TE 303.2
D1 2.00000000
d11 0.03000000
DELTA 1.89999998
MCREST 0.00000000
MCWRK 0.01500000
CHANNEL f1
NUC1 13C
P1 6.65
PL1 -4.00
SFO1 75.4752953
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 85.00
PL2 -4.00
PL12 17.69
PL13 17.00
SFO2 300.1312005
F2 - Processing paramete
SI 32768
SF 75.4677490
WDW EM
SSB 0
LB 1.00
GB 0
PC 1.40
XAH1 13C
190 180 170 160 150 140 130 120 110 100 ppm

v.

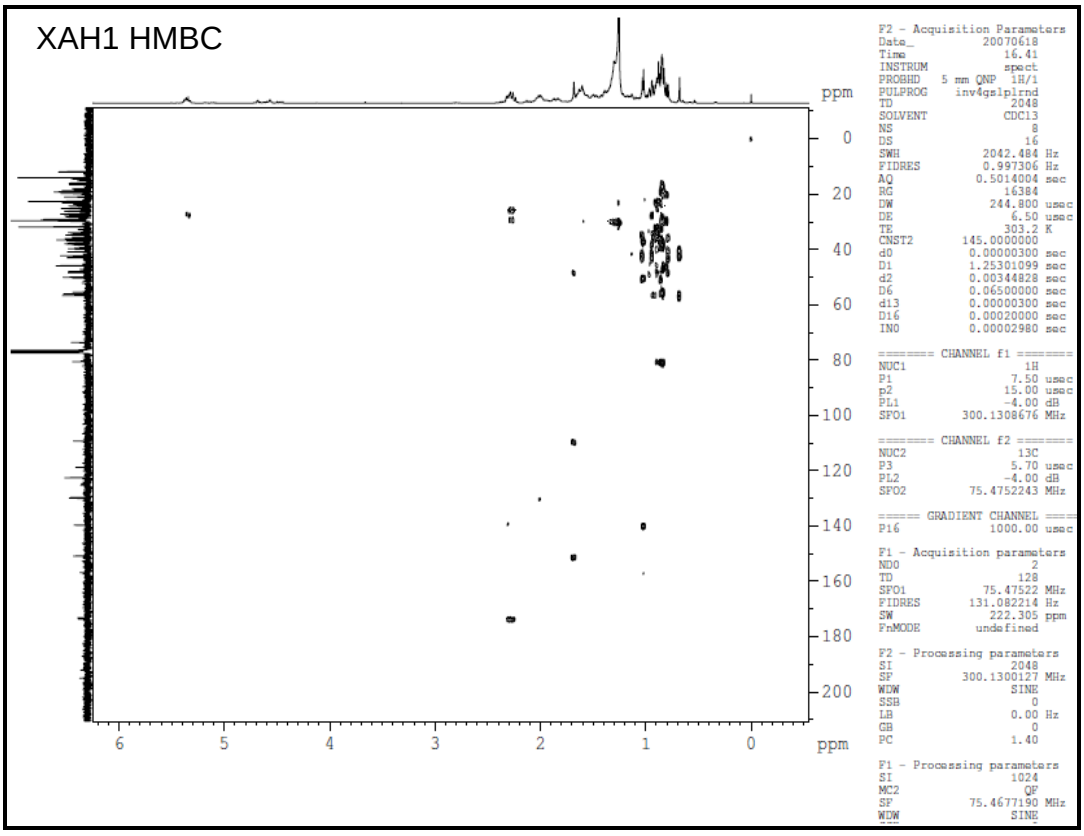


XAH ^{13}C dept

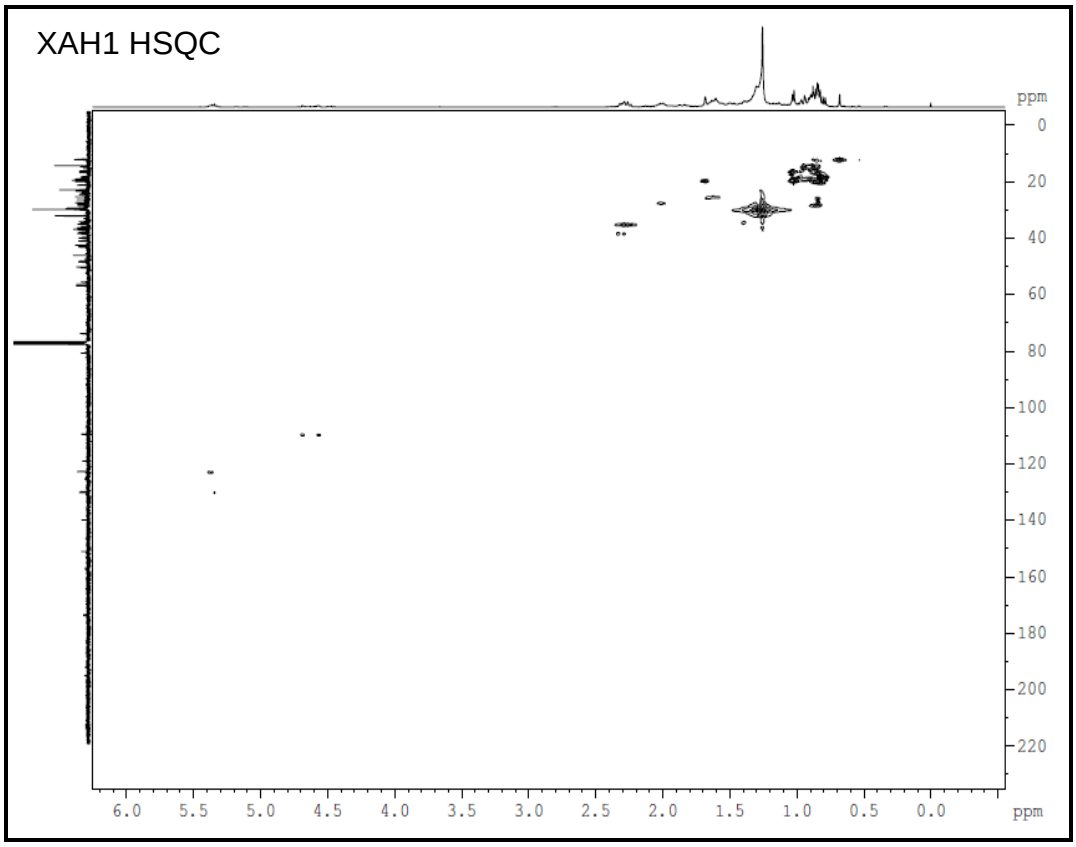
vi.



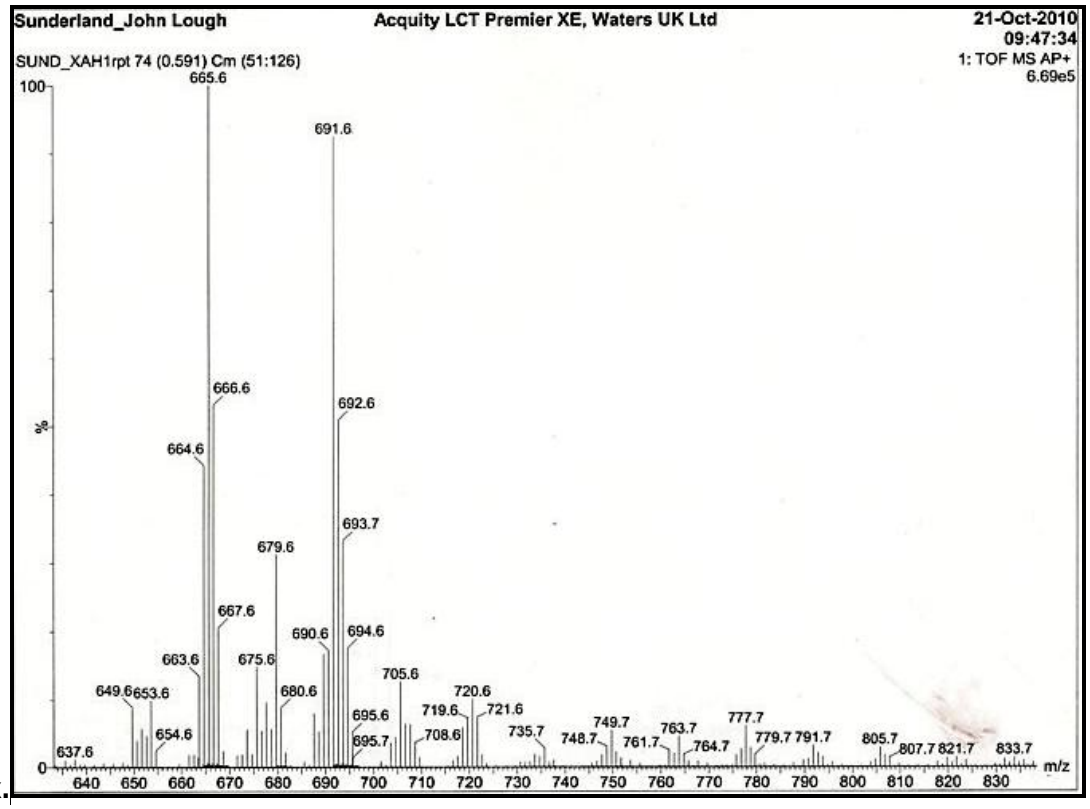
vii.


XAH1 HMBC
ppm
0
20
40
60
80
100
120
140
160
180
200
6
5
4
3
2
1
0
ppm
F2 - Acquisition Parameters
Date_ 20070618
Time 16.41
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG inv4gs1plrnd
TD 2048
SOLVENT CDCl3
NS 8
DS 16
SWH 2042.484 Hz
FIDRES 0.997306 Hz
AQ 0.5014004 sec
RG 16384
DW 244.800 usec
DE 6.50 usec
TE 303.2 K
CNST2 145.0000000
d0 0.00000300 sec
D1 1.25301099 sec
d2 0.00344828 sec
D6 0.06500000 sec
d13 0.00000300 sec
D16 0.00020000 sec
IN0 0.00002980 sec
======== CHANNEL f1 ========
NUC1 1H
P1 7.50 usec
p2 15.00 usec
PL1 -4.00 dB
SFO1 300.1308676 MHz
======== CHANNEL f2 ========
NUC2 13C
P3 5.70 usec
PL2 -4.00 dB
SFO2 75.4752243 MHz
====== GRADIENT CHANNEL ====
P16 1000.00 usec
F1 - Acquisition parameters
ND0 2
TD 128
SFO1 75.47522 MHz
FIDRES 131.082214 Hz
SW 222.305 ppm
FnMODE undefined
F2 - Processing parameters
SI 2048
SF 300.1300127 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
PC 1.40
F1 - Processing parameters
SI 1024
MC2 QF
SF 75.4677190 MHz
WDW SINE

viii.

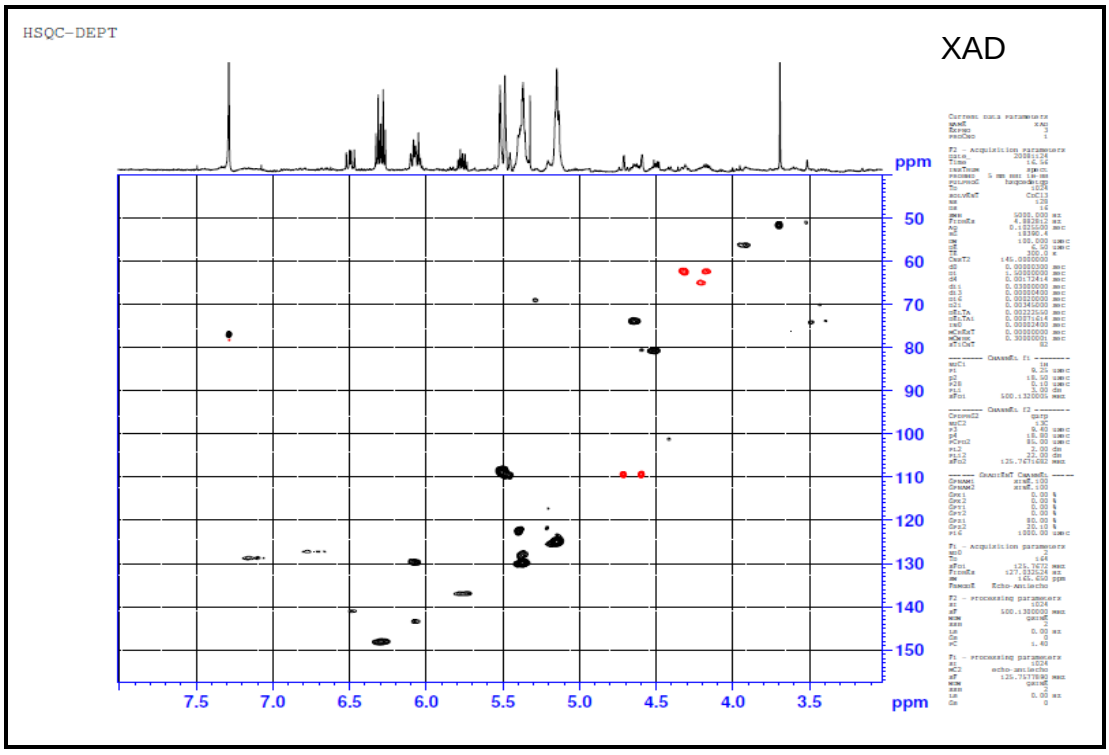

XAH1 HSQC
ppm
0
20
40
60
80
100
120
140
160
180
200
220
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm

ix.

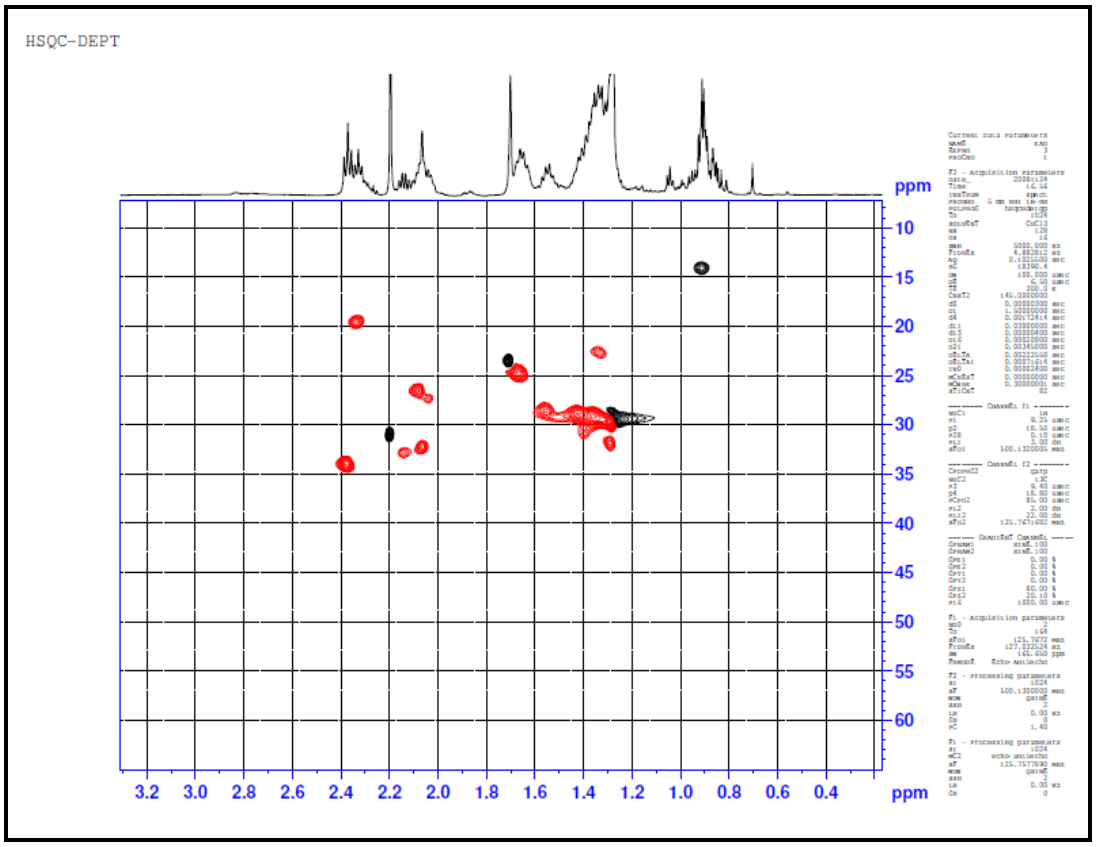

Sunderland_John Lough
Acquity LCT Premier XE, Waters UK Ltd
21-Oct-2010
09:47:34
SUND_XAH1rpt 74 (0.591) Cm (51:126)
1: TOF MS AP+
6.69e5
100
%
0
m/z
665.6
691.6
666.6
692.6
664.6
693.7
679.6
667.6
690.6
694.6
663.6
675.6
705.6
649.6
653.6
680.6
720.6
695.6
719.6
721.6
654.6
708.6
735.7
748.7
749.7
761.7
763.7
764.7
777.7
779.7
791.7
805.7
807.7
821.7
833.7
637.6
640 650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800 810 820 830

1. XAD

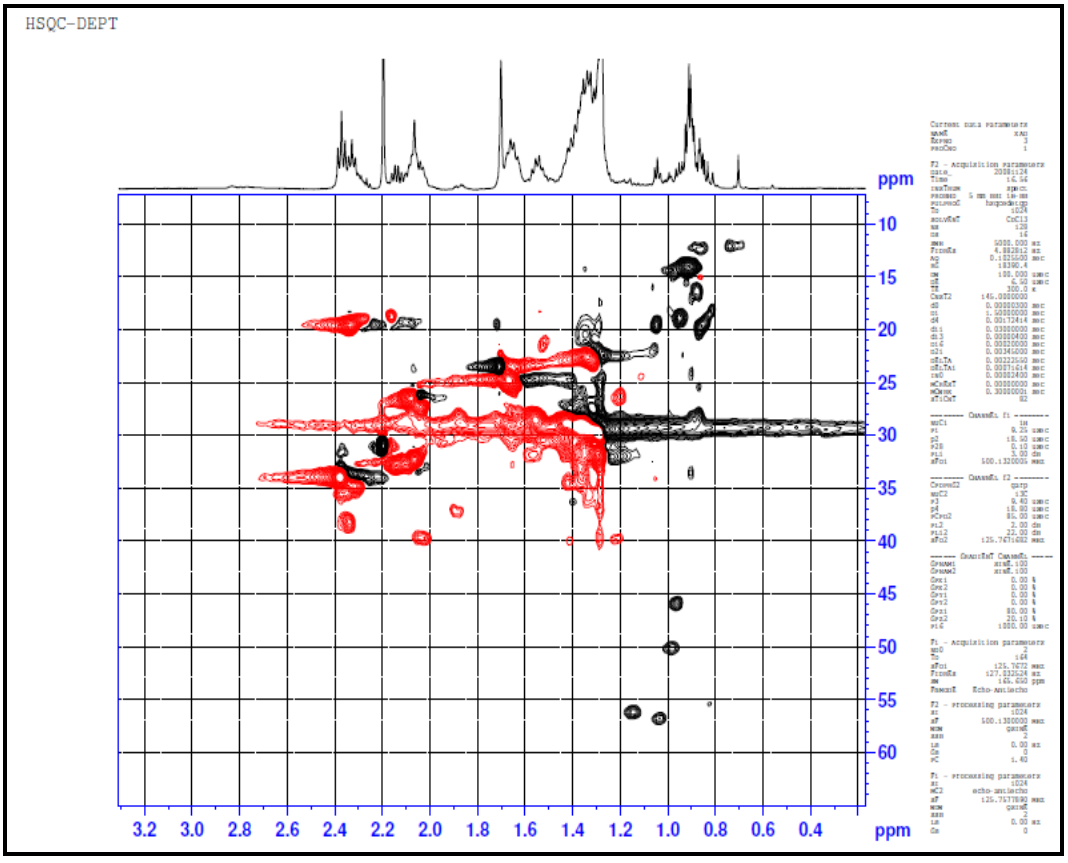
i.


HSQC-DEPT
XAD
ppm
50
60
70
80
90
100
110
120
130
140
150
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
ppm

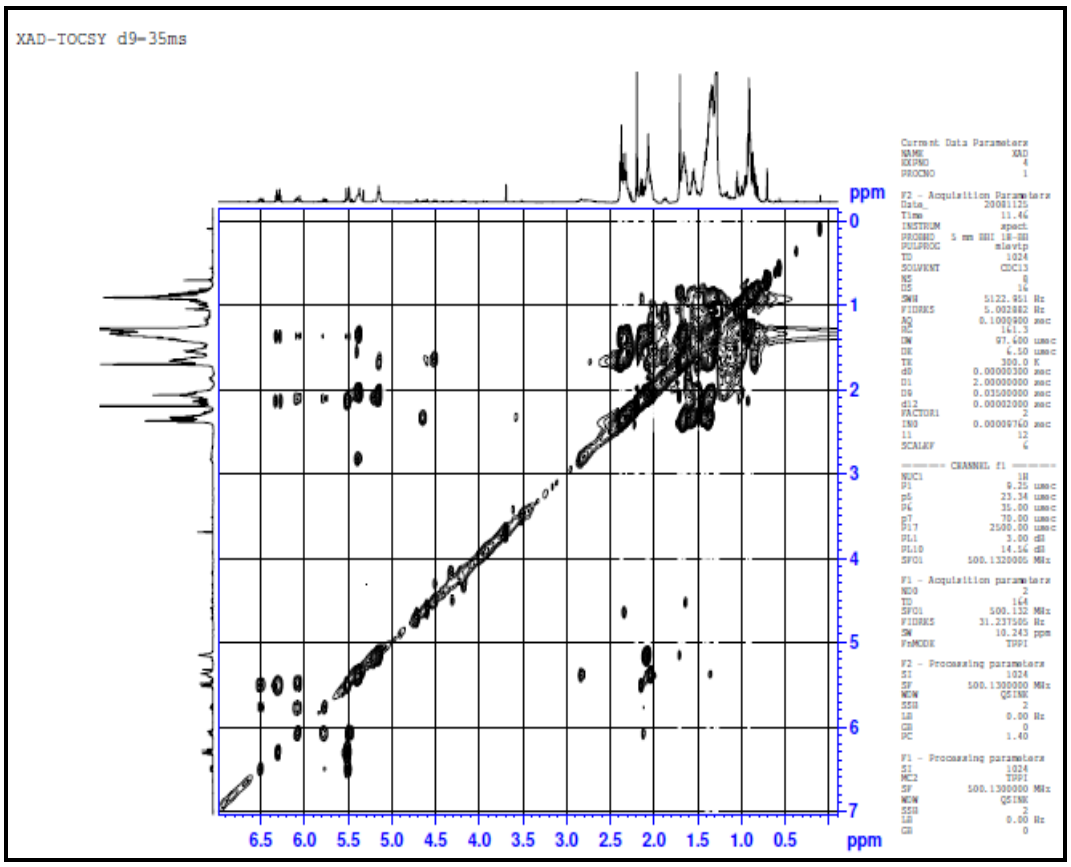
ii.


HSQC-DEPT
ppm
10
15
20
25
30
35
40
45
50
55
60
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
ppm

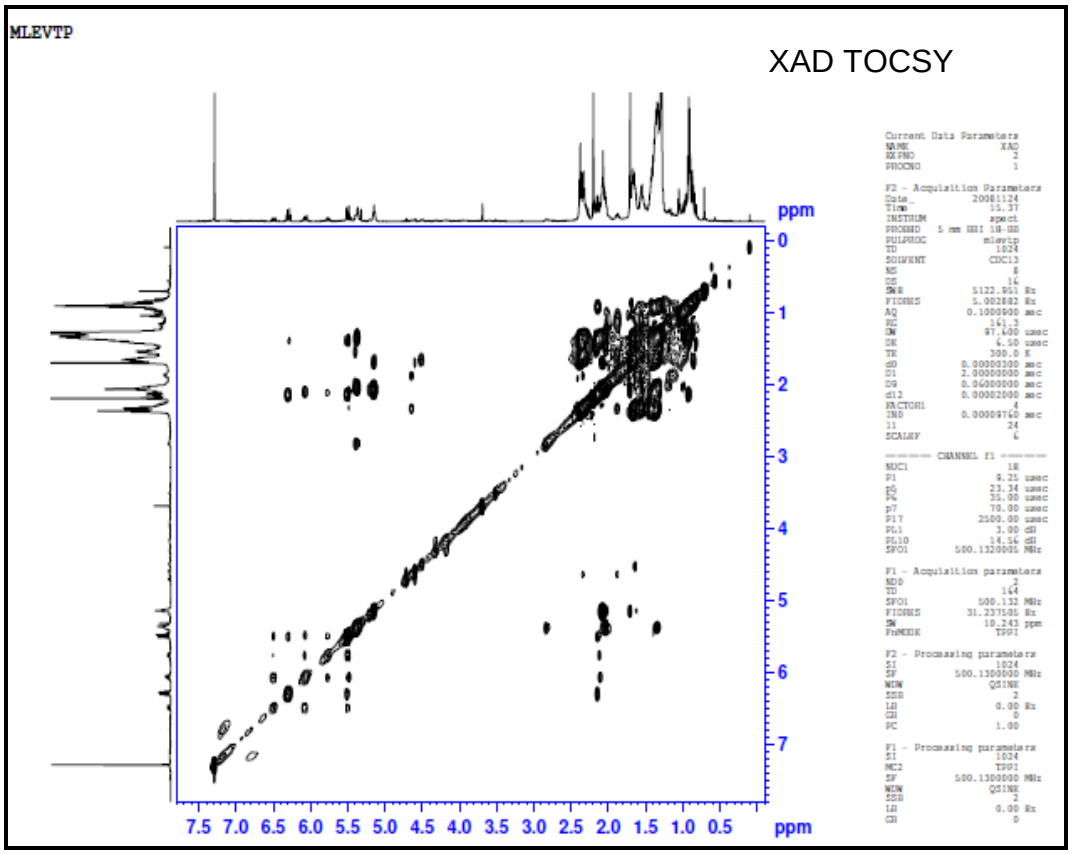
iii.


HSQC-DEPT
ppm
10
15
20
25
30
35
40
45
50
55
60
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
ppm

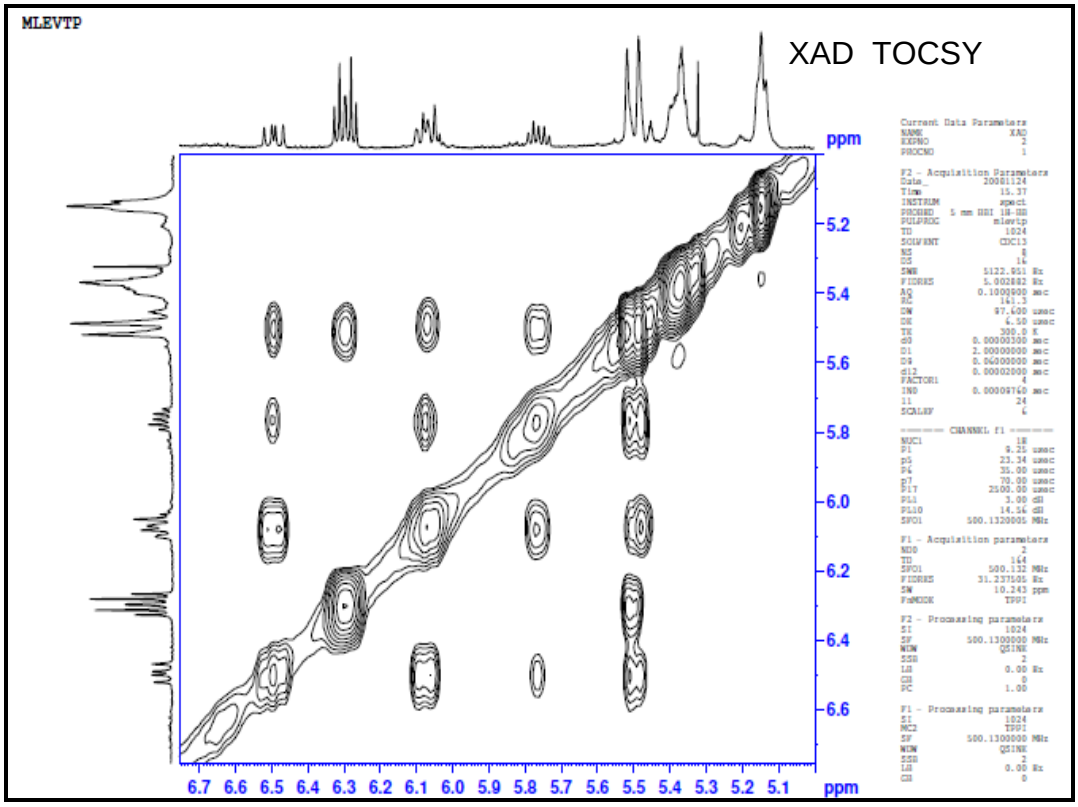
iv.


XAD-TOCSY d9=35ms
ppm
6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

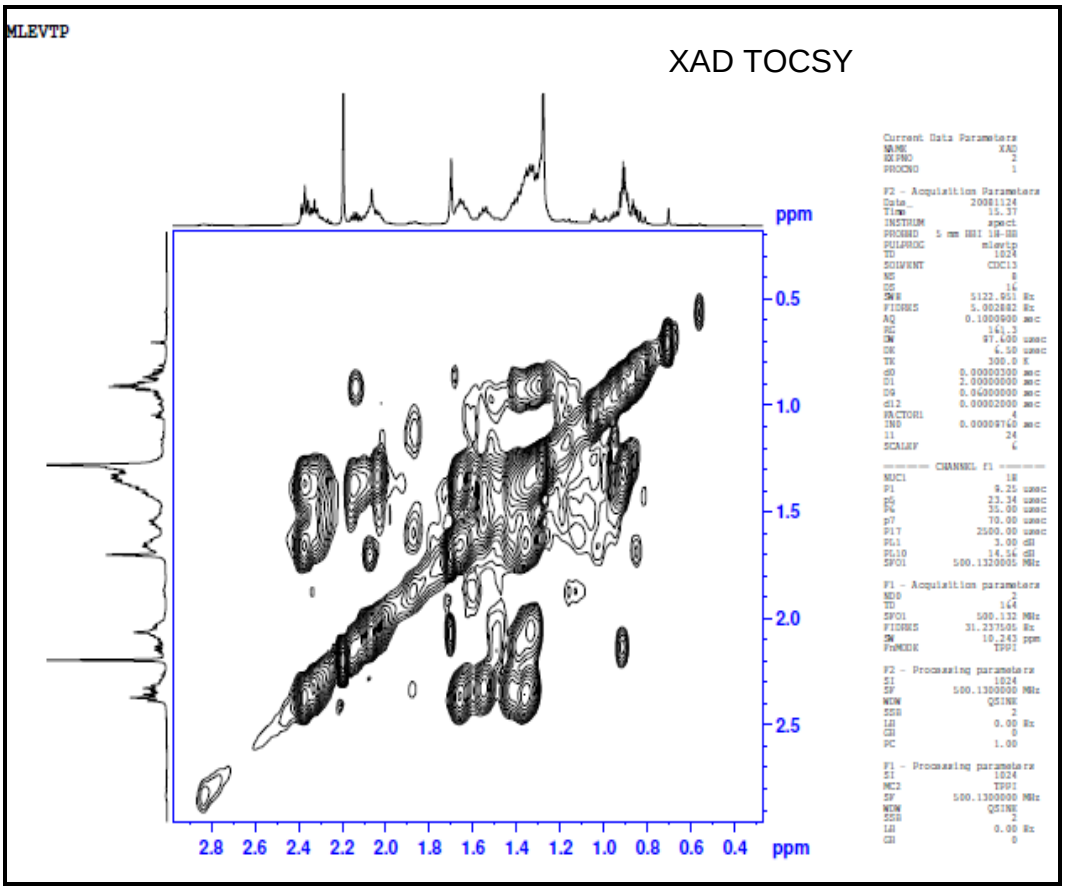
v.


MLEVTP
XAD TOCSY
ppm
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

vi.

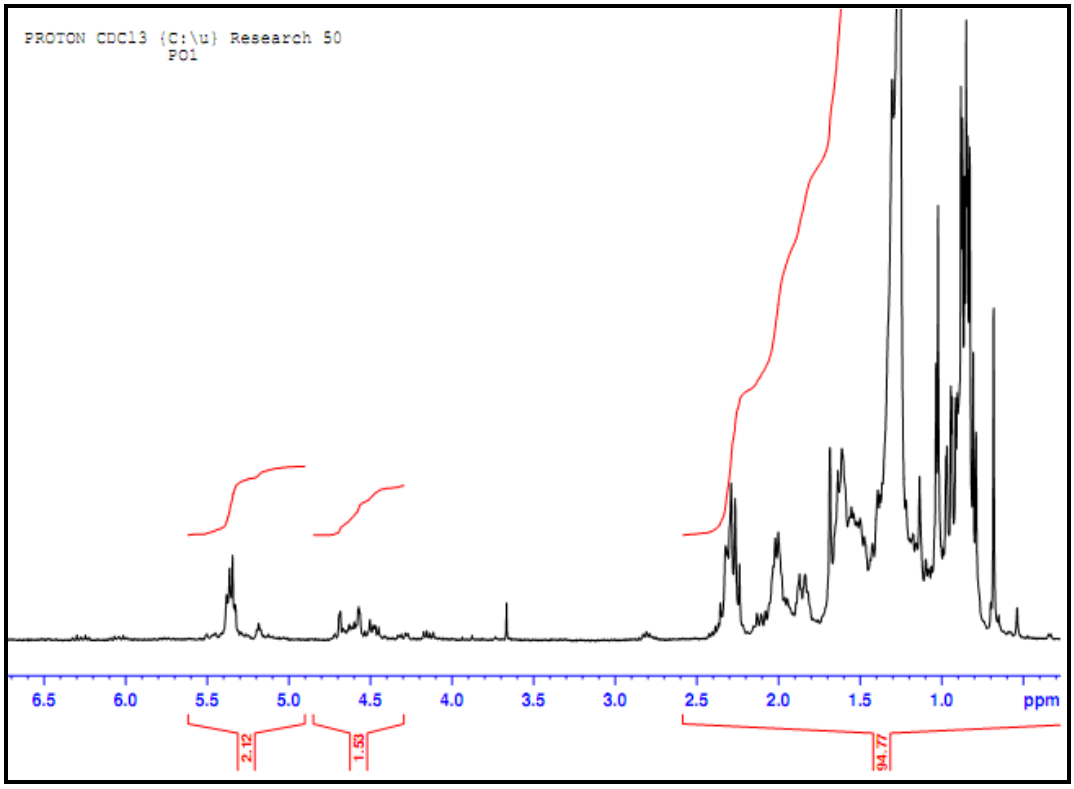

MLEVTP
XAD TOCSY
ppm
5.2
5.4
5.6
5.8
6.0
6.2
6.4
6.6
6.7 6.6 6.5 6.4 6.3 6.2 6.1 6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1
ppm

vii.

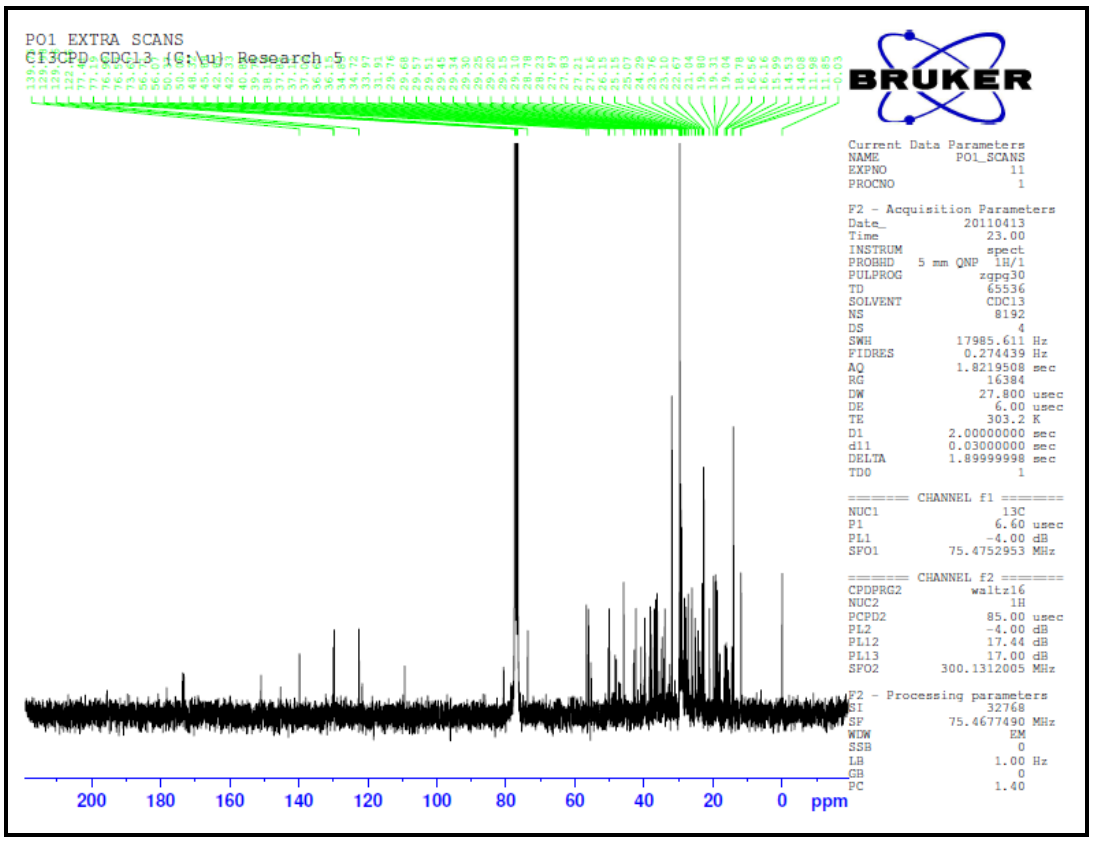

MLEVTP
XAD TOCSY
ppm
0.5
1.0
1.5
2.0
2.5
2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4
ppm

3. XD

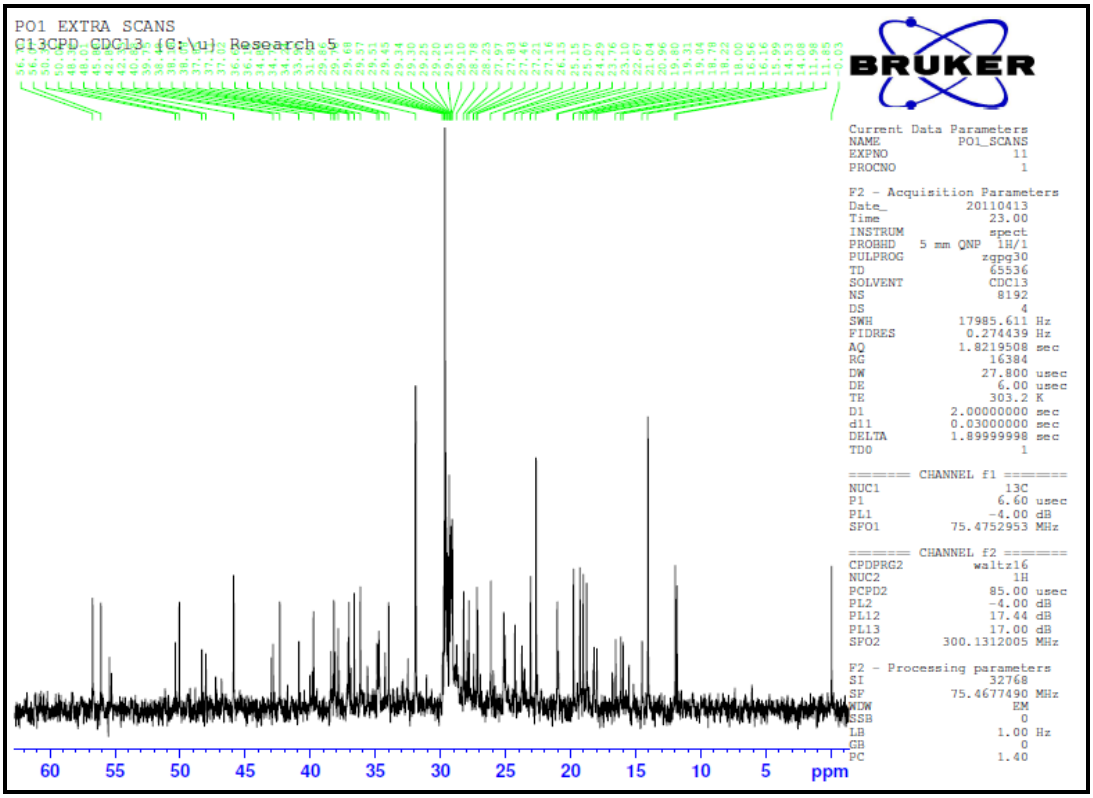
i.


PROTON CDCl3 (C:\u) Research 50
PO1
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
2.12
1.53
94.77

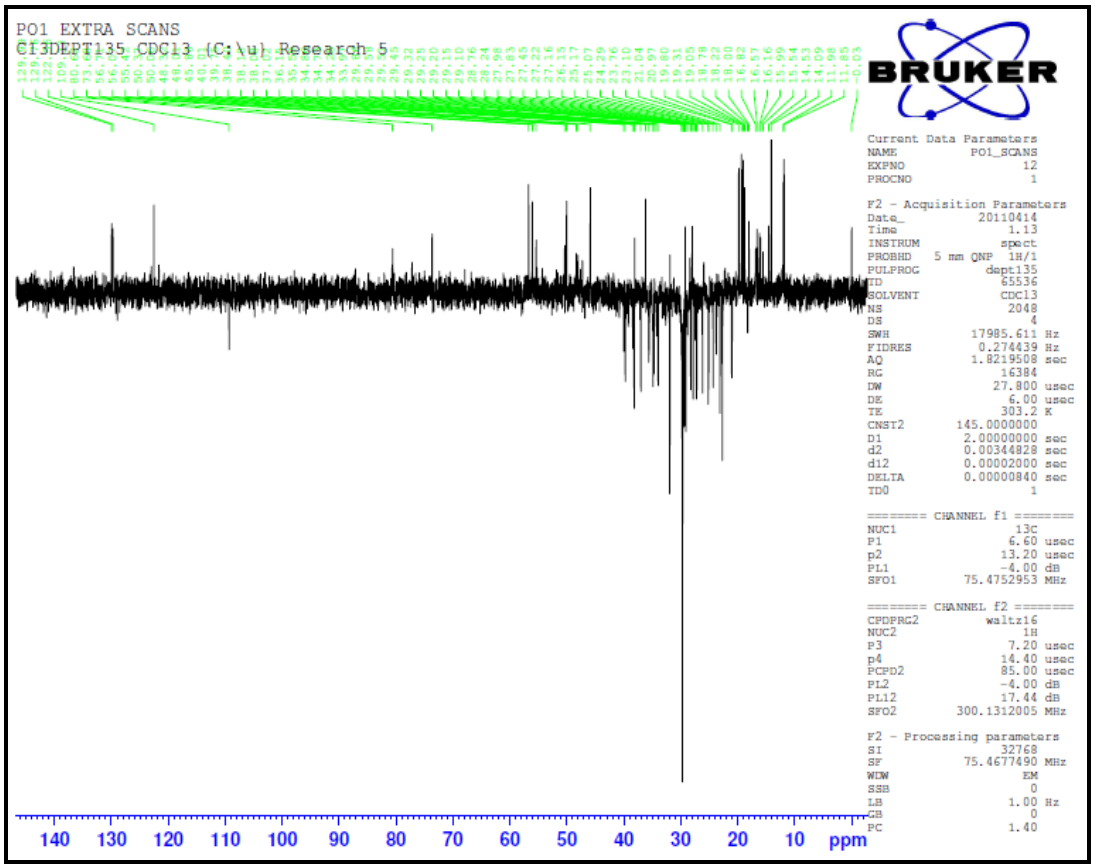
ii.


PO1 EXTRA SCANS
CF3CPD CDCl3 (C:\u) Research 5
BRUKER
Current Data Parameters
NAME PO1_SCANS
EXPNO 11
PROCNO 1
F2 - Acquisition Parameters
Date_ 20110413
Time 23.00
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 8192
DS 4
SWH 17985.611 Hz
FIDRES 0.274439 Hz
AQ 1.8219508 sec
RG 16384
DW 27.800 usec
DE 6.00 usec
TE 303.2 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1
======= CHANNEL f1 =======
NUC1 13C
P1 6.60 usec
PL1 -4.00 dB
SFO1 75.4752953 MHz
======= CHANNEL f2 =======
CPDPRG2 waltz16
NUC2 1H
PCPD2 85.00 usec
PL2 -4.00 dB
PL12 17.44 dB
PL13 17.00 dB
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677490 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
200
180
160
140
120
100
80
60
40
20
0
ppm

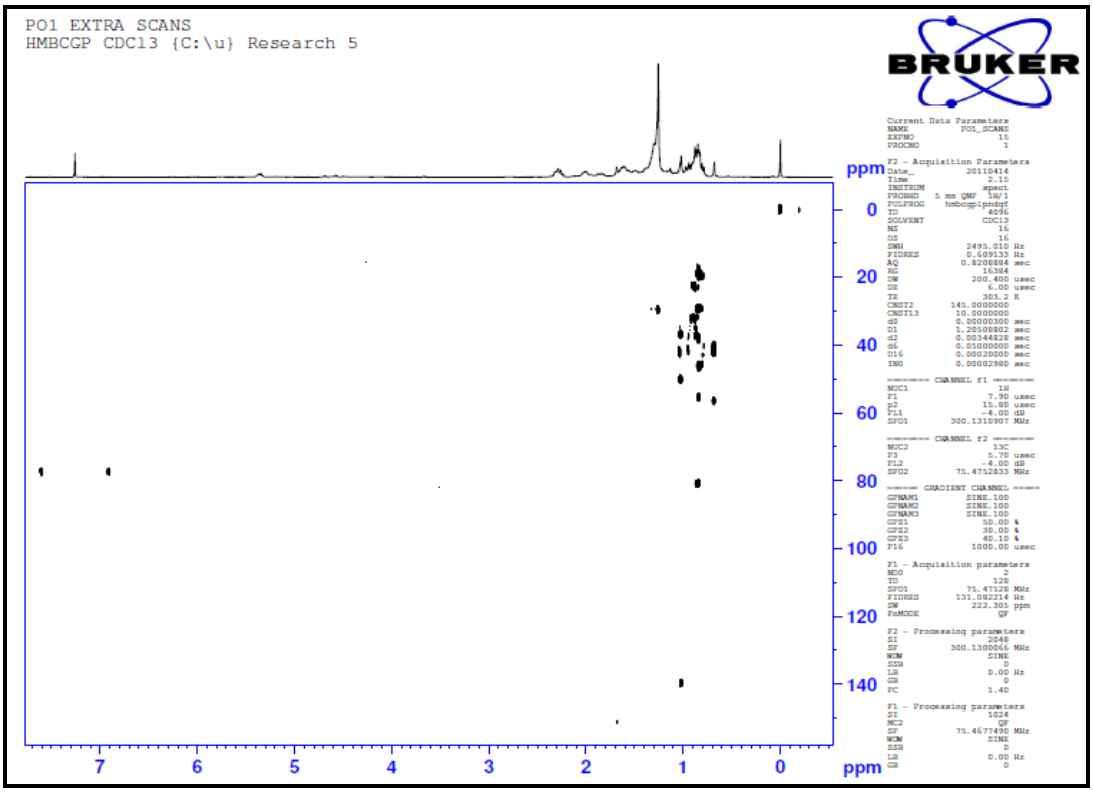
iii.


