A STUDY OF CARBONATE-RICH BRINES FROM SUA PAN TO CHARACTERIZE ORGANIC CONTAMINANTS IN THE SODA ASH PROCESS

THESIS

Submitted in fulfilment of the requirements for the degree of MASTER OF SCIENCE of Rhodes University

by

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DECLARATION

In accordance with the conditions for the award of the degree of Master of science, I declare that the work presented in this thesis is my own original research. Any assistance with experimental procedure or technique has been declared elsewhere when necessary. Neither the whole nor any part of this thesis has been, is being or will be submitted for a higher degree to any other university.

Manjusha Joseph

DEDICATION

To my parents, Joseph and Lilly

and

my brother Manesh

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ABSTRACT

Botswana Ash (Pty) Ltd which is situated in Sua Pan, north east Bostwana, is one of Africa's largest suppliers of salt and soda ash. For a number of years, the company has been experiencing problems which have resulted in the final soda ash product being contaminated and discoloured. The problems experienced at Sua Pan have been reported also to occur in other salt works all over the world. It has been suggested that contamination in many salt works could be possibly be due to the microbial activity by halophilic algae and bacteria that grow in the solar ponds.

This study was undertaken to investigate the nature of the contaminating organic compounds present in the brine, to identify the compounds, and to establish how these components vary during the various stages of the soda ash processing.

For this study, two sets of brine samples were used; the first set was collected before the summer rains and the second set was collected after the summer rains. Solid bicarbonate and soda ash samples were also used. Extractions, desalting, UV and HPLC analysis and oxidative biotransformations using four enzymes, were used for developing profiles and characterizing the brine components. From these studies, we were able to confirm that the components of the brine are organic in nature. A thorough study of one of the compounds isolated from solid bicarbonate and soda ash was conducted using UV, HPLC, IR, NMR, HPLC-MS, GC-MS and TLC. The results of these analyses, show that the isolated compound was benzyl butyl phthalate which is generally regarded to be humic in nature. This compound was found to be present in all the brine samples collected after the summer rains including the well brine, suggesting this compound occurs naturally and is not formed during the processing.

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LIST OF ABBREVIATIONS

BBP = Benzyl butyl phthalate

BSA = Bovine Serum Albumin

C = Carbon

CS = Cooling sump

E = Evaporation pond

GC = Gas chromatography

HPLC = High performance liquid chromatography

IR = Infra red

MS = Mass spectrometry

MWCO = Molecular Weight Cut Off

NMR = Nuclear Magnetic Resonance

NS = N-brine storage pond

ST = T-brine storage pond

TLC = Thin layer chromatography

TOC = Total Organic Carbon

TON = Total Organic Nitrogen

UK = United Kingdom

USA = United States of America

UV = Ultra Violet

X = Crystalliser pond

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CHAPTER 1

INTRODUCTION

This thesis describes a study of the soda ash organic contamination problem that has been experienced at Botswana Ash (Pty) Ltd in Sua Pan, Botswana. The aims of this study are described at the end of this chapter.

1.1 SOLAR EVAPORITES

Minerals which are found in environments such as enclosed basins and saline lakes, where annual evaporation exceeds total inflow of water, are known as solar evaporites. These minerals are formed from brines by solar evaporation and crystallize into deposits which may be commercially exploited (Gavish 1980). A variety of products such as gypsum (calcium sulphate), thernadite (sodium sulphate), potash, lithium, uranium and silicates are obtained from solar evaporites, but the most important products are halite (sodium chloride) and soda ash (sodium carbonate). The production of salt and soda ash are quite large in many countries throughout the world. The annual production of salt is about 200 million tonnes per annum and soda ash approximately 29 million tonnes per year (Glasby 1986; Taylor 1991).

1.1.1 Salt

Among the marine evaporites, salt is by far the most valuable mineral resource. Salt was one of the first observed and studied of all chemical substances (Keyser 1976). Salt from salt lakes has been an article of trade for centuries. Salt from North African sabkas, for example was mined by Arab caravans some 2000 years ago. Nevertheless, it is only relatively recently that the full economic values or potentialities of salt lakes have been realized. Borax, soda ash (sodium bicarbonate), sodium sulphate and halite (sodium chloride) have long been obtained from salt lake sediments (Williams 1981).

1.1.2 Soda ash

Soda ash (sodium carbonate) is an important industrial chemical, because of its chemical properties, and its use in the manufacture of other compounds such as sodium bicarbonate, sodium hydroxide and sodium nitrate. A potential large-scale use of one of its derivatives, sodium bicarbonate ($NaHCO_3$), is as a cleanser of sulphur dioxide from stack gases of power plants (Smith *et al.*, 1973). The manufactured soda ash which is usually made by the Solvay process from salt, limestone and ammonia is used by most countries, with the exception of the United States and a few in Africa (Smith *et al.*, 1973; Coetzee & Hammerbeck 1976).

Natural soda ash occurs mostly in the form of trona ($Na_2CO_3.NaHCO_3.2H_20$) and trona deposits have been found in the US in the Green River formation of Eocene and Wyoming and Lake Magadi, Kenya, where new trona is generated at about the same rate as annual production of soda ash (Smith *et al.*, 1973; Coetzee & Hammerback 1976; Eugster 1980). Other resources of soda ash include salt and limestone. Searles and Owens lake in California contain deposits of sodium carbonate which are of Quatenary age and consist of porous crystalline bodies saturated with brines (Smith *et al.*, 1973). World consumption of soda ash is ± 35 million tons.

1.2 SOLAR SALT-WORKS

The outcome anticipated for every solar saltworks is efficient and continuous production of high quality salt at design capacity. High quality salt has clear, solid crystals whose size range remains stable and whose contaminants remain within acceptable limits for world markets (Davis 1993).

Solar salt is normally produced by pumping seawater from one evaporation pond into another, allowing carbonates and gypsum to precipitate and finally draining NaCl-saturated brine into crystallizer ponds where sodium chloride precipitates. Before all the NaCl has crystallized out, the mother liquor (bittern) has to be drained off to reduce contamination of the sodium chloride with bromides and other salts that begin to precipitate at these elevated salinities (Tackaert & Sorgeloos 1993).

New and well-established solar saltworks often experience difficulties that result in production of salt at quantities considerably less than design capacity, and/or at reduced quality. Difficulties in concentrating ponds include brine leakage and massive accumulations of organic matter. In crystallizer ponds difficulties include hollow salt crystals that retain contaminants and increasing concentrations of contaminants in the harvested salt over time. In large part, these problems are functions of biological systems not favourable to salt production (Davis 1993). In systems where there is insufficient biological activity, brine leakage and inadequate water colour in the ponds occur due to failure to manufacture sufficient biological materials necessary to produce mats to seal ponds and plankton populations to properly colour brine (Davis 1980). In Dampier saltfields, Western Australia, biologically-related salt production problems have been reported, where soft hopper crystals high in calcium were produced due to high viscous brines. The microbial mats present in the ponds turned into unstable gelatinous masses due to a massive production of polysaccharide slime (Roux 1996). Due to these various biological problems, the development of biological management systems for solar evaporite production has become increasingly important over recent years and extensive studies have been carried out on the microbial ecology of solar evaporite systems (Borowitzka 1981).