PO1 EXTRA SCANS
C13CPD CDCl3 (C:\u) Research 5
BRUKER
Current Data Parameters
NAME PO1_SCANS
EXPNO 11
PROCNO 1
F2 - Acquisition Parameters
Date_ 20110413
Time 23.00
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 8192
DS 4
SWH 17985.611 Hz
FIDRES 0.274439 Hz
AQ 1.8219508 sec
RG 16384
DW 27.800 usec
DE 6.00 usec
TE 303.2 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1
======= CHANNEL f1 =======
NUC1 13C
P1 6.60 usec
PL1 -4.00 dB
SFO1 75.4752953 MHz
======= CHANNEL f2 =======
CPDPRG2 waltz16
NUC2 1H
PCPD2 85.00 usec
PL2 -4.00 dB
PL12 17.44 dB
PL13 17.00 dB
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677490 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
60 55 50 45 40 35 30 25 20 15 10 5 ppm

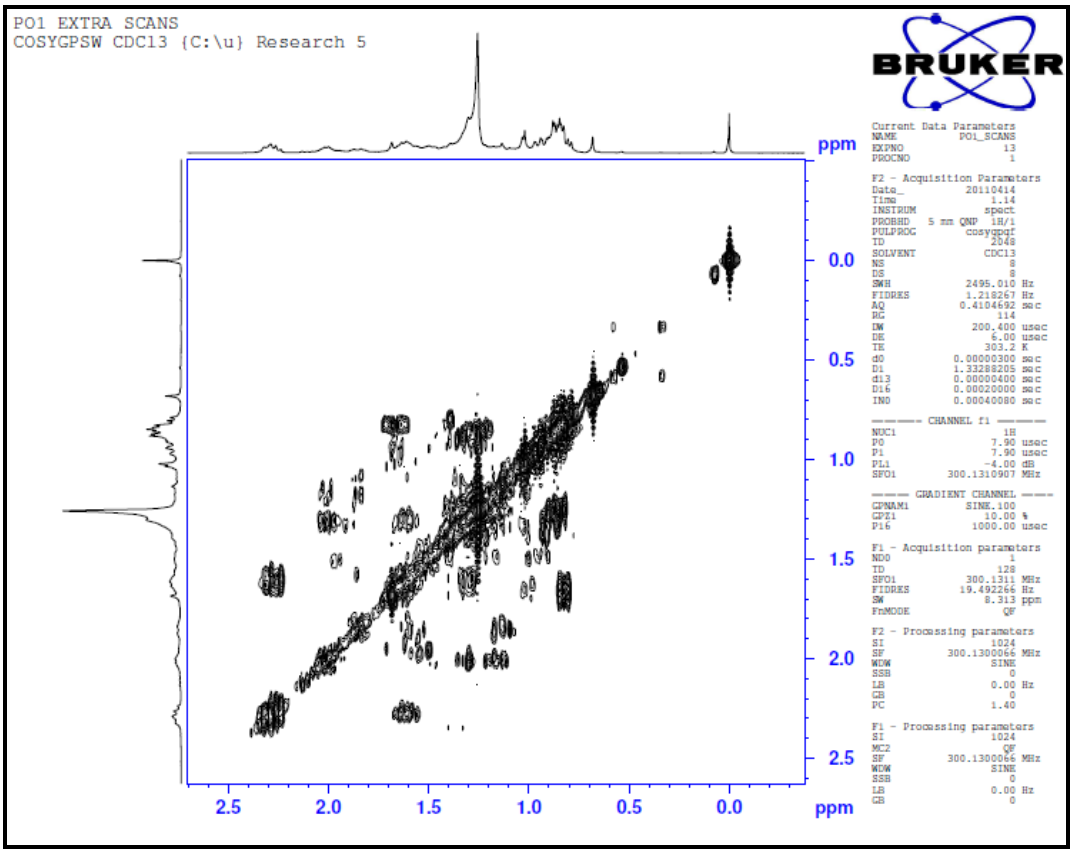
iv.


PO1 EXTRA SCANS
C13DEPT135 CDCl3 {C:\u} Research 5
BRUKER
Current Data Parameters
NAME PO1_SCANS
EXPNO 12
PROCNO 1
F2 - Acquisition Parameters
Date_ 20110414
Time 1.13
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG dept135
TD 65536
SOLVENT CDCl3
NS 2048
DS 4
SWH 17985.611 Hz
FIDRES 0.274439 Hz
AQ 1.8219508 sec
RG 16384
DW 27.800 usec
DE 6.00 usec
TE 303.2 K
CNST2 145.0000000
D1 2.00000000 sec
d2 0.00344828 sec
d12 0.00002000 sec
DELTA 0.00000840 sec
TD0 1
======== CHANNEL f1 ========
NUC1 13C
P1 6.60 usec
p2 13.20 usec
PL1 -4.00 dB
SFO1 75.4752953 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
P3 7.20 usec
p4 14.40 usec
PCPD2 85.00 usec
PL2 -4.00 dB
PL12 17.44 dB
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677490 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

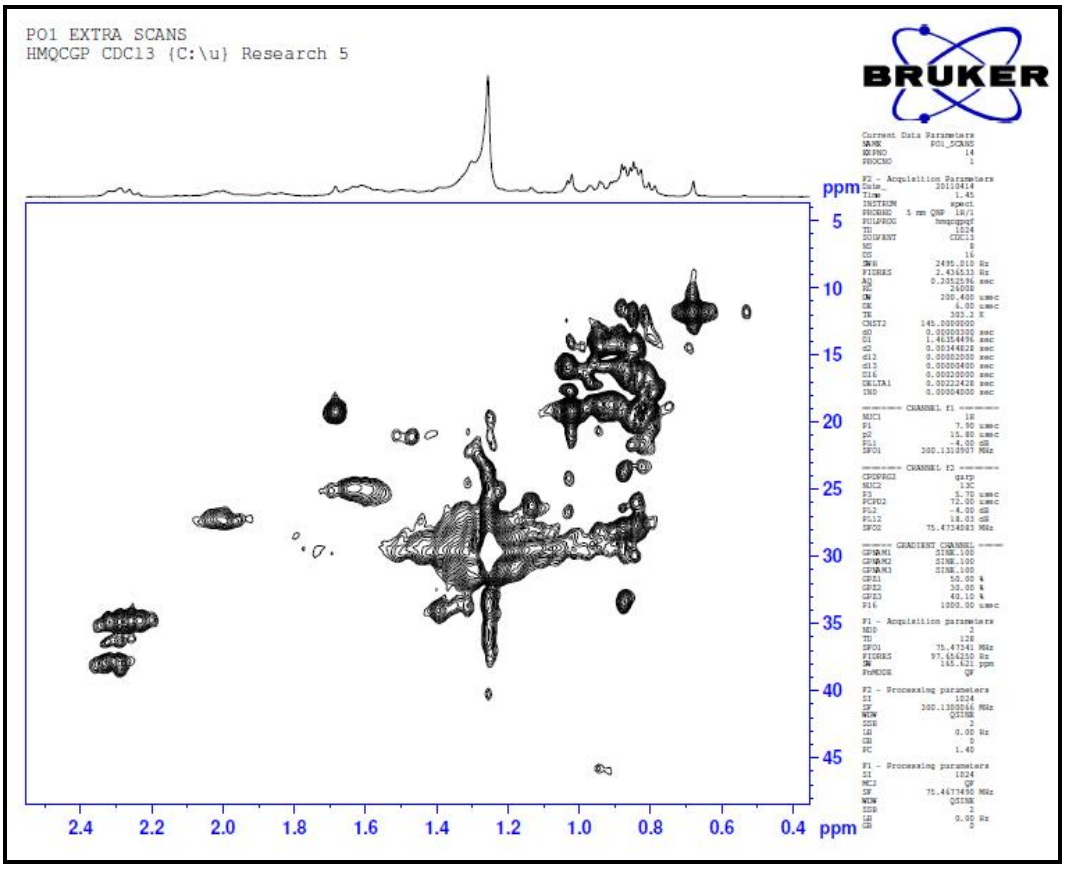
v.


PO1 EXTRA SCANS
HMBCGP CDCl3 {C:\u} Research 5
BRUKER
ppm
0
20
40
60
80
100
120
140
7 6 5 4 3 2 1 0 ppm

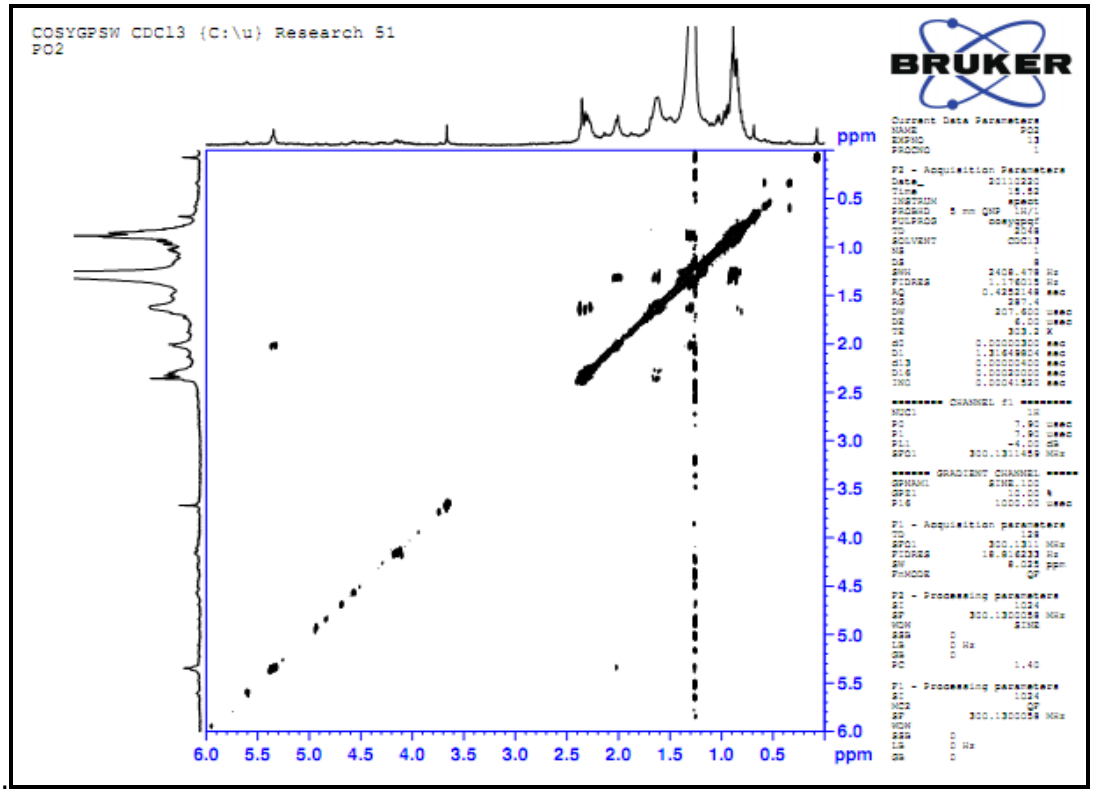
vi.


PO1 EXTRA SCANS
COSYGPSW CDCl3 (C:\u) Research 5
BRUKER
ppm
0.0
0.5
1.0
1.5
2.0
2.5
2.5
2.0
1.5
1.0
0.5
0.0
ppm

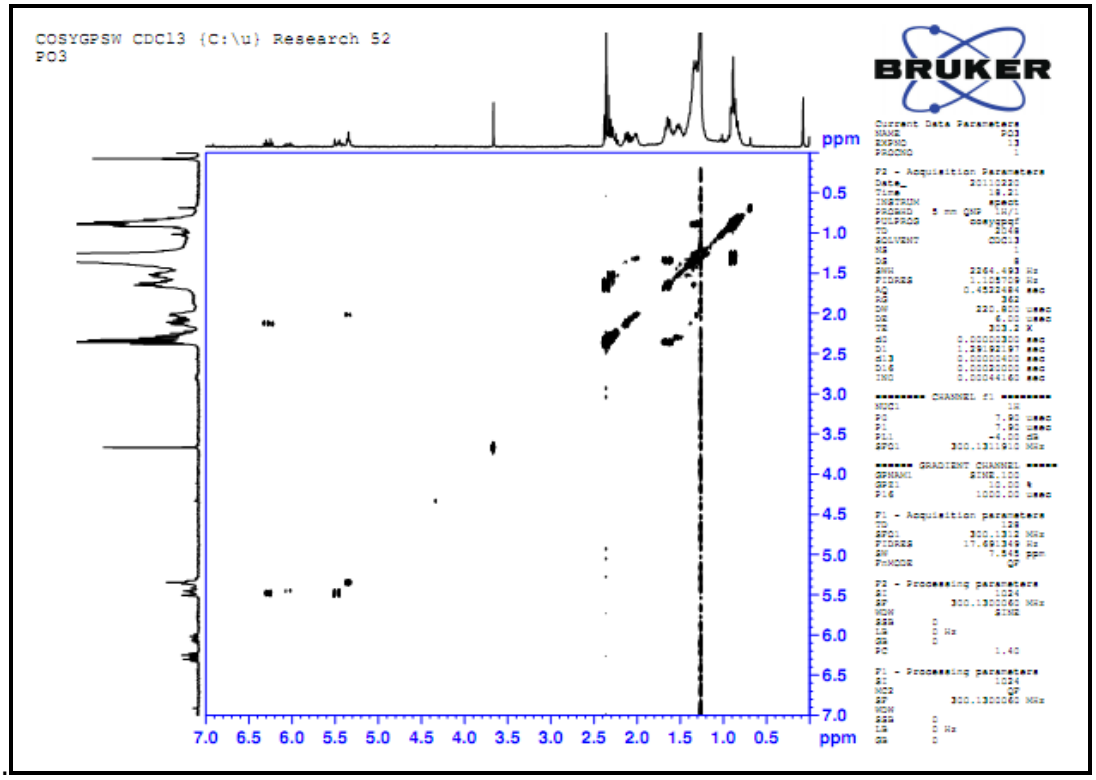
vii.


PO1 EXTRA SCANS
HMQCGP CDCl3 (C:\u) Research 5
BRUKER
ppm
5
10
15
20
25
30
35
40
45
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
ppm

4. XD2

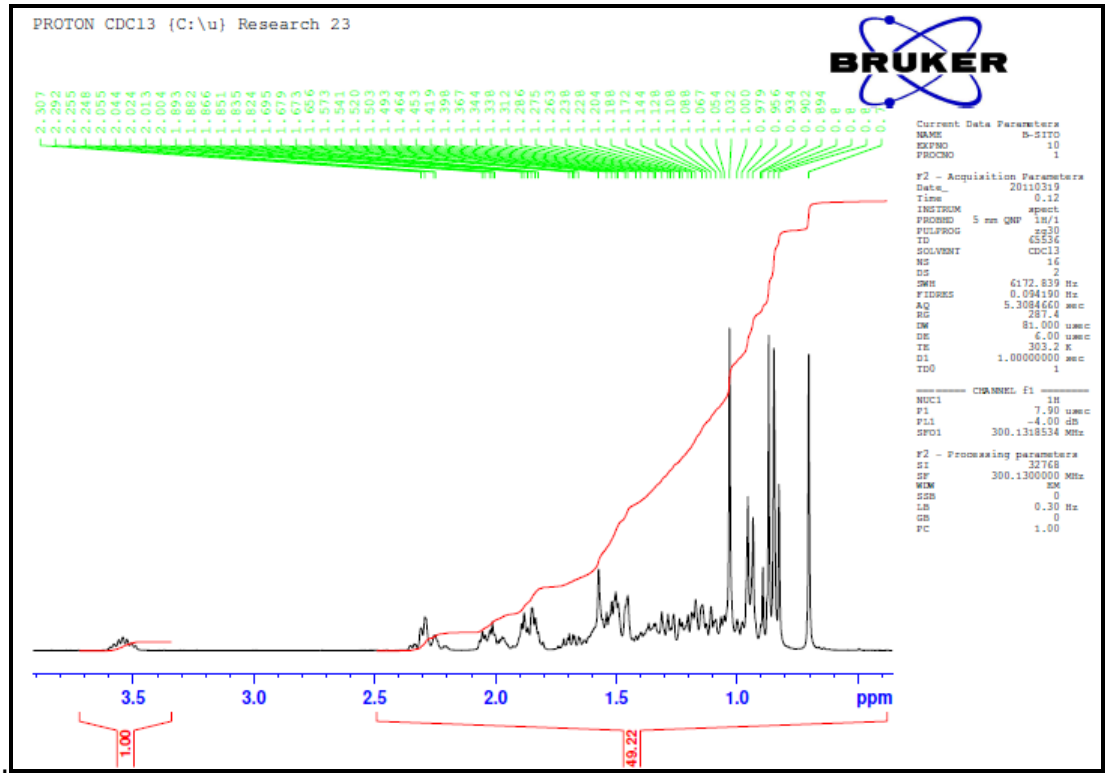
i. 
COSYGPSW CDCl3 (C:\u) Research 51
PO2
BRUKER
ppm
6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

5. XD3

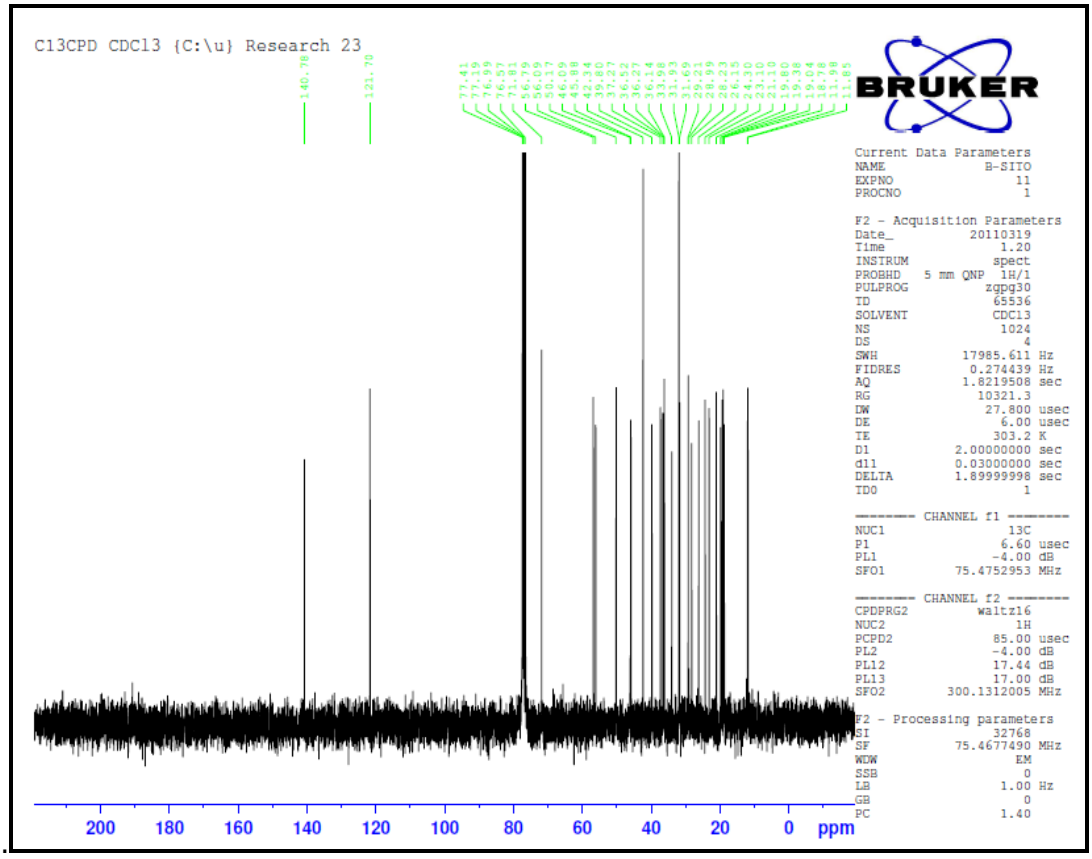
i. 
COSYGPSW CDCl3 (C:\u) Research 52
PO3
BRUKER
ppm
7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

6. β-sitosterol

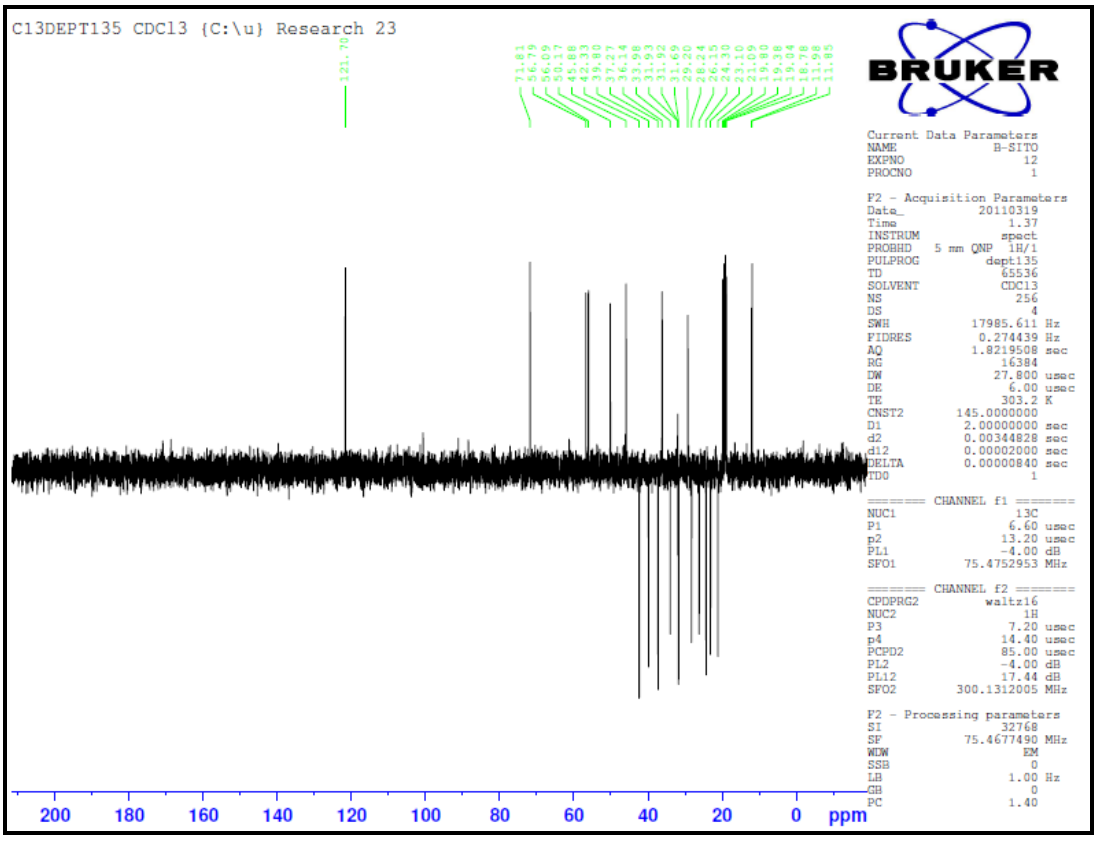
i.



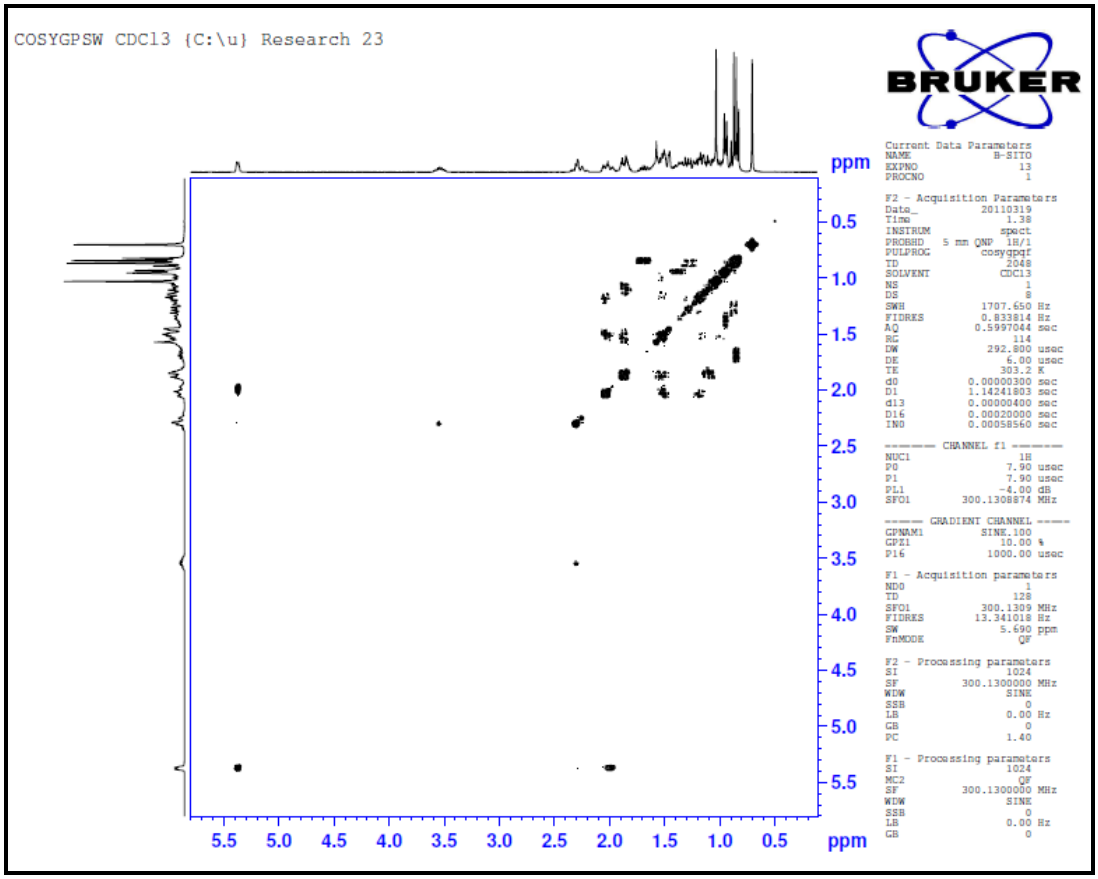
ii.



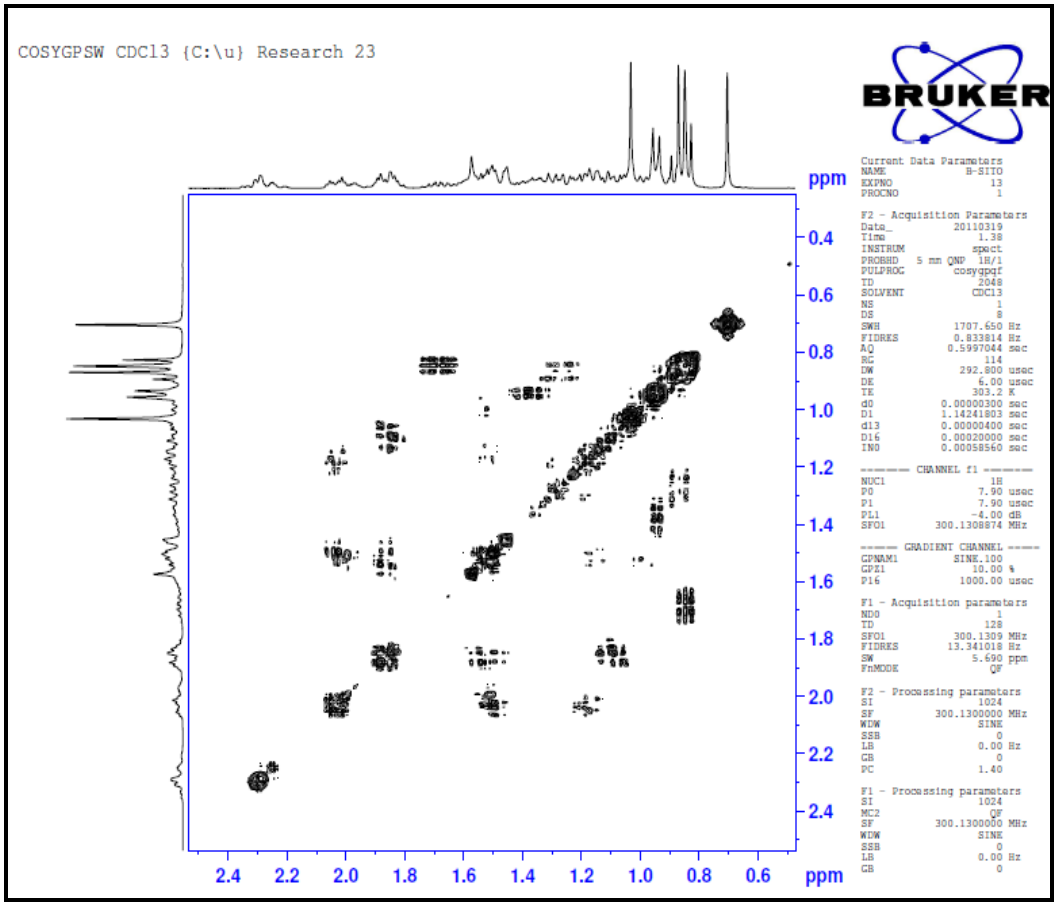
iii.


C13DEPT135 CDCl3 {C:\u} Research 23
BRUKER
200 180 160 140 120 100 80 60 40 20 0 ppm

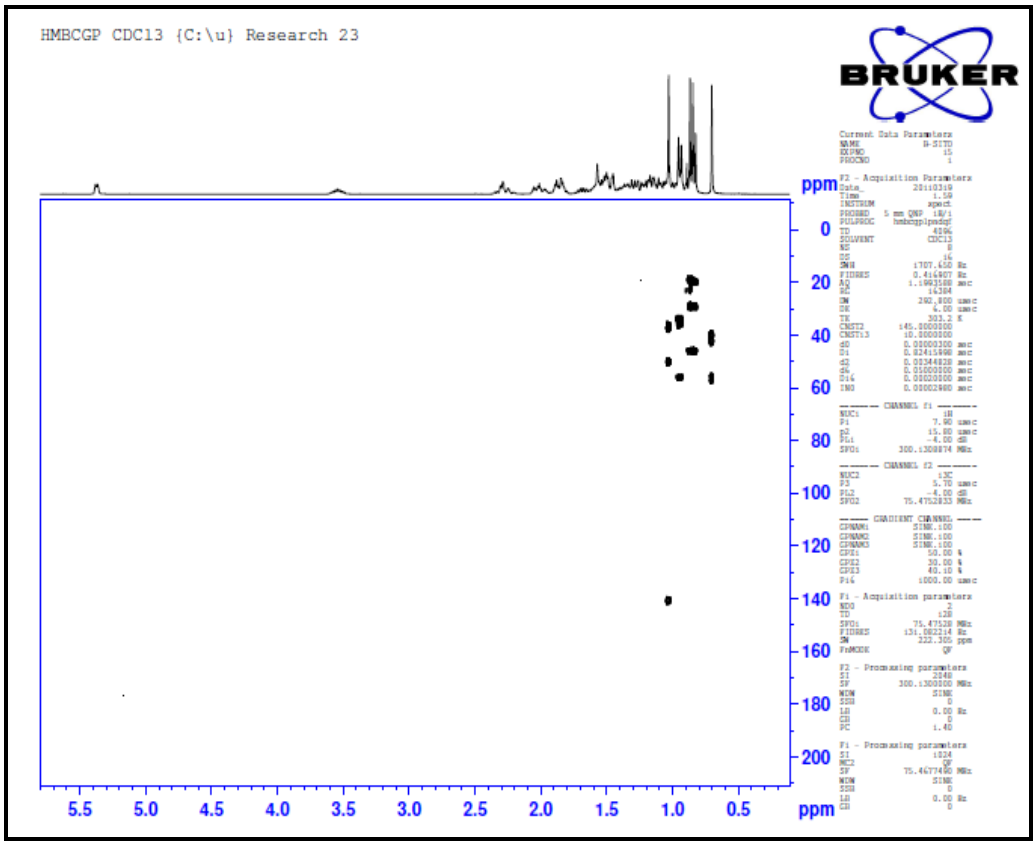
iv.


COSYGPSW CDCl3 {C:\u} Research 23
BRUKER
ppm
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

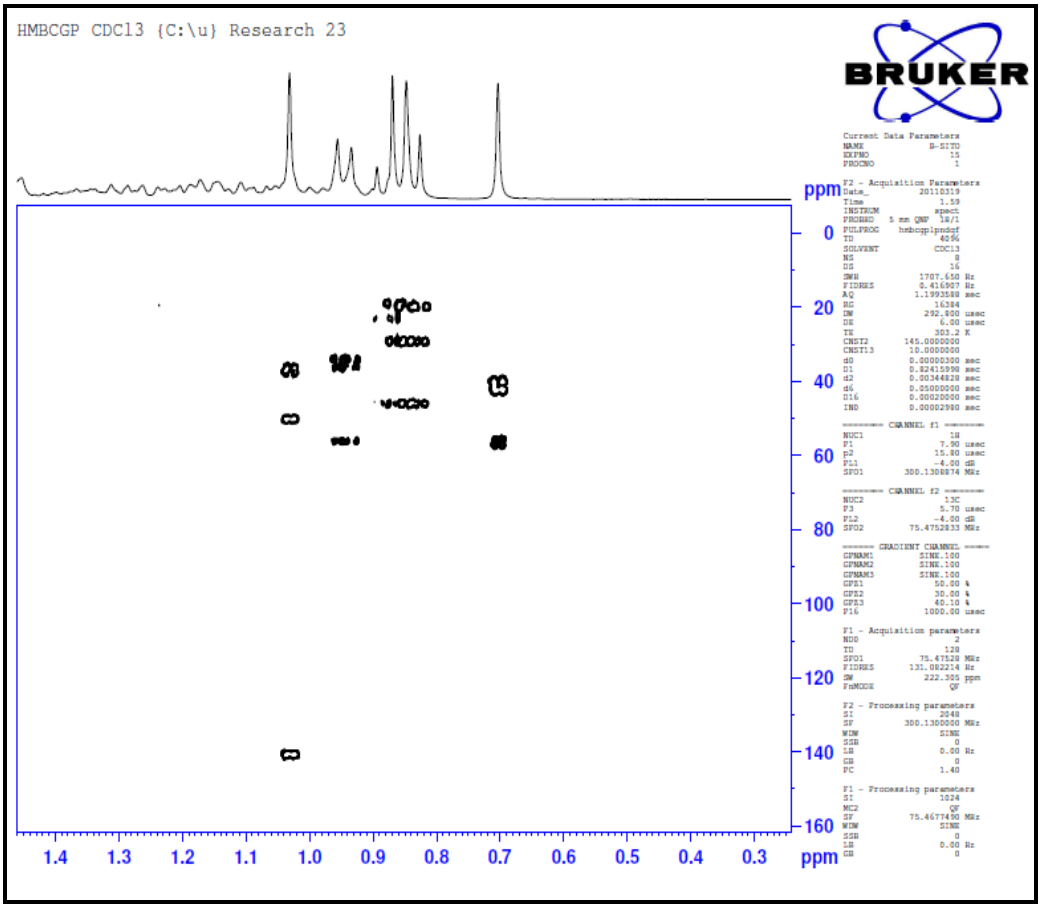
v.


COSYGPSW CDCl3 {C:\u} Research 23
BRUKER
ppm
2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 ppm

vi.

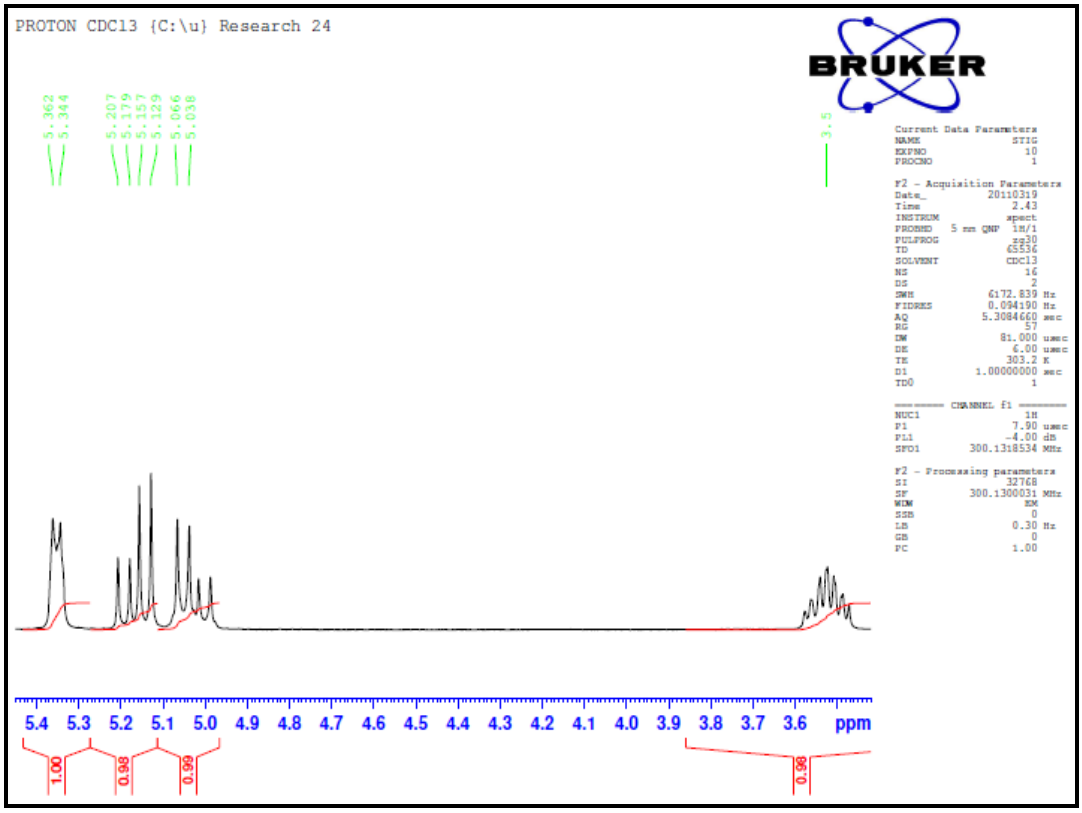

HMBCGP CDCl3 {C:\u} Research 23
BRUKER
ppm
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

vii.

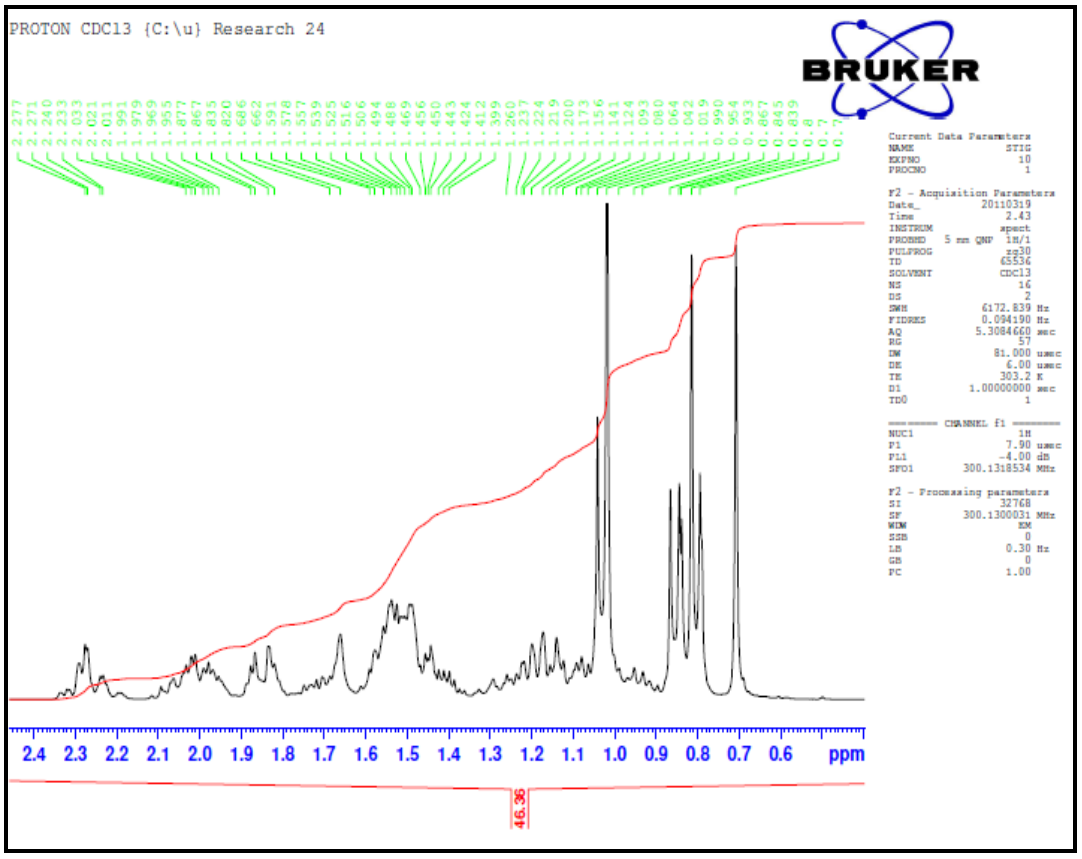


7. Stigmasterol.

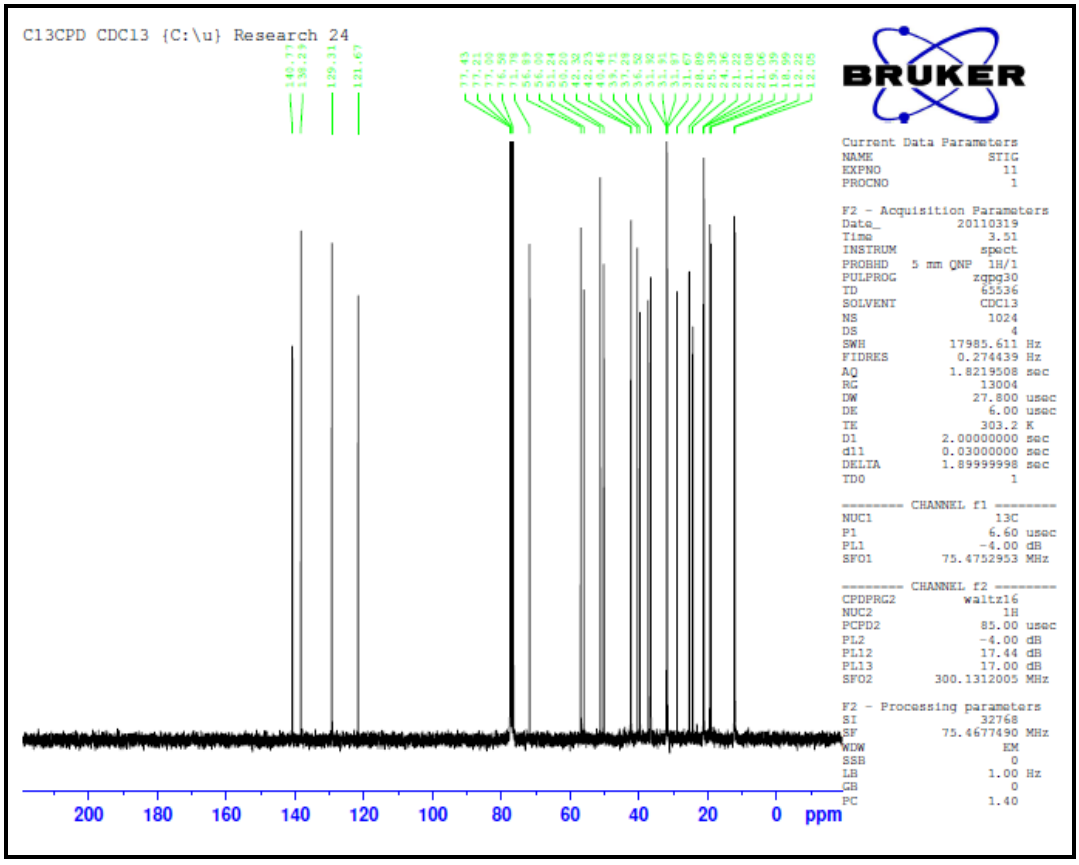
i.



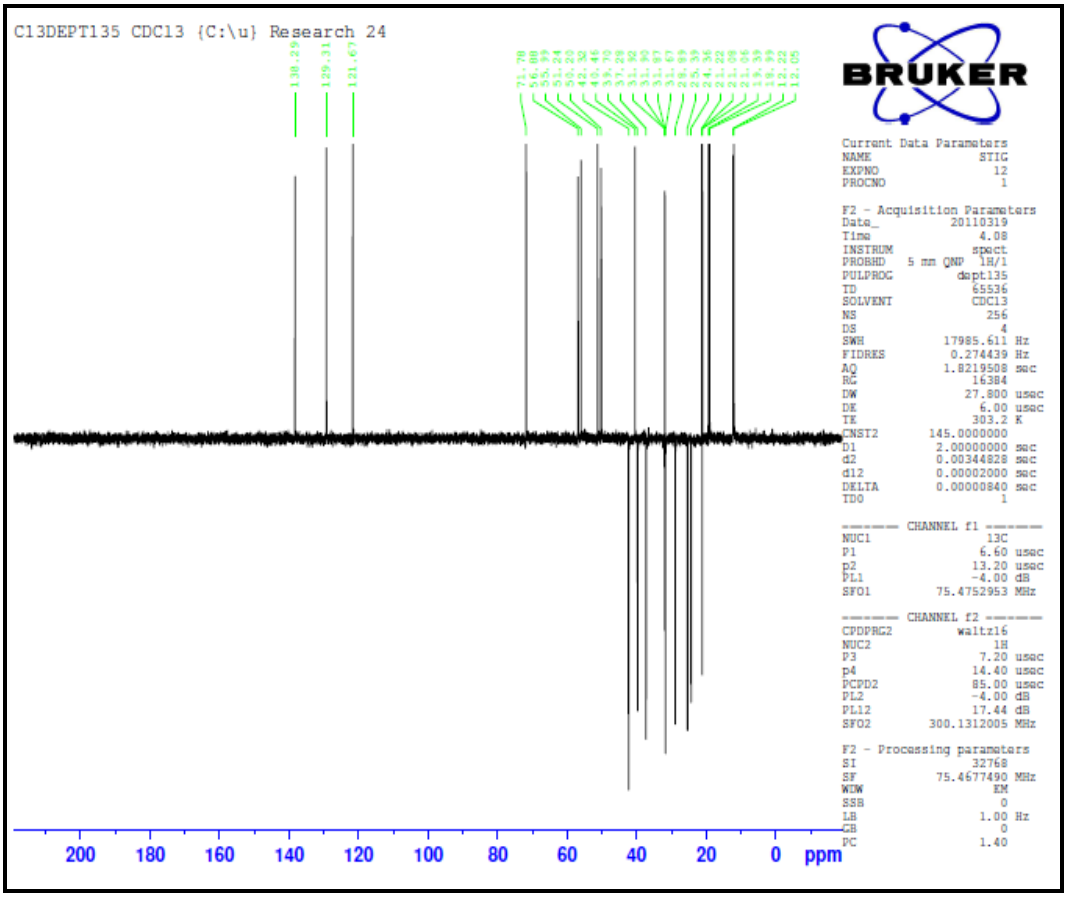
ii.


PROTON CDC13 {C:\u} Research 24
BRUKER
2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 0.9 0.8 0.7 0.6 ppm
46.36

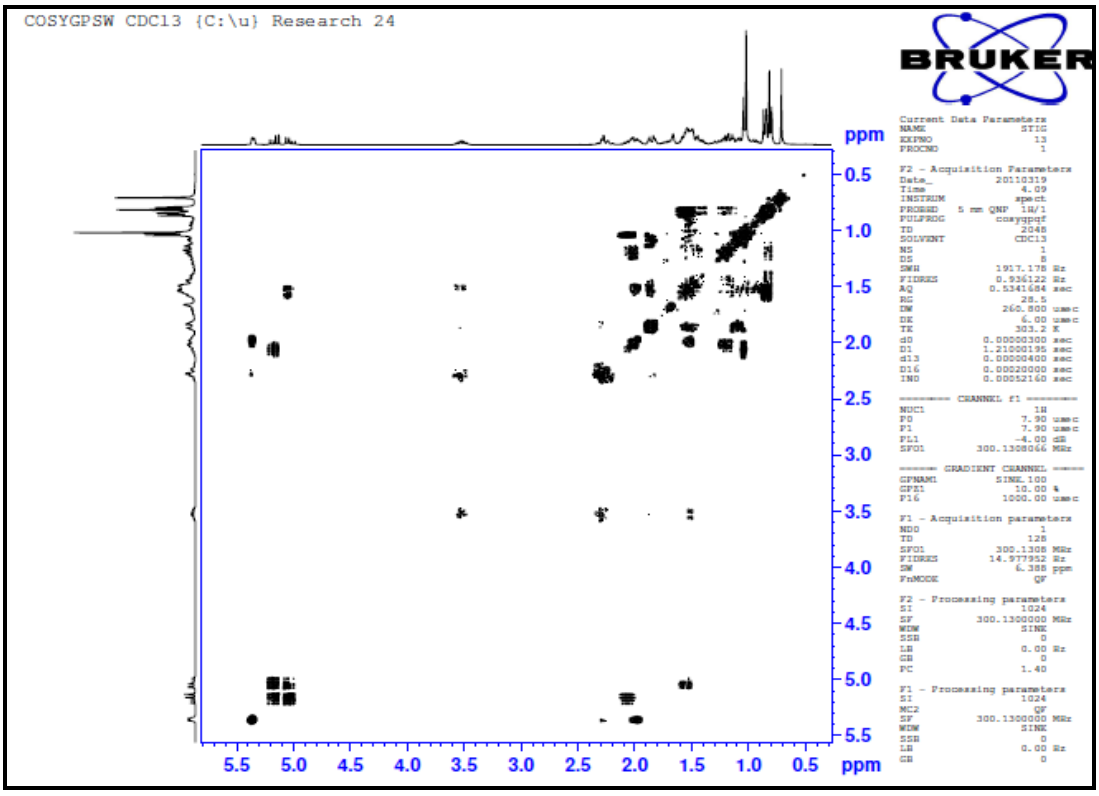
iii.


C13CPD CDC13 {C:\u} Research 24
BRUKER
200 180 160 140 120 100 80 60 40 20 0 ppm

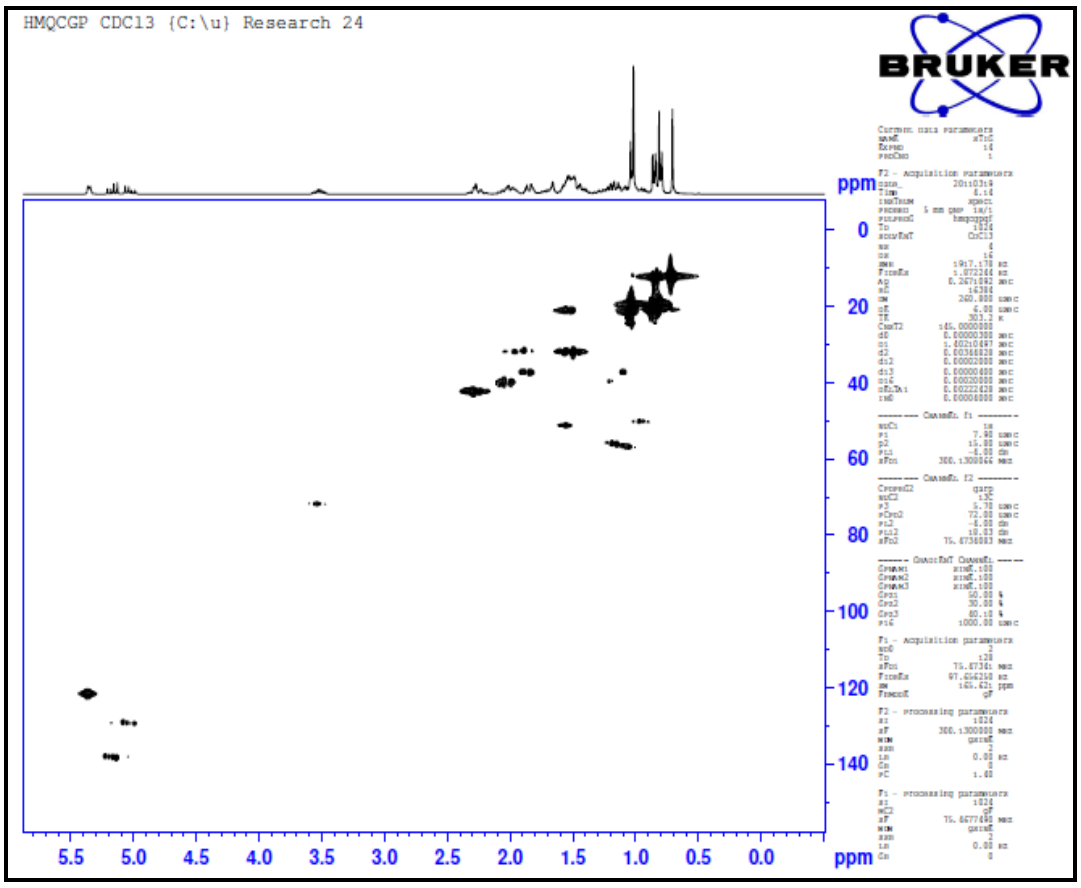
iv.


C13DEPT135 CDC13 {C:\u} Research 24
BRUKER
Current Data Parameters
NAME STIG
EXPNO 12
PROCNO 1
F2 - Acquisition Parameters
Date_ 20110319
Time 4.08
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG dept135
TD 65536
SOLVENT CDC13
NS 256
DS 4
SWH 17985.611 Hz
FIDRES 0.274439 Hz
AQ 1.8219508 sec
RG 16384
DW 27.800 usec
DE 6.00 usec
TE 303.2 K
CNST2 145.0000000
D1 2.00000000 sec
d2 0.00344828 sec
d12 0.00002000 sec
DELTA 0.00000840 sec
TD0 1
CHANNEL f1
NUC1 13C
P1 6.60 usec
p2 13.20 usec
PL1 -4.00 dB
SFO1 75.4752953 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
P3 7.20 usec
p4 14.40 usec
PCPD2 85.00 usec
PL2 -4.00 dB
PL12 17.44 dB
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677490 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
200 180 160 140 120 100 80 60 40 20 0 ppm

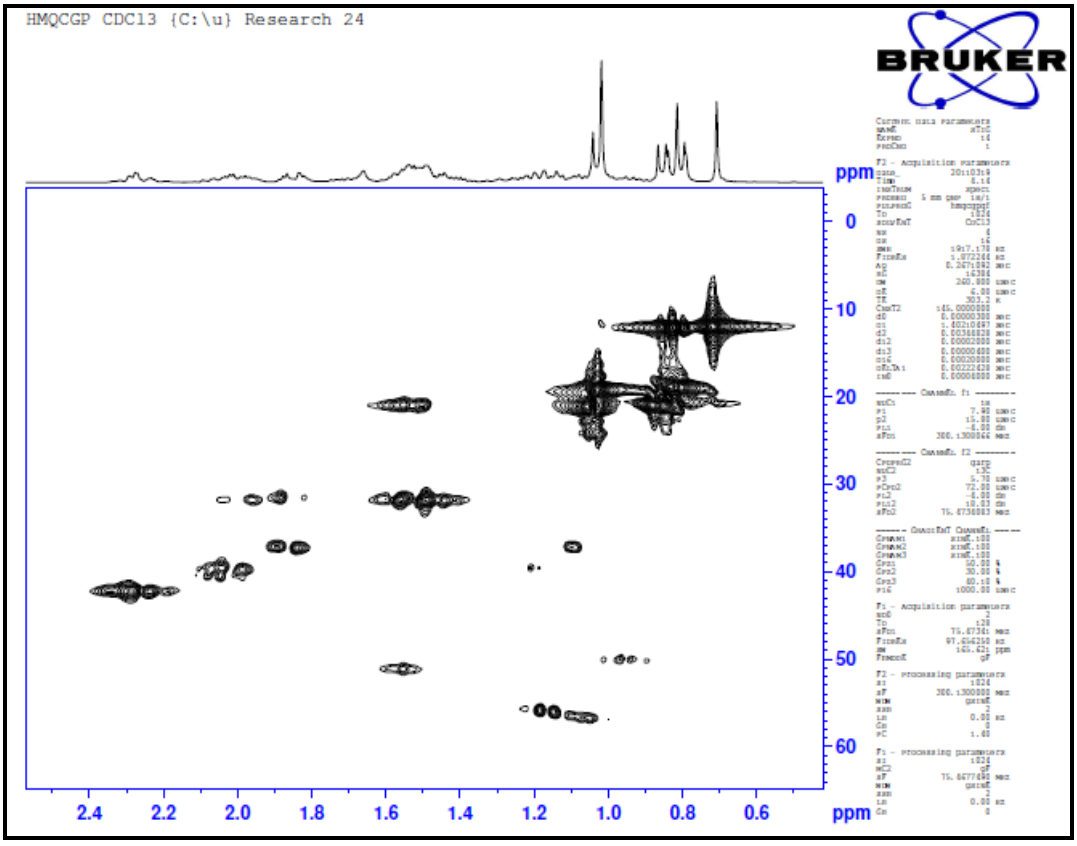
v.


COSYGPSW CDC13 {C:\u} Research 24
BRUKER
Current Data Parameters
NAME STIG
EXPNO 13
PROCNO 1
F2 - Acquisition Parameters
Date_ 20110319
Time 4.09
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG cosygpqf
TD 2048
SOLVENT CDC13
NS 1
DS 8
SWH 1917.178 Hz
FIDRES 0.936122 Hz
AQ 0.5341684 sec
RG 28.5
DW 260.800 usec
DE 6.00 usec
TE 303.2 K
d0 0.00000300 sec
D1 1.21000195 sec
d13 0.00000400 sec
D16 0.00020000 sec
IN0 0.00052160 sec
CHANNEL f1
NUC1 1H
P0 7.90 usec
P1 7.90 usec
PL1 -4.00 dB
SFO1 300.1308066 MHz
GRADIENT CHANNEL
GPNAM1 SINE.100
GPZ1 10.00 %
P16 1000.00 usec
F1 - Acquisition parameters
ND0 1
TD 128
SFO1 300.1308 MHz
FIDRES 14.977952 Hz
SW 6.388 ppm
FnMODE QF
F2 - Processing parameters
SI 1024
SF 300.1300000 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
PC 1.40
F1 - Processing parameters
SI 1024
MC2 QF
SF 300.1300000 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
ppm
0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

vi.

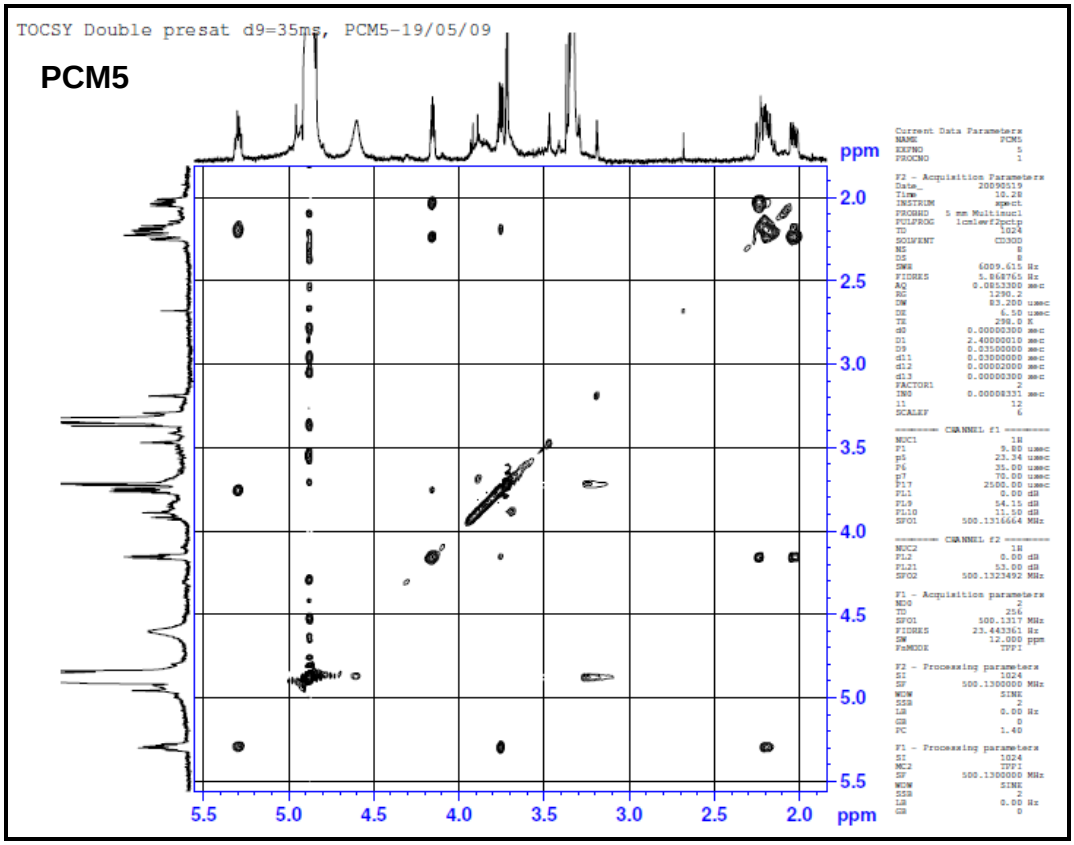

HMQCGP CDC13 {C:\u} Research 24
BRUKER
ppm
0
20
40
60
80
100
120
140
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm

vii.

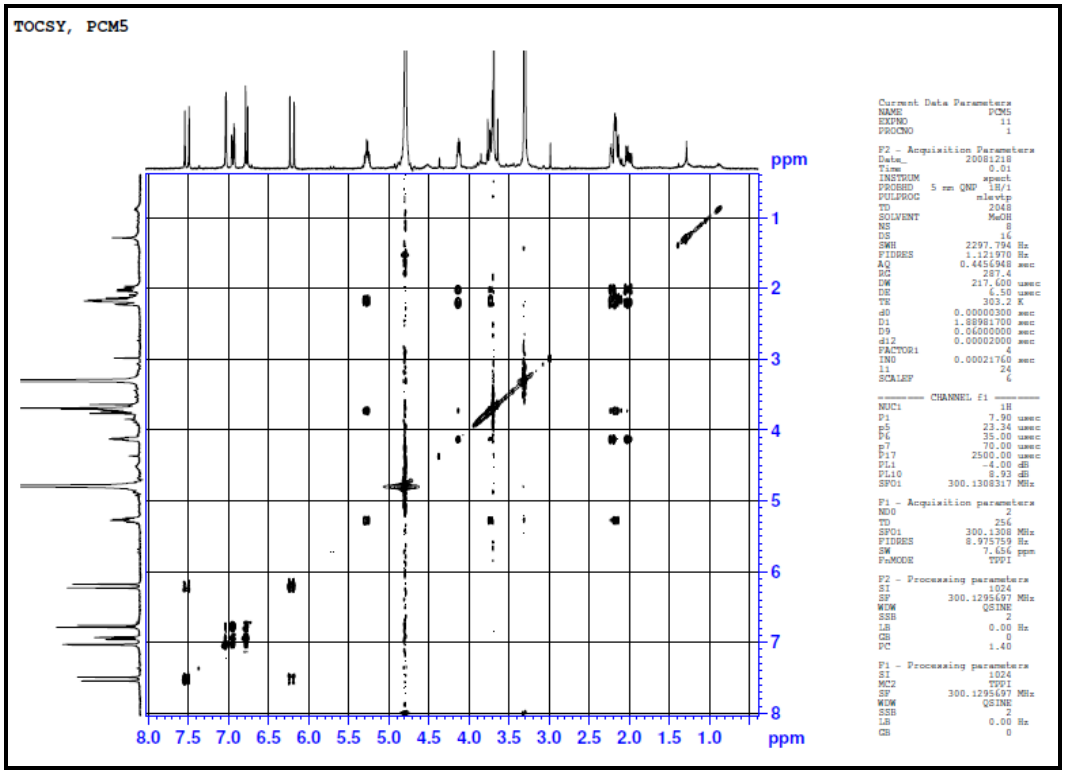

HMQCGP CDC13 {C:\u} Research 24
BRUKER
ppm
0
10
20
30
40
50
60
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
ppm

8. PCM 5

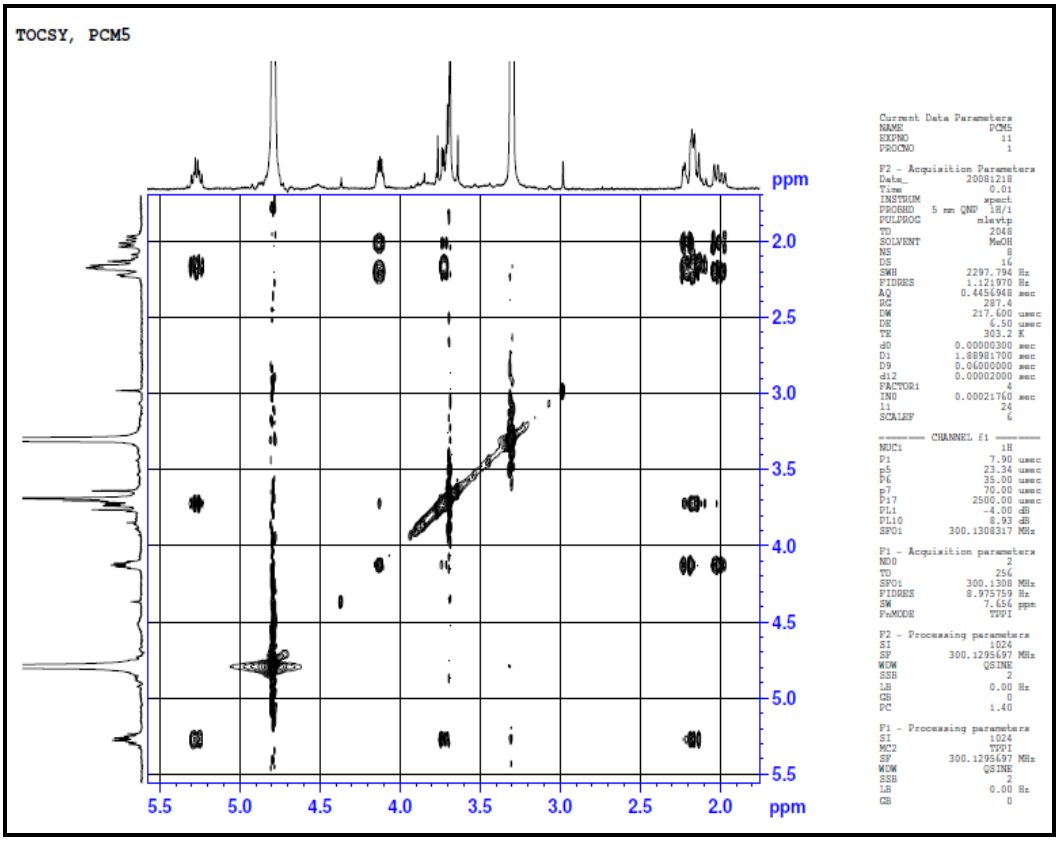
i.


TOCSY Double presat d9=35ms, PCM5-19/05/09
PCM5
ppm
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 ppm

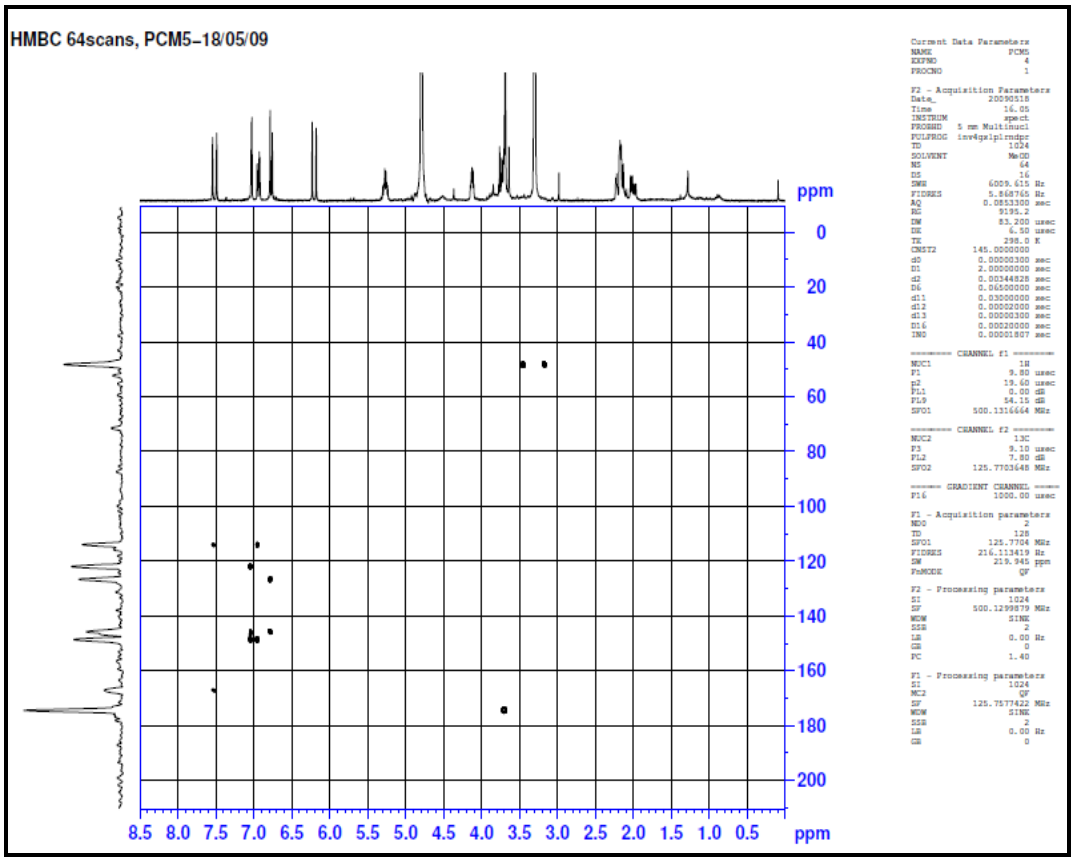
ii.


TOCSY, PCM5
ppm
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm

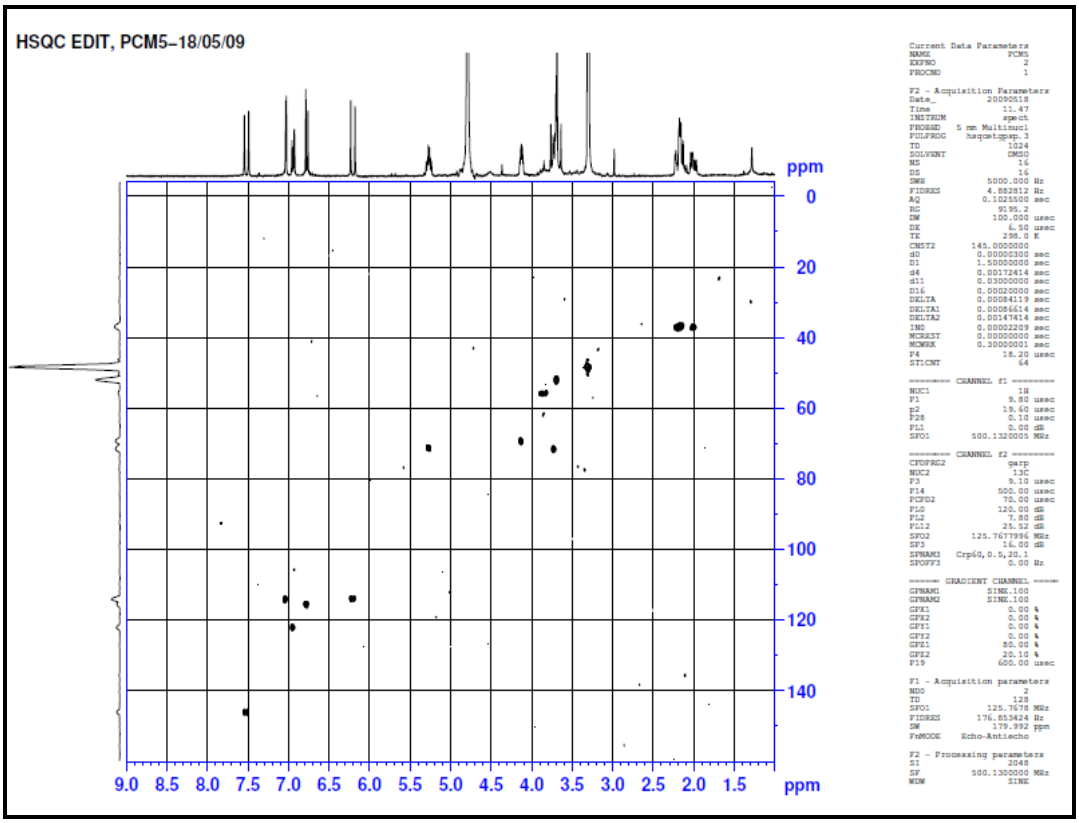
iii.


TOCSY, PCM5
ppm
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 ppm

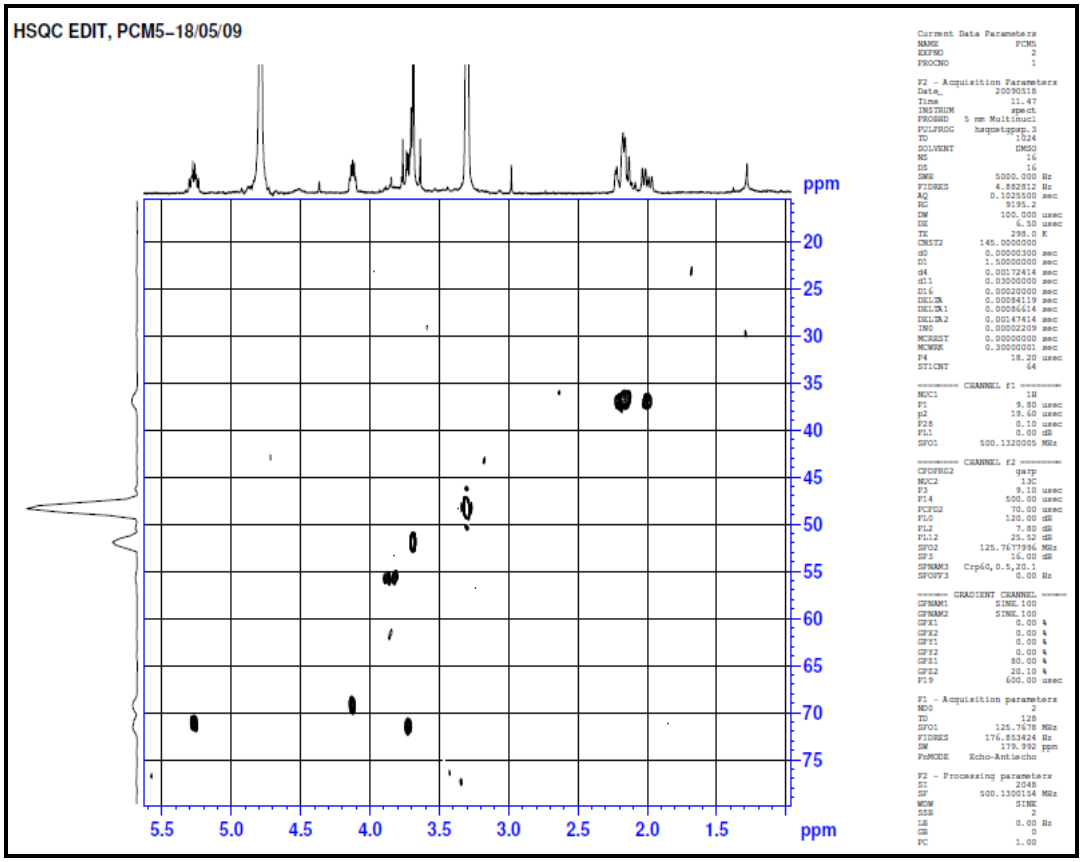
iv.


HMBC 64scans, PCM5–18/05/09
ppm
0
20
40
60
80
100
120
140
160
180
200
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

v.

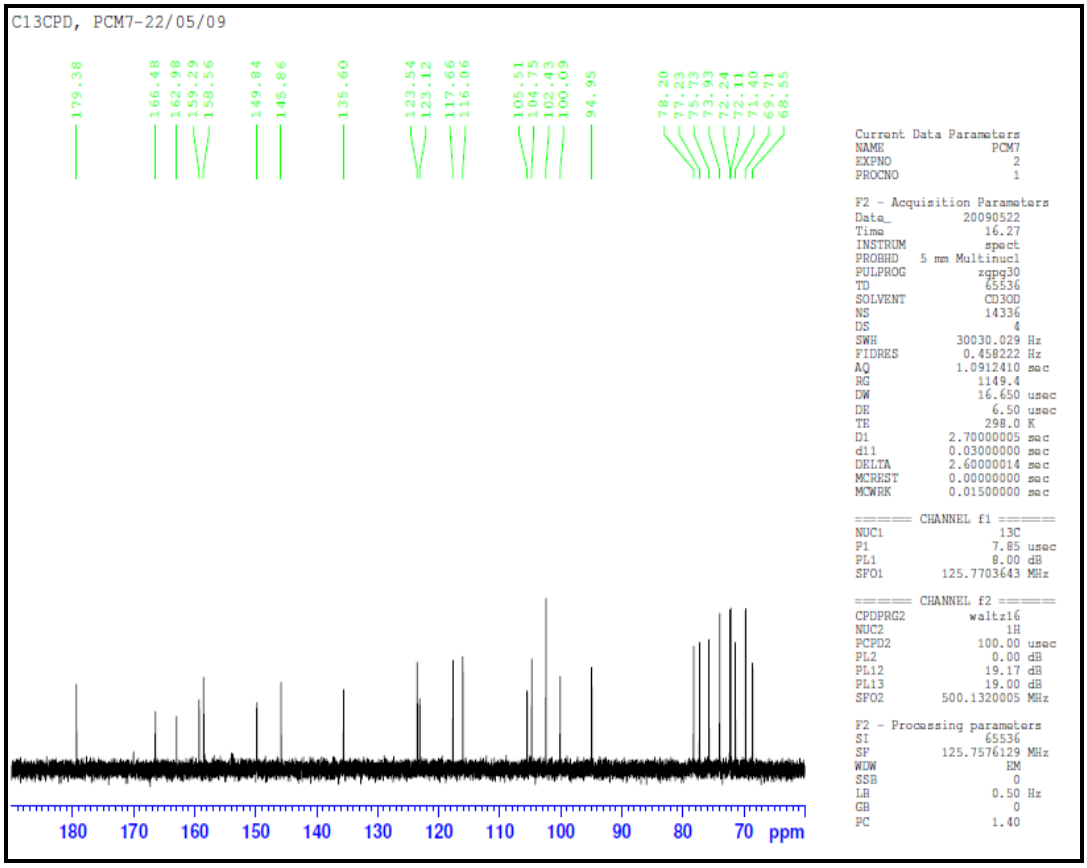

HSQC EDIT, PCM5–18/05/09
ppm
0
20
40
60
80
100
120
140
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5
ppm

vi.

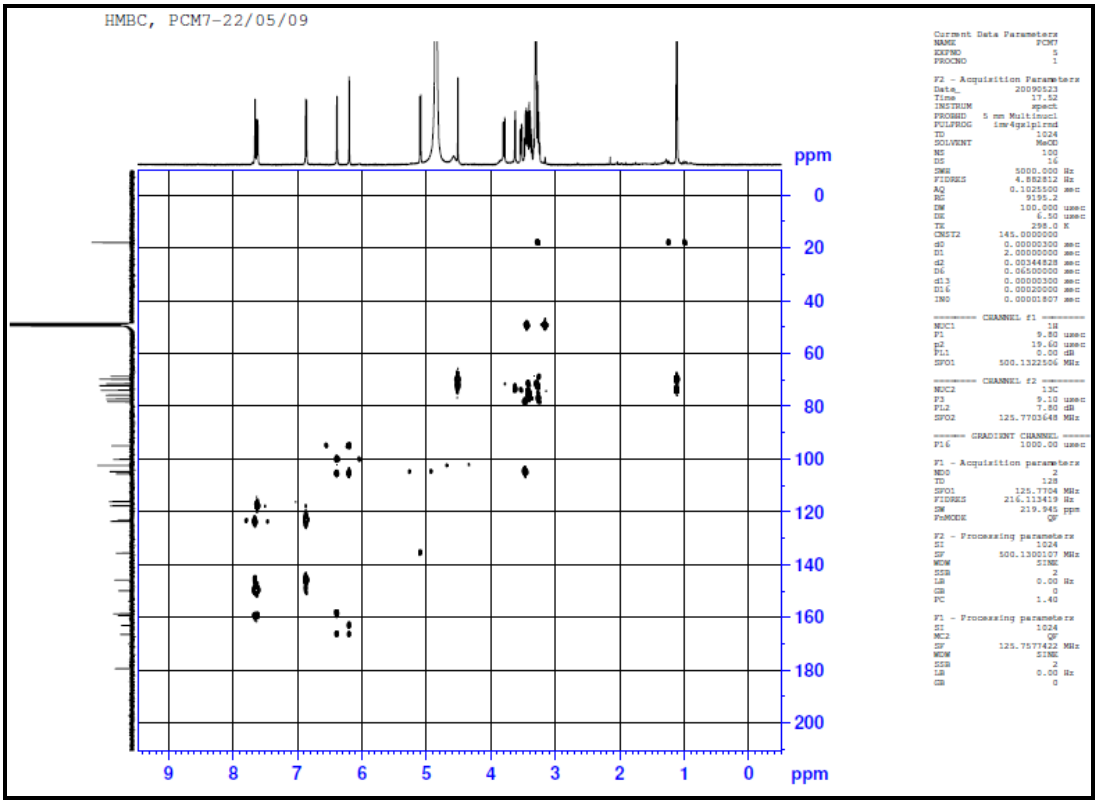

HSQC EDIT, PCM5–18/05/09
ppm
20
25
30
35
40
45
50
55
60
65
70
75
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5
ppm

9. PCM 7

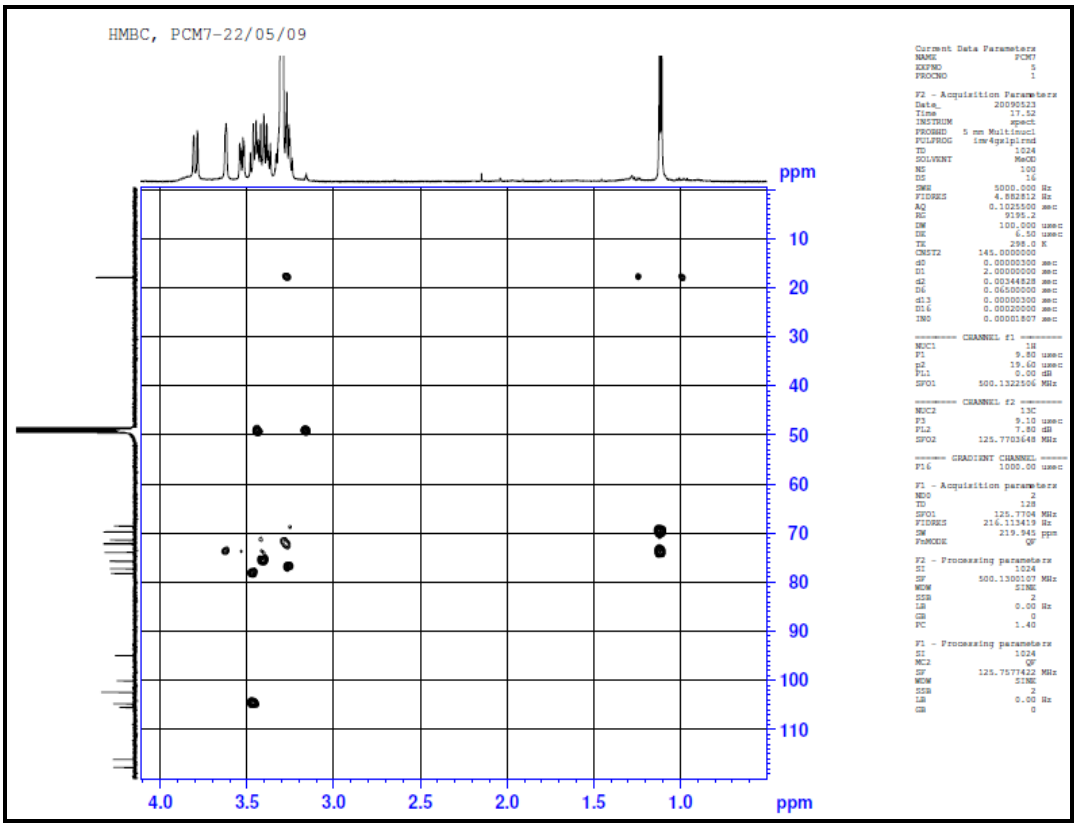
i.


C13CPD, PCM7-22/05/09
179.38
166.48
162.98
159.29
158.56
149.84
145.86
135.60
123.54
123.12
117.66
116.06
105.51
104.75
102.43
100.09
94.95
78.20
77.23
75.73
73.93
72.24
72.11
71.40
69.71
68.55
180 170 160 150 140 130 120 110 100 90 80 70 ppm
Current Data Parameters
NAME PCM7
EXPNO 2
PROCNO 1
F2 - Acquisition Parameters
Date_ 20090522
Time 16.27
INSTRUM spect
PROBHD 5 mm Multinucl
PULPROG zgpg30
TD 65536
SOLVENT CD3OD
NS 14336
DS 4
SWH 30030.029 Hz
FIDRES 0.458222 Hz
AQ 1.0912410 sec
RG 1149.4
DW 16.650 usec
DE 6.50 usec
TE 298.0 K
D1 2.70000005 sec
d11 0.03000000 sec
DELTA 2.60000014 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
====== CHANNEL f1 ======
NUC1 13C
P1 7.85 usec
PL1 8.00 dB
SFO1 125.7703643 MHz
====== CHANNEL f2 ======
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 0.00 dB
PL12 19.17 dB
PL13 19.00 dB
SFO2 500.1320005 MHz
F2 - Processing parameters
SI 65536
SF 125.7576129 MHz
WDW EM
SSB 0
LB 0.50 Hz
GB 0
PC 1.40

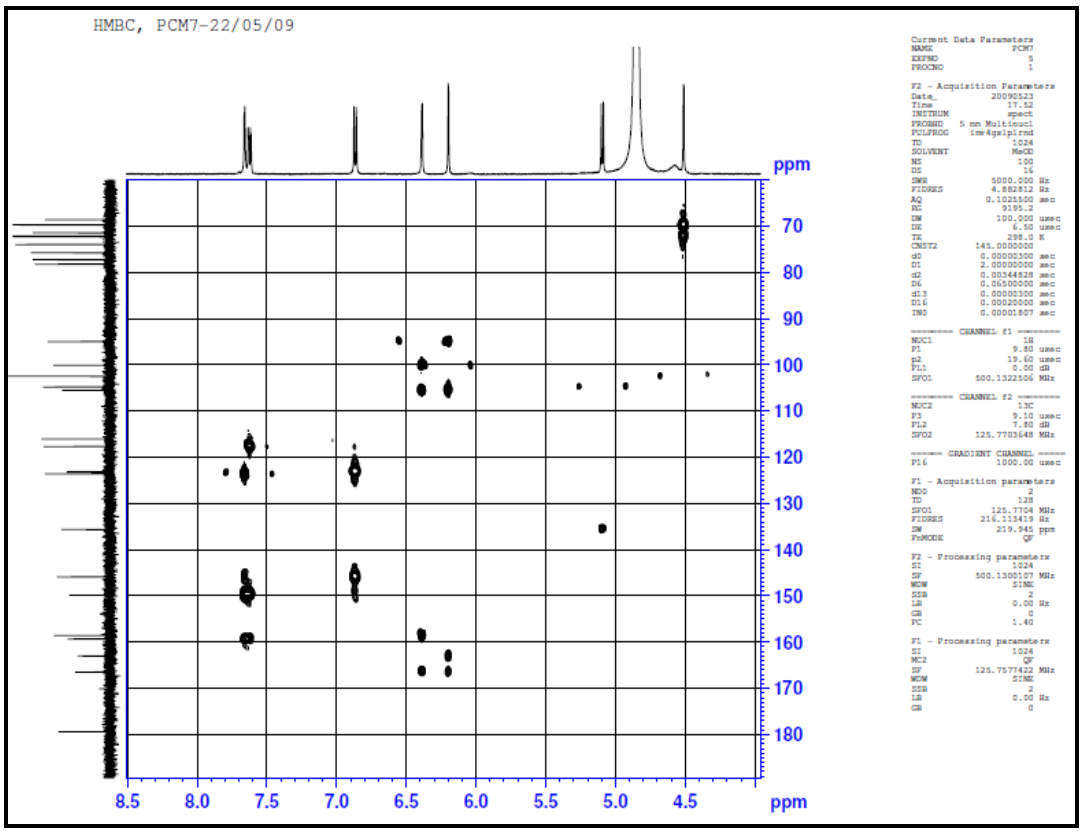
ii.


HMBC, PCM7-22/05/09
ppm
0
20
40
60
80
100
120
140
160
180
200
9 8 7 6 5 4 3 2 1 0 ppm

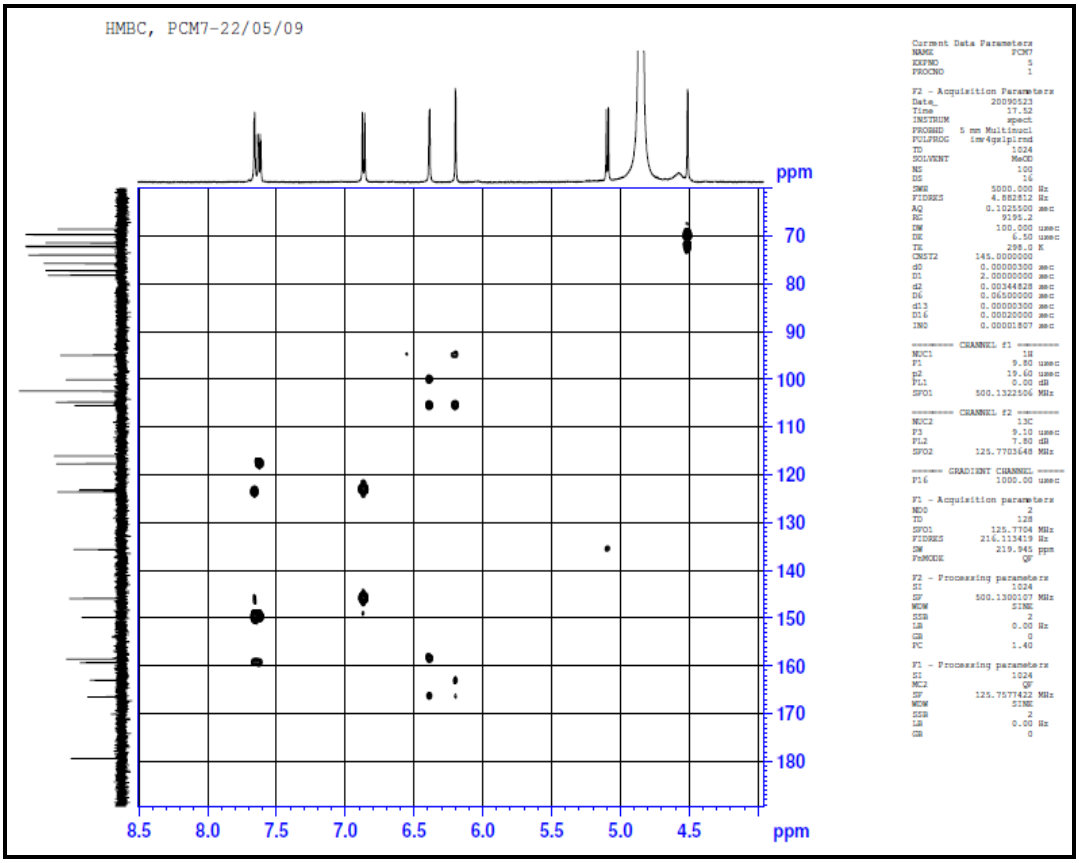
iii.


HMBC, PCM7-22/05/09
ppm
10
20
30
40
50
60
70
80
90
100
110
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

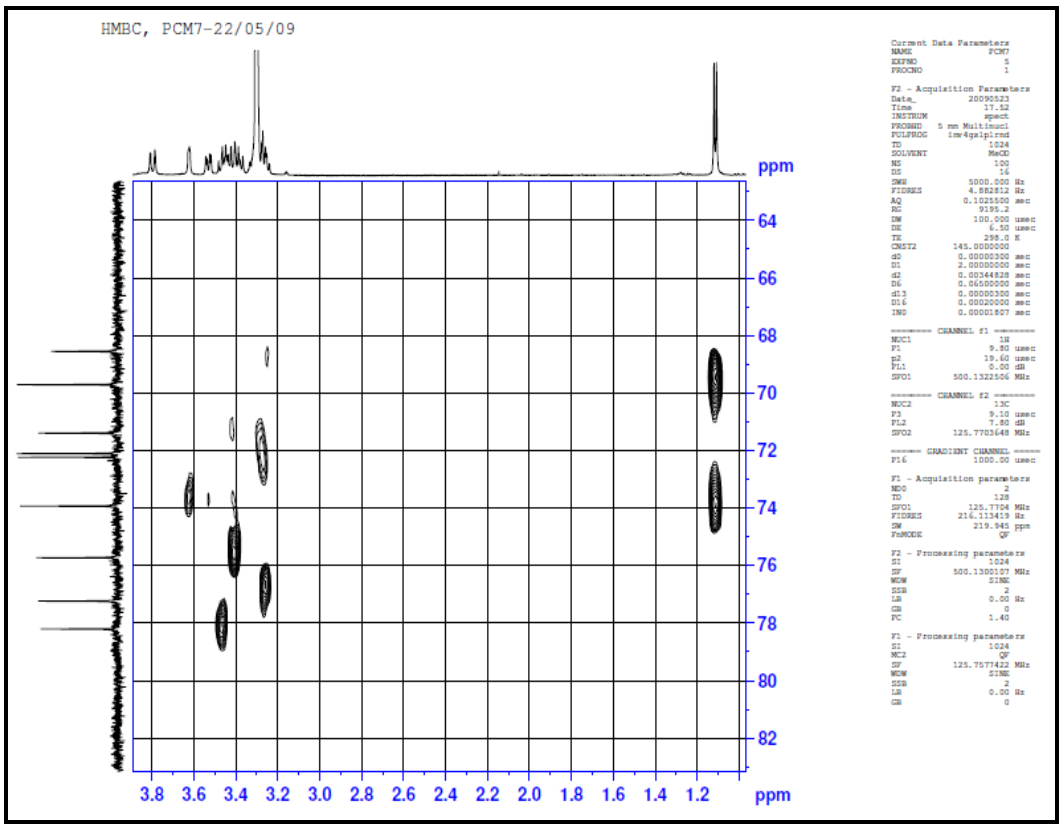
iv.