1.2.1 Microbial ecology of Hypersaline environments

Extreme environments are generally inhabited by a limited variety of forms of life. Hypersaline environments, such as concentrated brines, the Dead sea or the north arm of the Great Salt Lake, Utah, are rich in radiant energy and organic nutrients and are colonized by a limited variety of bacteria and unicellular algae that have evolved the ability to exploit moderately saline environments (Baas-Becking 1931; Dennis & Shimmin 1997; Oren 1988).

1.2.1.1 Algae

Dunaliella is a salt-tolerant, unicellular, motile green alga without a ridged cell wall, belonging to the order Volvocales and Division Chlorophycophyta. Two halophilic species were originally described and these are *D. salina*, whose cells accumulate carotenoids when grown in high salt concentrations and therefore appear red, and *D. viridis*, which remains green in all salt concentrations. *D. salina* has attracted the interest of science and industry for its capacity to produce large quantities of glycerol and beta-carotene which is used for protection against high levels of irradiation (Avron & Ben-Amotz 1992; Giordano *et al.*, 1994; Vilchez *et al.*, 1997). Both species are present in salt lakes around the world, but red *D. salina* cells usually predominate, often giving extremely saline lakes a characteristic red colour (Borowitzka 1981; Giordano *et al.*, 1994).

One aspect of the metabolism of unicellular algae that has attracted attention is their ability to liberate dissolved organic carbon (DOC) into the environment. DOC is found in varying concentrations in all natural water sources. It is a complex mixture of compounds formed as a result of the breakdown of animal and plant materials in the environment. The composition of the mixture is strongly dependent on the environment source (Hama & Handa 1980; Azam *et al.*, 1983; Jorgensen 1987; Hedges & Ertel 1992; Salonen *et al.*, 1992 Aiken & Cotsaris 1995). The release of DOC has practical importance in the environment of the hypersaline ponds of solar salt-works, because organic matter in salt crystallizing ponds is detrimental to salt production. Undesirable effects include interference with crystal growth, resulting in small, hollow crystals that retain contaminants. Salt crystallizing ponds gain part of their organic matter from the ponds that supply them with brine, but they also contain resident *D. salina* populations that probably account for a significant proportion of the organic matter (Fogg 1983; Giordano *et al.*, 1994). The quantity of these extracellular releases differ with species, light intensity, bicarbonate ion concentration, senescence, population density, exposure to darkness and nutrient supply (Watt 1969; Kaplan & Bott 1982). Among the different substances released are protein, polysaccharides, nucleic acids, lipids and low molecular weight metabolites. Polysaccharides make up approximately 80% of the total exudation under less extreme conditions (Choudry 1983; Thepenier & Gudin 1985; Beckett *et al.*, 1987). The mechanisms of exudation in algae are not well known, but there is growing evidence that small molecules such as sugars, amino acids, and organic acids are transported by simple diffusion through the cell membrane. The release rate depends upon the permeability coefficient of the membrane for the substance and the concentration gradient across the membrane (Myklestad 1995).

The organic material originating from *Dunaliella* populations is reported to be a major source of nourishment for halobacteria in the Dead Sea (Fogg 1983; Giordano *et al.*, 1994; Myklestad 1995). Exopolysaccharides are commercially useful for producing gels and modifying the rheological properties of aqueous systems (Tavernier *et al.*, 1997). They form the basis of an economically important and expanding global industry (Zilinskas & Lundin 1993). Key products are agars (Arminsen & Galatas 1987; Selby & Whistler 1993), agaroses (Arminsen & Galatas 1987), algins (McHugh 1987; Clare 1993) and carrageenans (Stanley 1987; Therkelsen 1993). These are used as ingredients in food, pharmaceuticals and various other industrial processes (Renn 1997).

1.2.1.2 Halobacteria

Halophilic or halotolerant bacteria are found both in the kingdom of the archaeobacteria (Archaea), and in the eubacteria. These are the two major physiological groups of aerobic heterotrophic bacteria generally found in hypersaline environments (Edgerton & Brimblecomb 1981; Vincenzini *et al.*, 1990; Oren *et al.*,1992). Most of these extremely halophilic bacteria contain a rhodopsin-like pigment (Brock & Petersen 1976; Rodriquez-Valera *et al.*, 1980). Halobacteria require high salt environments, with a minimum of 1.5M sodium chloride needed for growth and, in most cases, an optimum of 3.5 to 4.5M sodium chloride (Borowitzka 1981). Above 3M, only halobacteria are found as a thriving component of a typical hypersaline environment that includes green alage (*Dunaliella spp*), brine flies, and brine shrimp (Norton 1992). These levels far exceed the saltiness of seawater, which is about 0.6M in dissolved salts. The brines which halobacteria inhabit in nature may also be rich in other ions, including potassium, magnesium, sulphate, carbonate, and hydroxide.

Sodium chloride influences many physiological functions of halophilic bacteria. Hypersaline habitats are intensely stressful, the significant stress factors being osmotic stress, low oxygen, and habitat disappearance (Norton 1992). Osmoregulation prevents swelling, deformation, bursting and lysis of halophilic bacteria as a result of osmotic shock (Mudryk & Donderski 1991). Halobacteria are unique among brine-living forms in using potassium ions for protection against osmotic stress. They may develop intracellular K^+ concentrations up to 5M and can be growth-limited by shortage of K^+.

Concentrated brines have low dissolved oxygen capacity, which becomes even lower as the brine temperature rises. Many strains have unusual structural flexibility in their outer layers. This flexibility allows cells to assume a variety of flattened shapes, including squares, rectangles, flattened discs, and triangles. Such morphological characteristics undoubtedly can help bacterial cells efficiently exchange nutrients and gases. A third environmental stress that many halobacteria manage to survive is the disappearance of salt lakes and salterns, which often dry up completely for a season or so. Once rains comes, the halobacteria reestablish themselves. The organisms' ability to be captured and survive in brine inclusions is perhaps their most critical survival skill (Norton 1992).

1.3 SUA PAN SALTWORKS

Botswana Ash (Pty) Ltd, which is situated at Sua Pan, north-east Botswana, is one of Africa's major suppliers of soda ash (Na_2CO_3) and salt (NaCl). Sua Pan, which is located 80km from Francistown, is one of the most easterly of a group of large, flat, unvegetated areas in the Makgadikgadi and has proved to be the most significant resource of natural soda ash so far discovered in Southern Africa. The Makgadikgadi is a flat area lying at the centre of a basin of internal drainage, and has a lowest point of approximately 890m above sea level (Gould 1986; Ehlers 1998) (Figure 1.1). Sua Pan covers an area approximately 200 km from east to west and 120 km from north to south, lying 50 km from east to west and 100 km from north to south (Gould 1986).

The production plant, which has a capacity of 300 000 tons of soda ash per annum and can produce 650, 000 tons of table salt as a by-product, was commissioned in 1991. Soda ash and salt produced by Botswana Ash are derived from an ancient brine resource beneath the Makgadikgadi playa which are thought to have originated from the drying up of what once was the largest lake in Africa. The brine itself is straw coloured and has a mild odour typical of humic derivatives (Gould 1986). More than 80% of the soda ash production at Sua Pan is consumed by the South African industry (Ehlers 1998).