HMBC, PCM7-22/05/09
ppm
70
80
90
100
110
120
130
140
150
160
170
180
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
ppm

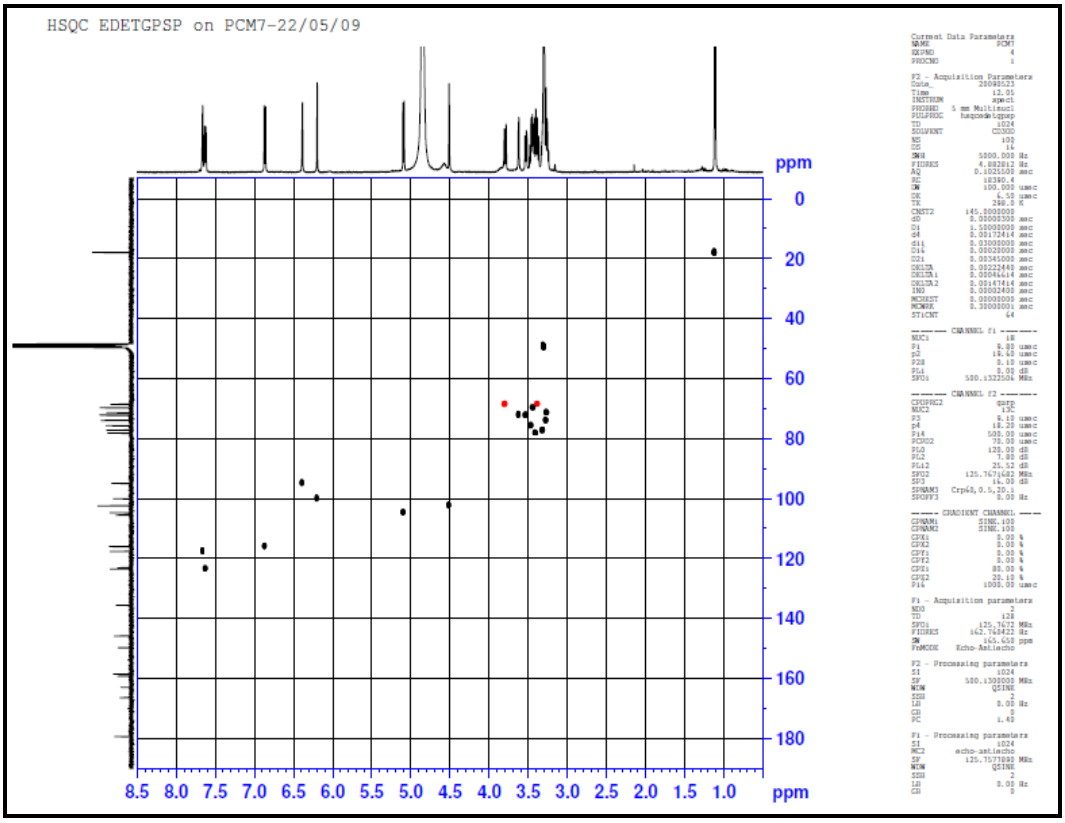
v.


HMBC, PCM7-22/05/09
ppm
70
80
90
100
110
120
130
140
150
160
170
180
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
ppm

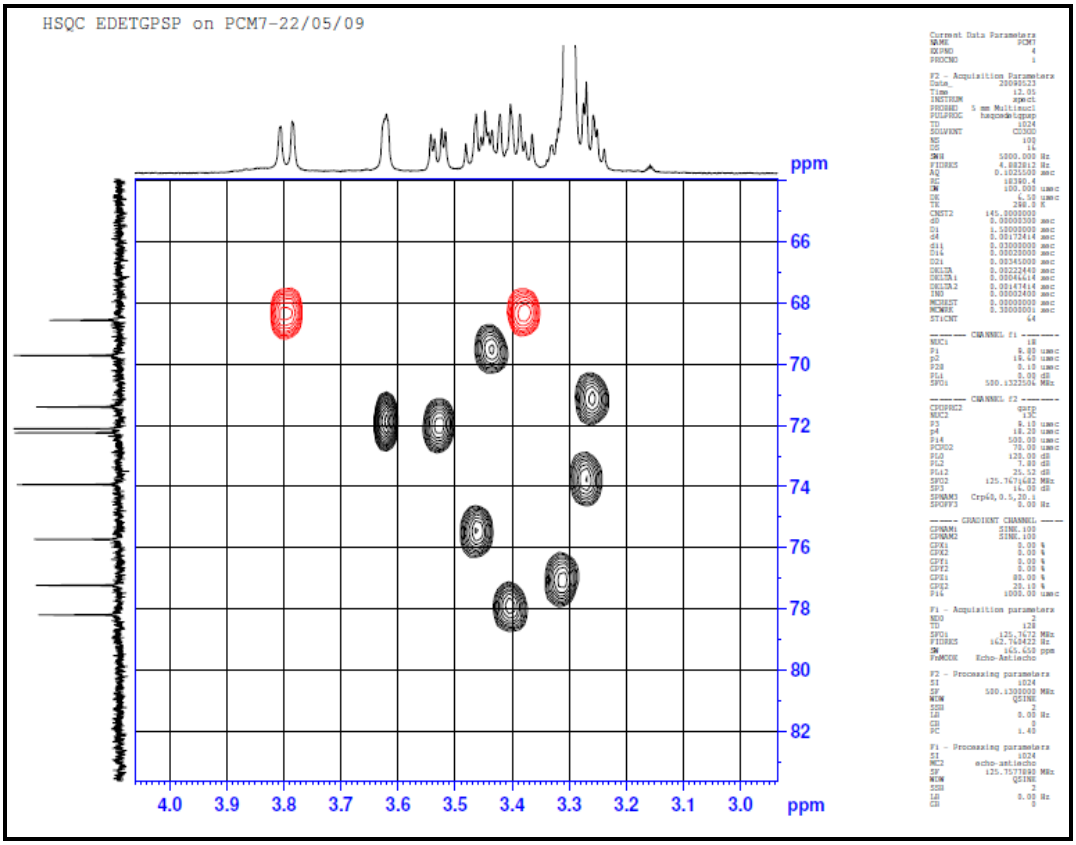
vi.


HMBC, PCM7-22/05/09
ppm
64
66
68
70
72
74
76
78
80
82
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
ppm

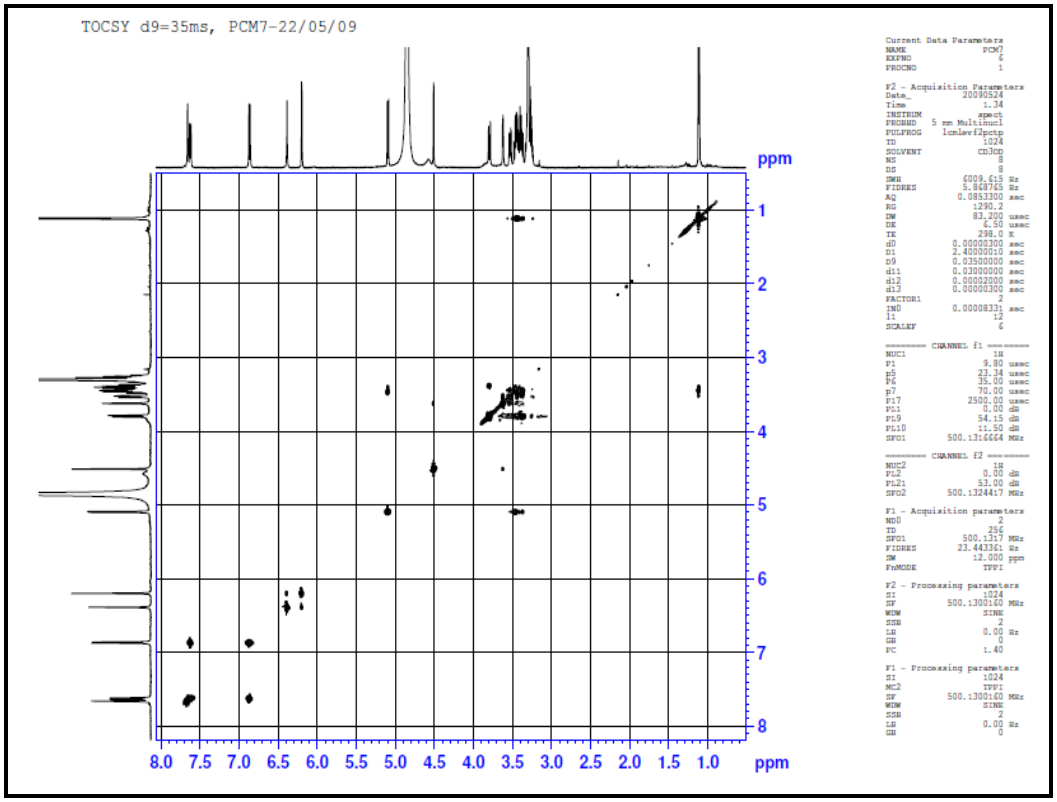
vii.


HSQC EDETGPSP on PCM7-22/05/09
ppm
0
20
40
60
80
100
120
140
160
180
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
ppm

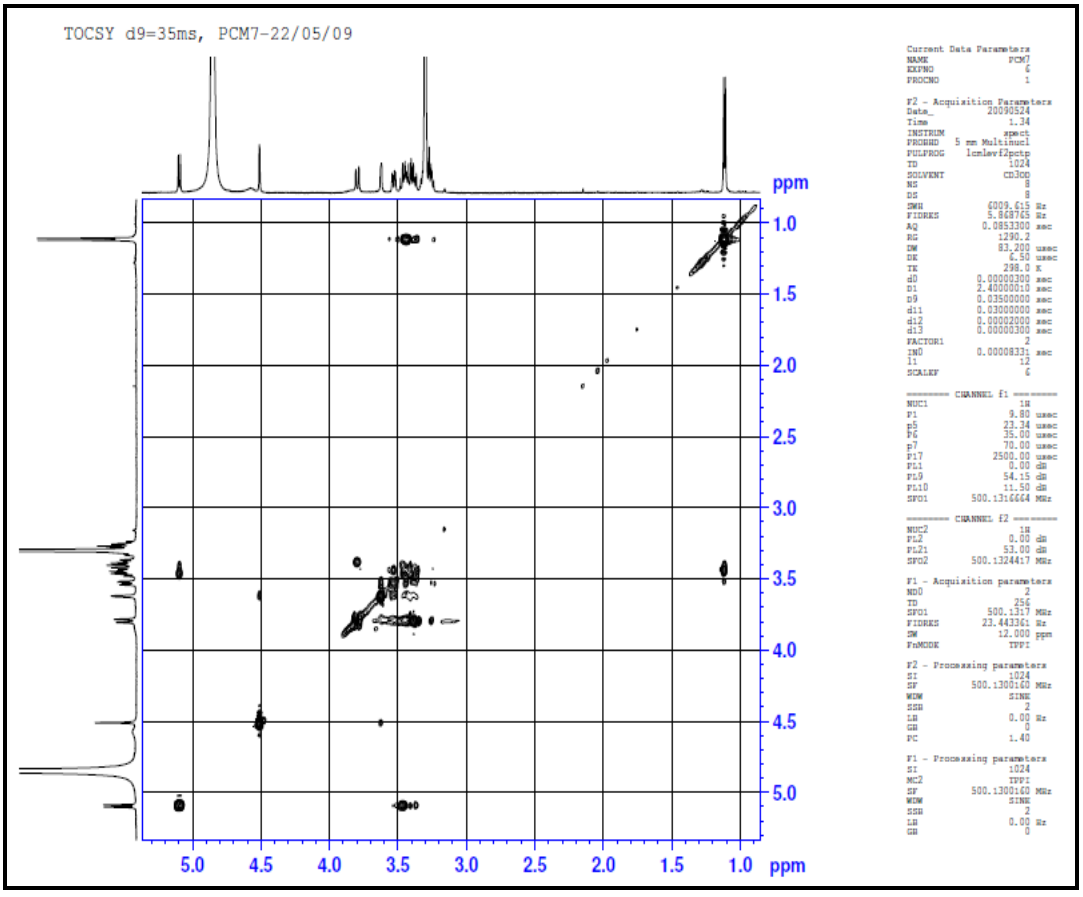
viii.


HSQC EDETGPSP on PCM7-22/05/09
ppm
66
68
70
72
74
76
78
80
82
4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0
ppm

ix.

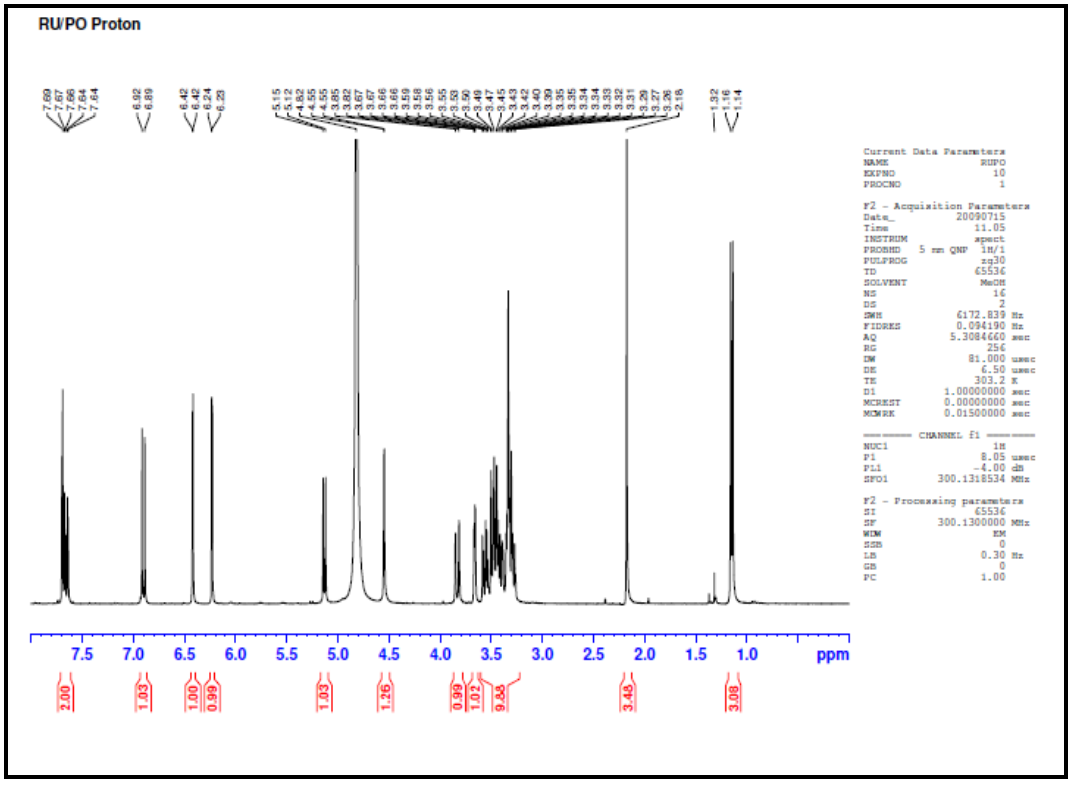

TOCSY d9=35ms, PCM7-22/05/09
ppm
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm

x.

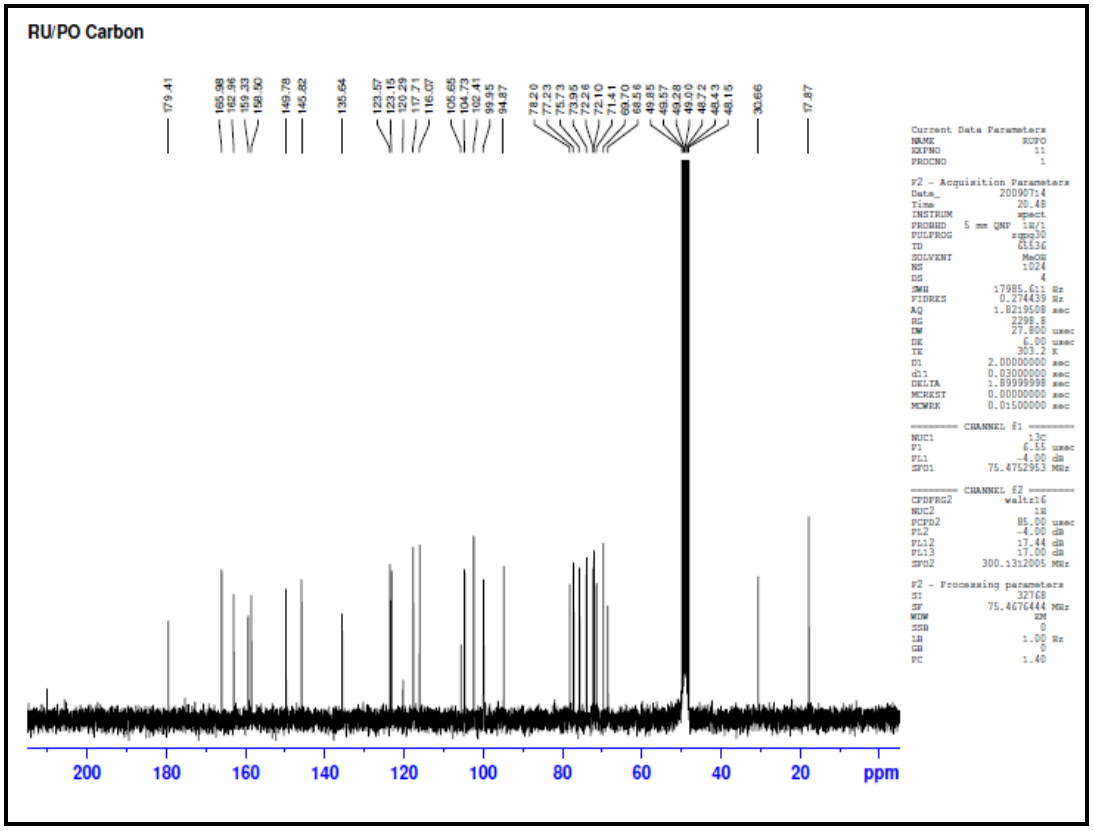

TOCSY d9=35ms, PCM7-22/05/09
ppm
5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm

10. Rutin reference standard

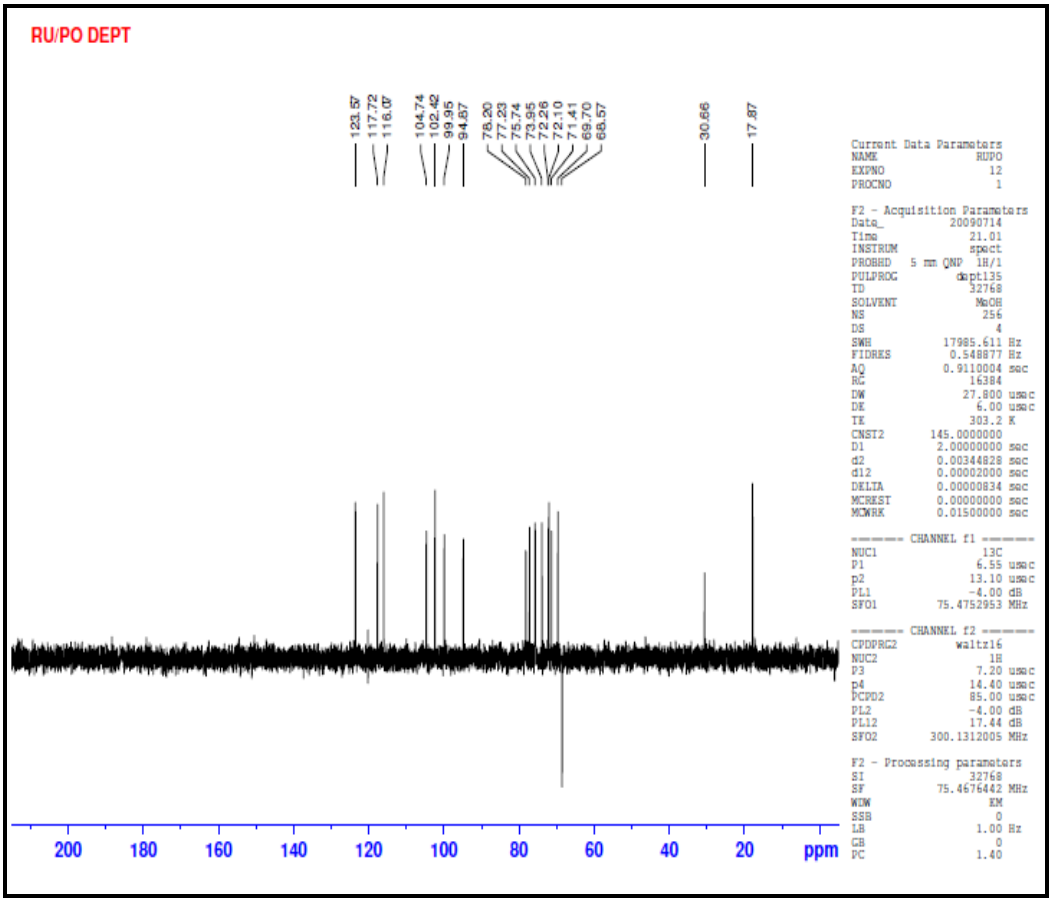
i.



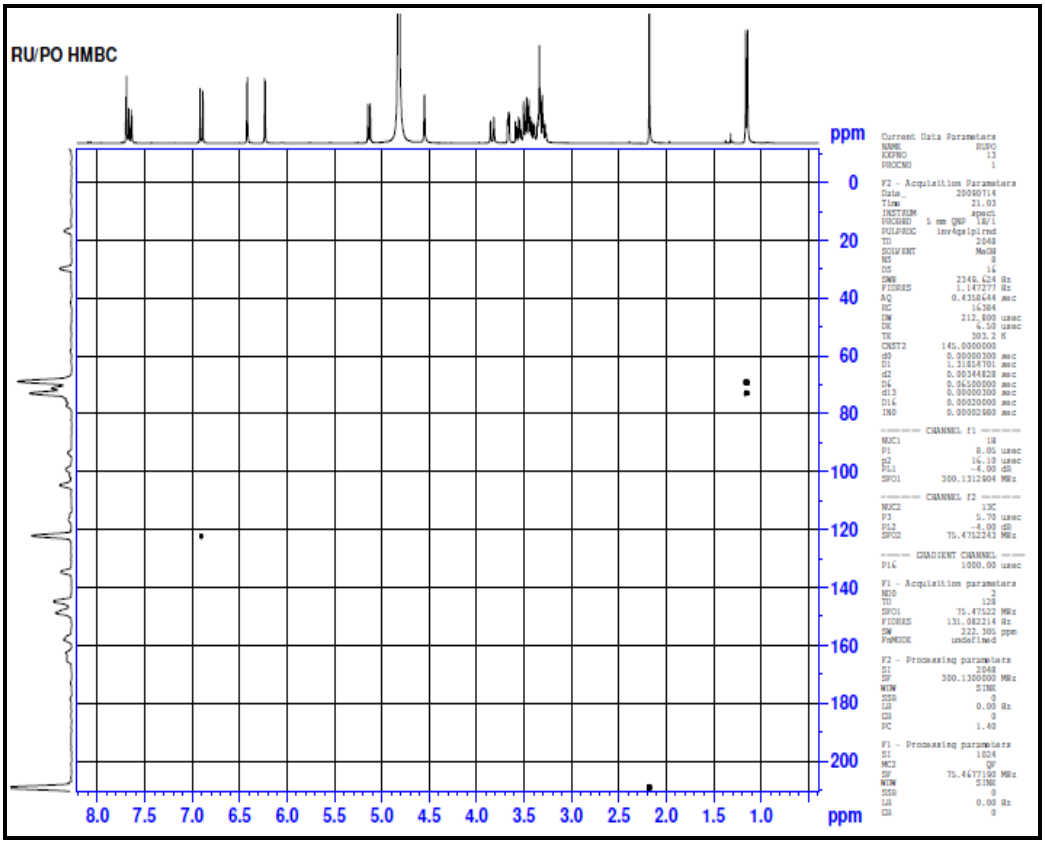
ii.



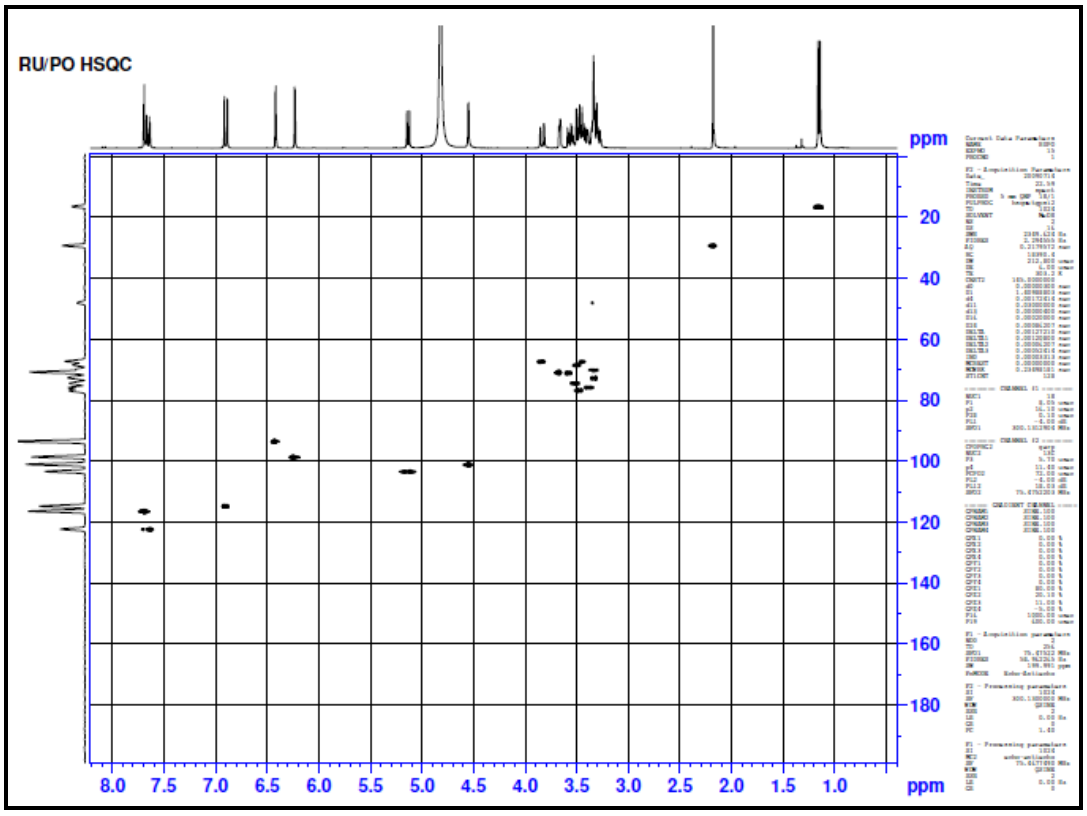
iii.


RU/PO DEPT
123.57
117.72
116.07
104.74
102.42
99.95
94.87
78.20
77.23
75.74
73.95
72.26
72.10
71.41
69.70
68.57
30.66
17.87
200
180
160
140
120
100
80
60
40
20
ppm

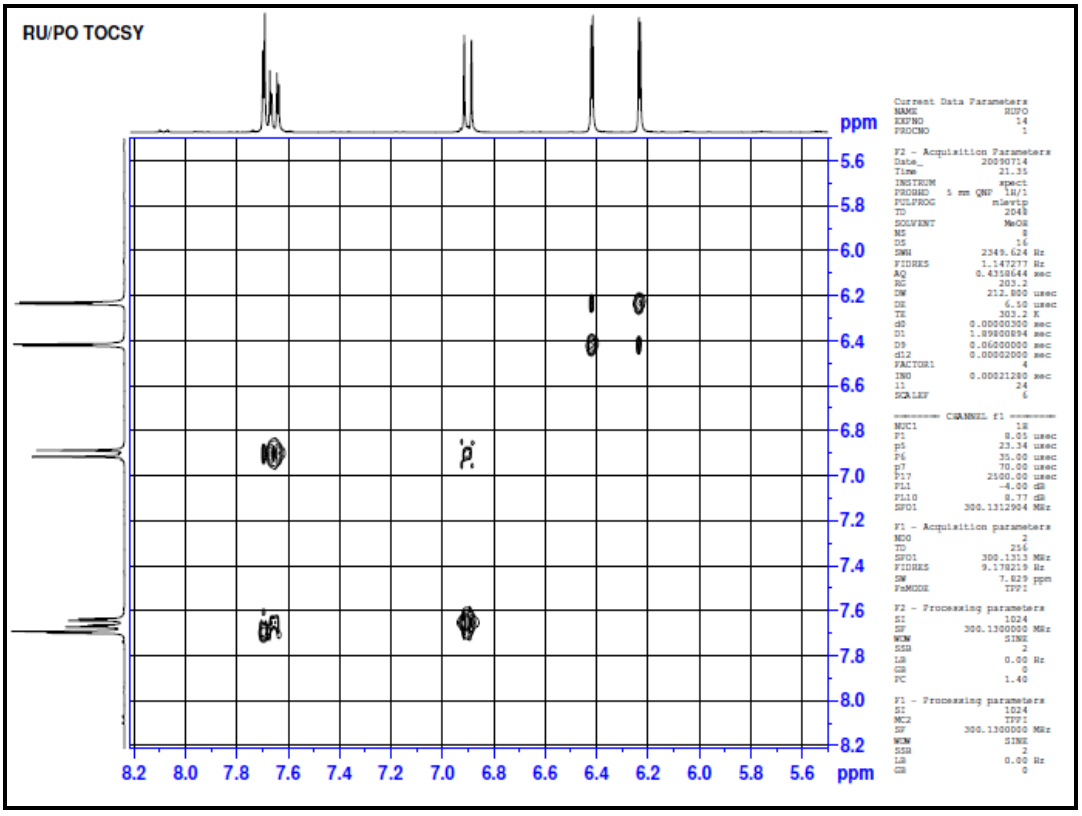
iv.


RU/PO HMBC
ppm
0
20
40
60
80
100
120
140
160
180
200
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

v.

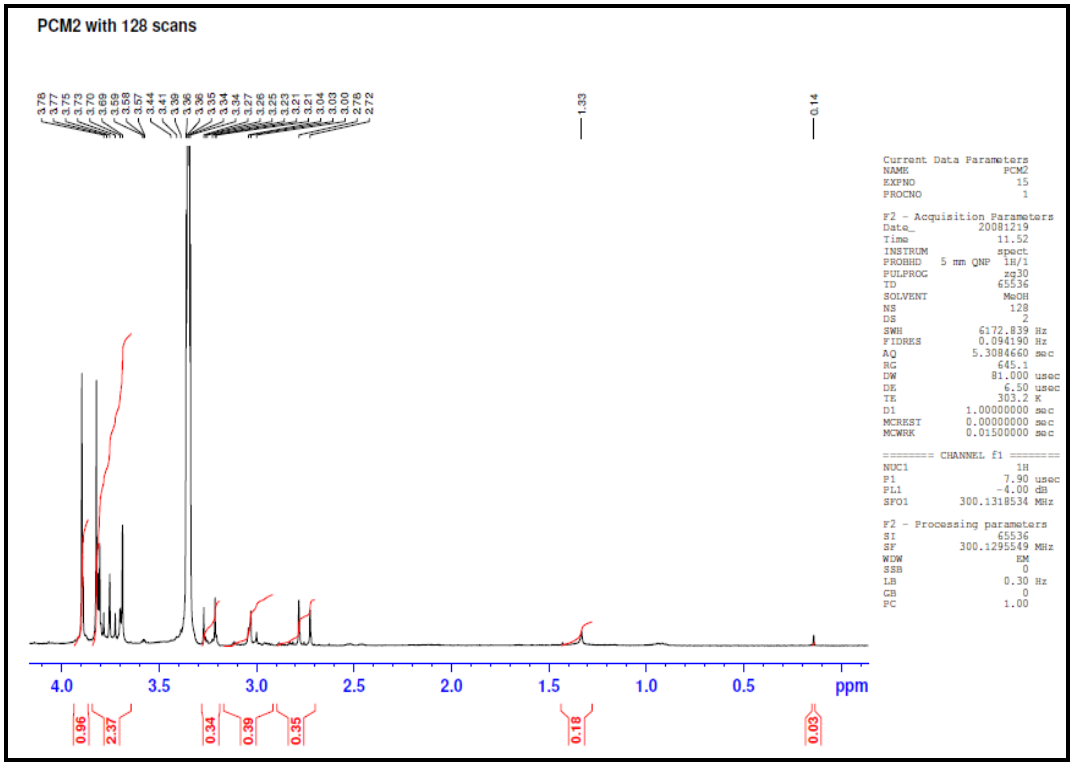

RU/PO HSQC
ppm
20
40
60
80
100
120
140
160
180
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
ppm

vi.

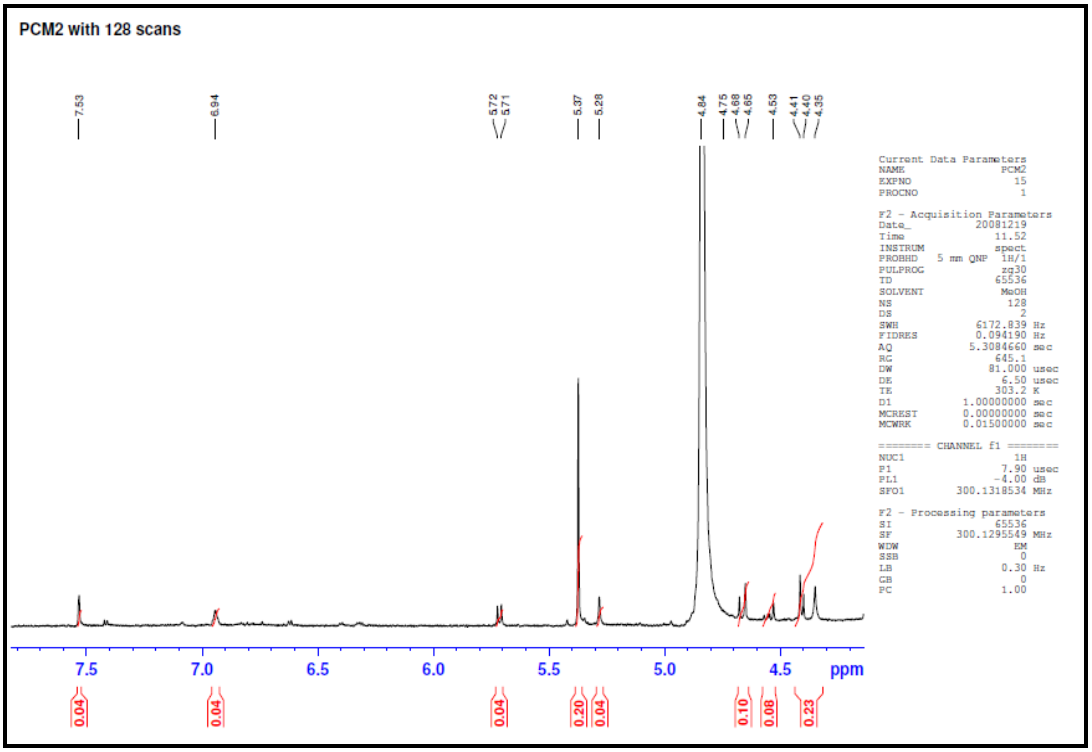

RU/PO TOCSY
ppm
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6
ppm

11. PCM2

i.

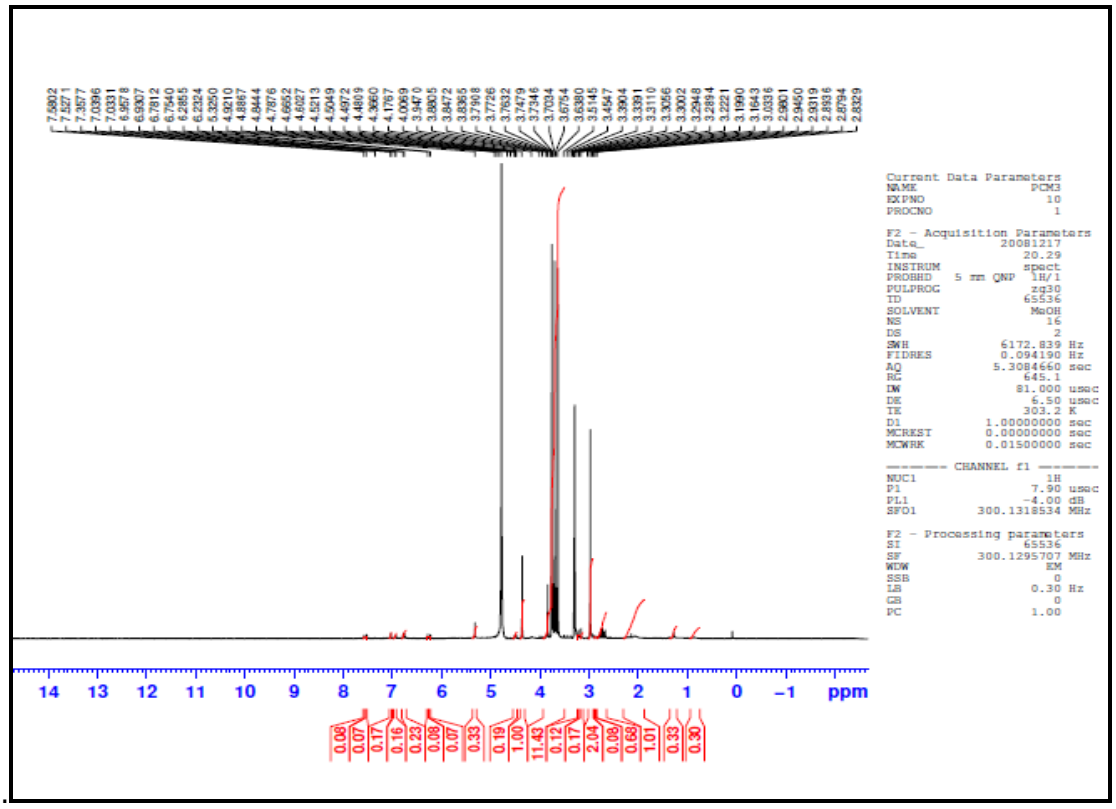

PCM2 with 128 scans
3.76 3.77 3.75 3.73 3.70 3.69 3.59 3.58 3.57 3.44 3.41 3.39 3.36 3.36 3.35 3.34 3.34 3.27 3.26 3.25 3.23 3.21 3.21 3.04 3.03 3.00 2.76 2.72
1.33
0.14
Current Data Parameters
NAME PCM2
EXPNO 15
PROCNO 1
F2 - Acquisition Parameters
Date_ 20081219
Time 11.52
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg30
TD 65536
SOLVENT MeOH
NS 128
DS 2
SWH 6172.839 Hz
FIDRES 0.094190 Hz
AQ 5.3084660 sec
RG 645.1
DW 81.000 usec
DE 6.50 usec
TE 303.2 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 7.90 usec
PL1 -4.00 dB
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 65536
SF 300.1295549 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
0.96 2.37 0.34 0.39 0.35 0.18 0.03

ii.

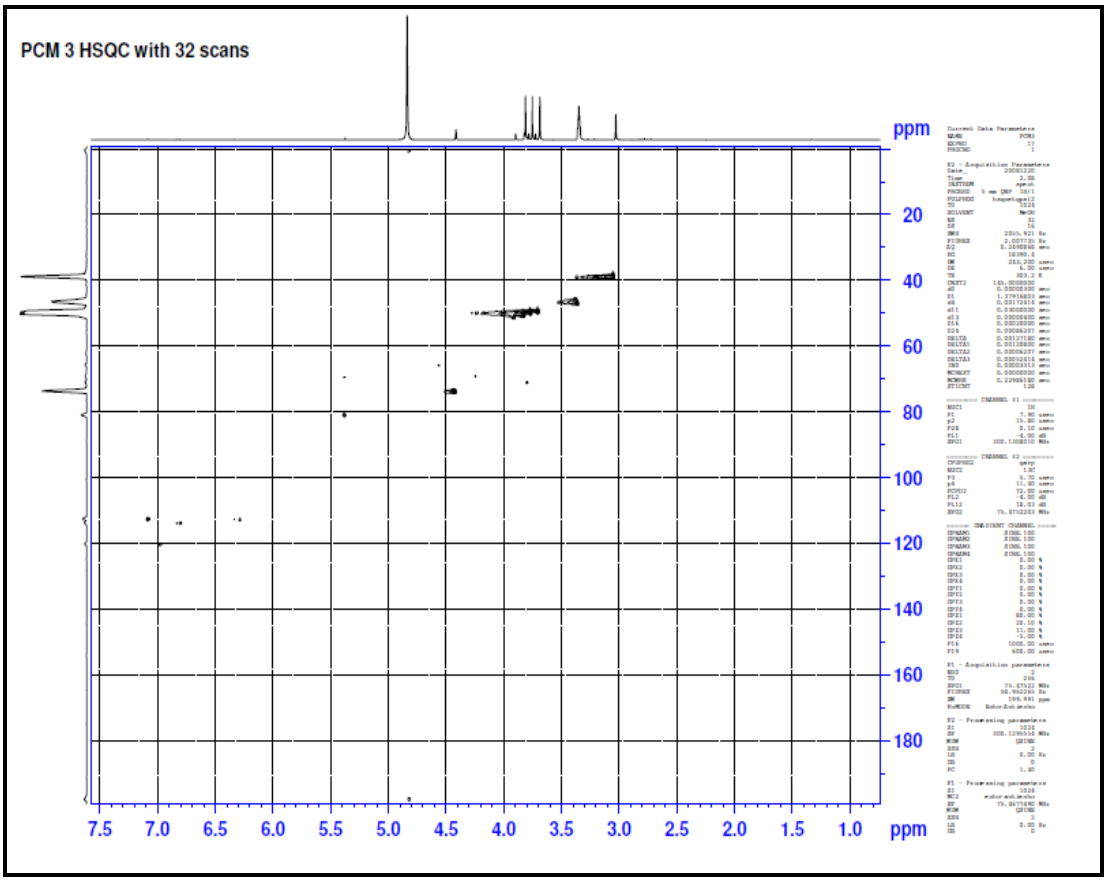

PCM2 with 128 scans
7.53
6.94
5.72 5.71
5.37
5.28
4.84
4.75 4.68 4.65
4.53
4.41 4.40 4.35
Current Data Parameters
NAME PCM2
EXPNO 15
PROCNO 1
F2 - Acquisition Parameters
Date_ 20081219
Time 11.52
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg30
TD 65536
SOLVENT MeOH
NS 128
DS 2
SWH 6172.839 Hz
FIDRES 0.094190 Hz
AQ 5.3084660 sec
RG 645.1
DW 81.000 usec
DE 6.50 usec
TE 303.2 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 7.90 usec
PL1 -4.00 dB
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 65536
SF 300.1295549 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
7.5 7.0 6.5 6.0 5.5 5.0 4.5 ppm
0.04 0.04 0.04 0.20 0.04 0.10 0.06 0.23

1. PCM

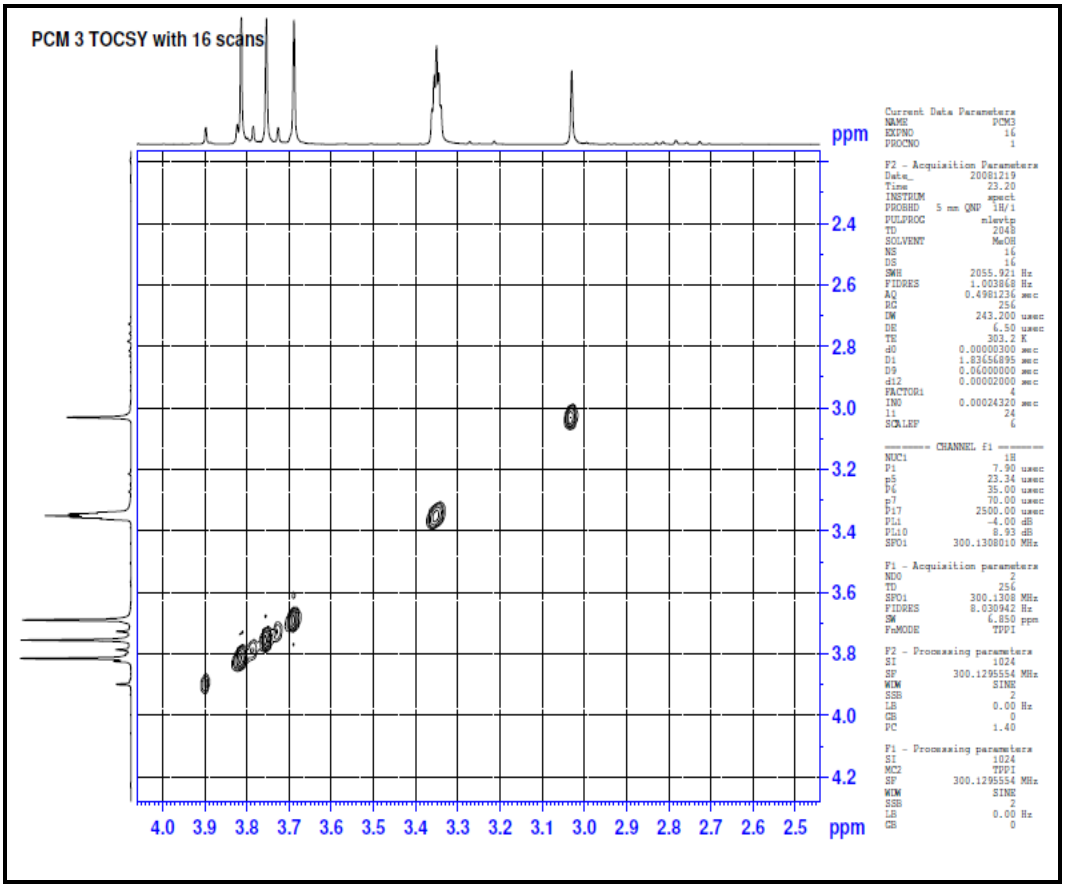
i.


Current Data Parameters
NAME PCM3
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Data_ 20081217
Time 20.29
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg30
TD 65536
SOLVENT MeOH
NS 16
DS 2
SWH 6172.839 Hz
FIDRES 0.094190 Hz
AQ 5.3084660 sec
RG 645.1
DW 81.000 usec
DE 6.50 usec
TE 303.2 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
CHANNEL f1
NUC1 1H
P1 7.90 usec
PL1 -4.00 dB
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 65536
SF 300.1295707 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
ppm

ii.

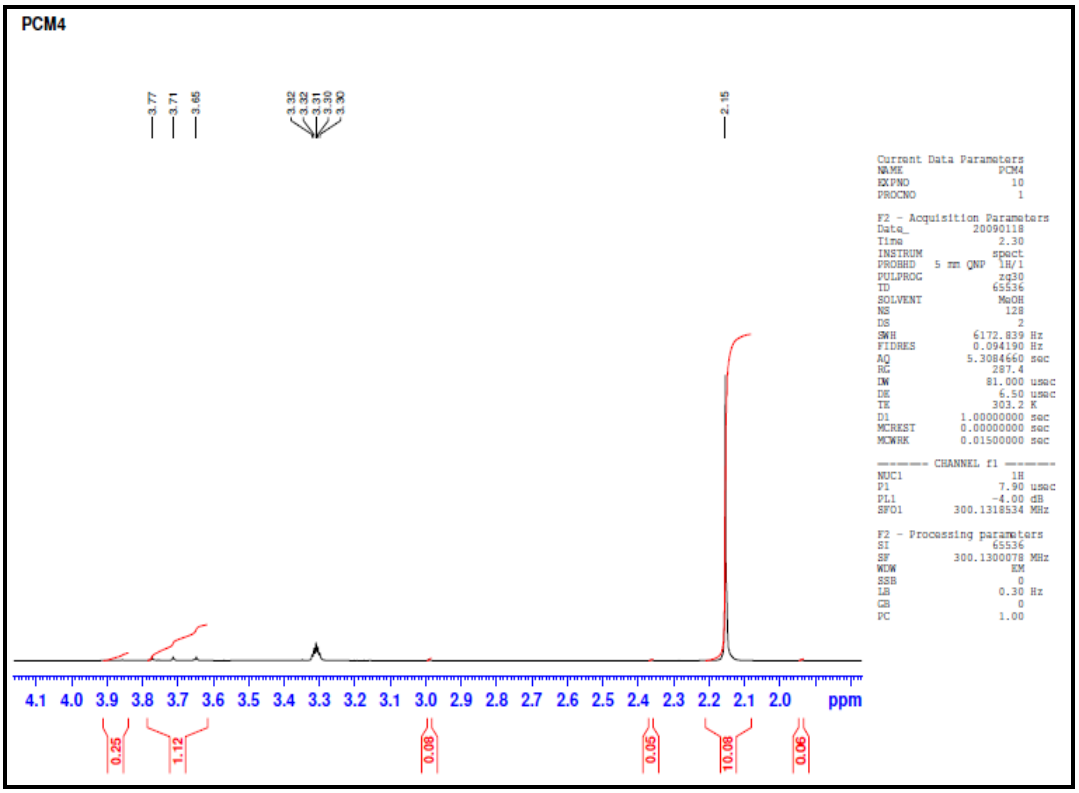

PCM 3 HSQC with 32 scans
ppm
ppm

iii.

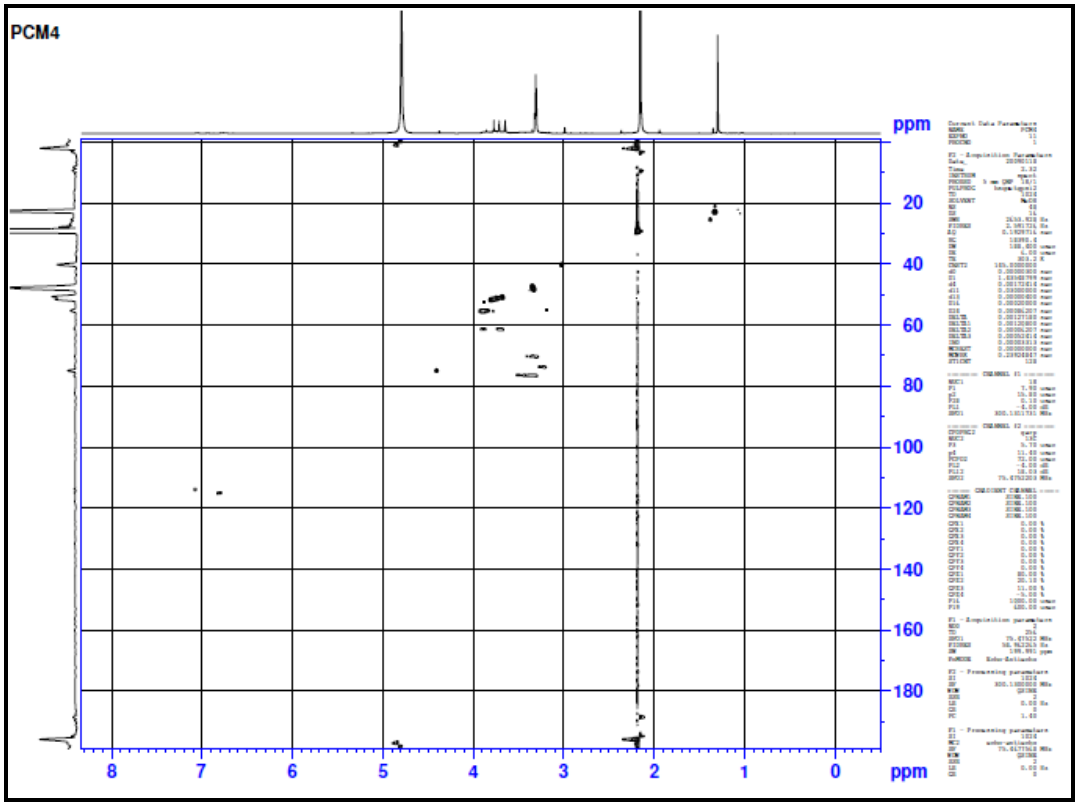

PCM 3 TOCSY with 16 scans
ppm
4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 ppm
2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2
Current Data Parameters
NAME PCM3
EXPNO 16
PROCNO 1
F2 - Acquisition Parameters
Date_ 20081219
Time 23.20
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG mlevtp
TD 2048
SOLVENT MeOH
NS 16
DS 16
SWH 2055.921 Hz
FIDRES 1.003868 Hz
AQ 0.4981236 sec
RG 256
DW 243.200 usec
DE 6.50 usec
TE 303.2 K
d0 0.00000300 sec
D1 1.83656895 sec
D9 0.06000000 sec
d12 0.00002000 sec
FACTOR1 4
IN0 0.00024320 sec
TI 24
SCALEF 6
CHANNEL f1
NUC1 1H
P1 7.90 usec
p3 23.34 usec
P6 35.00 usec
p7 70.00 usec
P17 2500.00 usec
PL1 -4.00 dB
PL10 8.93 dB
SFO1 300.1308010 MHz
F1 - Acquisition parameters
ND0 2
TD 256
SFO1 300.1308 MHz
FIDRES 8.030942 Hz
SW 6.850 ppm
FnMODE TPPI
F2 - Processing parameters
SI 1024
SF 300.1295554 MHz
WDW SINE
SSB 2
LB 0.00 Hz
GB 0
PC 1.40
F1 - Processing parameters
SI 1024
MC2 TPPI
SF 300.1295554 MHz
WDW SINE
SSB 2
LB 0.00 Hz
GB 0

12. PCM4

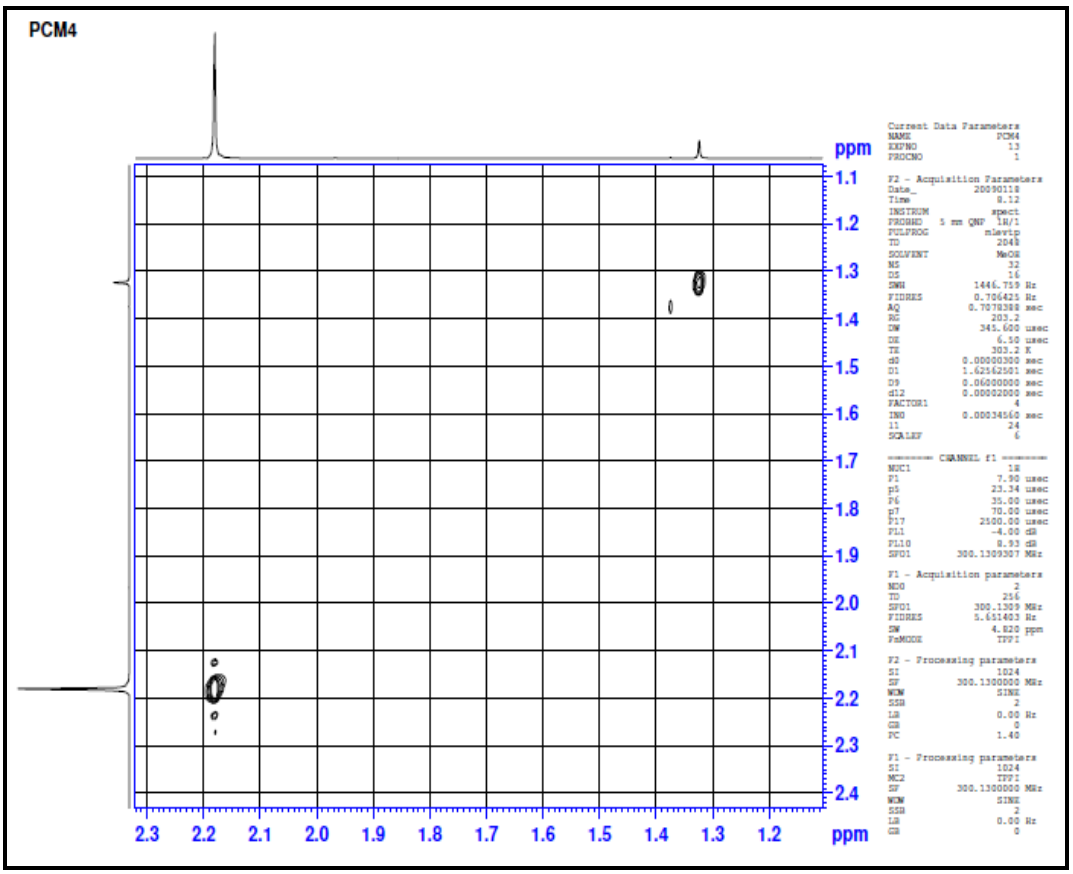
i.


PCM4
3.77
3.71
3.65
3.32
3.32
3.31
3.30
3.30
2.15
4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 ppm
0.25
1.12
0.08
0.05
10.08
0.06
Current Data Parameters
NAME PCM4
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20090118
Time 2.30
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg30
TD 65536
SOLVENT MeOH
NS 128
DS 2
SWH 6172.839 Hz
FIDRES 0.094190 Hz
AQ 5.3084660 sec
RG 287.4
DW 81.000 usec
DE 6.50 usec
TE 303.2 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
CHANNEL f1
NUC1 1H
P1 7.90 usec
PL1 -4.00 dB
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 65536
SF 300.1300078 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

ii.

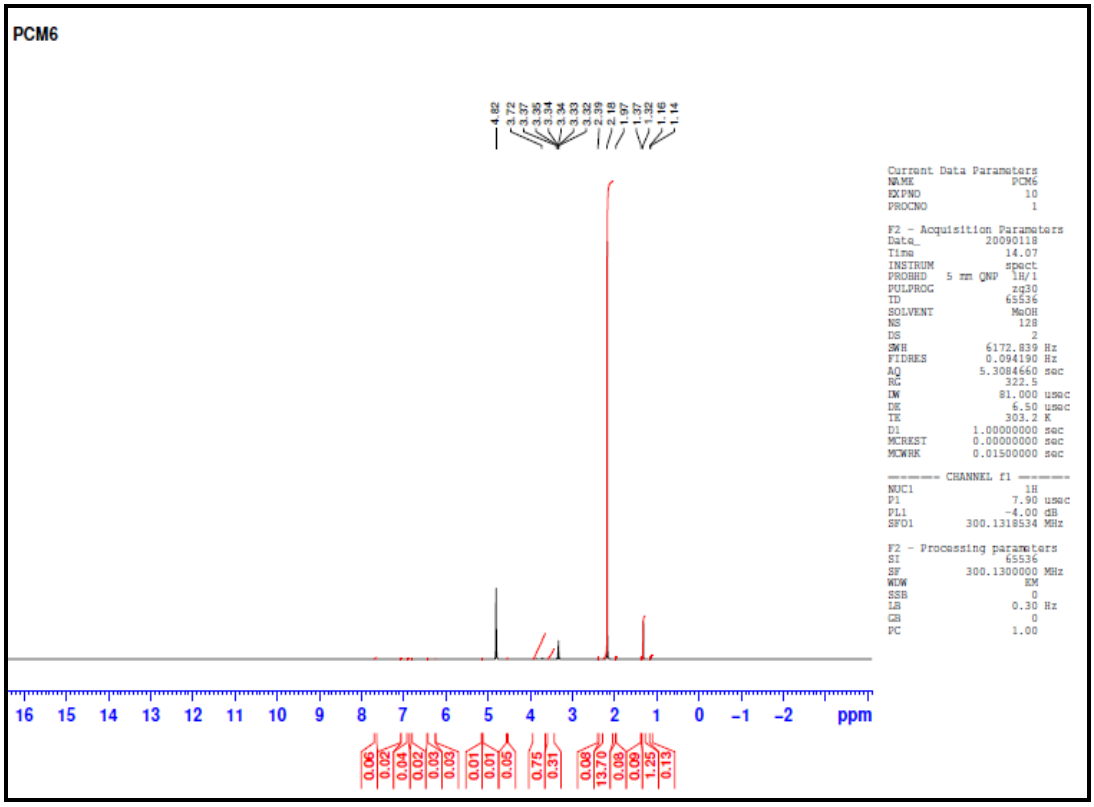


iii.

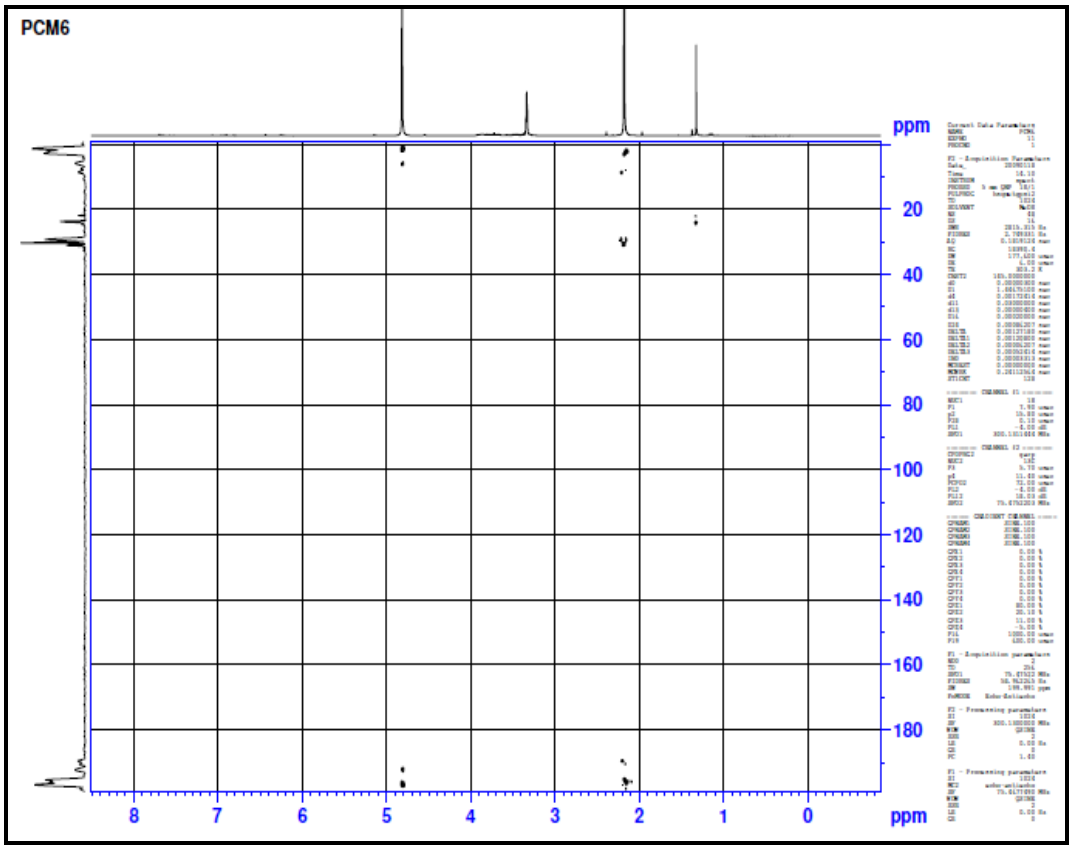


13.PCM6

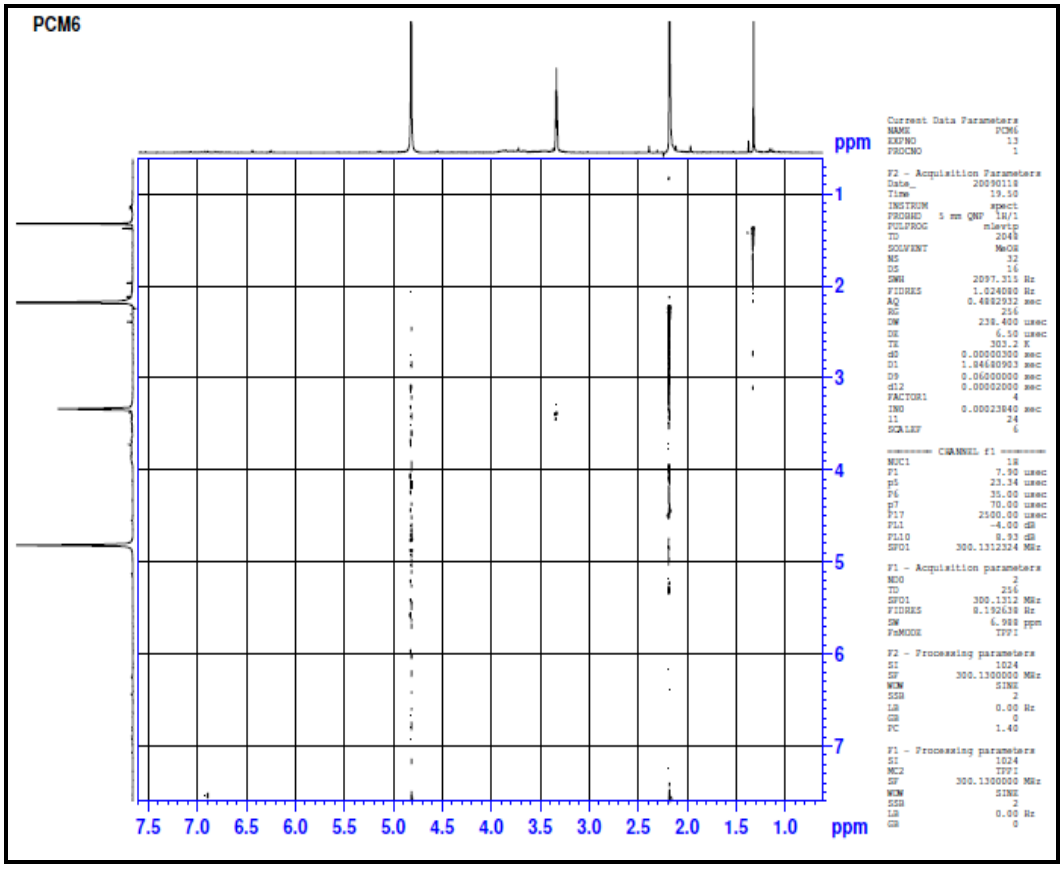
i.


PCM6
4.82
3.72
3.37
3.35
3.34
3.34
3.33
3.32
2.39
2.18
1.97
1.37
1.32
1.16
1.14
Current Data Parameters
NAME PCM6
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20090118
Time 14.07
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg30
TD 65536
SOLVENT MeOH
NS 128
DS 2
SWH 6172.839 Hz
FIDRES 0.094190 Hz
AQ 5.3084660 sec
RG 322.5
DW 81.000 usec
DE 6.50 usec
TE 303.2 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
CHANNEL f1
NUC1 1H
P1 7.90 usec
PL1 -4.00 dB
SFO1 300.1318534 MHz
F2 - Processing parameters
SI 65536
SF 300.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 ppm
0.06 0.02 0.04 0.02 0.03 0.03 0.01 0.01 0.05 0.75 0.31 0.08 13.70 0.08 0.09 1.25 0.13

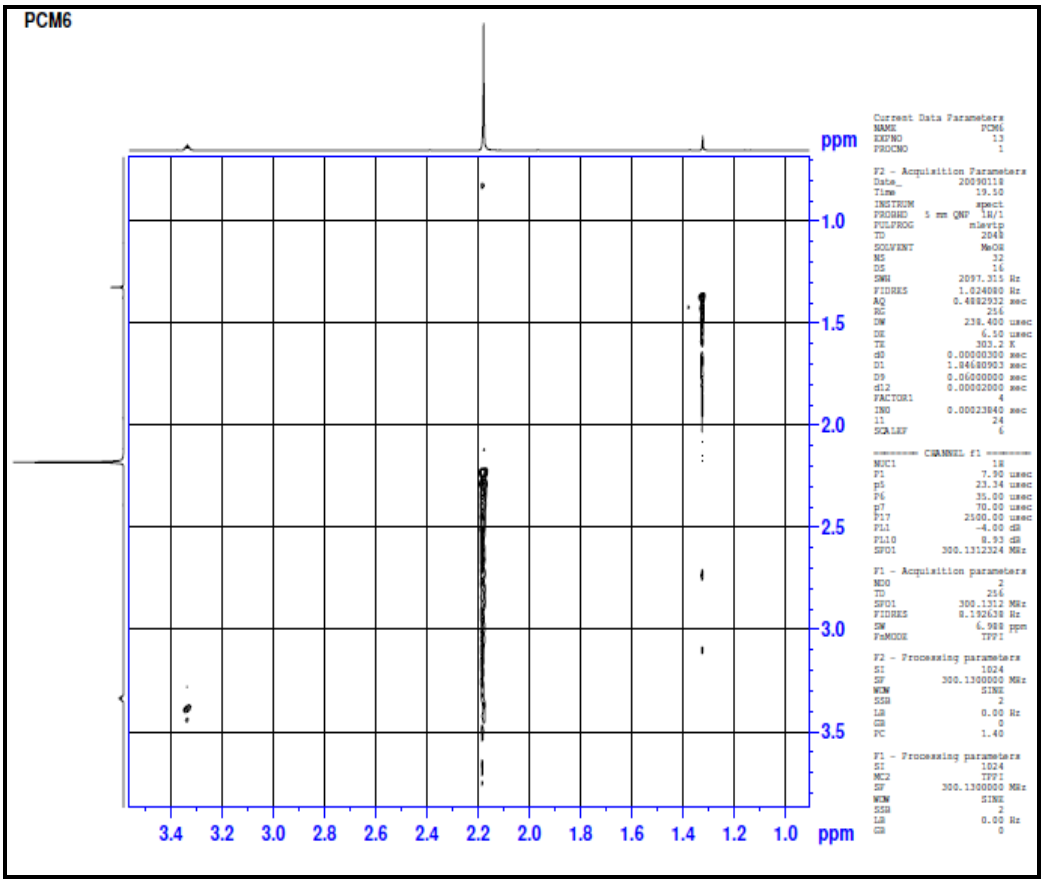
ii.


PCM6
ppm
20
40
60
80
100
120
140
160
180
8 7 6 5 4 3 2 1 0 ppm

iii.


PCM6
ppm
1
2
3
4
5
6
7
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm

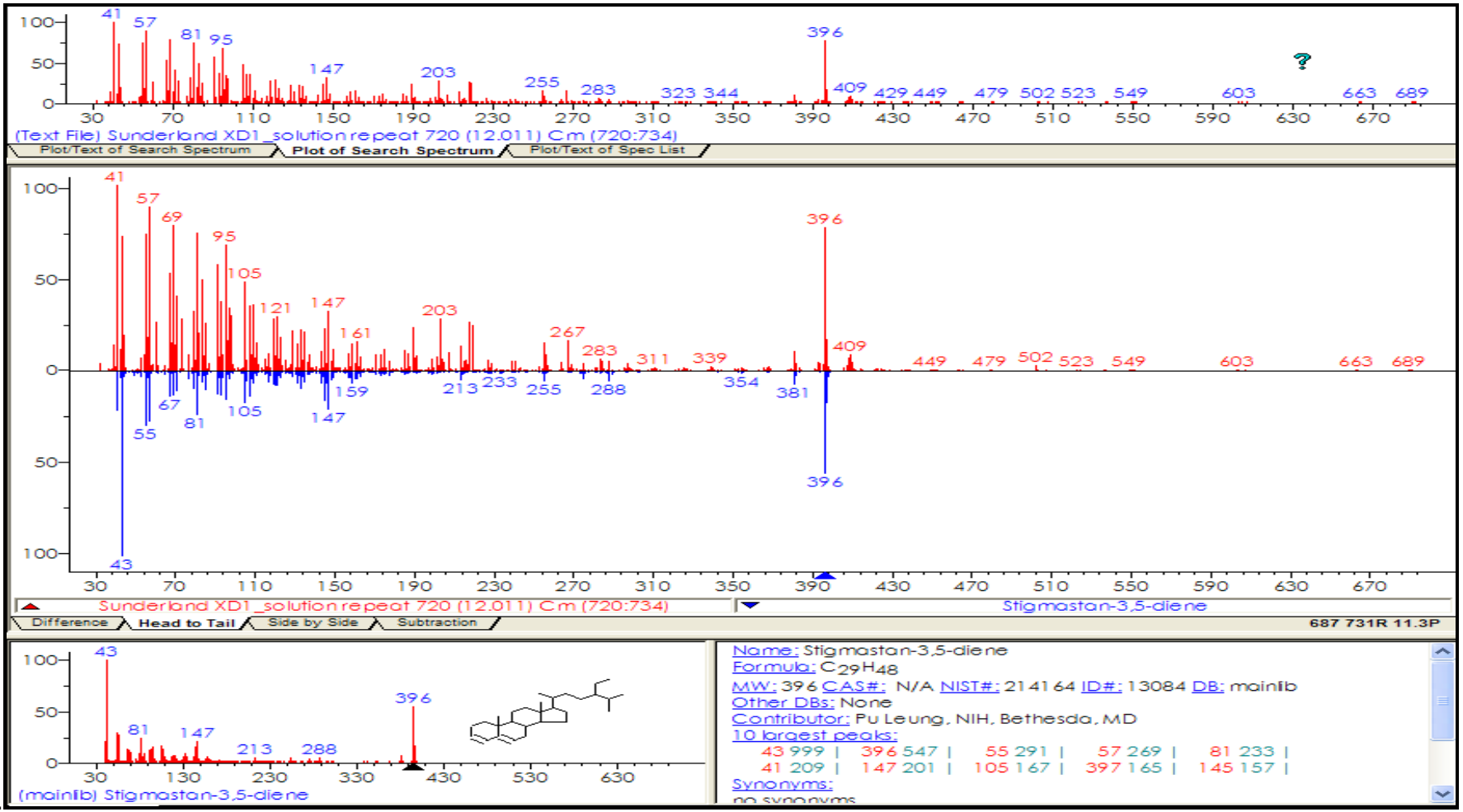
iv.


PCM6
ppm
1.0
1.5
2.0
2.5
3.0
3.5
3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 ppm

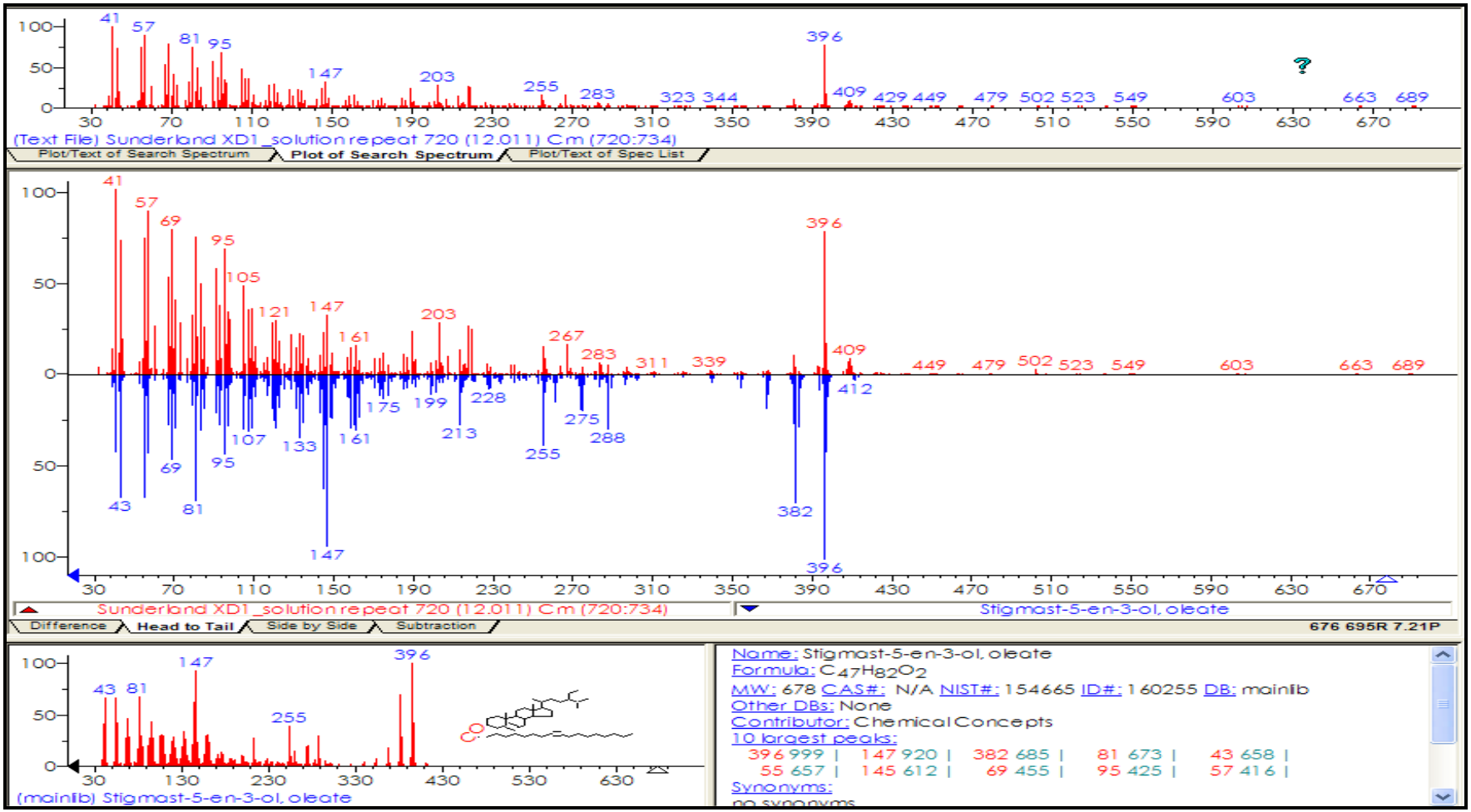
Appendix E : Mass spectral data. (TOF MS EI+)

1. XD1

i.

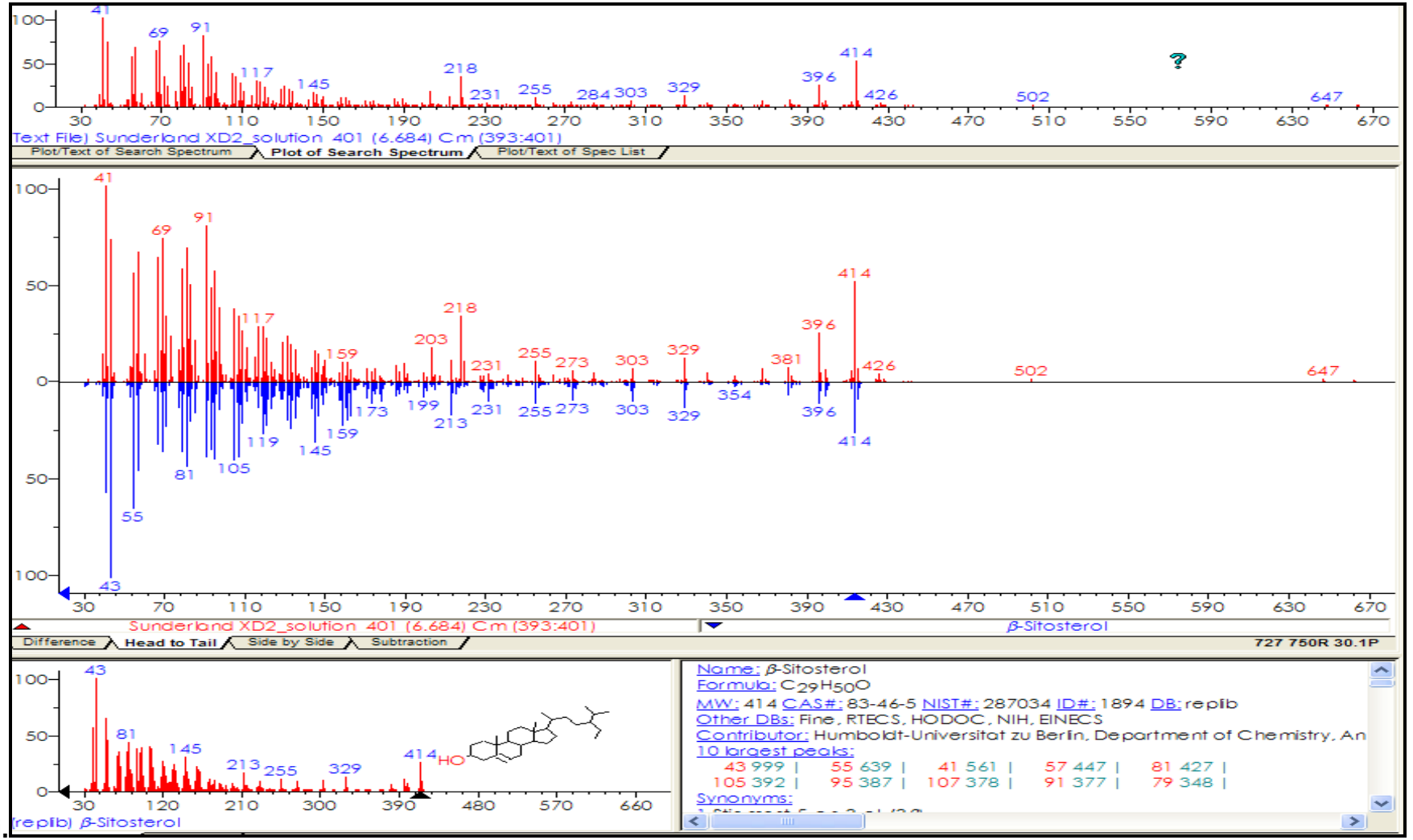


ii.

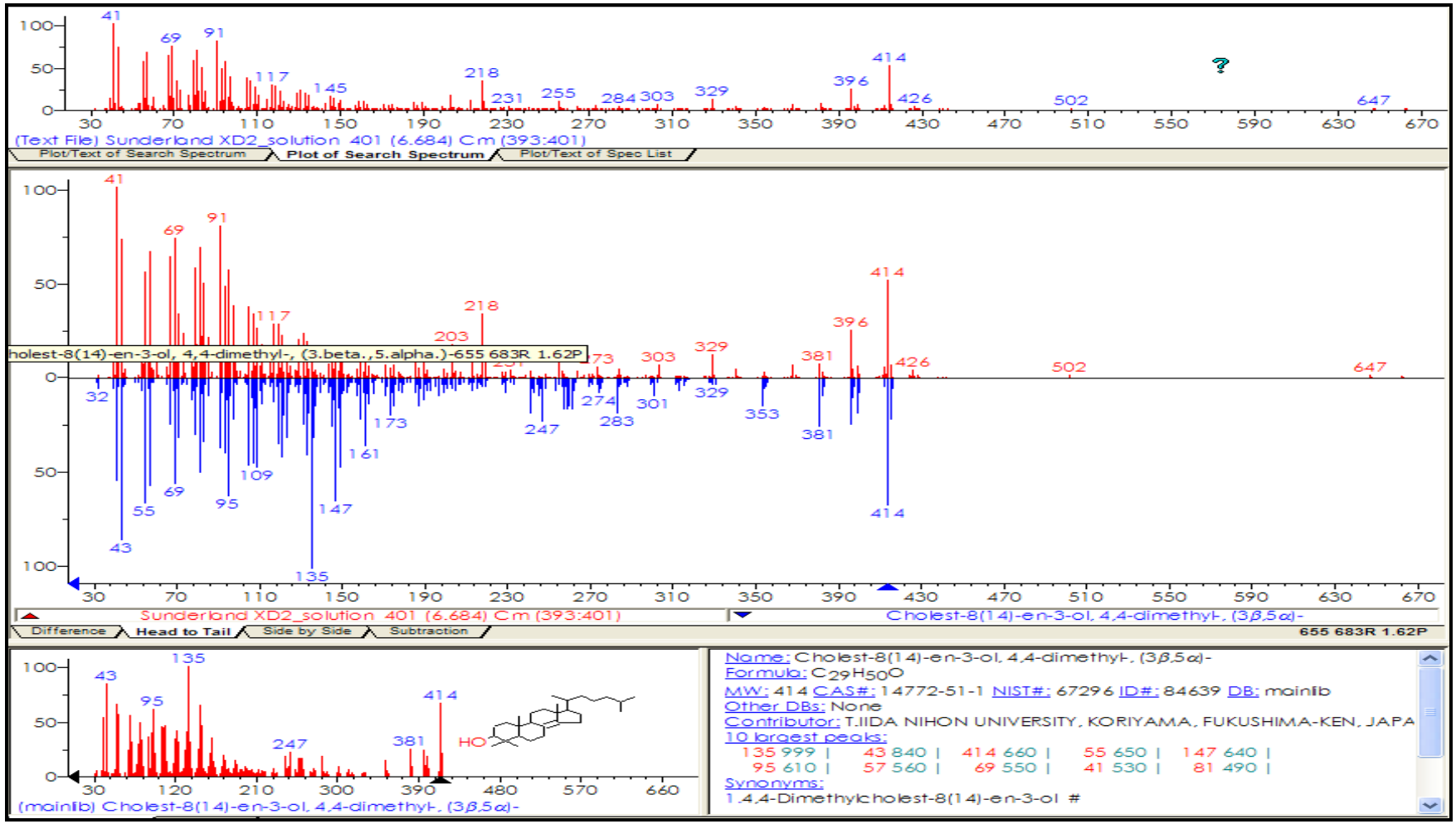

(Text File) Sunderland XD1_solution repeat 720 (12.011) Cm (720:734)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
Sunderland XD1_solution repeat 720 (12.011) Cm (720:734)
Stigmast-5-en-3-ol, oleate
Difference
Head to Tail
Side by Side
Subtraction
676 695R 7.21P
(mainlib) Stigmast-5-en-3-ol, oleate
Name: Stigmast-5-en-3-ol, oleate
Formula: C47H82O2
MW: 678 CAS#: N/A NIST#: 154665 ID#: 160255 DB: mainlib
Other DBs: None
Contributor: Chemical Concepts
10 largest peaks:
396 999 | 147 920 | 382 685 | 81 673 | 43 658 |
55 657 | 145 612 | 69 455 | 95 425 | 57 416 |
Synonyms:
no synonyms

2. XD2.

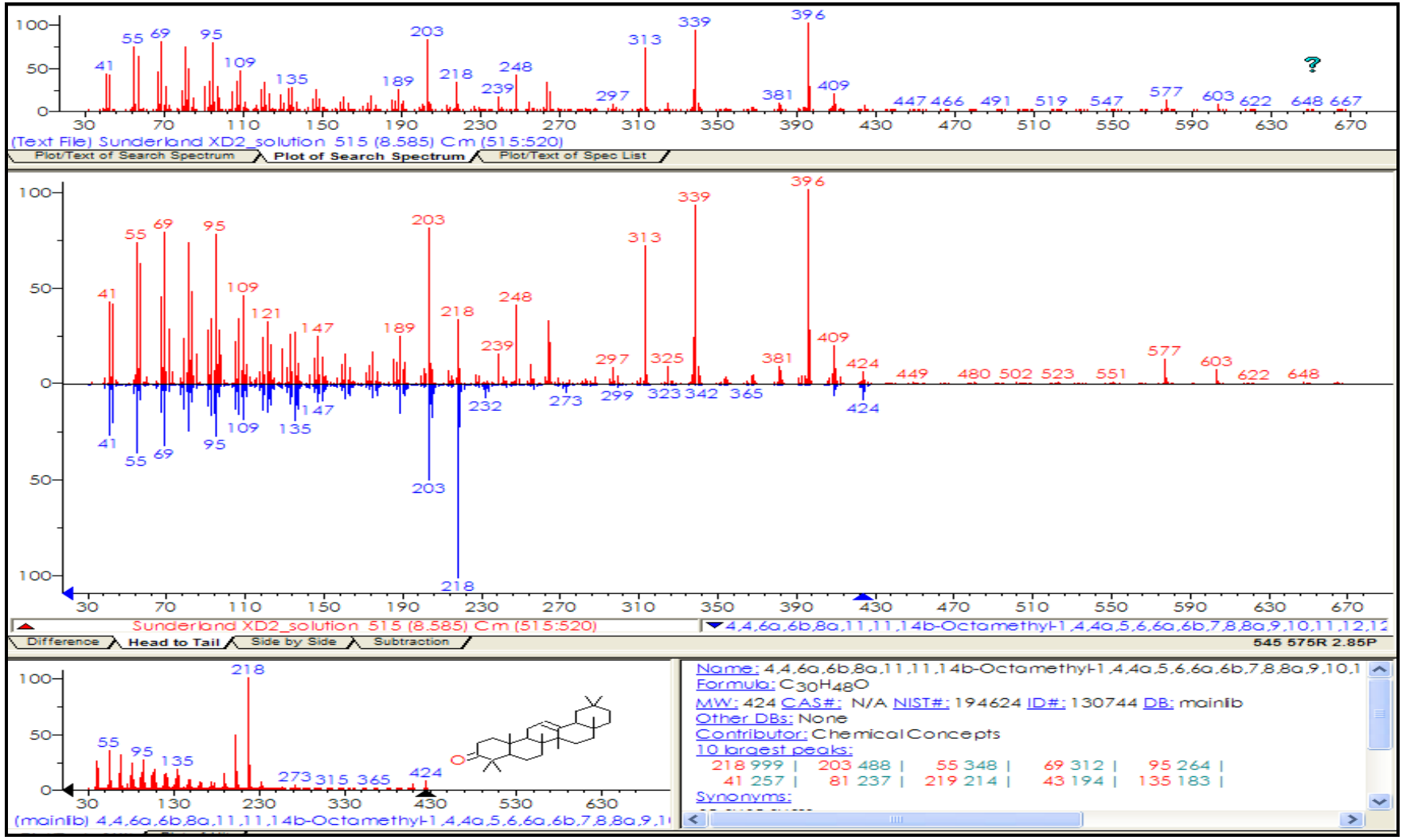
i.


Text File) Sunderland XD2_solution 401 (6.684) Cm (393:401)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
Sunderland XD2_solution 401 (6.684) Cm (393:401)
β-Sitosterol
Difference
Head to Tail
Side by Side
Subtraction
727 750R 30.1P
(replib) β-Sitosterol
Name: β-Sitosterol
Formula: C29H50O
MW: 414 CAS#: 83-46-5 NIST#: 287034 ID#: 1894 DB: replib
Other DBs: Fine, RTECS, HODOC, NIH, EINECS
Contributor: Humboldt-Universitat zu Berlin, Department of Chemistry, An
10 largest peaks:
43 999 | 55 639 | 41 561 | 57 447 | 81 427 |
105 392 | 95 387 | 107 378 | 91 377 | 79 348 |
Synonyms:

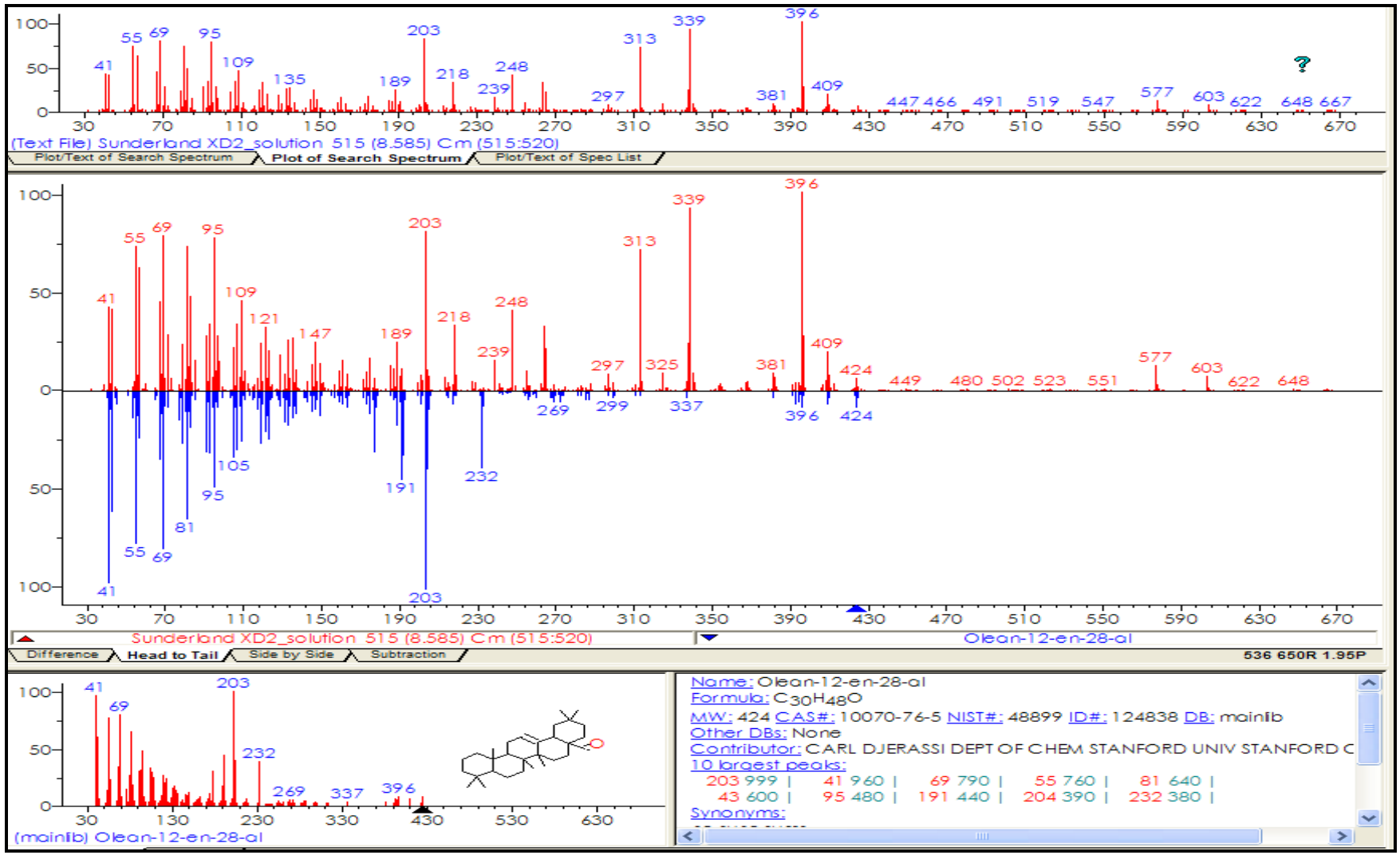
g.