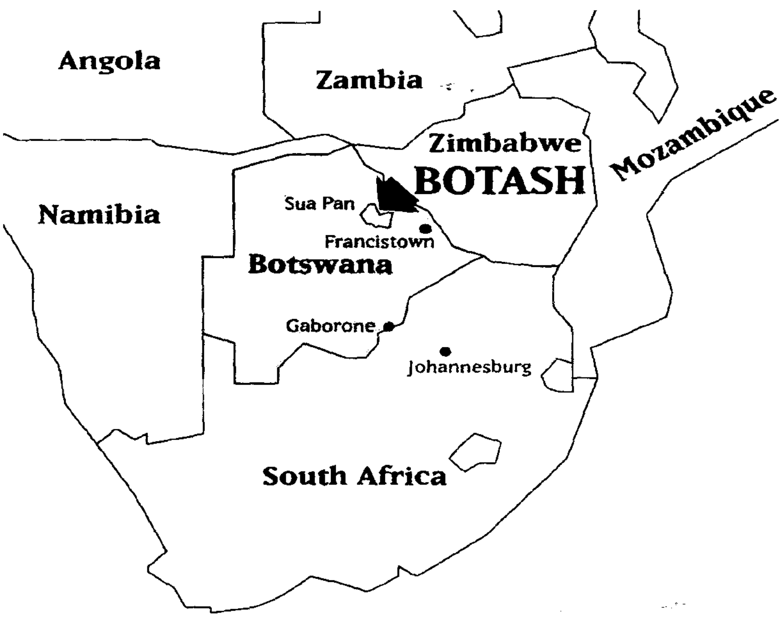


Figure 1.1. Map of Botswana showing the location of Sua Pan (Anon Botash Pty Ltd).

1.4 HISTORY OF SUA PAN

At the end of winter in certain years, effervesences of salt occur in pools at the mouth of the Nata River, the main river draining Sua Pan. The salt was traditionally exploited by the local inhabitants in a small scale, being transported as far as Serowe. The material was used both for domestic purposes and for feeding to livestock (Gould 1986). Van Straten (1958) conducted a detailed study of the brine deposits on the Nata delta and drew attention to the presence of sodium carbonate and bicarbonate as major components of the brine and to their potential economic importance. The presence of potassium salts was also found in the brine. Although the brines taken from different parts of the pan are similar in chemical composition, they are not identical. There are also variations in composition between the brines at different depths. The average chemical composition of the brines is shown in Table 1.1 (Gould 1986).

In 1988, an agreement was reached to build a soda ash and salt pan at Sua Pan to exploit the natural deposits of the pan and by 1991 the plant was in operation. In 1995, the company was restructured as Botswana Ash (Pty) Ltd and is owned by the Botswana Government (50%), Anglo American Corporation, AECI and De Beers (42%) and financial institutions (8%). Coarse salt is exported to South Africa for the use in production of chlorine and caustic soda.

Table 1.1. Chemical composition of Sua Pan brines (Gould 1986).

Chemical	**Concentration (g/l)**
Na^+	67.4
K^+	2.25
Cl^-	79.74
CO_3^{2-}	13.05
HCO_3^-	7
SO_4^{2-}	9
NaCl	128
Na_2CO_3	23.05
$NaHCO_3$	9.64
$NaSO_4$	13.76
KCl	4.3

1.4.1 SUA PAN PROCESS

1.4.1.1 Well Field Capacity

Botswana Ash produces soda ash and salt which are derived from an ancient brine resource beneath the Makgadikgadi playa, found in the easterly section of Sua Pan, consisiting of a large 200 x 120 km system of dry lakes. The brine abstraction wellfield is situated in Sua Pan to the north and northeast of a peninsula of land known as Suaspit, covering an area of approximately 200 km^2. The wellfield comprises 98 production wells distributed between 58 pumping nodes. Brine is conveyed from each pumping node via a pipeline to a central header which ultimately feeds the solar evaporation ponds. The wellfield has an installed capacity of 2 400 m^3/h (Figure 1.2) (Anon Botash Pty Ltd).

1.4.1.2 Solar Evaporation Ponds

The solar ponds have a perimeter boundary covering more than 22 km^2 and provide an area of exposure to the raw wellfield brine which enables solar energy utilization to promote water evaporation from the brine surface. The wellfield brine flows into the primary evaporation/cooling pond EO and this together with the cooling sump (CS), operate as a system to provide a source of cooling brine to the various heat loads in the process plant(Anon Botash Pty Ltd).

Brine is continuously pumped into the factory's cooling reticulation at approximately 4 500 m^3/h and the warmed brine is returned partially as a recycle to pond EO to maintain the cooling system's inventory. The balance is pumped forward into the brine evaporation circuit proper. The shallow depth of pond EO enables the warmed brine contents to respond rapidly to cool night temperatures before being emptied into a relatively deep cooling storage whose contents respond slowly to the daytime temperatures.

The feed forward brine is further evaporated through ponds E_2, E_3, E_4 and E_5 resulting in the concentration of the brine soluble salts up to the point where sodium chloride reaches its saturation point and begins to crystallize out of solution. Brine at this condition is referred to as N-Brine and is retained in the N-Brine storage pond NS. Three crystalliser ponds, X_1, X_2 and X_3, receive their feedstock brine from N-brine storage. Additional evaporation from the brine over these areas results in salt being deposited on the crystalliser floors as well as the further enrichment of the brine in the other sodium salts.

Ultimately a point is reached where it becomes essential to pump the concentrated brine off in order to avoid excessive deposition of brine saturated secondary salts resulting in the contamination of the salt floor. Brine at this stage is referred to T-Brine, named after the Trona process in California from where Botswana Ash obtained its technology. A factory buffer storage of T-Brine is held in the two storage ponds ST1 and ST2. The area of exposure within the solar ponds can be reduced or enlarged by the controlled removal or addition of extra areas to accomodate season changes in climate or variation in well brine inflow. This ensures a consistent brine composition independent of climatic conditions (Anon Botash Pty Ltd) (Figure 1.3).