(Text File) Sunderland XD2_solution 401 (6.684) Cm (393:401)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
holest-8(14)-en-3-ol, 4,4-dimethyl-, (3.beta.,5.alpha.)-655 683R 1.62P
Sunderland XD2_solution 401 (6.684) Cm (393:401)
Cholest-8(14)-en-3-ol, 4,4-dimethyl-, (3β,5α)-
655 683R 1.62P
Difference
Head to Tail
Side by Side
Subtraction
(mainlib) Cholest-8(14)-en-3-ol, 4,4-dimethyl-, (3β,5α)-
Name: Cholest-8(14)-en-3-ol, 4,4-dimethyl-, (3β,5α)-
Formula: C29H50O
MW: 414 CAS#: 14772-51-1 NIST#: 67296 ID#: 84639 DB: mainlib
Other DBs: None
Contributor: T.IIDA NIHON UNIVERSITY, KORIYAMA, FUKUSHIMA-KEN, JAPA
10 largest peaks:
135 999 | 43 840 | 414 660 | 55 650 | 147 640 |
95 610 | 57 560 | 69 550 | 41 530 | 81 490 |
Synonyms:
1.4,4-Dimethylcholest-8(14)-en-3-ol #

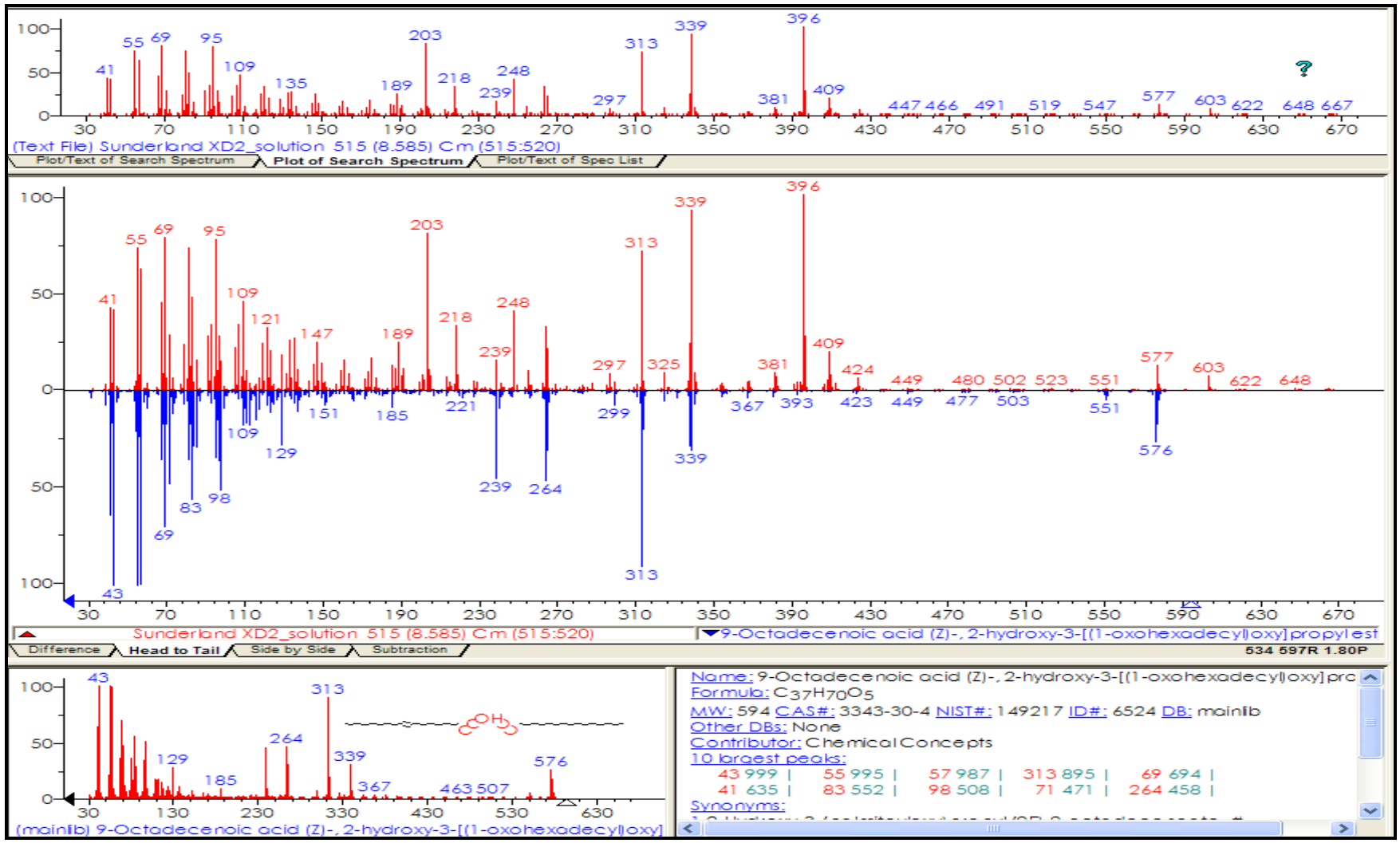
h.


(Text File) Sunderland XD2_solution 515 (8.585) Cm (515:520)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
Sunderland XD2_solution 515 (8.585) Cm (515:520)
4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12
Difference
Head to Tail
Side by Side
Subtraction
545 575R 2.85P
(mainlib) 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,1
Name: 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,1
Formula: C30H48O
MW: 424 CAS#: N/A NIST#: 194624 ID#: 130744 DB: mainlib
Other DBs: None
Contributor: Chemical Concepts
10 largest peaks:
218 999 | 203 488 | 55 348 | 69 312 | 95 264 |
41 257 | 81 237 | 219 214 | 43 194 | 135 183 |
Synonyms:

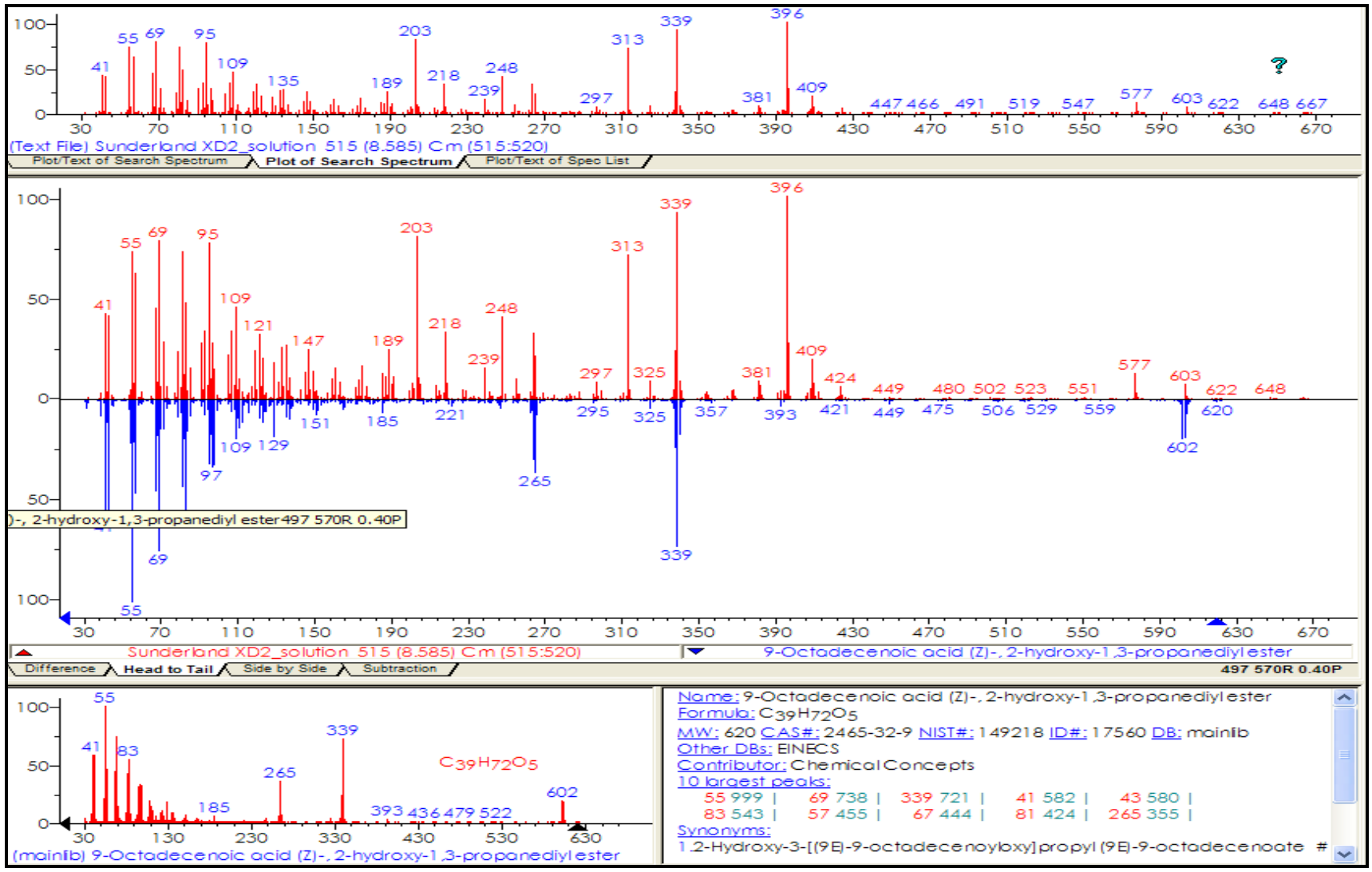
i.


(Text File) Sunderland XD2_solution 515 (8.585) Cm (515:520)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
Sunderland XD2_solution 515 (8.585) Cm (515:520)
Olean-12-en-28-al
Difference
Head to Tail
Side by Side
Subtraction
536 650R 1.95P
(mainlib) Olean-12-en-28-al
Name: Olean-12-en-28-al
Formula: C30H48O
MW: 424 CAS#: 10070-76-5 NIST#: 48899 ID#: 124838 DB: mainlib
Other DBs: None
Contributor: CARL DJERASSI DEPT OF CHEM STANFORD UNIV STANFORD C
10 largest peaks:
203 999 | 41 960 | 69 790 | 55 760 | 81 640 |
43 600 | 95 480 | 191 440 | 204 390 | 232 380 |
Synonyms:

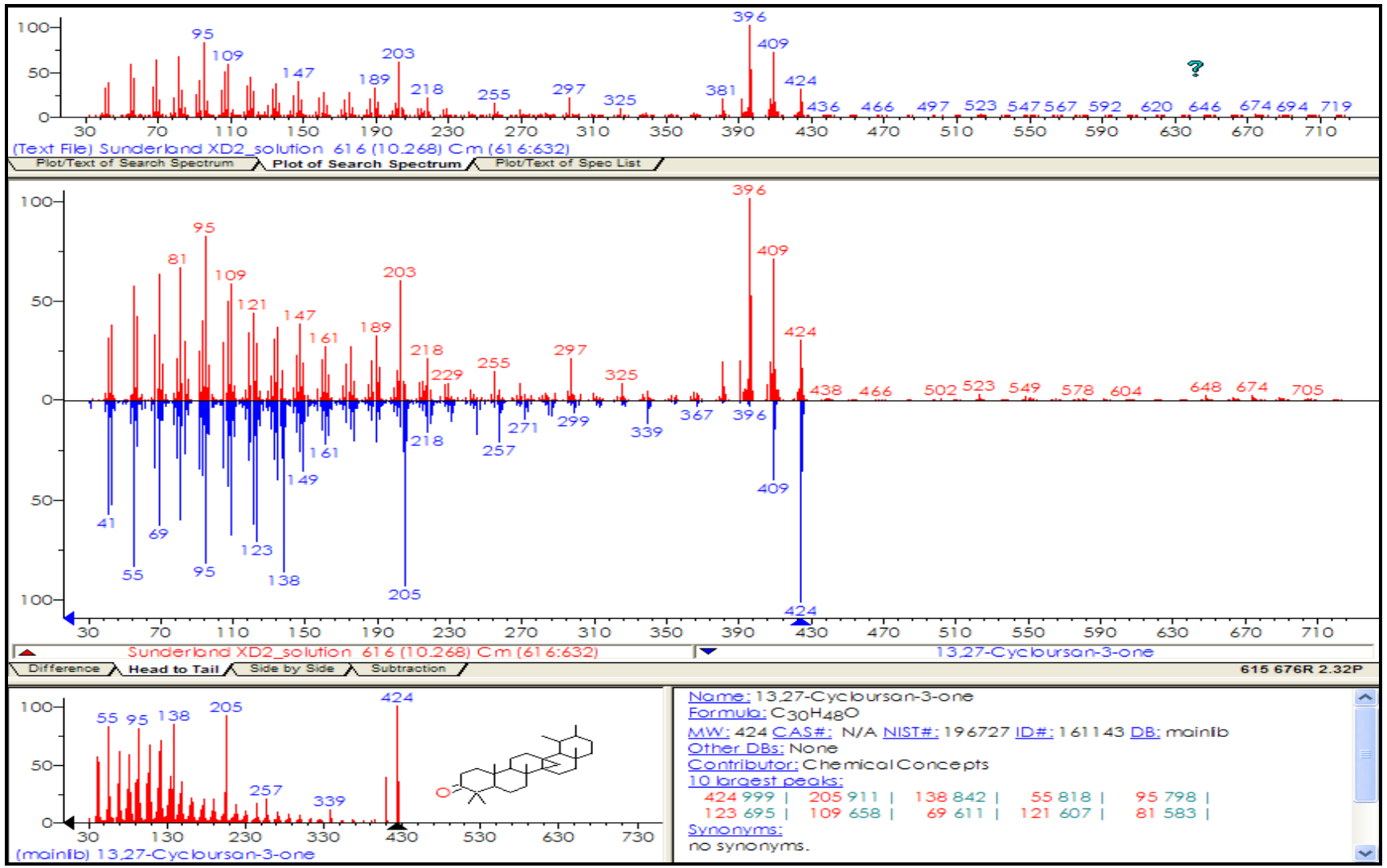
j.


(Text File) Sunderland XD2_solution 515 (8.585) Cm (515:520)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
Sunderland XD2_solution 515 (8.585) Cm (515:520)
9-Octadecenoic acid (Z)-, 2-hydroxy-3-[(1-oxohexadecyl)oxy]propyl est
Difference
Head to Tail
Side by Side
Subtraction
534 597R 1.80P
(mainlib) 9-Octadecenoic acid (Z)-, 2-hydroxy-3-[(1-oxohexadecyl)oxy]
Name: 9-Octadecenoic acid (Z)-, 2-hydroxy-3-[(1-oxohexadecyl)oxy]pro
Formula: C37H70O5
MW: 594 CAS#: 3343-30-4 NIST#: 149217 ID#: 6524 DB: mainlib
Other DBs: None
Contributor: Chemical Concepts
10 largest peaks:
43 999 | 55 995 | 57 987 | 313 895 | 69 694 |
41 635 | 83 552 | 98 508 | 71 471 | 264 458 |
Synonyms:

k.

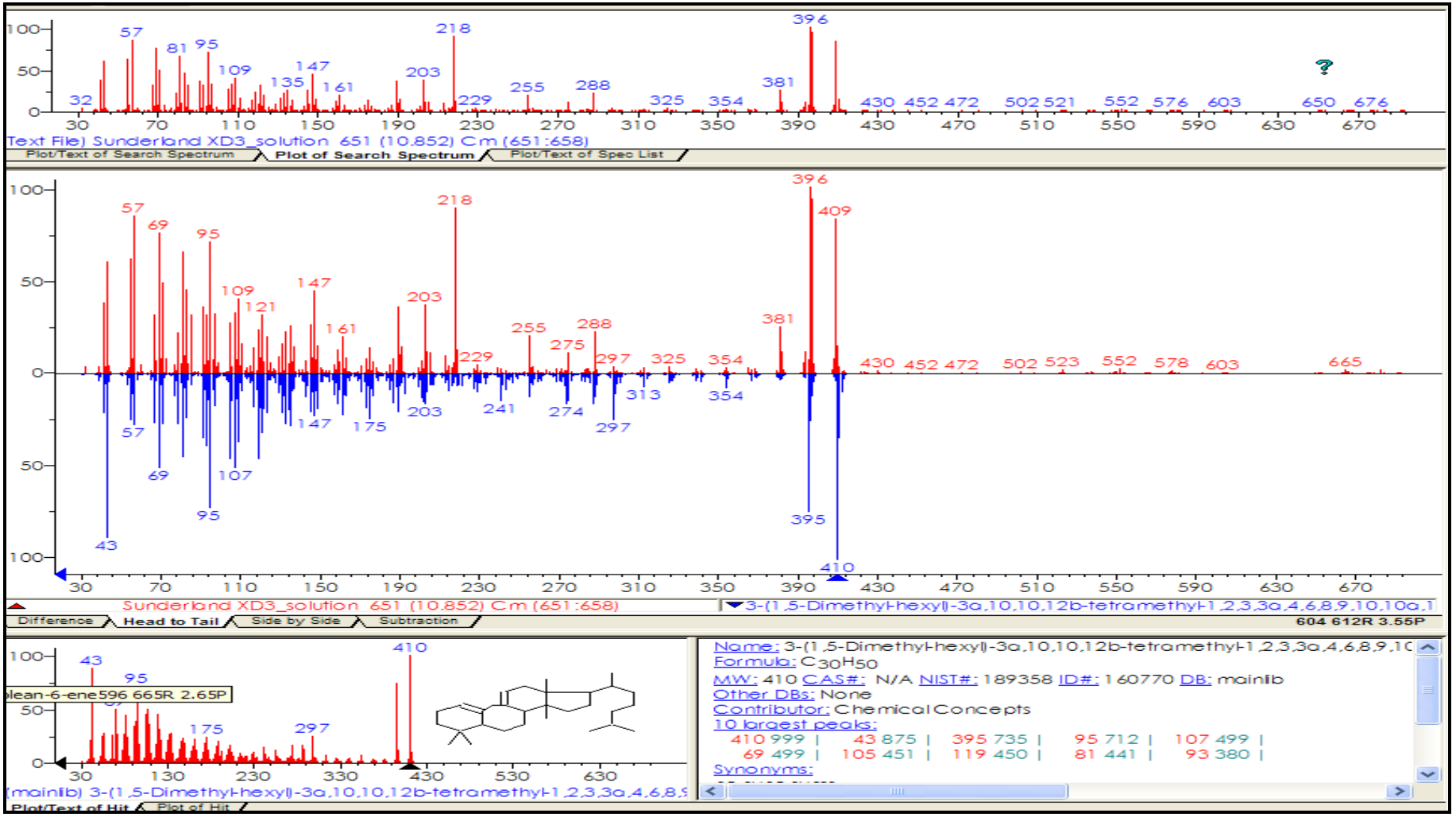

(Text File) Sunderland XD2_solution 515 (8.585) Cm (515:520)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
)-, 2-hydroxy-1,3-propanediyl ester 497 570R 0.40P
Sunderland XD2_solution 515 (8.585) Cm (515:520)
9-Octadecenoic acid (Z)-, 2-hydroxy-1,3-propanediyl ester
Difference
Head to Tail
Side by Side
Subtraction
497 570R 0.40P
C39H72O5
(mainlib) 9-Octadecenoic acid (Z)-, 2-hydroxy-1,3-propanediyl ester
Name: 9-Octadecenoic acid (Z)-, 2-hydroxy-1,3-propanediyl ester
Formula: C39H72O5
MW: 620 CAS#: 2465-32-9 NIST#: 149218 ID#: 17560 DB: mainlib
Other DBs: EINECS
Contributor: ChemicalConcepts
10 largest peaks:
55 999 | 69 738 | 339 721 | 41 582 | 43 580 |
83 543 | 57 455 | 67 444 | 81 424 | 265 355 |
Synonyms:
1,2-Hydroxy-3-[(9E)-9-octadecenoyloxy]propyl (9E)-9-octadecenoate #

I.

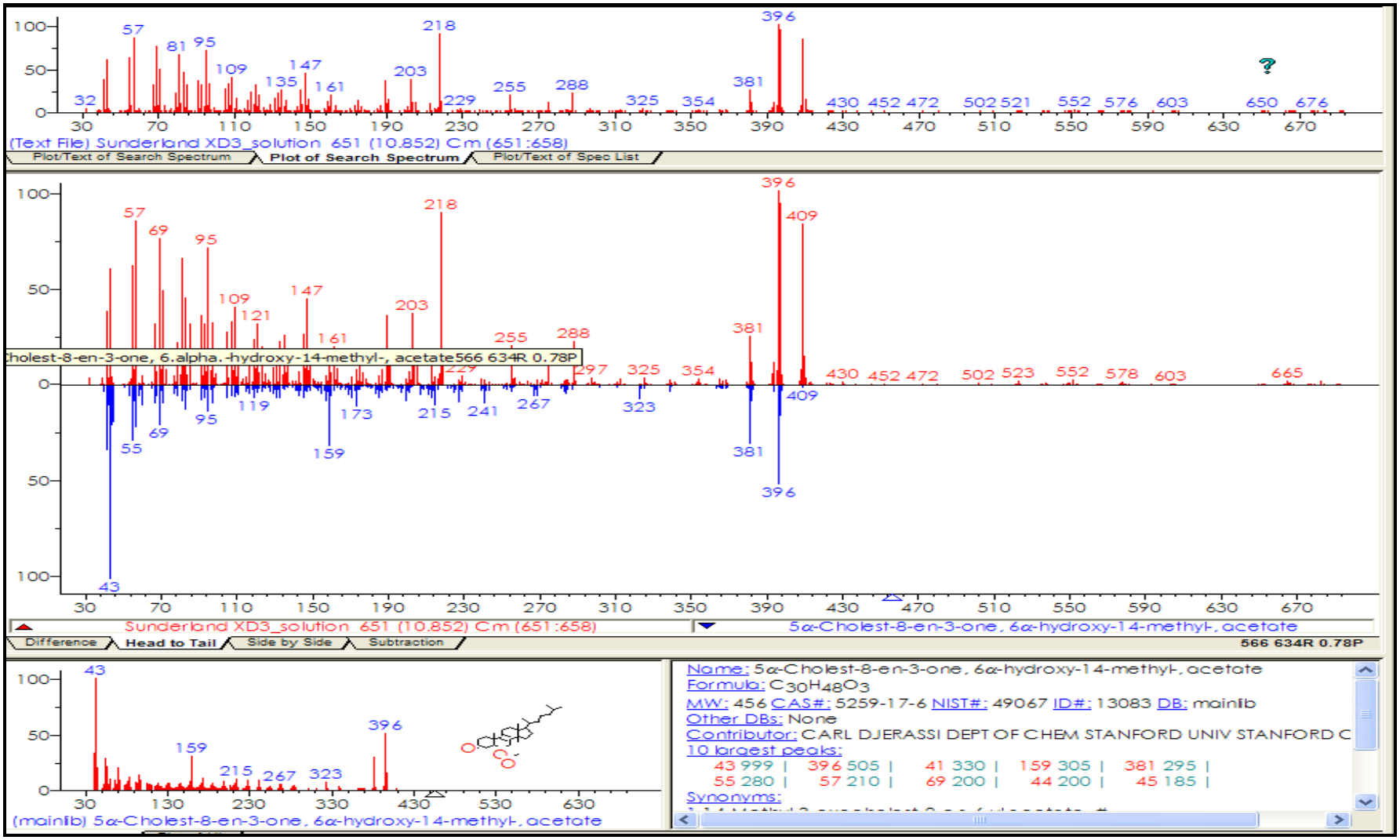

(Text File) Sunderland XD2_solution 616 (10.268) Cm (616:632)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
Sunderland XD2_solution 616 (10.268) Cm (616:632)
13,27-Cyclоursan-3-one
Difference
Head to Tail
Side by Side
Subtraction
615 676R 2.32P
(mainlib) 13,27-Cyclоursan-3-one
Name: 13,27-Cyclоursan-3-one
Formula: C30H48O
MW: 424 CAS#: N/A NIST#: 196727 ID#: 161143 DB: mainlib
Other DBs: None
Contributor: ChemicalConcepts
10 largest peaks:
424 999 | 205 911 | 138 842 | 55 818 | 95 798 |
123 695 | 109 658 | 69 611 | 121 607 | 81 583 |
Synonyms:
no synonyms.

3. XD3

i.

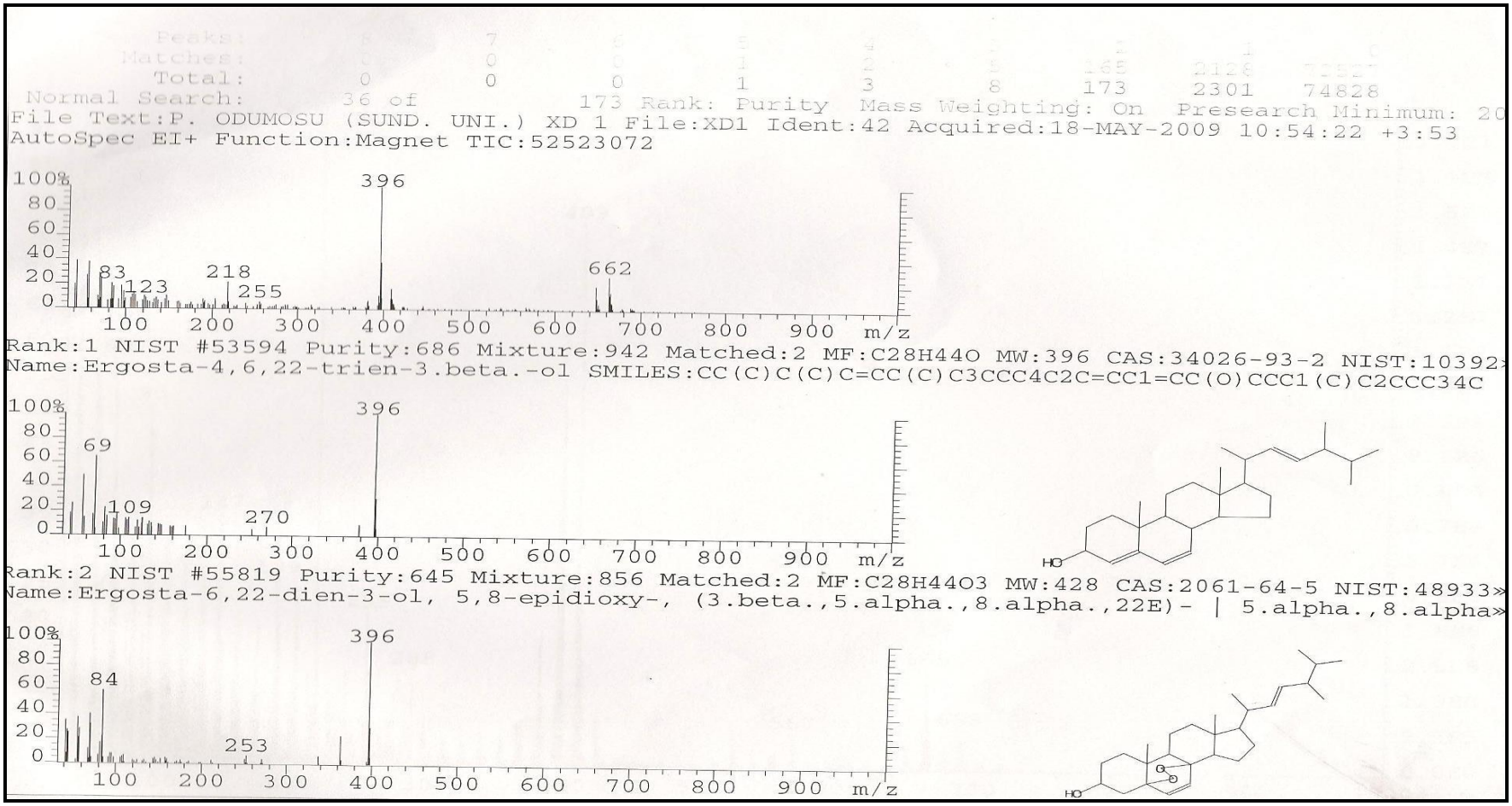

Text File) Sunderland XD3_solution 651 (10.852) Cm (651:658)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
Sunderland XD3_solution 651 (10.852) Cm (651:658)
3-(1,5-Dimethyl-hexyl)-3a,10,10,12b-tetramethyl-1,2,3,3a,4,6,8,9,10,10a,1
Difference
Head to Tail
Side by Side
Subtraction
604 612R 3.55P
(mainlib) 3-(1,5-Dimethyl-hexyl)-3a,10,10,12b-tetramethyl-1,2,3,3a,4,6,8,9
Plot/Text of Hit
Plot of Hit
Name: 3-(1,5-Dimethyl-hexyl)-3a,10,10,12b-tetramethyl-1,2,3,3a,4,6,8,9,1C
Formula: C30H50
MW: 410 CAS#: N/A NIST#: 189358 ID#: 160770 DB: mainlib
Other DBs: None
Contributor: ChemicalConcepts
10 largest peaks:
410 999 | 43 875 | 395 735 | 95 712 | 107 499 |
69 499 | 105 451 | 119 450 | 81 441 | 93 380 |
Synonyms:

ii.

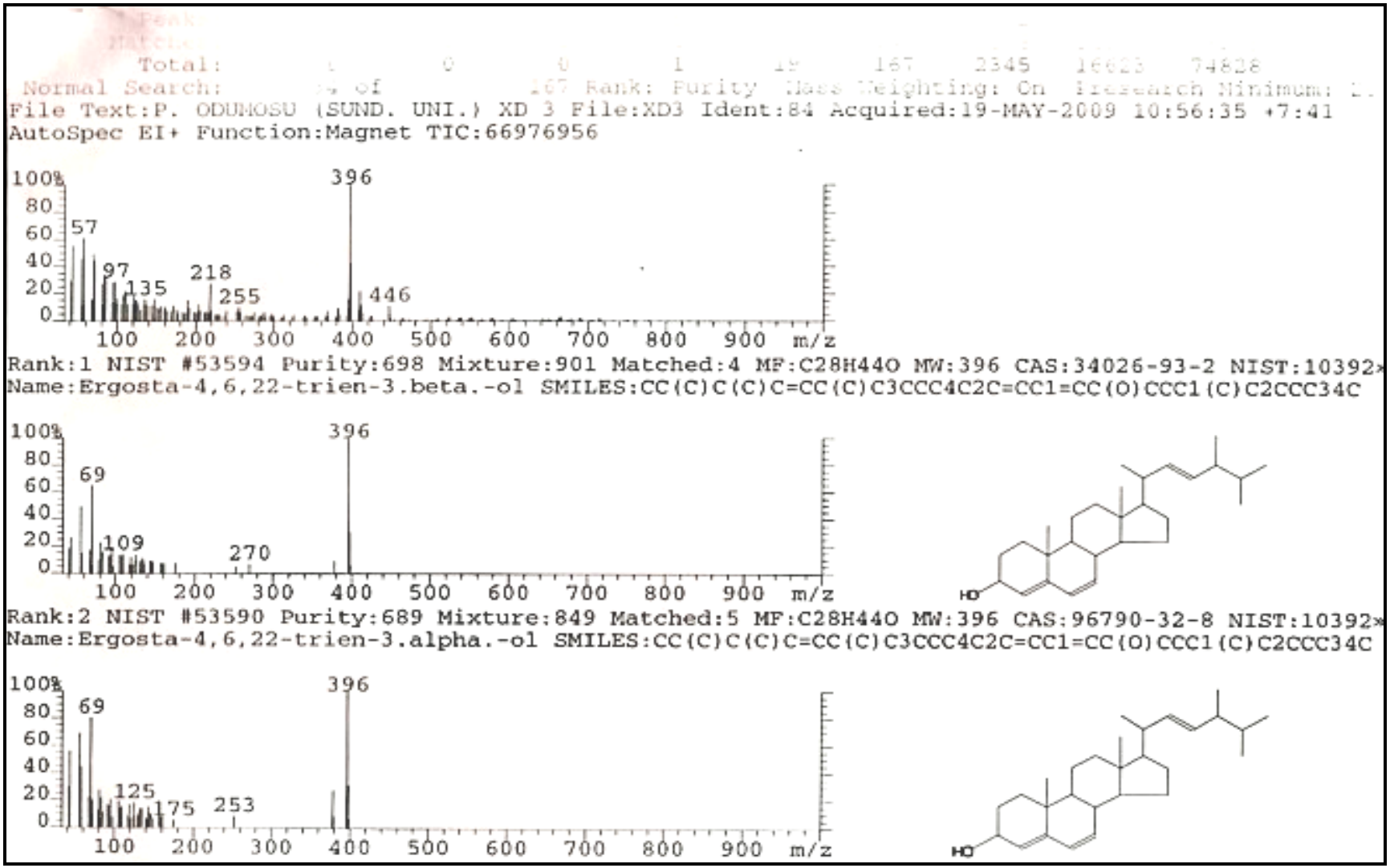

(Text File) Sunderland XD3_solution 651 (10.852) Cm (651:658)
Plot/Text of Search Spectrum
Plot of Search Spectrum
Plot/Text of Spec List
cholest-8-en-3-one, 6.alpha.-hydroxy-14-methyl-, acetate566 634R 0.78P
Sunderland XD3_solution 651 (10.852) Cm (651:658)
5α-Cholest-8-en-3-one, 6α-hydroxy-14-methyl-, acetate
Difference
Head to Tail
Side by Side
Subtraction
566 634R 0.78P
(mainlib) 5α-Cholest-8-en-3-one, 6α-hydroxy-14-methyl-, acetate
Name: 5α-Cholest-8-en-3-one, 6α-hydroxy-14-methyl-, acetate
Formula: C30H48O3
MW: 456 CAS#: 5259-17-6 NIST#: 49067 ID#: 13083 DB: mainlib
Other DBs: None
Contributor: CARL DJERASSI DEPT OF CHEM STANFORD UNIV STANFORD C
10 largest peaks:
43 999 | 396 505 | 41 330 | 159 305 | 381 295 |
55 280 | 57 210 | 69 200 | 44 200 | 45 185 |
Synonyms:

4. XAD fractions analysed by EI MS +

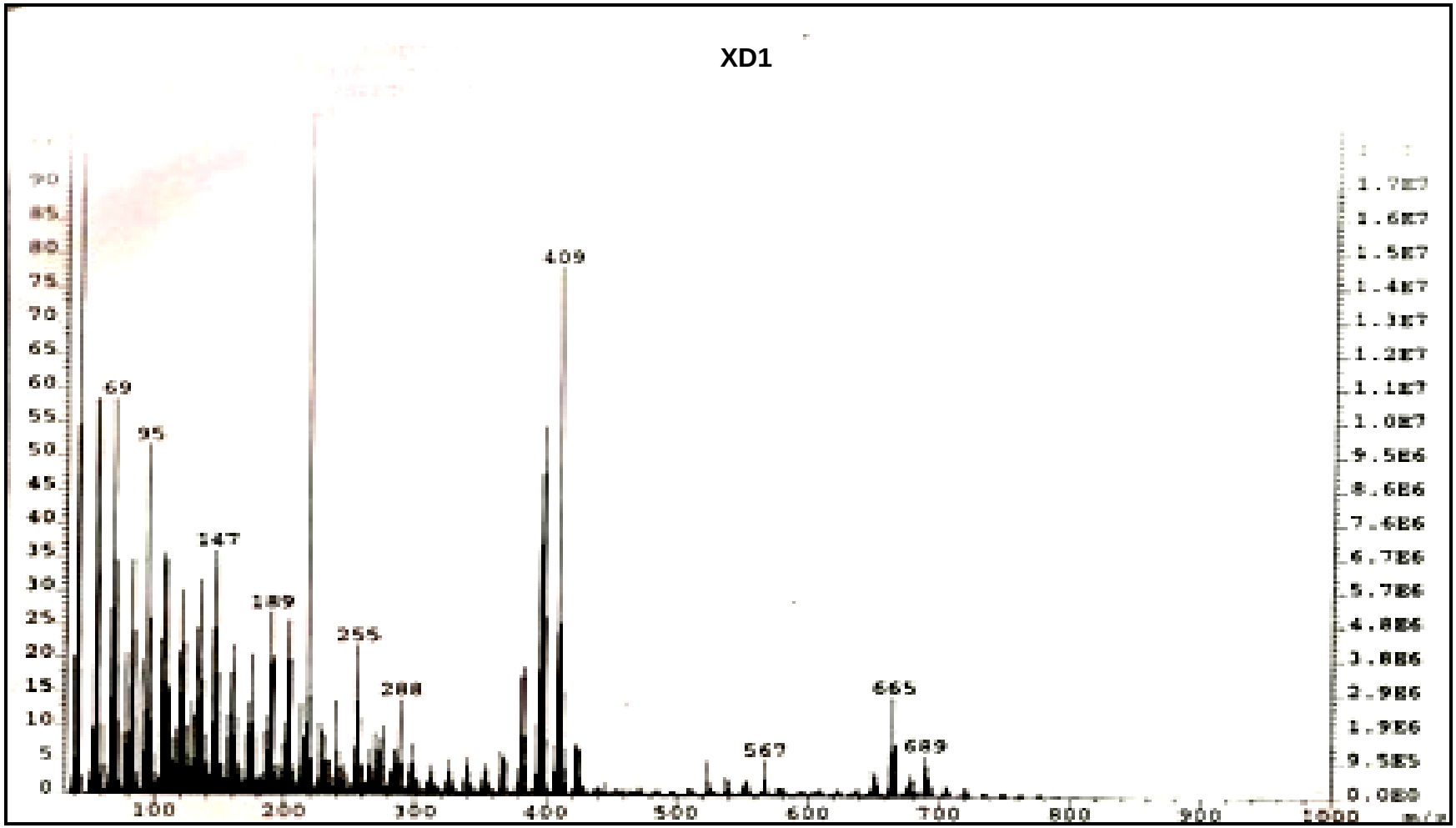
i.



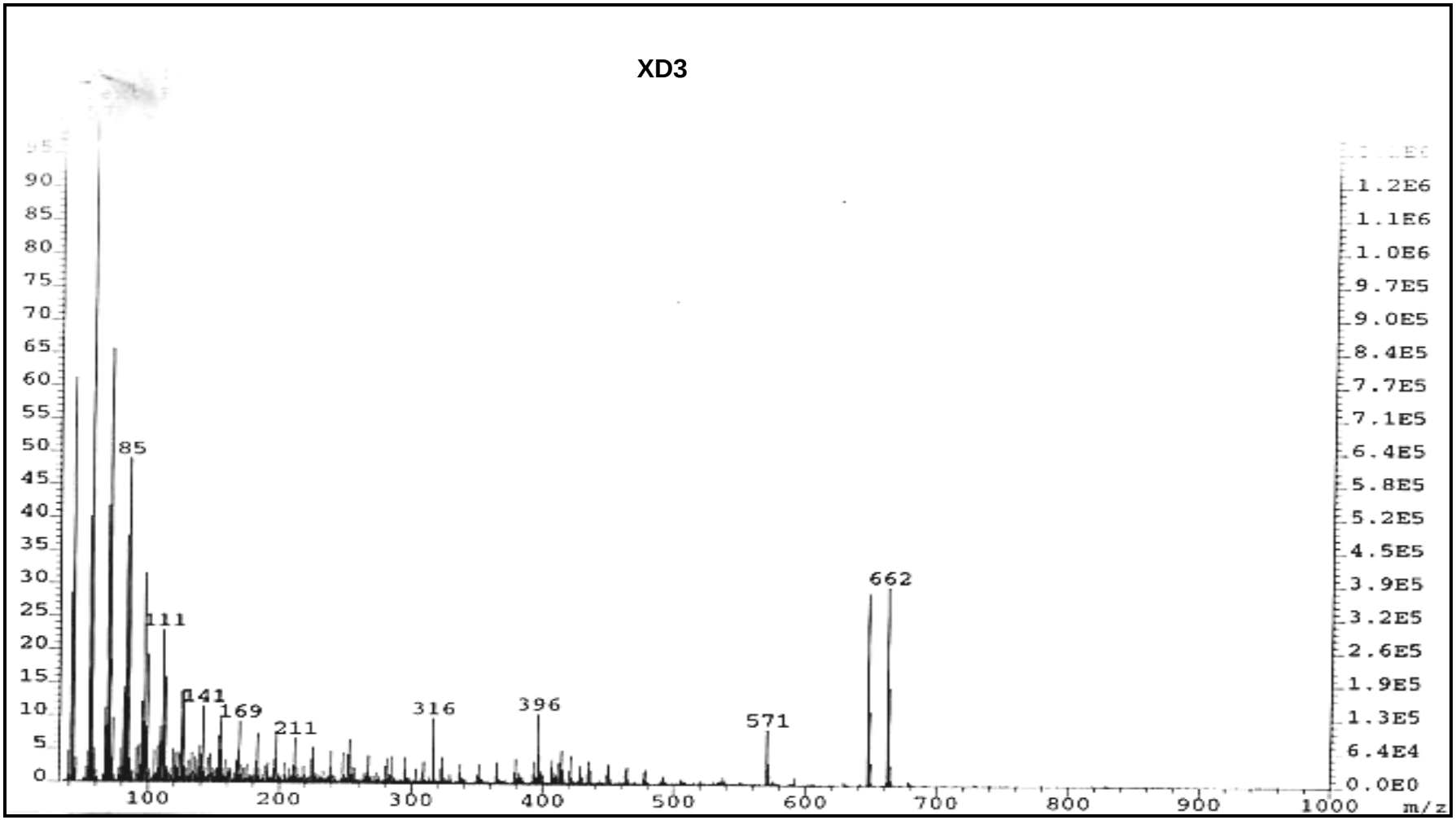
ii.

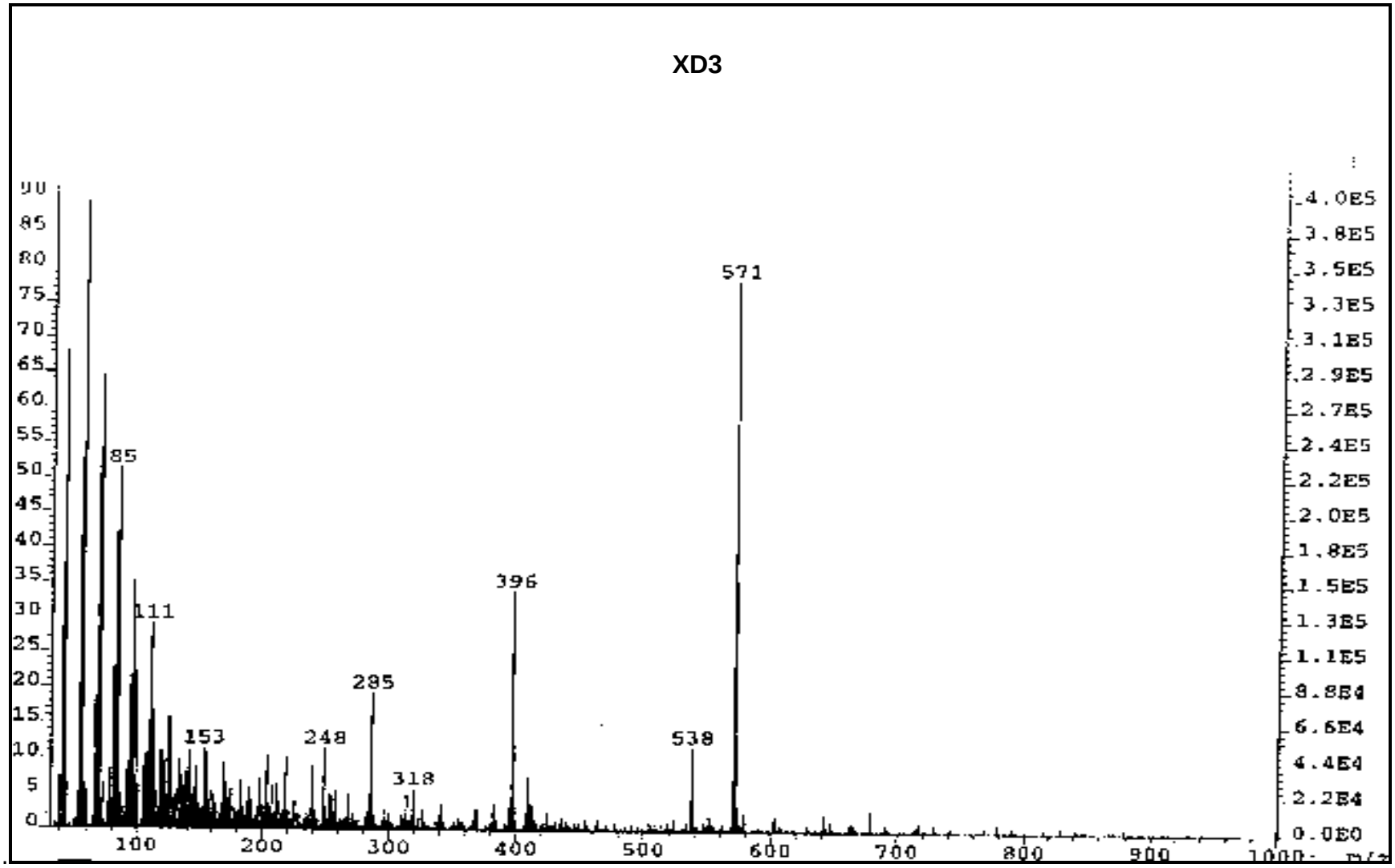

Total:
Normal Search:
File Text:P. ODUMOSU (SUND. UNI.) XD 3 File:XD3 Ident:84 Acquired:19-MAY-2009 10:56:35 +7:41
AutoSpec EI+ Function:Magnet TIC:66976956
100%
80
60
40
20
0
57
97
135
218
255
396
446
100 200 300 400 500 600 700 800 900 m/z
Rank:1 NIST #53594 Purity:698 Mixture:901 Matched:4 MF:C28H44O MW:396 CAS:34026-93-2 NIST:10392
Name:Ergosta-4,6,22-trien-3.beta.-ol SMILES:CC(C)C(C)C=CC(C)C3CCC4C2C=CC1=CC(O)CCC1(C)C2CCC34C
69
109
270
Rank:2 NIST #53590 Purity:689 Mixture:849 Matched:5 MF:C28H44O MW:396 CAS:96790-32-8 NIST:10392
Name:Ergosta-4,6,22-trien-3.alpha.-ol SMILES:CC(C)C(C)C=CC(C)C3CCC4C2C=CC1=CC(O)CCC1(C)C2CCC34C
125
175
253
HO

iii.


XD1
69
95
147
189
255
288
409
567
665
689
100
200
300
400
500
600
700
800
900
1000

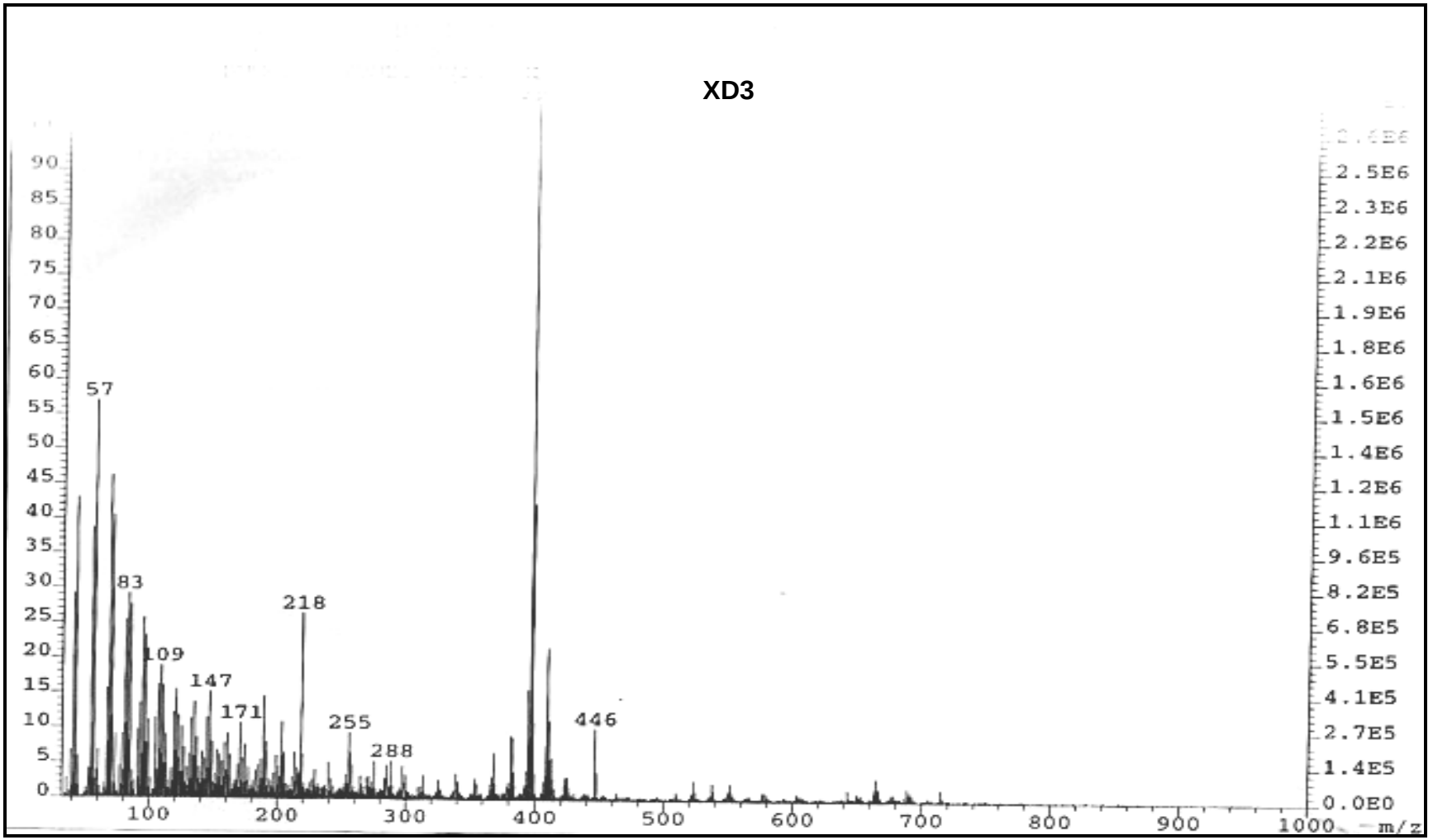
iv.