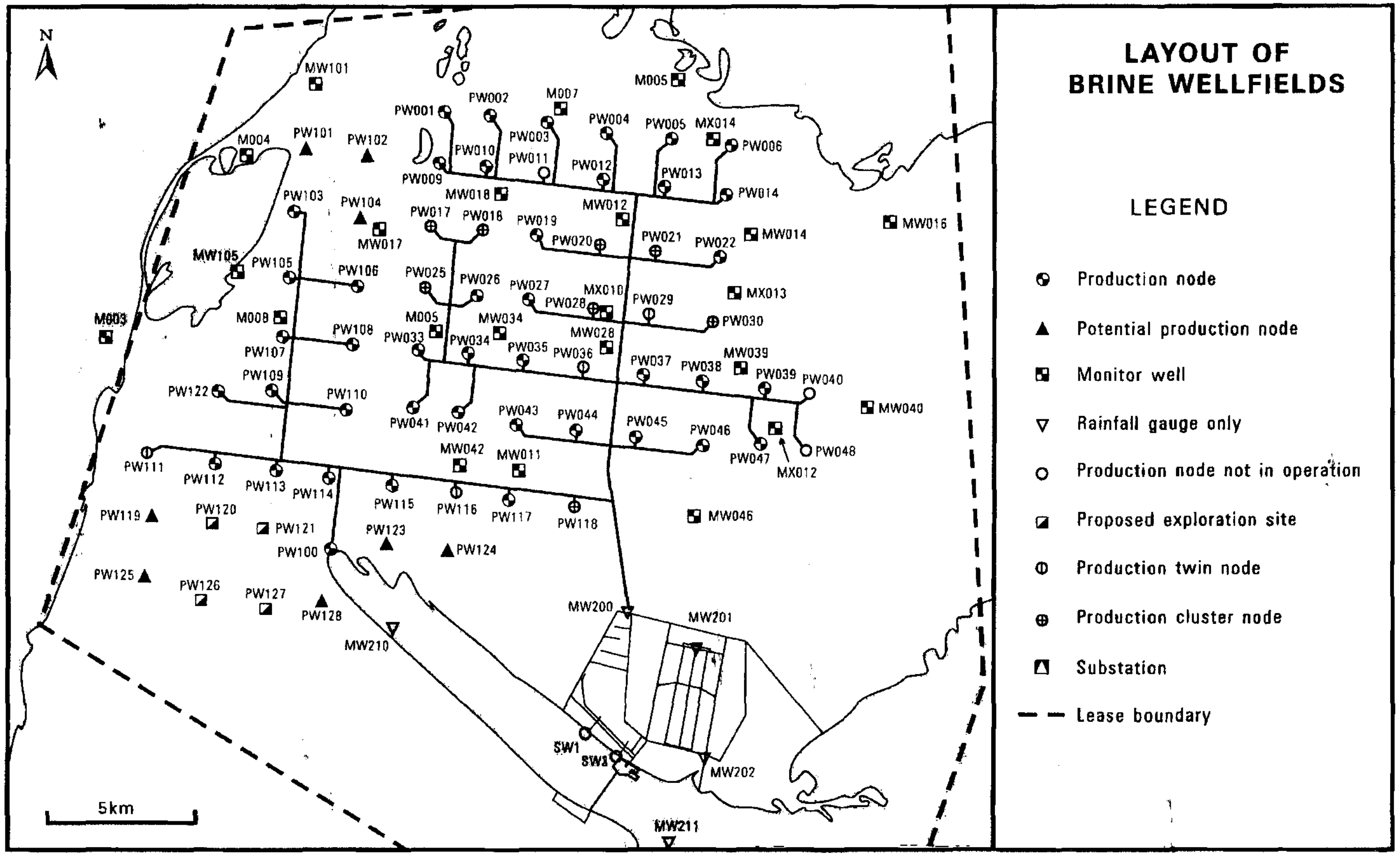


Figure 1.2. Diagram showing the layout of the wellfield (Anon Botash Pty Ltd).

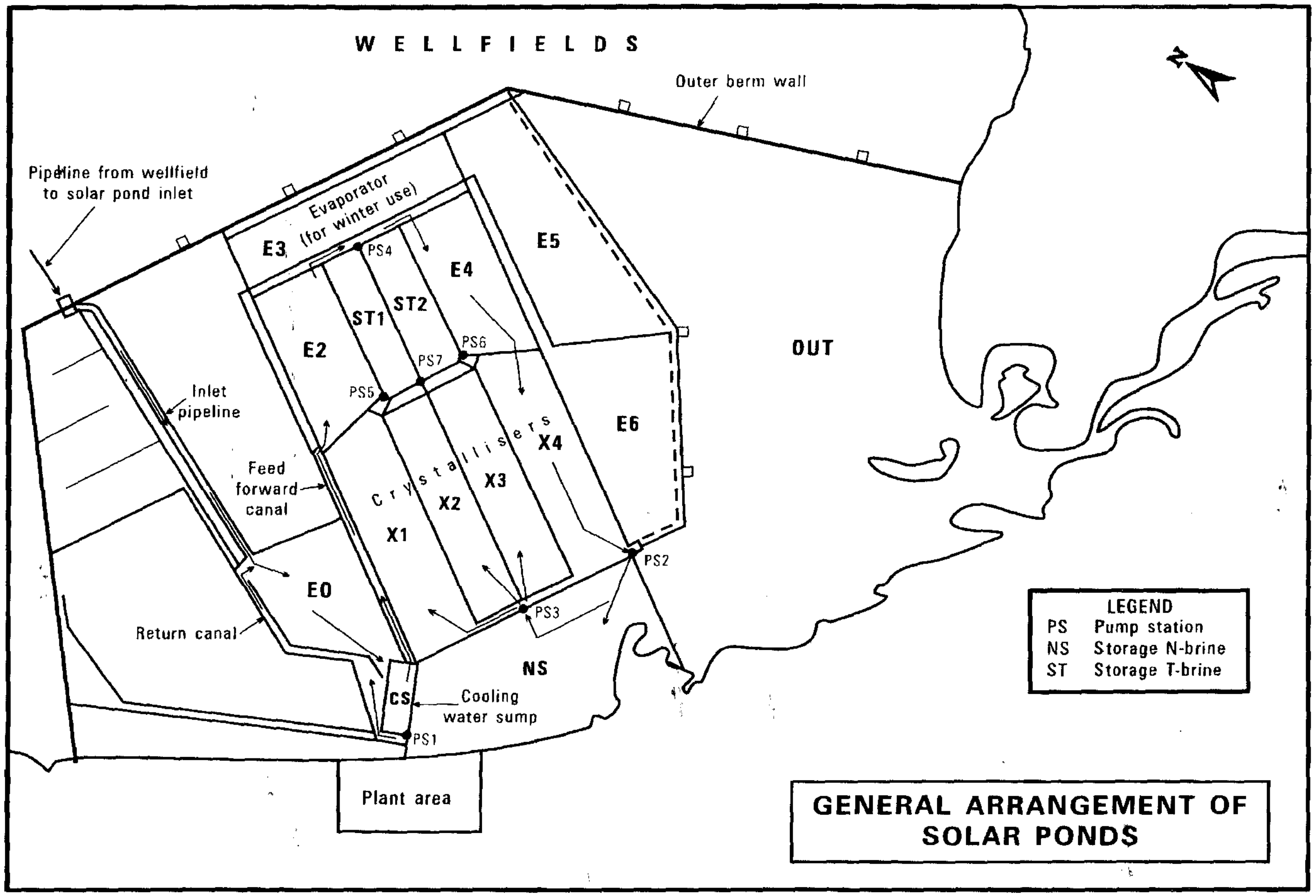


Figure 1.3. Diagram showing the arrangement of the solar ponds (Anon Botash Pty Ltd).

1.4.1.3 Soda Ash Production Process

The technology developed to process brine at Searles Lake in California is used to produce soda ash and the process requires the following sequence of events. Figure 1.4 shows a flow diagram of the soda ash process.

A. Bicarbonate precipitation:

Particulates in the T-Brine from the storage ponds are removed by filtering. This is carried out prior to heating of the brine in a series of plate heat exchangers. The temperature of the brine is raised to 45^0C by counter-current heat exchange with the process heated sodium bicarbonate slurry. This is done to ensure optimal growth of the sodium bicarbonate crystals in the carbonation vessels. The sodium carbonate present in the brine is converted to sodium bicarbonate by reaction with carbon dioxide gas in pressurised vessels at 80 kPa (g). An elevation in the slurry temperature to about 55^0C is caused by the heat evolved during the carbonation process and this reaction is carried out in two stages in order to achieve a conversion efficiency of 95%. Approximately 70% of the carbon dioxide used in the process is obtained by recycling of the carbon dioxide from the calciners. The carbon dioxide purification plant which produces high purity carbon dioxide from the boiler flue gas supplies the balance. The maximum yield of sodium bicarbonate crystals is obtained by cooling the slurry exiting the secondary carbonator to 32^0C. The crystallisers ensure that supersaturation is removed before the separation of the crystals from their mother liquor(Anon Botash Pty Ltd).

B. Bicarbonate thickening:

The sodium bicarbonate slurry leaving the third crystalliser vessel contains only 6-7 % solids by mass. The bulk of the mother liquor is decanted from the solids in a thickener in order to obtain efficient filtration of the slurry. The sodium bicarbonate crystals settle and are then raked to the thickener before being pumped to the filter feed steady head tank as a slurry containing about 25% solids. The spent brine (bitterns), containing about 20% of the original T-brine carbonate alkalinity and above 70% of the original well brine sodium chloride, overflows the thickener and can be used in the salt washing process or is pumped out to the bitterns disposal pond.

C. Bicarbonate filtration:

To minimise the risk of a total plant shortage in the event of a breakdown, processing of the sodium bicarbonate slurry is carried out in two separate lines (A & B) which operate independently. The thickened sodium bicarbonate slurry flows to two horizontal belt filters. The slurry is first de-watered to separate the mother liquor which contains high levels of the impurities, sodium chloride and sodium sulphate. A three stage counter-current wash of the sodium bicarbonate cake using softened water is then carried out. Efficient washing of the cake ensures that the soda ash produced contains less than 1 500 ppm sodium chloride and less than 2 000 ppm sodium sulphate. The wash stages are followed by a drying zone where air is sucked through the cake in order to reduce its moisture content to less than 18%. A low moisture content is essential because it results in improved purity and also minimises the energy requirements for calcining (Anon Botash Pty Ltd).

D. Calcining:

Sodium bicarbonate cake is converted into light soda ash by feeding the filter cake to steam heated rotary kilns which are capable of converting 40 tonnes per hectare (tph) of cake into soda ash. The filter cake is blended with recycled soda ash prior to calcining so as to reduce the moisture content of the feed in order to prevent choking and scaling in the calciner. About 28 tph of steam (2 400 kPa and 230°C) is used to heat the contents of the calciner to 200°C, thus producing 21 tph of ash per stream. The ash is then transferred by means of inclined conveyors to the light ash silos which have a capacity of 200 tons each. The CO_2 liberated in the reaction is cooled before being returned to the carbonators by rotary screw compressors (Anon Botash Pty Ltd).

E. Compaction:

Light soda ash at a density of about 750 kg/m^3 is received by the compaction plant and is compressed into flakes with a density of 1000 kg/m^3 in the compactors. The flakes are then fed through a series of crushers and screens to produce dense ash of the required size distribution. A bulk store which has been designed to hold 40 000 tons of ash receives the product. A proportion of the screened undersize material is conveyed to the light ash bagging plant.

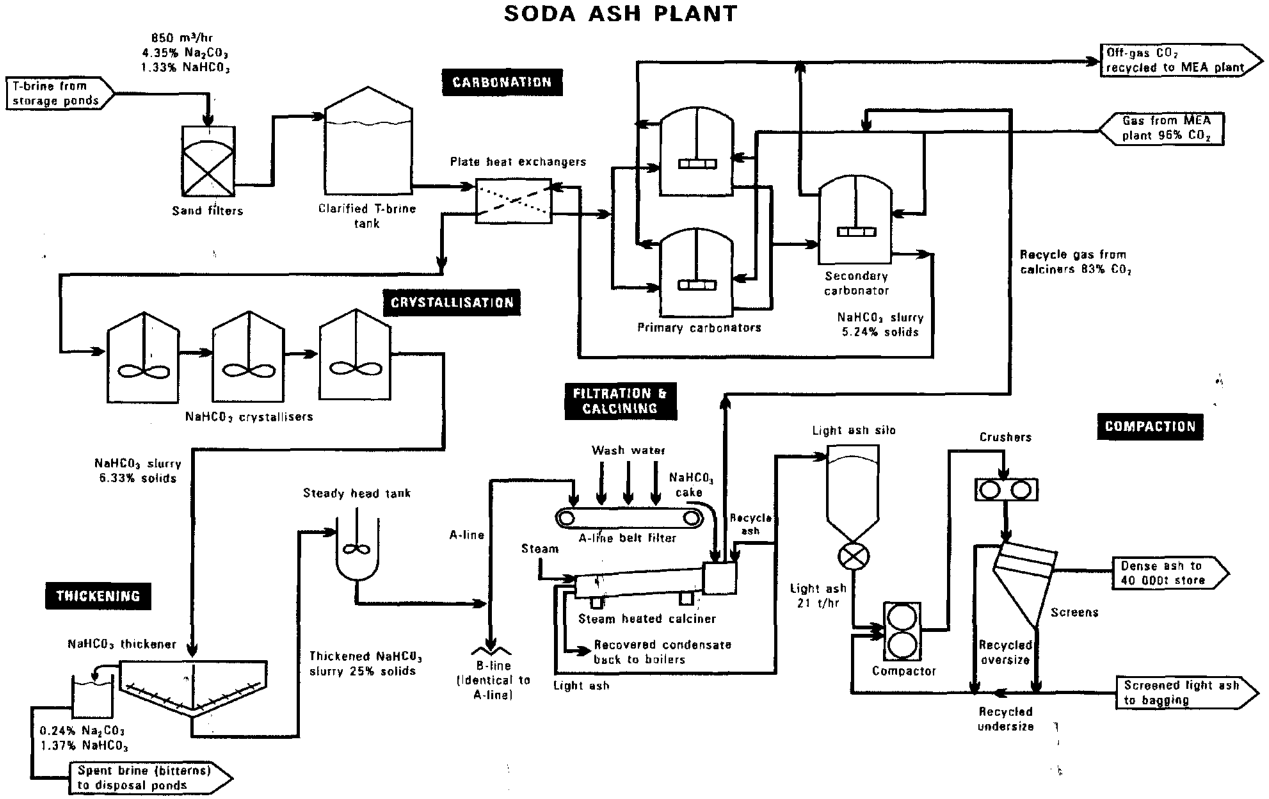


Figure 1.4. Diagram showing the soda ash plant (Anon Botash Pty Ltd).