XD3
85
111
141
169
211
316
396
571
662
100
200
300
400
500
600
700
800
900
1000
m/z
1.2E6
1.1E6
1.0E6
9.7E5
9.0E5
8.4E5
7.7E5
7.1E5
6.4E5
5.8E5
5.2E5
4.5E5
3.9E5
3.2E5
2.6E5
1.9E5
1.3E5
6.4E4
0.0E0

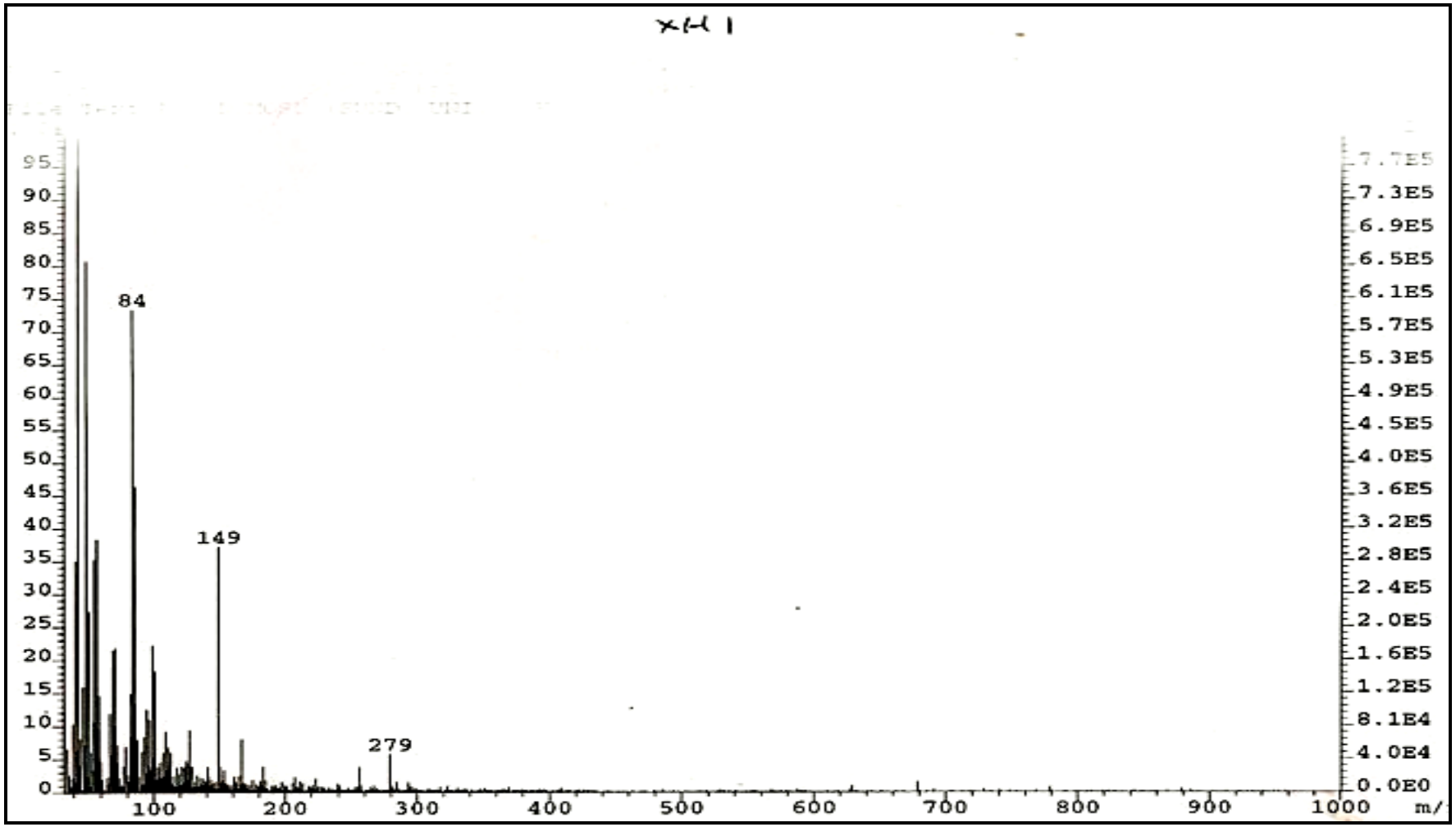

XD3
85
111
153
248
285
318
396
538
571
100
200
300
400
500
600
700
800
900
1000
4.0E5
3.8E5
3.5E5
3.3E5
3.1E5
2.9E5
2.7E5
2.4E5
2.2E5
2.0E5
1.8E5
1.5E5
1.3E5
1.1E5
8.8E4
6.6E4
4.4E4
2.2E4
0.0E0

V. .

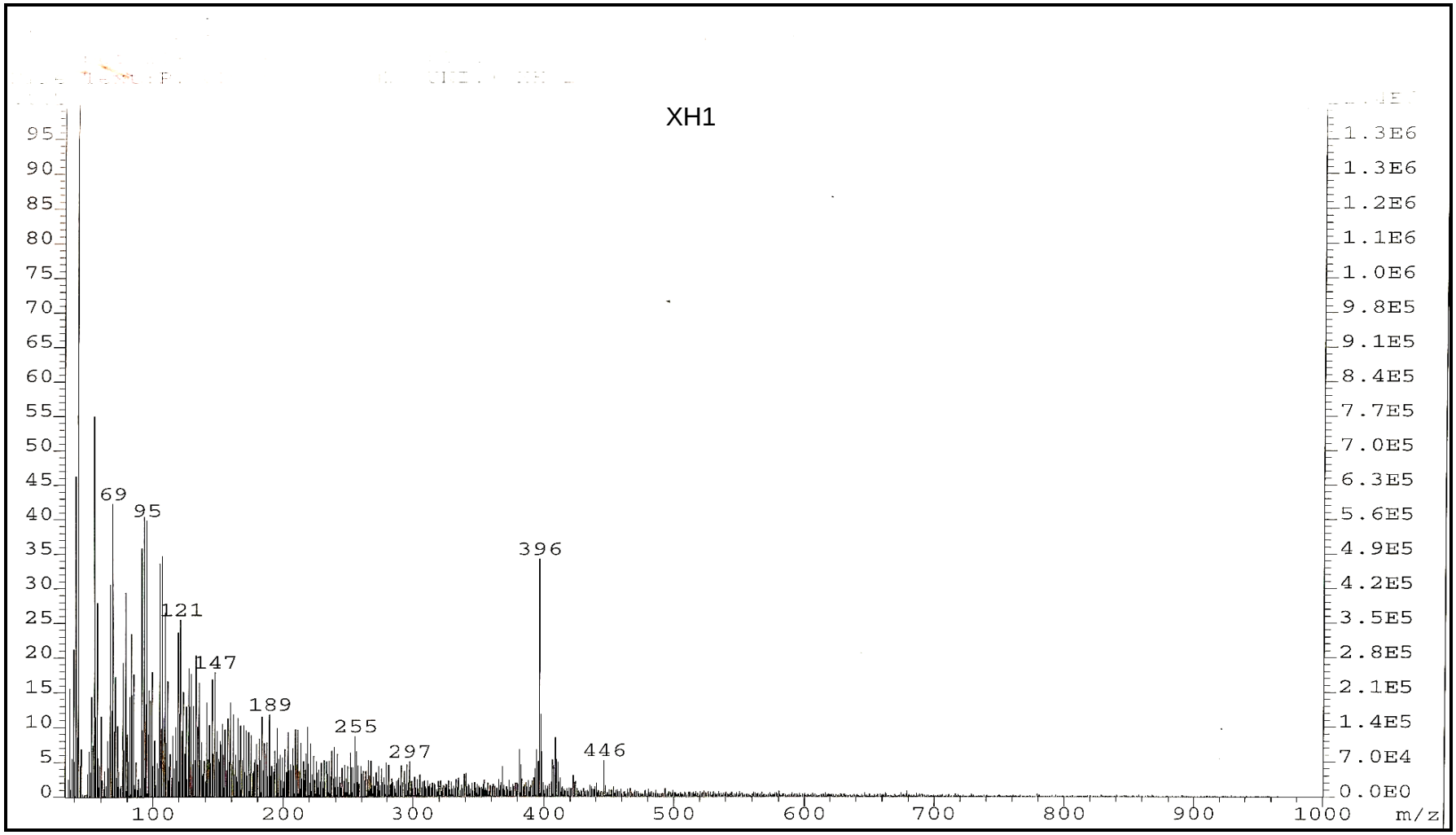
vi.


XD3
57
83
109
147
171
218
255
288
446
100
200
300
400
500
600
700
800
900
1000
m/z

vii.

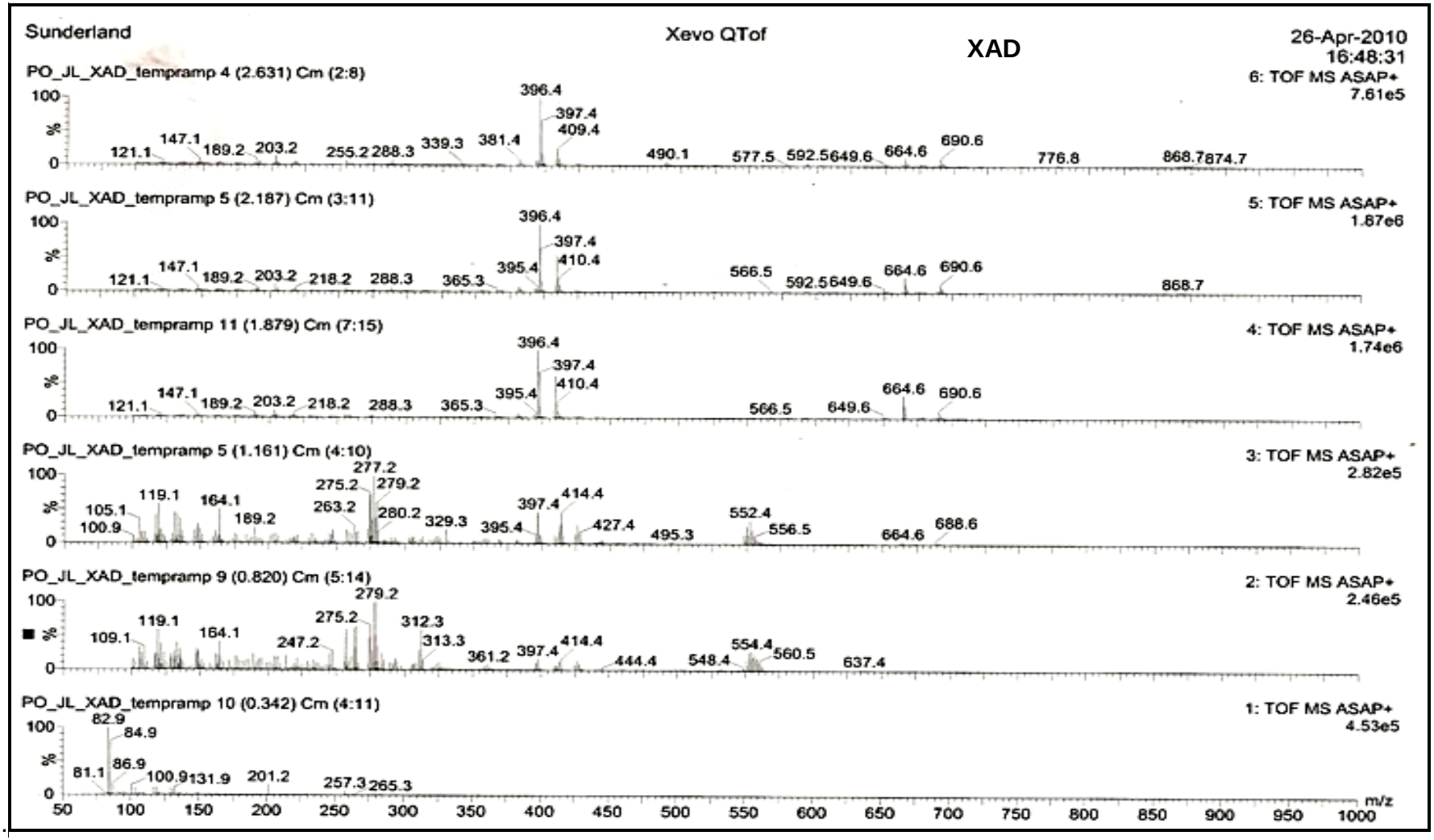

XH 1
84
149
279
100
200
300
400
500
600
700
800
900
1000
m/
7.7E5
7.3E5
6.9E5
6.5E5
6.1E5
5.7E5
5.3E5
4.9E5
4.5E5
4.0E5
3.6E5
3.2E5
2.8E5
2.4E5
2.0E5
1.6E5
1.2E5
8.1E4
4.0E4
0.0E0

viii.

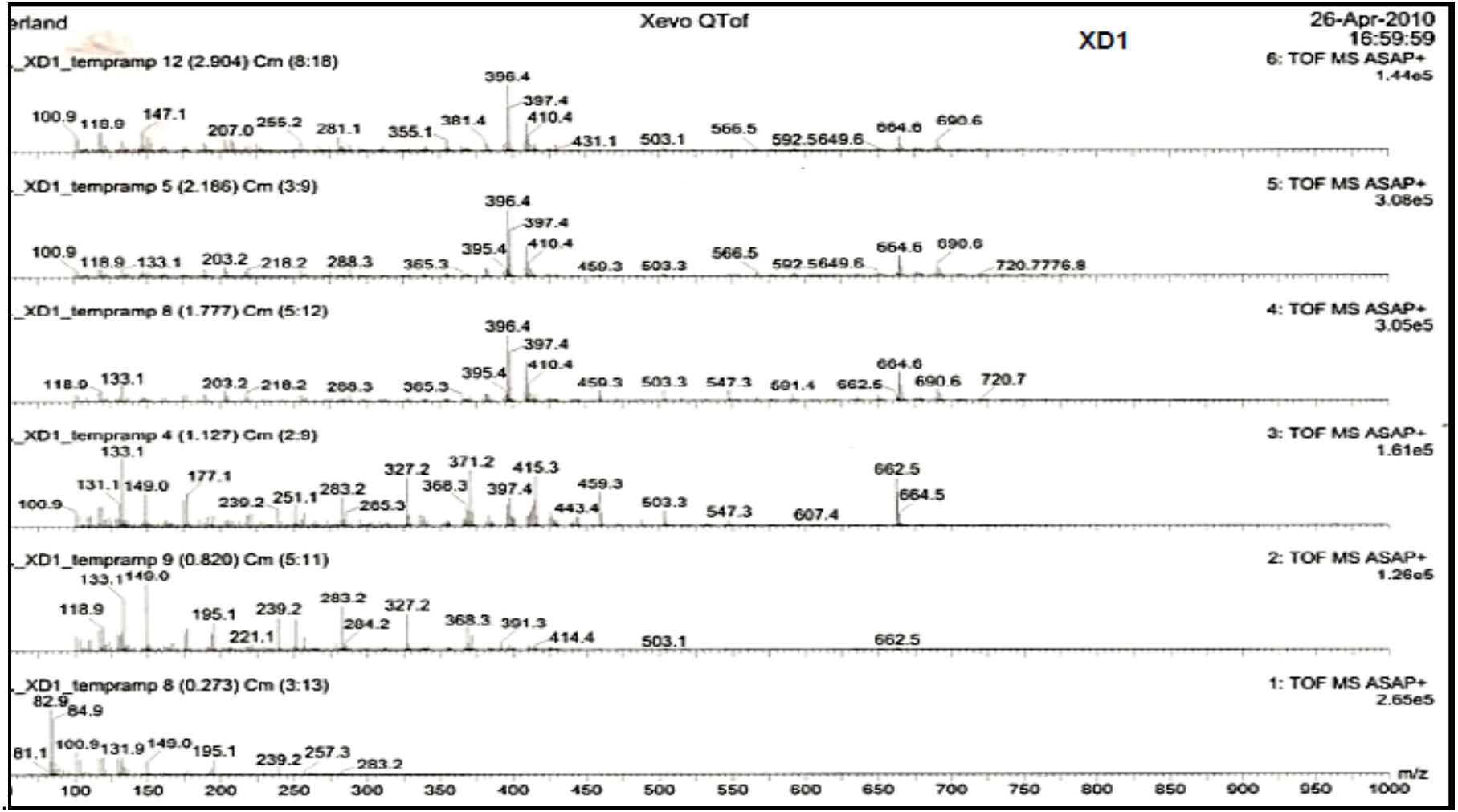

XH1
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
69
95
121
147
189
255
297
396
446
100
200
300
400
500
600
700
800
900
1000
m/z
1.3E6
1.3E6
1.2E6
1.1E6
1.0E6
9.8E5
9.1E5
8.4E5
7.7E5
7.0E5
6.3E5
5.6E5
4.9E5
4.2E5
3.5E5
2.8E5
2.1E5
1.4E5
7.0E4
0.0E0

5) MS data for LC fractions obtained by TOF MS ASAP +

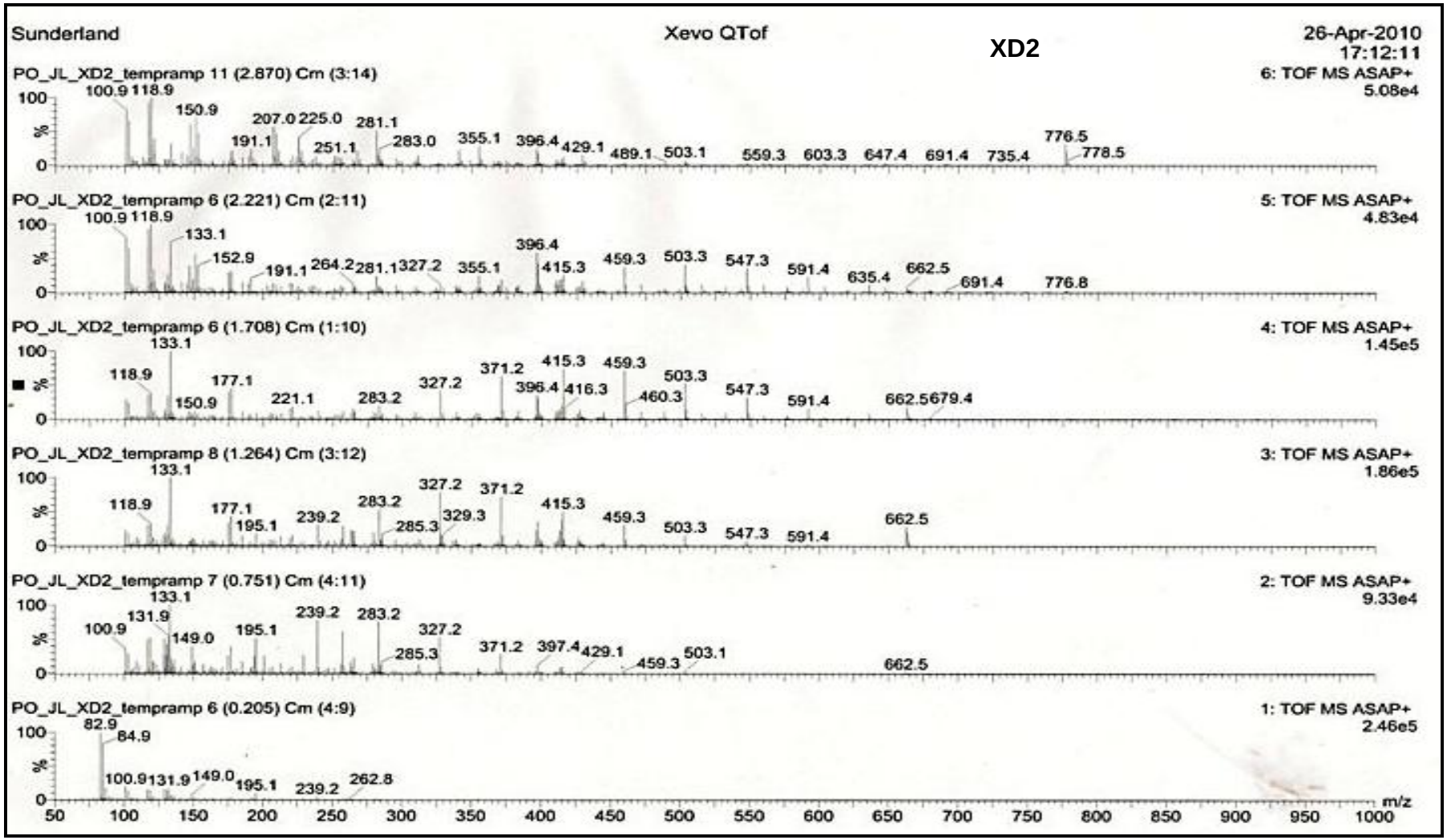
i.



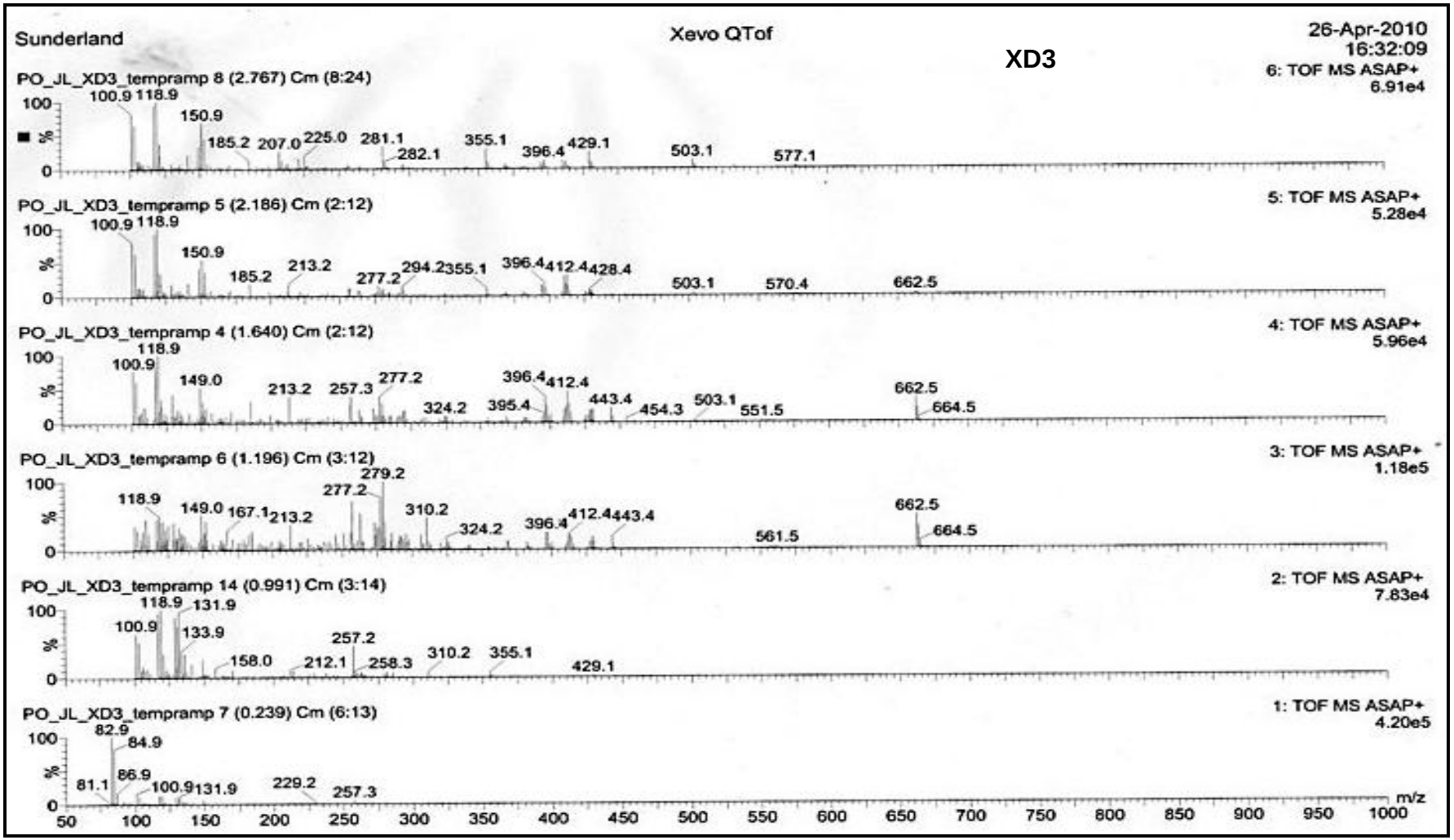
ii.


erland
Xevo QTof
XD1
26-Apr-2010
16:59:59
_XD1_tempramp 12 (2.904) Cm (8:18)
6: TOF MS ASAP+
1.44e5
396.4
397.4
410.4
100.9
118.9
147.1
207.0
255.2
281.1
355.1
381.4
431.1
503.1
566.5
592.5649.6
664.6
690.6
_XD1_tempramp 5 (2.186) Cm (3:9)
5: TOF MS ASAP+
3.08e5
396.4
397.4
410.4
100.9
118.9
133.1
203.2
218.2
288.3
365.3
395.4
459.3
503.3
566.5
592.5649.6
664.6
690.6
720.7776.8
_XD1_tempramp 8 (1.777) Cm (5:12)
4: TOF MS ASAP+
3.05e5
396.4
397.4
410.4
118.9
133.1
203.2
218.2
288.3
365.3
395.4
459.3
503.3
547.3
591.4
662.5
664.6
690.6
720.7
_XD1_tempramp 4 (1.127) Cm (2:9)
3: TOF MS ASAP+
1.61e5
133.1
131.1
149.0
177.1
100.9
239.2
251.1
283.2
285.3
327.2
368.3
371.2
397.4
415.3
443.4
459.3
503.3
547.3
607.4
662.5
664.5
_XD1_tempramp 9 (0.820) Cm (5:11)
2: TOF MS ASAP+
1.26e5
133.1
149.0
118.9
195.1
221.1
239.2
283.2
284.2
327.2
368.3
391.3
414.4
503.1
662.5
_XD1_tempramp 8 (0.273) Cm (3:13)
1: TOF MS ASAP+
2.65e5
82.9
84.9
81.1
100.9
131.9
149.0
195.1
239.2
257.3
283.2
m/z
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000

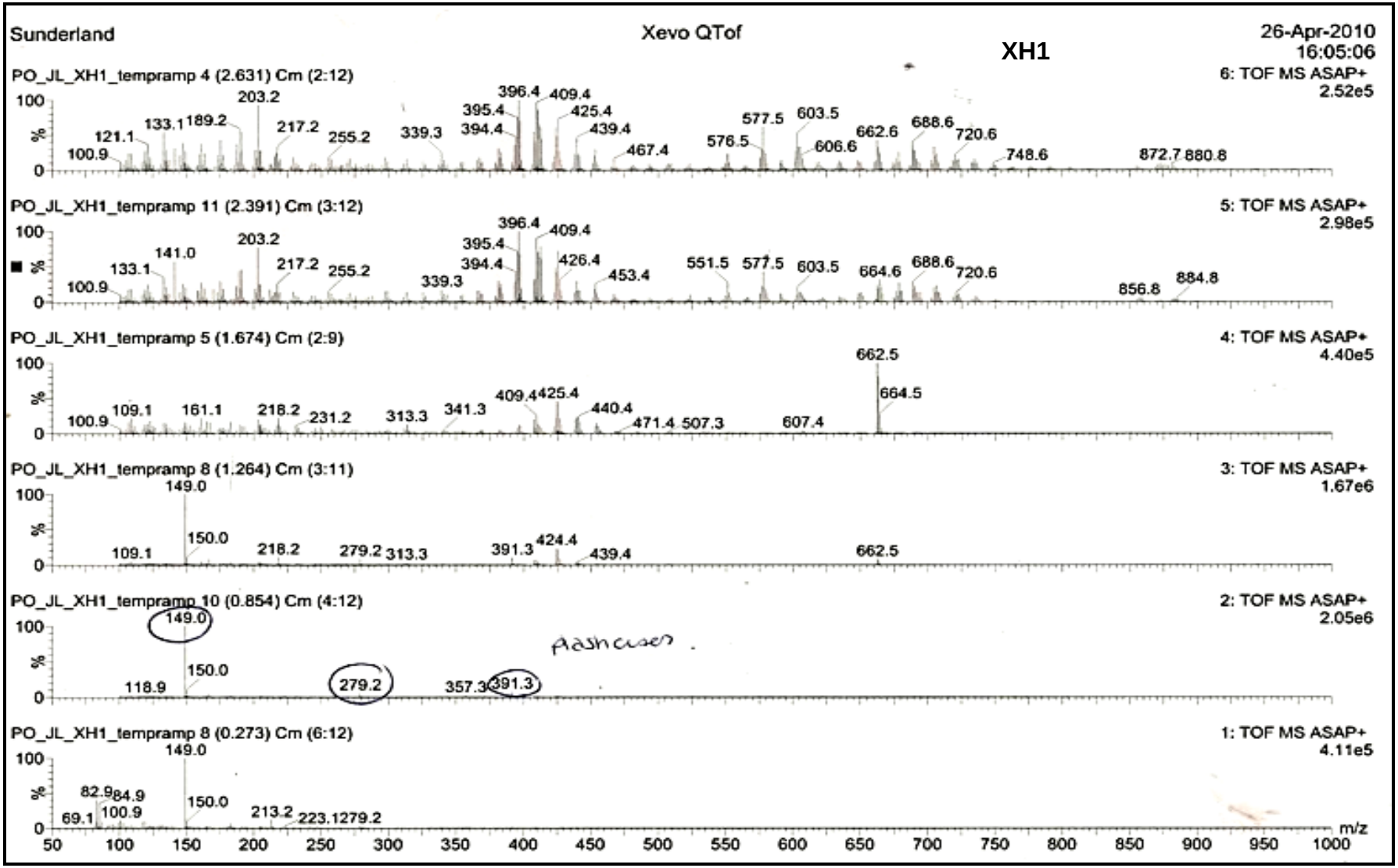
iii.


Sunderland
Xevo QTof
XD2
26-Apr-2010
17:12:11
PO_JL_XD2_tempramp 11 (2.870) Cm (3:14)
6: TOF MS ASAP+
5.08e4
PO_JL_XD2_tempramp 6 (2.221) Cm (2:11)
5: TOF MS ASAP+
4.83e4
PO_JL_XD2_tempramp 6 (1.708) Cm (1:10)
4: TOF MS ASAP+
1.45e5
PO_JL_XD2_tempramp 8 (1.264) Cm (3:12)
3: TOF MS ASAP+
1.86e5
PO_JL_XD2_tempramp 7 (0.751) Cm (4:11)
2: TOF MS ASAP+
9.33e4
PO_JL_XD2_tempramp 6 (0.205) Cm (4:9)
1: TOF MS ASAP+
2.46e5
%
m/z
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000

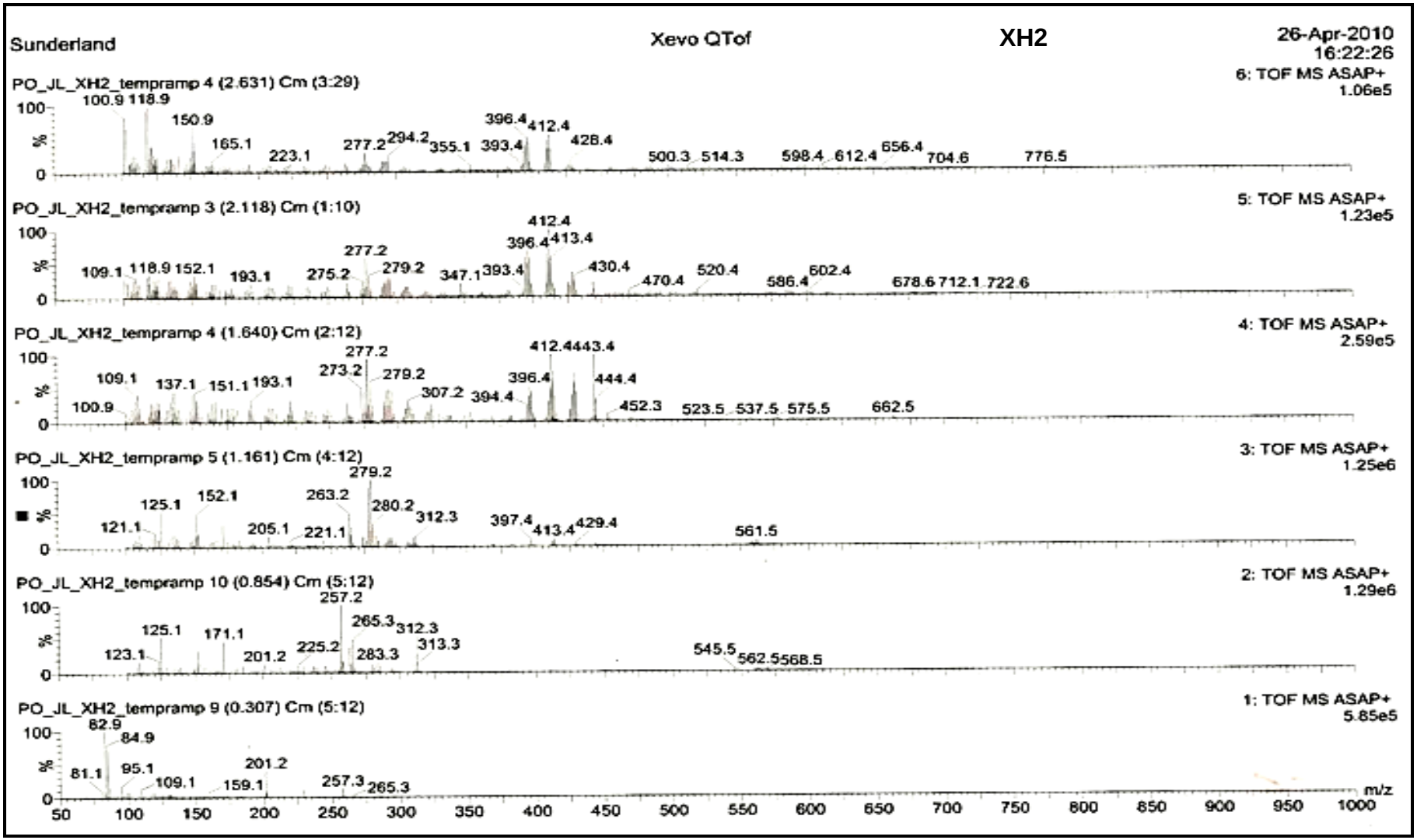
iv.


Sunderland
Xevo QTof
XD3
26-Apr-2010
16:32:09
PO_JL_XD3_tempramp 8 (2.767) Cm (8:24)
6: TOF MS ASAP+
6.91e4
PO_JL_XD3_tempramp 5 (2.186) Cm (2:12)
5: TOF MS ASAP+
5.28e4
PO_JL_XD3_tempramp 4 (1.640) Cm (2:12)
4: TOF MS ASAP+
5.96e4
PO_JL_XD3_tempramp 6 (1.196) Cm (3:12)
3: TOF MS ASAP+
1.18e5
PO_JL_XD3_tempramp 14 (0.991) Cm (3:14)
2: TOF MS ASAP+
7.83e4
PO_JL_XD3_tempramp 7 (0.239) Cm (6:13)
1: TOF MS ASAP+
4.20e5
%
m/z
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000

v.

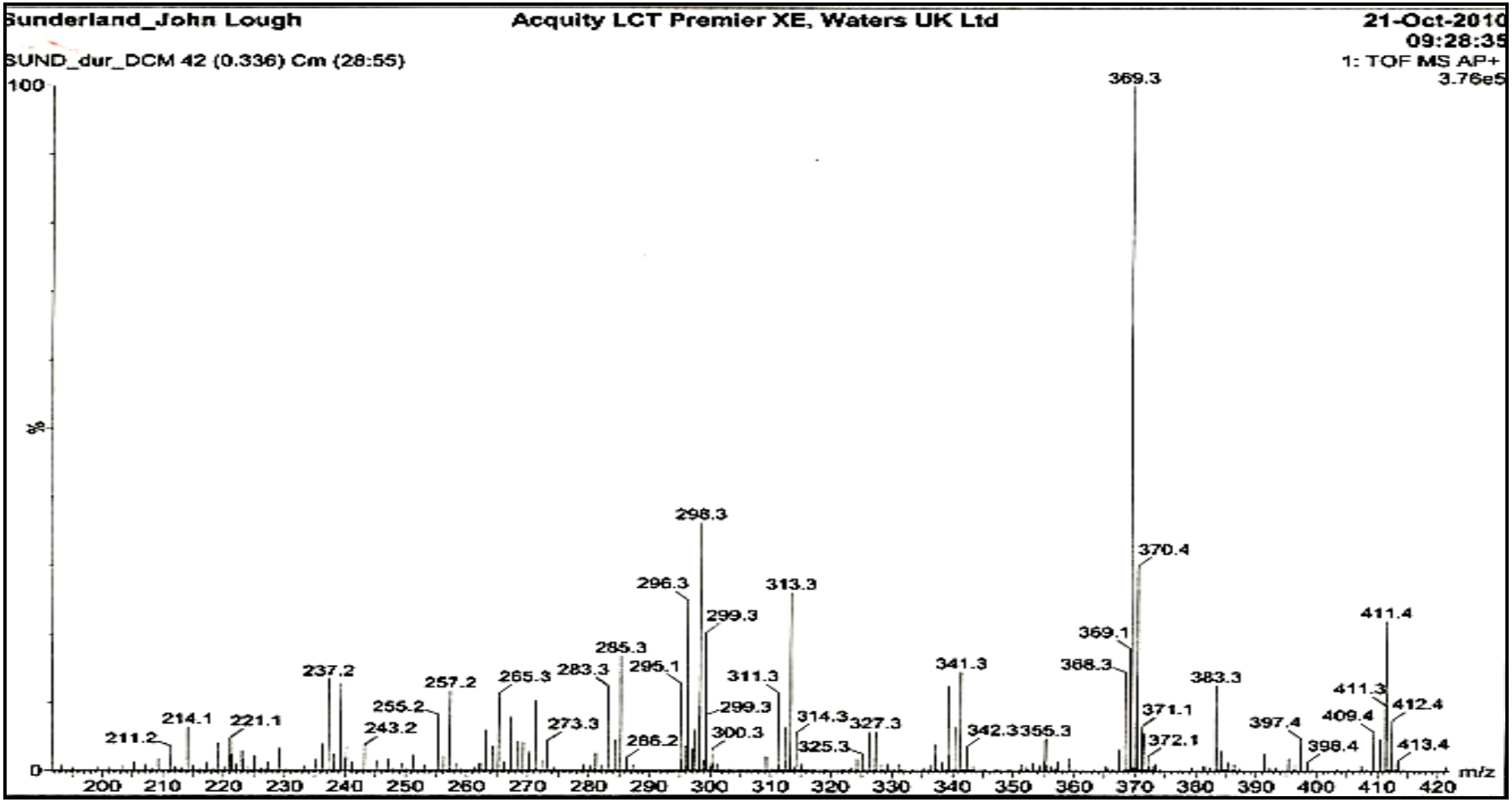

Sunderland
Xevo QTof
XH1
26-Apr-2010
16:05:06
PO_JL_XH1_tempramp 4 (2.631) Cm (2:12)
6: TOF MS ASAP+
2.52e5
PO_JL_XH1_tempramp 11 (2.391) Cm (3:12)
5: TOF MS ASAP+
2.98e5
PO_JL_XH1_tempramp 5 (1.674) Cm (2:9)
4: TOF MS ASAP+
4.40e5
PO_JL_XH1_tempramp 8 (1.264) Cm (3:11)
3: TOF MS ASAP+
1.67e6
PO_JL_XH1_tempramp 10 (0.854) Cm (4:12)
2: TOF MS ASAP+
2.05e6
Ash cluster
PO_JL_XH1_tempramp 8 (0.273) Cm (6:12)
1: TOF MS ASAP+
4.11e5
m/z

vi.

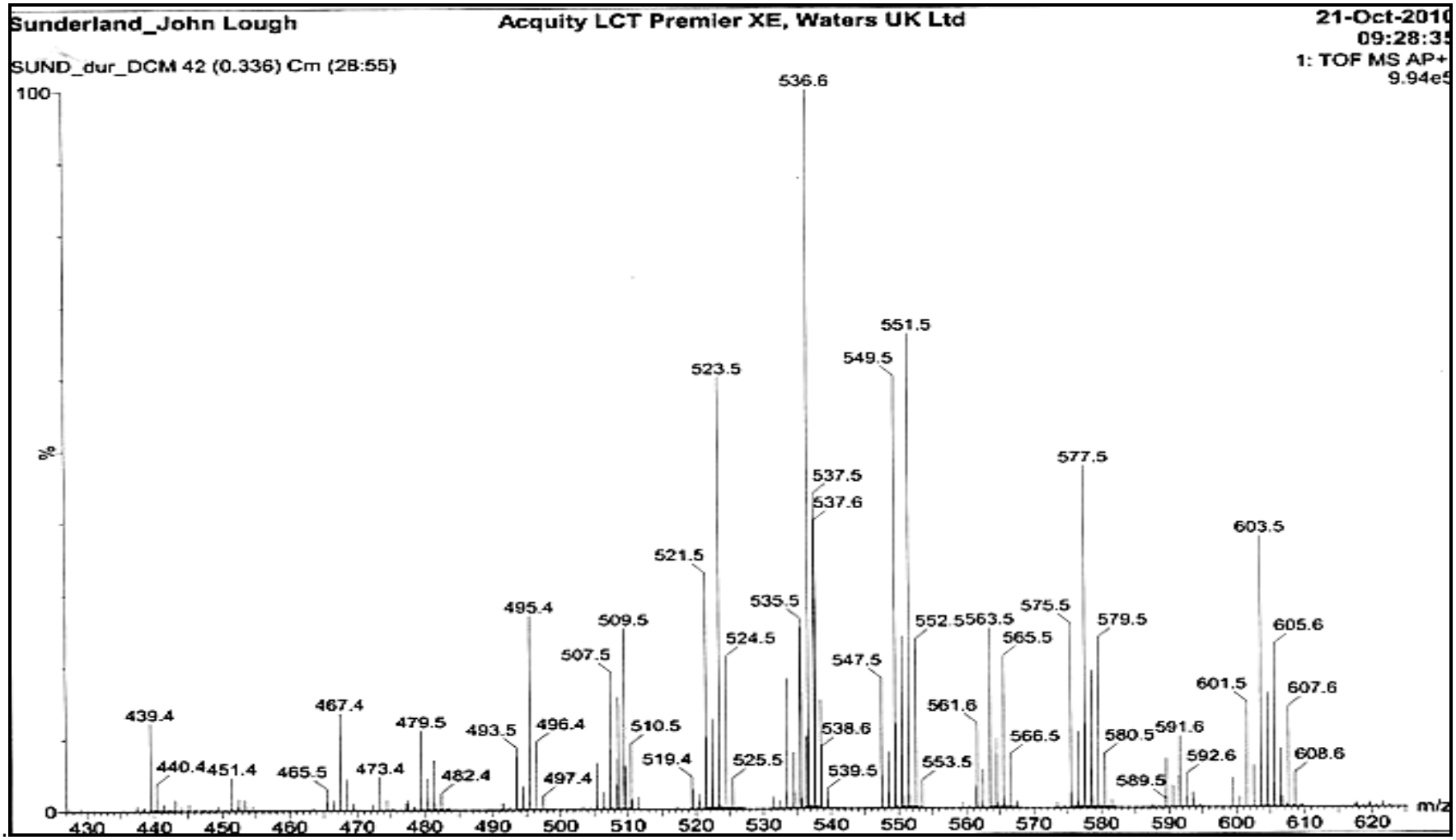

Sunderland
Xevo QTof
XH2
26-Apr-2010
16:22:26
PO_JL_XH2_tempramp 4 (2.631) Cm (3:29)
6: TOF MS ASAP+
1.06e5
PO_JL_XH2_tempramp 3 (2.118) Cm (1:10)
5: TOF MS ASAP+
1.23e5
PO_JL_XH2_tempramp 4 (1.640) Cm (2:12)
4: TOF MS ASAP+
2.59e5
PO_JL_XH2_tempramp 5 (1.161) Cm (4:12)
3: TOF MS ASAP+
1.25e6
PO_JL_XH2_tempramp 10 (0.854) Cm (5:12)
2: TOF MS ASAP+
1.29e6
PO_JL_XH2_tempramp 9 (0.307) Cm (5:12)
1: TOF MS ASAP+
5.85e5
%
m/z

6. TOF MS AP + data (Acquity LCT Premier XE)

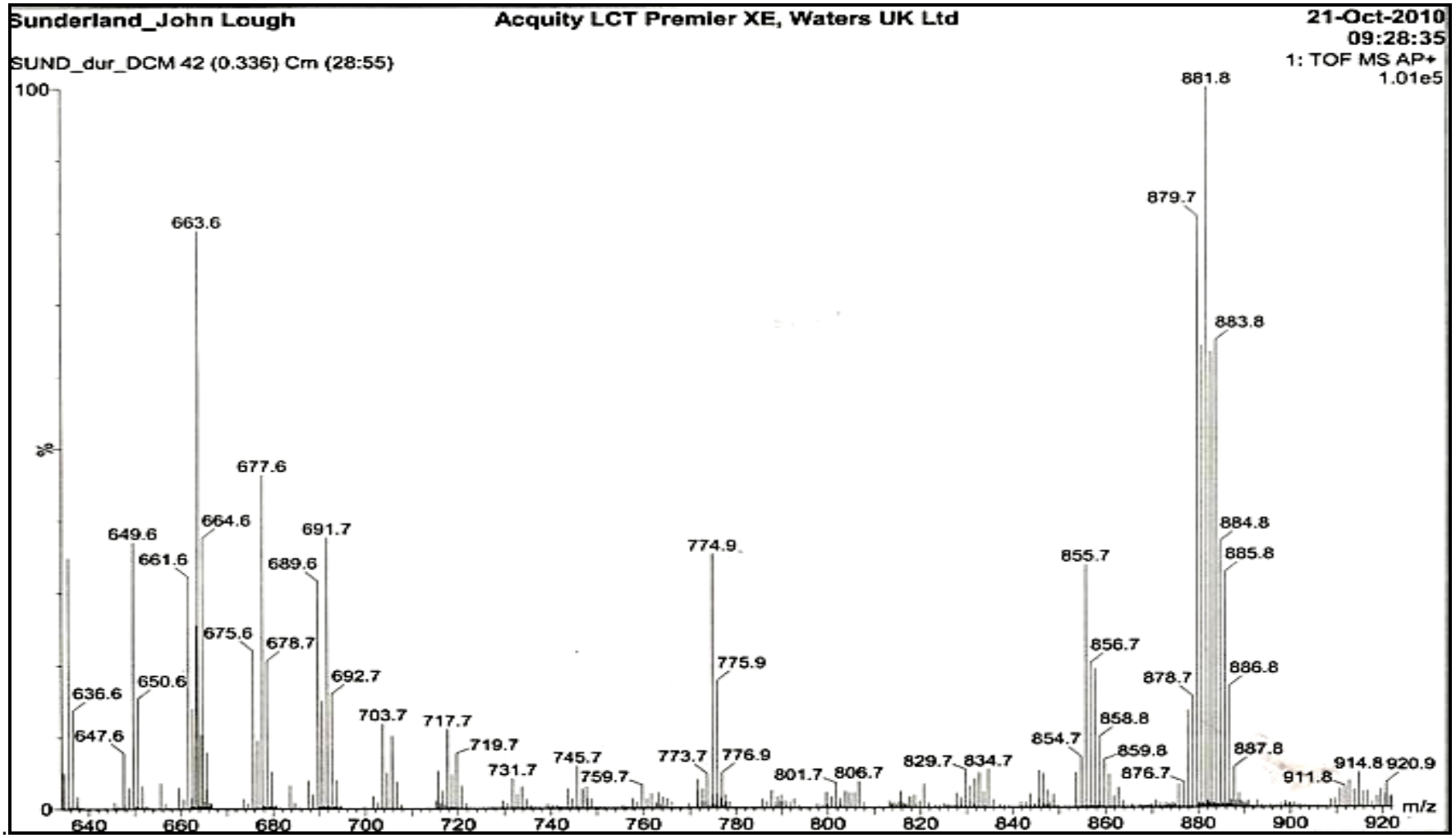
i.