1.5 PRODUCT QUALITY

After the soda-ash plant was started in Sua Pan, a number of problems were experienced resulting in a poor soda ash product quality. A preliminary investigation of the problems was undertaken at Sua Pan by Rhodes University (Rose 1993). A complex interaction of various biological, organic and physical factors have been identified as being possibly involved in their cause (Rose 1993). These problems include:

1. Fouling of well gravel packs with reduction in pumping capacity. Aggressive aeration of the brine during pumping resulted in the precipitation of humic substances which had a complex organic mixture containing possibly both humic and fulvic acids, as well as bacterial growth in the well gravel pack.
2. Microbial growth of *Dunaliella* species, a halophilic red alga in the evaporation ponds resulting in the production of exopolysaccharides (EPS). Large amounts of nutrients in the well-brine supported the growth of *Dunaliella* spp which resulted in high production of exopolysacharides.
3. Brine leakage from the evaporation ponds. Lack of effective biological management resulting in an unbalanced biological system in the ponds and the growth of algal mats on the floor of the ponds. This is a product of complex interaction of planktonic, bottom dwelling and sediment inhabiting microorganisms.
4. Discolouration of the final salt and soda ash products. The actual cause of the soda ash discolouration has not been identified but the beta-carotene produced by the *Dunaliella spp* could be a possible cause.

1.6 ORGANIC CONTAMINATION AT SUA PAN

Botswana Ash has been experiencing difficulties in maintaining a consistent soda ash product quality. It has been identified that the raw well brine contains brine soluble organic compounds and a further increase in the amount of the organics is caused by biological activity in the solar ponds where the brine is concentrated for salt and soda ash production. This results in a soda ash product with very high levels of Total Organic Carbon (TOC). Further modification of this organic material also takes place during the calcining (kilning) process where the bicarbonate is converted to soda ash at above 200°C.

1.7 BIOLOGICAL MANAGEMENT OF SOLAR SALT-WORKS

In the brine ponds of any solar saltworks (salina), living organisms constitute a biological system or ecosystem which is essential to salt production and is able to aid or decrease the production and quality of the product (Davis 1990; Tackaert & Sorgeloos 1993). Biological management for most solar saltworks involves considerable effort in solving problems caused by organisms living in the ponds (Davis 1993). The brine biological system is a number of interconnected compartments, consisting of planktonic, bottom-dwelling and fringe communities inhabiting the ponds. The planktonic community consists of algae, protozoa and bacteria. The bottom community consists of microorganisms, small molluscs and nematodes growing on the floor of the ponds as mats and deposits. The fringe community, which is of minor importance, includes the mangroves, grasses and salt brushes growing in the ponds or near the pond borders (Davis 1990).

The ponds of a solar saltworks can be divided into low, intermediate and high salinity ranges. Low salinity ponds contain the greatest organism diversity and biological productivity of organic matter. As salinity increases in a salina, there is a decrease in organism diversity and biological productivity, and an increase in dissolved and particulate organic matter. Of the diverse organisms in the ponds of low salinity, photosynthetic algae and bacteria manufacture organic materials from carbon dioxide, water, light energy and essential minerals.

In the ponds of intermediate salinity, brine shrimp, brine flies and blue-green algae develop. In a balanced biological system, non-mucilage producing planktonic blue-green algae thrive and impart dark colours to the brine whereas in an unbalanced or improperly managed salina, *Coccochloris elabens*, which is a planktonic blue-green algae, may predominate and damage the salina (Davis 1990).

In the high salinity ponds, the main organisms are the red halophilic bacteria and the salt-tolerant *Dunaliella*. A salina with a balanced biological system consists of microorganims with high biological productivity in the low salinity ponds, the brine animals and planktonic blue-green algae in the intermediate ponds and the red halophilic bacteria and the salt-tolerant *Dunaliella* in the high salinity ponds (Davis 1990).

In an unbalanced biological system, excessively deep ponds and high concentratins of combined nitrogen and phosphorous in water favour the development of the planktonic community, and the suppression of the bottom community. The imbalance between the two communities often causes leakage of brine, and higher than usual concentrations of suspended and dissolved organic materials.

1.8 HUMIC SUBSTANCES

Humic substances (HS) are associated with many soda ash deposits world wide (Garett 1992) and are complex, macromolecular, organic structures formed by microbial processes (humification) from plant remains and other debris, either in aquatic systems (aquagenic HS) or in soils (pedogenic HS) (Norwood & Christman 1987; Burba *et al.*, 1995; Jahnel & Frimmel 1995; De Paolis & Kukkonen 1997; Dunkelog *et al.*, 1997; Klavins & Apsite 1997; Hofrichter *et al.*, 1998; Santos & Duarte 1998; Schmitt-Kopplin *et al.*, 1998). Due to their complex structures, it is necessary to classify the structures into groups with specific ranges of molecular weight, functional group content, elemental composition etc, in order to facilitate the application of analytical techniques (Fujitake *et al.*, 1998). Humic substances when extracted, normally consist of aromatic and aliphatic constituents with a variety of functional groups, but the structure of the extracted material varies considerably depending on the methods chosen for extraction and analysis (Knauber *et al.*, 1998).

HS are a group of compounds which are highly active in a wide variety of interactions including ion exchange, sorption, complexation, and solubilization (Wershaw *et al.*, 1981). The ability of humic substances to undergo redox reactions and to complex with metal ions is attributed to a wide variety of functional groups, such as carboxyl, carbonyl, hydroxyl, phenol and quinone groups (Klavins & Apsite1997; Sanchez-Monedero *et al.*, 1999).

HS are ubiquitous throughout the environment and constitute the bulk of organic material in soil and approximately half of the dissolved organic carbon (DOC) in terrestrial streams (Alberts *et al.*, 1992; El-Sayed *et al.*, 1993; Hambsch *et al.*, 1993; Schlautman & Morgan 1993; Burba *et al.*, 1995; Shin & Lim 1996; Thomas & Eaton 1996; Afcharian *et al.*, 1997; Volk *et al.*, 1997; Christensen *et al.*, 1998). Because the concentration of humic substances in water rarely exceeds 5 mg/l DOC, very large volumes of water (1000L) must be processed in order to obtain gram quantities of humic substances (Thurman & Malcolm 1980; McCarthy & Zachara 1989; Fujimura *et al.*, 1994).

Humic substances can be divided into humic and fulvic acids based on their hydrophilic properties. The humic acids (HA) are soluble in alkaline solution, but precipitate in strong acid solutions. The fulvic acids (FA) are soluble over the entire pH range and typically account for 80-90% of the humic substances in surface waters (Van Breemen *et al.*, 1979; Kipton *et al.*, 1996). It has been reported that both humic and fulvic acids have a phenolic acid skeleton with attached carbohydrate, aliphatic and amino acid groups (Hayes & Swift 1978; Schnitzer 1978; Stevenson 1982; Reuter *et al.*, 1983; Lead *et al.*, 1994). In general FA's have a lower molecular weight than HA's and they also have higher oxygen content and lower carbon content and are thought to be less phenolic than humic acids (Liao *et al.*, 1982; Aiken *et al.*, 1985; Beckett *et al.*, 1987; Watanabe & Kuwatsuka 1993).

Lignin, a phenolic polymer and a major component of woody tissue, is thought to be an important precursor for humic substances. The lignin/phenol content of humic substances has been extensively investigated via oxidative chemical degradation (Hayes & Swift 1978). Alkaline solutions of cupric oxide (CuO) as well as other oxidizing agents (Hayes & Swift 1978) have been used to degrade lignin and humic substances with the resulting production of phenolic aldehydes and acids

(Norwood & Christman 1987).

Views of the biological role of humic substances in aquatic environments have changed significantly over the past few years, from that of biologically inert compounds to that of potentially important carbon and energy sources for bacteria, and ultimately for microbial food webs. Humic substances play an important biological role as substrates for the growth of natural bacterial assemblages in a variety of aquatic environments (Moran & Hodson 1994).

1.8.1 Isolation and characterization of humic substances

A number of methods have been used for the isolation of humic substances. These methods include solvent extraction, dialysis, ultrafiltration, precipitation, freeze-drying, use of macroporous resins such as Amberlite XAD-2 or XAD-8 and gel filtration chromatography using Sephadex gels (Aiken 1985; Kuiters & Mulder 1993; Shaw *et al.*, 1994; Tao 1994). The use of macroporous resins is popular for the isolation of humic substances from large volumes of water (Aiken 1988; Sirotkina *et al.*, 1974; Miles *et al.*, 1983; Sorouradin *et al.*, 1993). Numerous chemical and spectroscopic methods have been used to characterize and compare the properties of different humic fractions (Stevenson 1994; Saiz-Jimenez 1996). Carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR) is a relatively new technique that is being widely used to carry out structural investigations of humic substances (Norwood & Christman 1987; Ricca & Severini 1993; Frund *et al.*, 1994; Contè *et al.*, 1997; Piccolo & Conte 1997). This technique makes possible direct observations of the macromolecular carbon skeletons (Schnitzer & Preston 1983). Infrared spectroscopy is another useful technique which is used for characterizing humic substances.

This technique provides information about the functional groups present in a compound (Cegarra *et al.*, 1994; Peuravuori *et al.*, 1999). Gas chromatography/mass spectrometry is also a powerful analytical technique for studying the chemical nature of aquatic and terrestrial humic substances (Saiz-Jimenez & De Leeuw 1984; Saiz-Jimenez & De Leeuw 1986; Abbt-Braun *et al.*, 1989; Schulten & Gleixner 1999).

Recent studies of FA's by NMR methods have revealed an extremely variable composition depending on the nature of the environment (Hatcher & Spiker 1988). NMR spectroscopy is also very useful for assessing the structural features of soil organic matter (SOM) (Wilson 1987; Preston 1996; Garver *et al.*, 1996; Piccolo & Conte 1997). Structural information is extremely helpful in understanding the interactions of organic and inorganic pollutants with humic substances (Ginwalla & Mikita 1992; Hatcher *et al.*, 1993; Tatsumi *et al.*, 1994; Shin *et al.*, 1996; Piccolo 1996; Zech & Guggenberger 1996).

1.9 ENZYME STUDIES

The application of enzymes as catalysts for chemical reactions has become a valuable technique. Enzymatic transformations carried out using partially purified enzymes or whole-cell catalysts are being used for the production of a wide range of compounds that are of economic value (Maloney *et al.*, 1986; Schulze & Wubbolts 1999).

Enzymes are highly attractive and selective catalysts because they possess the unique properties of producing high reaction rates under mild conditions. They have been used among other applications, for the removal of contaminants from wastewaters (Yang & Robb 1993). A method involving enzymatic coupling of aromatic compounds has been suggested for the removal of aromatic compounds from waste waters (Maloney *et al.*, 1986; Schulze & Wubbolts 1999). The potential advantages of enzyme-based treatment over conventional biological treatment and chemical/physical processes include application to a broad range of compounds; operation both at high and low concentrations of contaminants; operation over wide temperature, pH and salinity ranges; better defined system, with simpler process control; operation under milder, less corrosive, conditions; reduced consumption of oxidants and operation in a catalytic manner (Nicell *et al.*, 1993).

In nature, enzymatic oxidation and coupling of phenols are important biosynthetic pathways which lead to the formation of lignins, melanins, toxins, alkaloids, antibiotics and humic acids (Bollag *et al.*, 1979; Aitken *et al.*, 1994). Oxidoreductases comprise the large class of enzymes that catalyze biological oxidation/reduction reactions. As so many chemical and biochemical transformations involve oxidation/reduction processes, the notion of developing practical biocatalytic applications of oxidoreductase enzymes has been an attractive goal since the very early years of Biotechnology. One of the applications envisioned for these enzymes include oxidative degradation of pollutants such as polychlorinated biphenyls (PCBs) and phenolic compounds (Dec & Bollag 1994; Hummel 1999; May 1999).

Horseradish peroxidase (HRP), lignin peroxidase (LiP), laccase and polyphenol oxidase (PPO, also known as tyrosinase) are examples of oxidoreductase enzymes which can catalyze the oxidation of aromatic compounds (Nicell *et al.*, 1993; Aitken *et al.*, 1994; Al-Kassim & Taylor 1994; Presnell *et al.*, 1994; Adam *et al.*, 1995; Crecchio *et al.*, 1995; Miland *et al.*, 1996; Perez *et al.*, 1996).

Peroxidases are haemoproteins that are very widespread in nature and they possess a wide substrate specificity and can oxidize a large number of aromatic compounds in the presence of hydrogen peroxide (Colonna *et al.*, 1992; Chen & Nobe 1993; McEldoon *et al.*, 1995; Lin *et al.*, 1999; Richard-Forget & Gauillard 1997; Colonna *et al.*, 1999; Kazunga *et al.*, 1999; Liao *et al.*, 1999; Srinivas *et al.*, 1999). In their catalytic cycle they oxidize phenols with hydrogen peroxide, generating phenoxy radicals which then react to form insoluble polymers independent of the enzyme (Klibanov *et al.*, 1980; Dunford & Adeniran 1986; Al-Kassim *et al.*, 1994; Ghioureliotis & Nicell 1999). HRP can be used in the removal of a wide variety of aromatic compounds from aqueous solution and is effective over a wide range of concentrations (Klibanov *et al.*, 1983; Ryan *et al.*, 1994; Tatsumi *et al.*, 1996; Wright & Nicell 1999). LiP's are capable of oxidizing a wide variety of non phenolic aromatic substrates to give aryl cation radicals (Hammel *et al.*, 1993; Sayadi & Ellouz 1995; Ten-Have *et al.*, 1998; Lopretti *et al.*, 1998). They are ligninolytic enzymes which catalyze the H_2O_2-dependent oxidation of lignin model compounds, depolymerization of lignin and cleavage of dimeric lignin, halogenated phenolic compounds, polycyclic aromatic hydrocarbons etc (Tien & Kirk 1993; Yoshida *et al.*, 1996; Gojrup *et al.*, 1999).