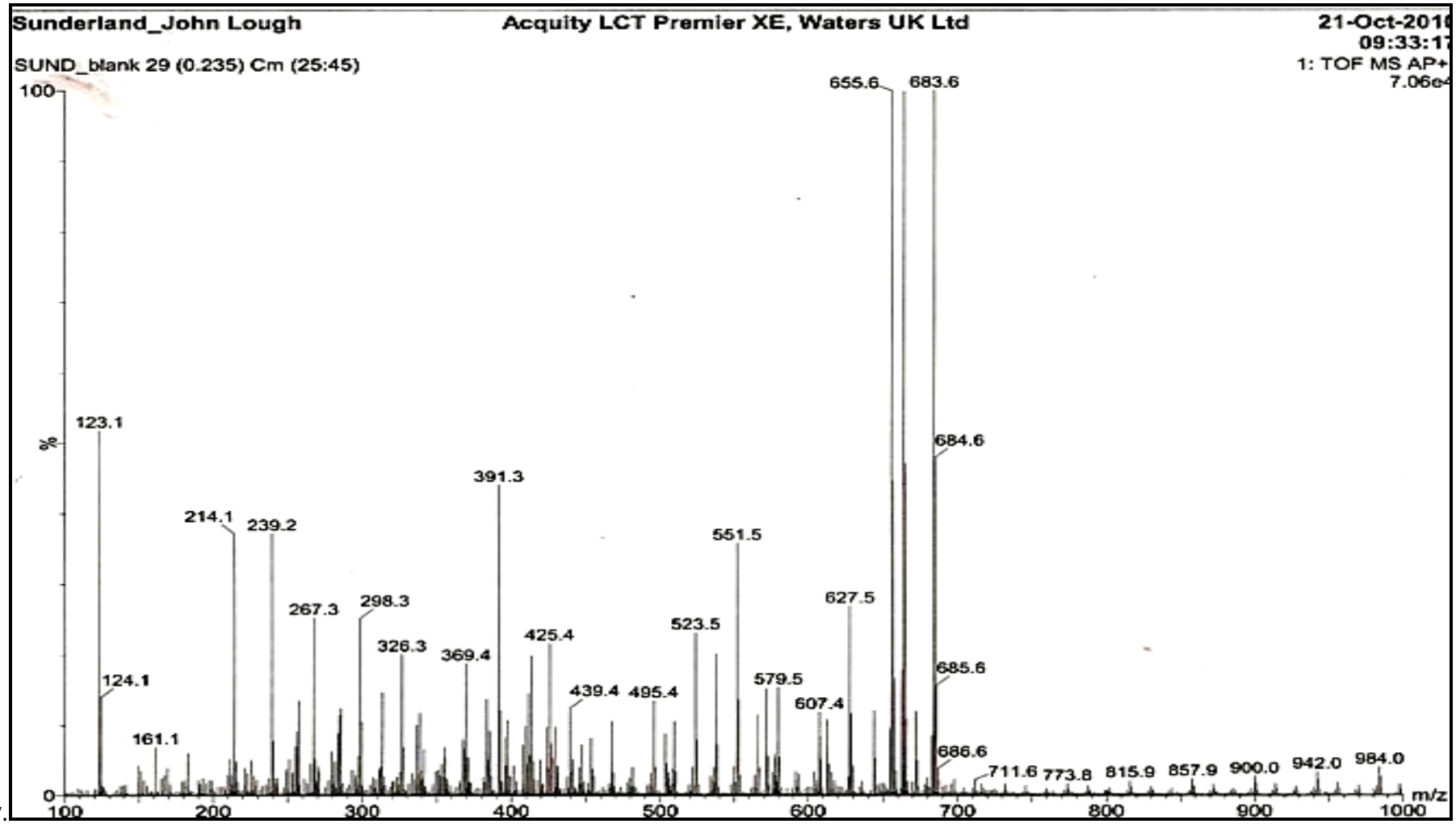
ii.

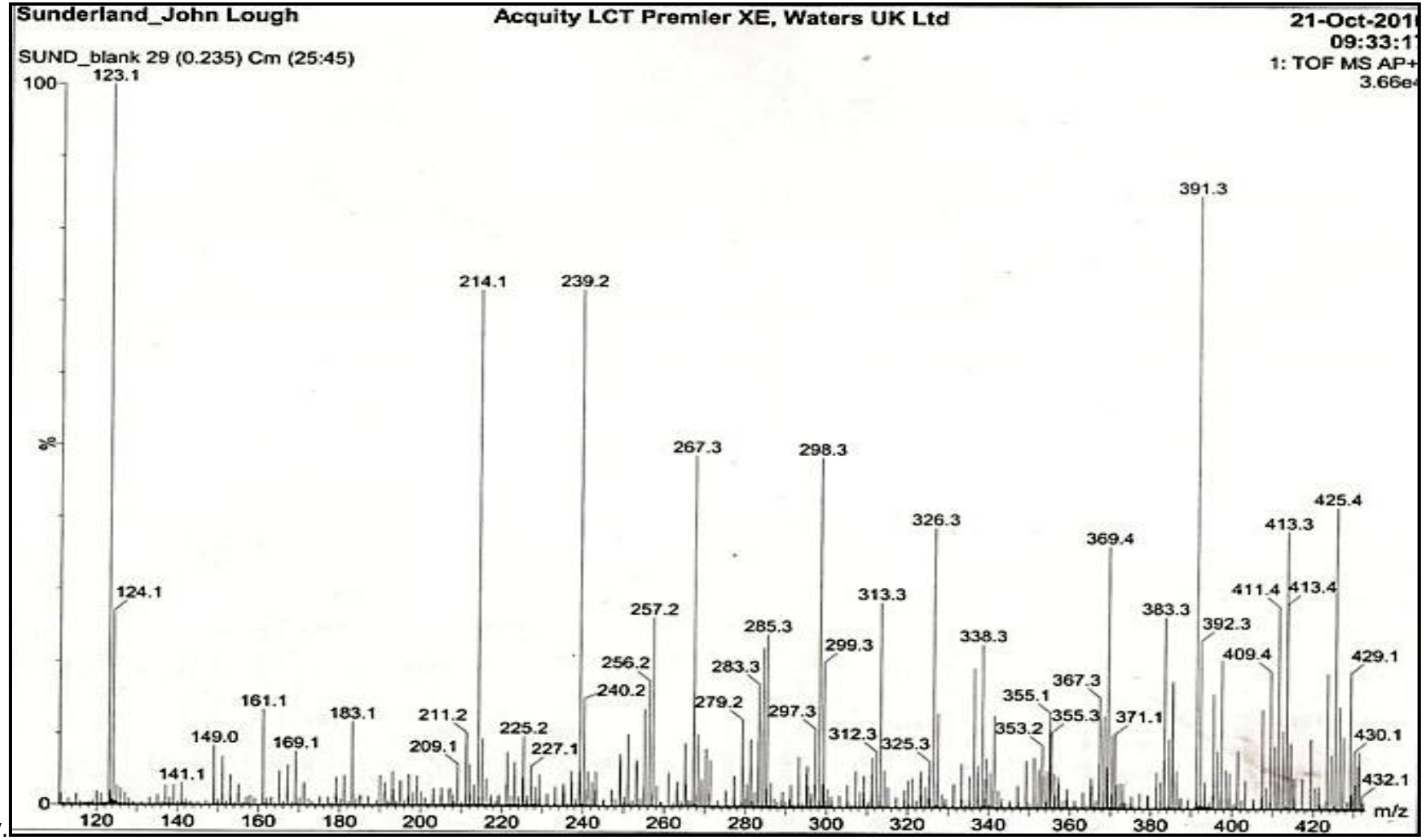

Sunderland_John Lough
Acquity LCT Premier XE, Waters UK Ltd
21-Oct-2010
09:28:35
SUND_dur_DCM 42 (0.336) Cm (28:55)
1: TOF MS AP+
9.94e5
100
%
0
m/z
536.6
551.5
549.5
523.5
577.5
537.5
537.6
603.5
521.5
535.5
575.5
495.4
509.5
552.5
563.5
579.5
605.6
524.5
565.5
507.5
547.5
601.5
607.6
439.4
467.4
561.6
479.5
493.5
496.4
510.5
538.6
591.6
566.5
580.5
440.4
451.4
465.5
473.4
482.4
519.4
525.5
553.5
592.6
608.6
497.4
539.5
589.5
430
440
450
460
470
480
490
500
510
520
530
540
550
560
570
580
590
600
610
620

iii.

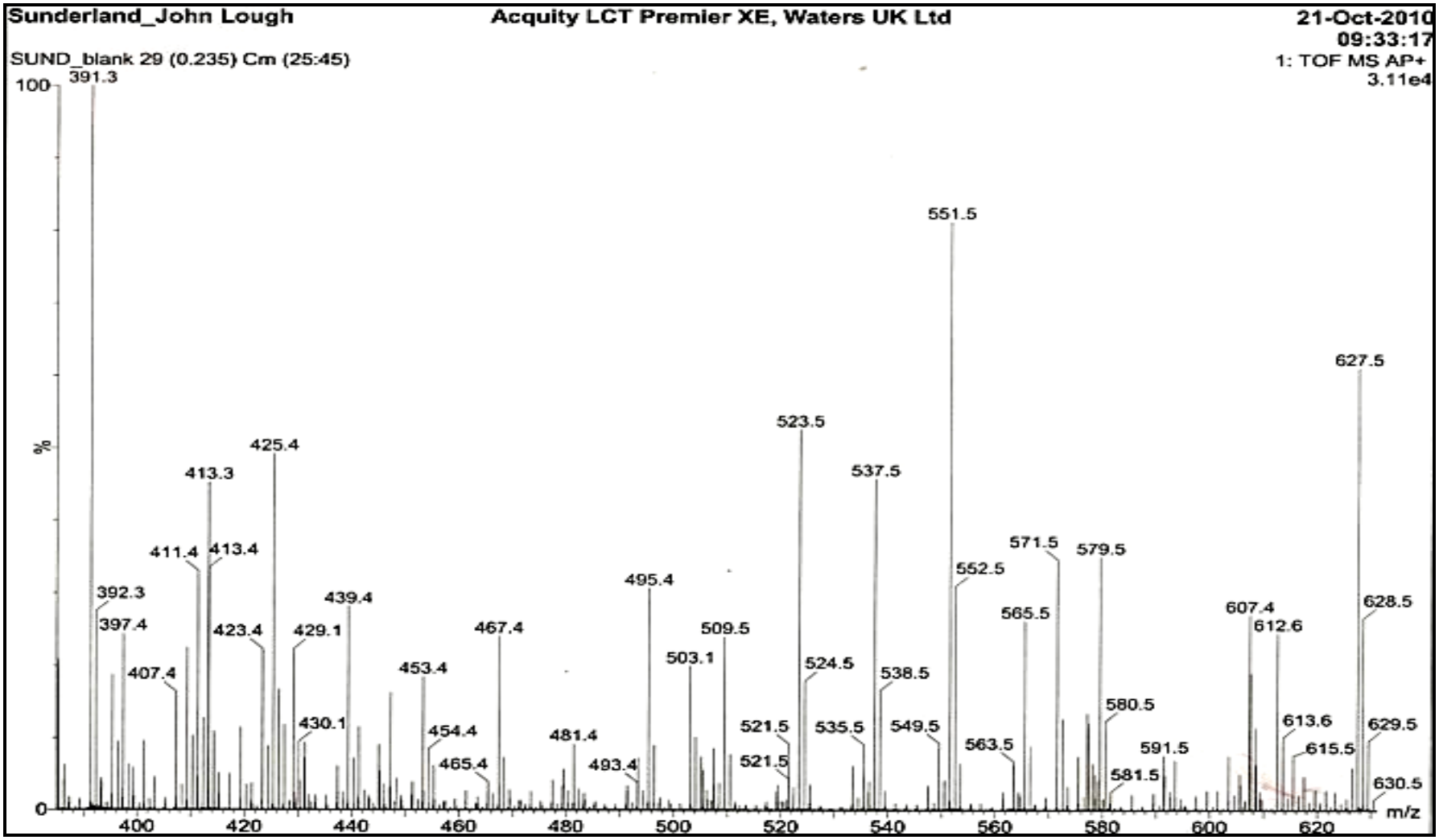

Sunderland_John Lough
Acquity LCT Premier XE, Waters UK Ltd
21-Oct-2010
09:28:35
SUND_dur_DCM 42 (0.336) Cm (28:55)
1: TOF MS AP+
1.01e5
100
%
0
881.8
879.7
663.6
883.8
677.6
664.6
691.7
649.6
661.6
689.6
774.9
855.7
884.8
885.8
675.6
678.7
856.7
775.9
886.8
636.6
650.6
692.7
878.7
703.7
717.7
858.8
647.6
719.7
854.7
859.8
887.8
745.7
773.7
776.9
829.7
834.7
914.8
920.9
731.7
759.7
801.7
806.7
876.7
911.8
640
660
680
700
720
740
760
780
800
820
840
860
880
900
920
m/z

iv.

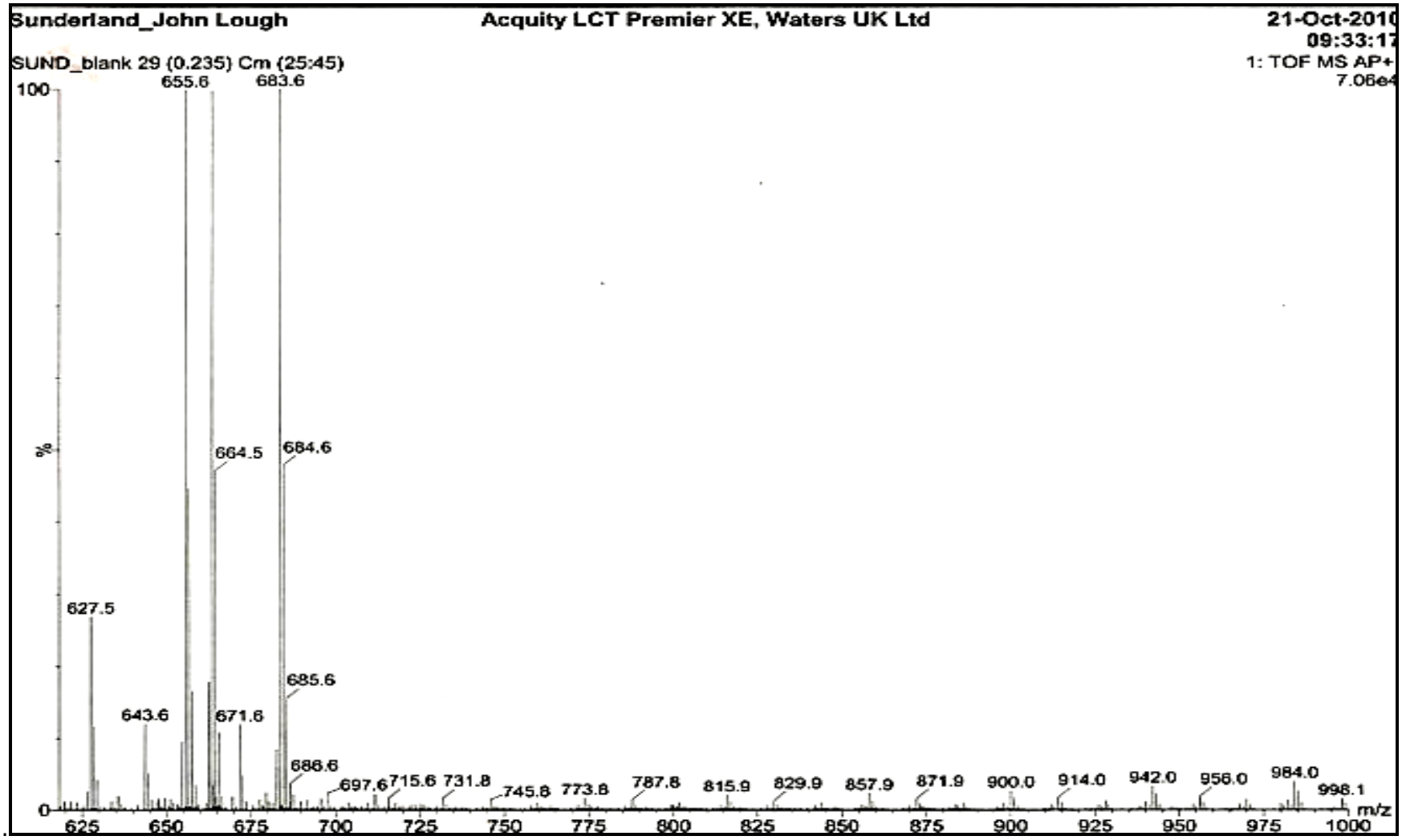

Sunderland_John Lough
Acquity LCT Premier XE, Waters UK Ltd
21-Oct-2010
09:33:17
SUND_blank 29 (0.235) Cm (25:45)
1: TOF MS AP+
7.06e4
100
%
0
123.1
124.1
161.1
214.1
239.2
267.3
298.3
326.3
369.4
391.3
425.4
439.4
495.4
523.5
551.5
579.5
607.4
627.5
655.6
683.6
684.6
685.6
686.6
711.6
773.8
815.9
857.9
900.0
942.0
984.0
m/z
100
200
300
400
500
600
700
800
900
1000

v. 
Sunderland_John Lough
Acquity LCT Premier XE, Waters UK Ltd
21-Oct-201
09:33:1
SUND_blank 29 (0.235) Cm (25:45)
1: TOF MS AP+
3.66e
100
%
0
123.1
124.1
141.1
149.0
161.1
169.1
183.1
209.1
211.2
214.1
225.2
227.1
239.2
240.2
256.2
257.2
267.3
279.2
283.3
285.3
297.3
298.3
299.3
312.3
313.3
325.3
326.3
338.3
353.2
355.1
355.3
367.3
369.4
371.1
383.3
391.3
392.3
409.4
411.4
413.3
413.4
425.4
429.1
430.1
432.1
m/z
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420

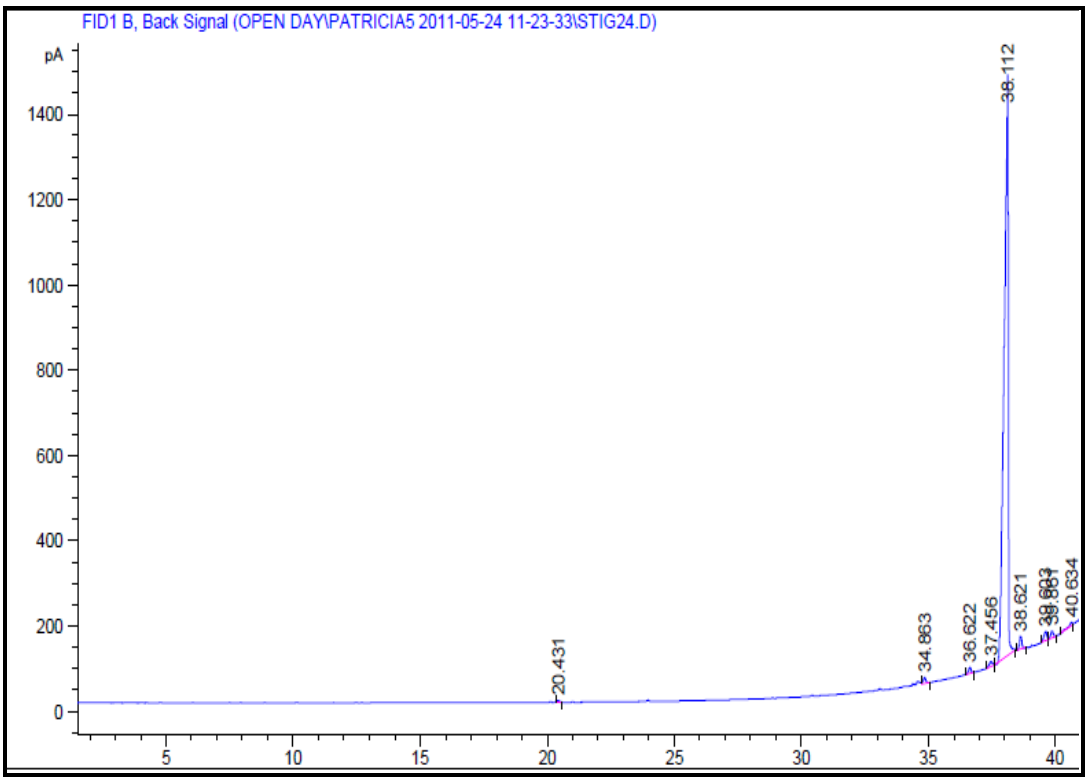
vi.

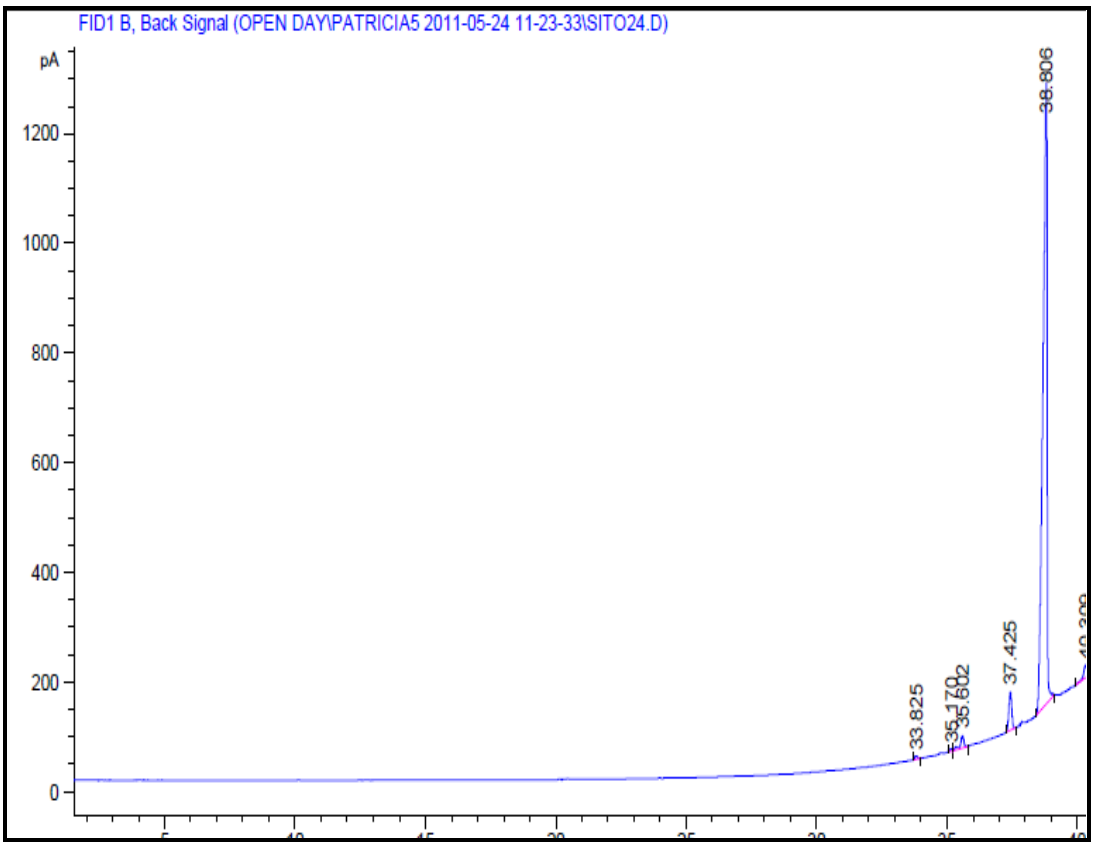

Sunderland_John Lough
Acquity LCT Premier XE, Waters UK Ltd
21-Oct-2010
09:33:17
SUND_blank 29 (0.235) Cm (25:45)
1: TOF MS AP+
3.11e4
100
%
0
391.3
551.5
627.5
523.5
425.4
537.5
413.3
571.5
579.5
411.4
413.4
552.5
495.4
392.3
439.4
565.5
607.4
628.5
397.4
423.4
429.1
467.4
509.5
612.6
503.1
524.5
538.5
453.4
407.4
580.5
430.1
613.6
629.5
521.5
535.5
549.5
454.4
481.4
563.5
591.5
615.5
465.4
493.4
521.5
581.5
630.5
m/z
400
420
440
460
480
500
520
540
560
580
600
620

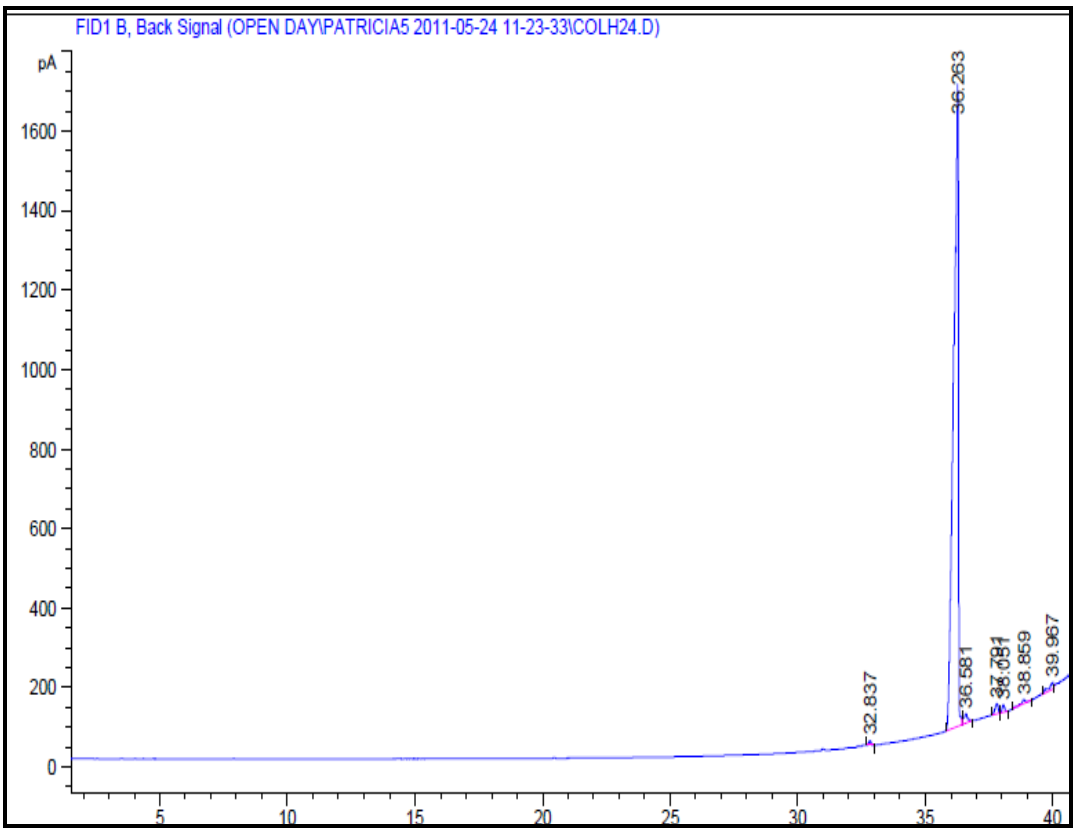
vii.

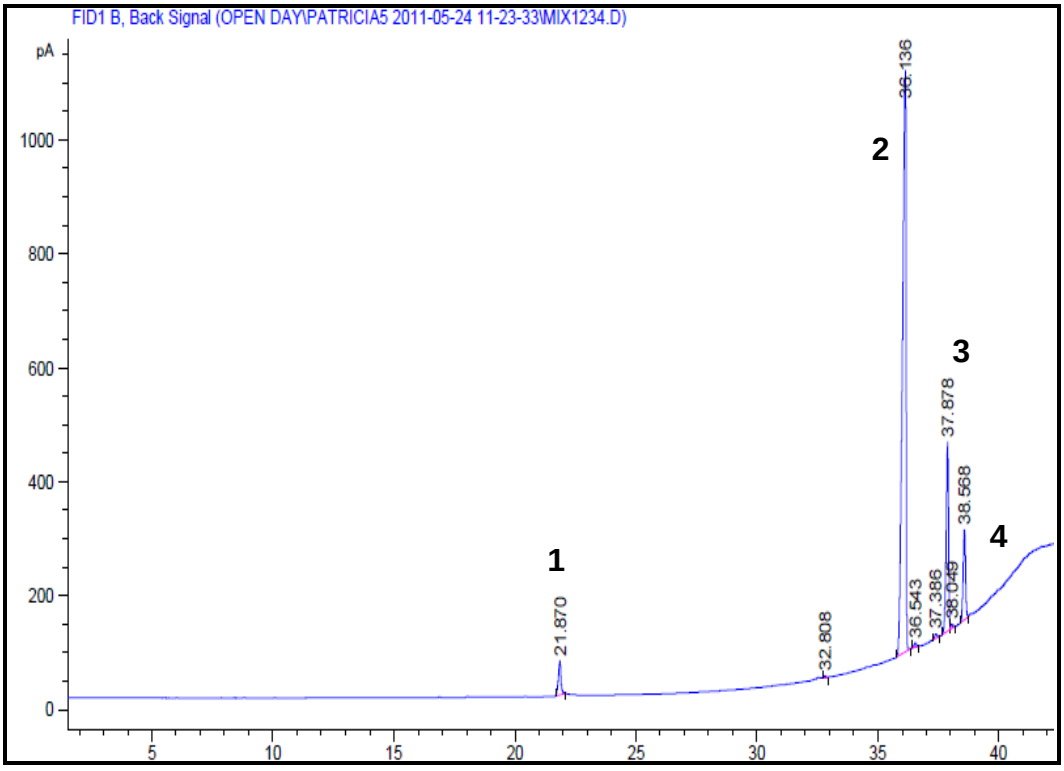

Sunderland_John Lough
Acquity LCT Premier XE, Waters UK Ltd
21-Oct-2010
09:33:17
SUND_blank 29 (0.235) Cm (25:45)
1: TOF MS AP+
7.06e4
100
%
0
655.6
683.6
664.5
684.6
627.5
685.6
643.6
671.6
686.6
697.6
715.6
731.8
745.8
773.8
787.8
815.9
829.9
857.9
871.9
900.0
914.0
942.0
956.0
984.0
998.1
m/z
625
650
675
700
725
750
775
800
825
850
875
900
925
950
975
1000

Appendix F : GC data

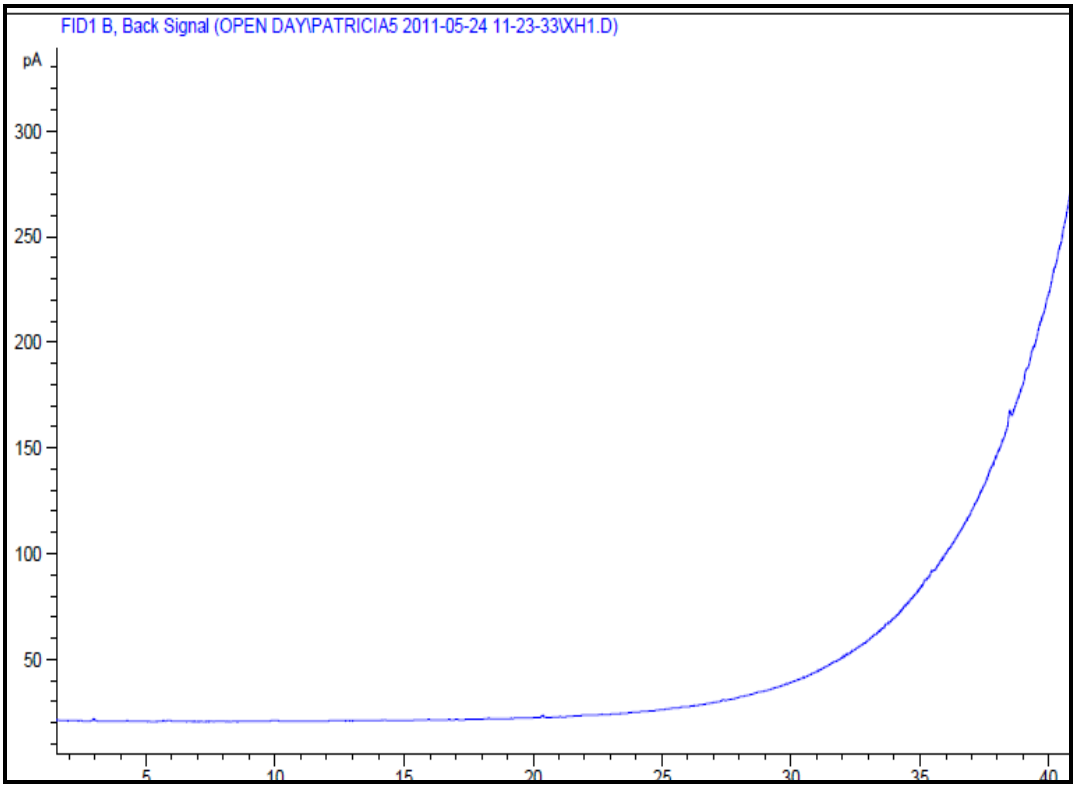
1. 

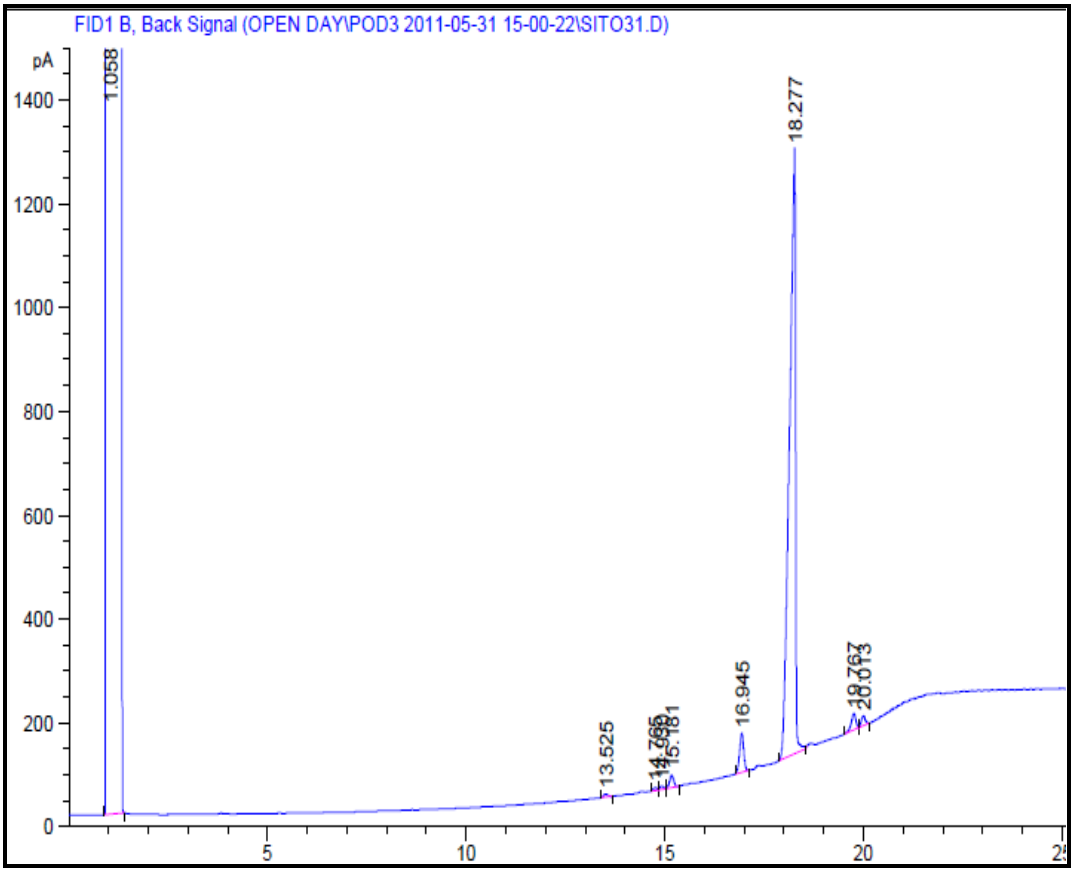
2. 

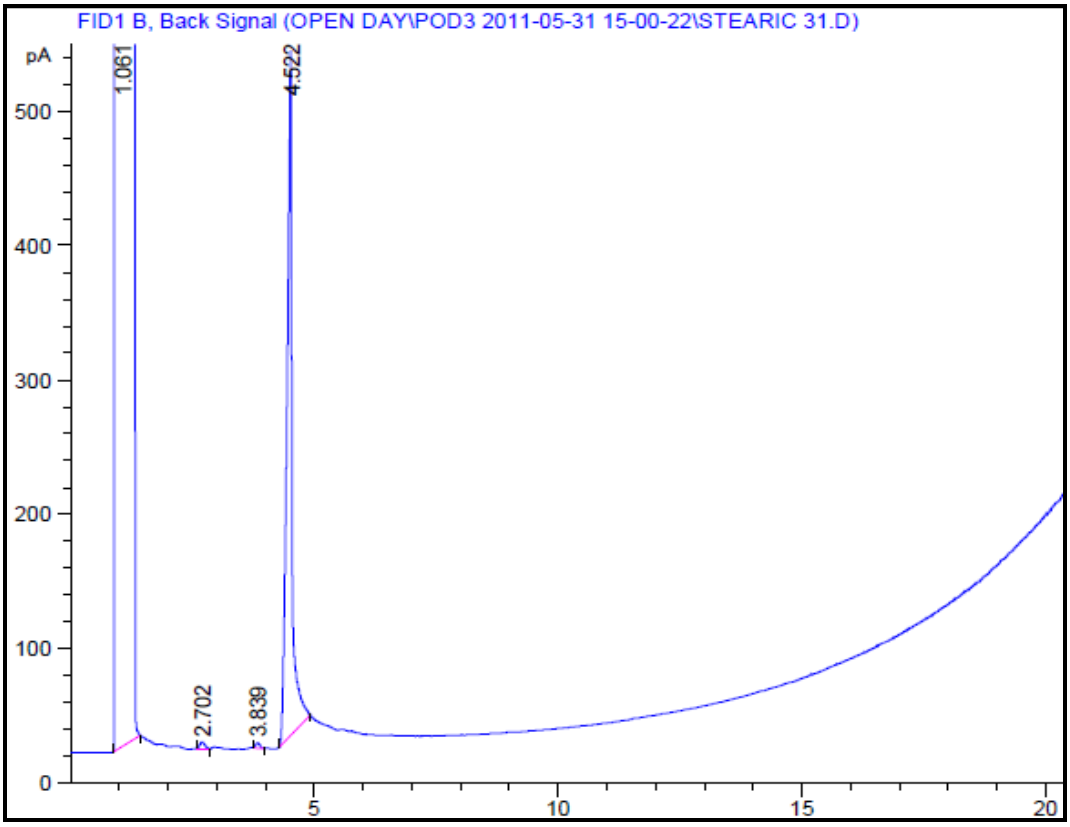
3. 

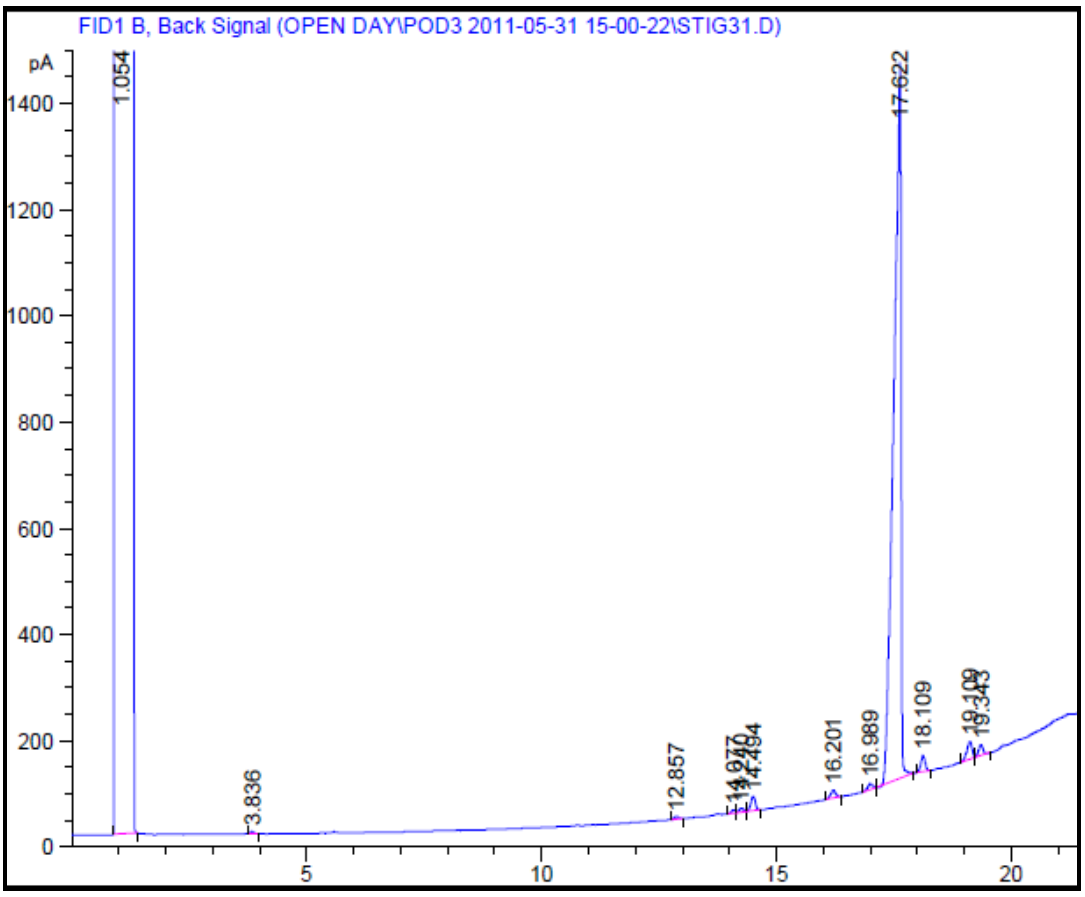
4. 

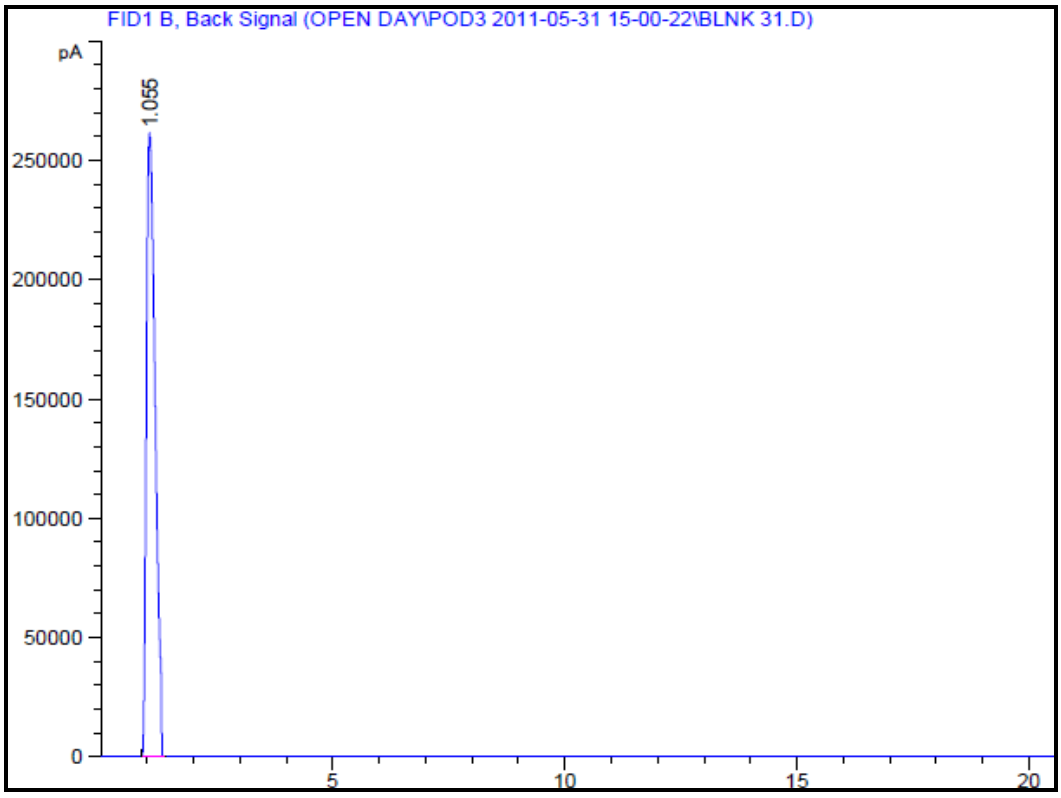
1- Stearic acid, 2- cholesterol, 3- stigmasterol, 4- sitosterol

5. 
FID1 B, Back Signal (OPEN DAY\PATRICIA5 2011-05-24 11-23-33\XH1.D)
pA
300
250
200
150
100
50
5
10
15
20
25
30
35
40

6. 
FID1 B, Back Signal (OPEN DAY\POD3 2011-05-31 15-00-22\SITO31.D)
pA
1400
1200
1000
800
600
400
200
0
1.058
13.525
14.765
15.181
16.945
18.277
18.767
19.613
5
10
15
20

7. 
FID1 B, Back Signal (OPEN DAY\POD3 2011-05-31 15-00-22\STEARIC 31.D)
pA
500
400
300
200
100
0
1.061
2.702
3.839
4.522
5
10
15
20

8. 
FID1 B, Back Signal (OPEN DAY\POD3 2011-05-31 15-00-22\STIG31.D)
pA
1400
1200
1000
800
600
400
200
0
1.054
3.836
12.857
16.201
16.989
17.622
18.109
5
10
15
20

9. 
FID1 B, Back Signal (OPEN DAY\POD3 2011-05-31 15-00-22\BLNK 31.D)
pA
1.055
250000
200000
150000
100000
50000
0
5
10
15
20

.