PPO's are copper-containing monooxygenases which were identified over a hundred years ago, and in contrast to peroxidases, utilise molecular oxygen as the oxidant. These enzymes are ubiquitous in nature and catalyze two reactions viz., the *ortho*-hydroxylation of phenols and the oxidation of catechols (1,2-dihydroxybenzenes) to *ortho*-quinones utilizing molecular oxygen (Della Longa *et al.*, 1996; Tse *et al.*, 1997; Warrington & Saville 1999; Gupta & Mukherjee 2000). The names 'tyrosinase', 'phenol oxidase', and 'polyphenolase' are also commonly used interchangeably with 'polyphenol oxidase'(Burton 1997; Pialis *et al.*, 1996). They are involved in the biosynthesis of a wide range of natural products including lignins, tannins, melanins, antibiotics and alkaloids, as well as the polymerization of naturally occurring phenols resulting in the formation of humic acid and humus in the soil (Randtke & Larson 1984).

Laccase is an extracellular lignin-modifying, copper-containing phenol oxidase which uses molecular oxygen as an oxidant (Perez *et al.*, 1996; Bourbonnais *et al.*, 1997). Laccase was first isolated from the latex of the Japanese lacquer tree (*Rhus vernicifera*) and subsequently from other lacquer trees (Chefetz *et al.*, 1998). Laccase contains three types of copper (Potthast *et al.*, 1995; Hublik & Schinner 2000. Palmieri *et al.*, 2000), and catalyzes the oxidation of various aromatic compounds such as *o*-diphenols, polyphenols, aminophenols and diamines while concomitantly reducing molecular oxygen to water (Dedeyan *et al.*, 2000; Dekker *et al.*, 2000; Xu *et al.*, 2000). This oxidative coupling reaction is important in the synthesis of numerous naturally occurring complexes such as humic substances, lignins, tannins, melanins and alkaloids (Gianfreda *et al.*, 1999). Laccase is distinguished from polyphenol oxidase because it also catalyzes the oxidation of hydroquinone and *p*-phenylene diamine (Lopretti *et al.*, 1998; Palmieri *et al.*, 2000) and it has a broader substrate specificity (Leonowicz *et al.*, 1988). It also plays a role in lignin degradation and has been implicated in the pigmentation of fungal spores and the development of spore-bearing structures (De Vries *et al.*, 1986; Milstein *et al.*, 1993; Perez *et al.*, 1996). Laccases are involved in the formation of humus and the incorporation of organic xenobiotics into soil organic matter (Leonwicz and Bollag 1987).

1.10 RESEARCH AIMS:

This research project was carried out to address the organic contamination problem at Sua Pan with the overall aim of isolating and characterizing the contaminant components, as well as developing a means to monitor the contamination. The specific objectives of this project include the following:

- Development of analytical methodology for characterizing the organics in the brine and soda ash samples.
- Development of profiles of the organic components of brine samples and establishment of how the components vary during the processing.
- Identification of the contaminant components of the soda ash, using chemical and biochemical methodologies.
- Establishment of a routine analytical method to monitor the contamination, based on the outcome of the analyses and the results of the method development.

CHAPTER 2

MATERIALS AND METHODS

2.1 PRELIMINARY WORK

Work on this project was started using three brine samples (W-Brine, N-Brine and T-Brine) which were available in the Biochemistry and Microbiology Department, Rhodes University. The brine samples contained in percentage weight, respectively, NaCl (12.51; 21.46; 20.90), KCl (0.26; 0.52; 0.82), Na_2SO_4 (1.44; 2.78; 3.75), Na_2CO_3 (2.23; 3.66; 4.25) and $NaHCO_3$ (0.70; 1.06; 0.99). NaCl was present in the brine samples in the highest amount. N-Brine and T-Brine contained about two fold higher concentrations of NaCl and $NaHCO_3$ than the well-brine. With the other components, W-Brine, N-Brine and T-Brine had concentrations in the ratio 1:2:3 respectively.

2.1.1 Solvent extractions of brine samples

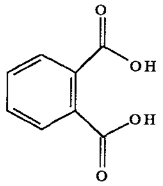
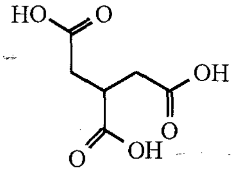
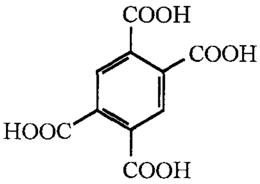
Solvent extraction was carried out using four different organic solvents which include butanol, ethyl acetate, chloroform and methyl ethyl ketone (BDH Laboratory Supplies and Sigma). 100ml of each brine sample was extracted with an equal volume of solvent in a closed separating funnel (250ml). The mixture was vigorously shaken over a period of three to four hours. The organic layer was collected, dried with anhydrous sodium sulphate (Na_2SO_4) (Merck) for an hour and filtered through Whatman No.1 filter paper. The filtrate was evaporated in a round-bottom flask (250ml) using a Buchi Rotavapor R-114. The residue was dissolved in 5ml of mobile phase consisting of water and acetonitrile (BDH Laboratory Supplies) (75:25) followed by centrifugation at 13 000 g for 5 minutes using a Heraeus biofuge.

2.1.2 High Performance Liquid Chromatography analysis of extracts

After solvent extraction, HPLC analysis was conducted using a LaChrom L-7400 (Merck, Hitachi, Germany) HPLC system, equipped with a variable UV/VIS detector and an 80 position auto sampler/auto injector (20µl), with a 4.6 x 250mm Spherisorb S5ODS1 Waters (U.S.A.) C_{18} Reverse phase column and guard column. The conditions were as follows: column temperature 25°C, detector wavelength 254nm, flow rate 1.0ml/min and run time 40 minutes. Analyses was carried out using a variety of mobile phases which include phosphate buffer (pH 6.4), acetonitrile/phosphate buffer (pH 2, 10:90), acetonitrile/water (10:90), methanol/water (2:98), and water/acetonitrile (75:25).

2.1.3 Standard humic compounds

Table 2.1. Standard humic substances (Liao *et al.* 1982).

Standard Compound	**Chemical Formula**	**Chemical Structure**
Malonic acid dimethyl ester	$C_5H_8O_4$	$CH_2(COOCH_3)_2$
Phthalic acid	$C_8H_6O_4$	
Tricarballyic acid	$C_6H_8O_6$	
1,2,4,5-Benzenetetracarboxylic acid	$C_{10}H_6O_8$	
Tridecanoic acid methyl ester	$C_{14}H_{28}O_2$	$CH_3\text{-}(CH_2)_{11}\text{-}COOCH_3$
Caprylic acid methyl ester	$C_9H_{18}O_2$	$CH_3\text{-}(CH_2)_6\text{-}COOCH_3$
Sebacic acid dimethyl ester	$C_{12}H_{22}O_4$	$H_3COOC\text{-}(CH_2)_8\text{-}COOCH_3$

Seven standard humic substances were identified from the literature (Table 2.1) and these standards were obtained from Sigma and were used for confirmatory analysis. HPLC analysis of these compounds was conducted under conditions described in Section 2.1.2, to determine the presence of any of these standard humic compounds in the brine samples.

2.1.4 Desalting of brine samples

Brine samples were desalted using a Sephadex G-25 (Sigma) column. 1g of Sephadex G-25 was hydrated in double distilled water and the mixture was poured into a column. The water was drained out and 20ml of each brine sample was passed through the column, collected, and the eluent was freeze-dried overnight in an Edwards freeze dryer (Edwards, Sussex, U.K.). Freeze-dried samples were dissolved in small amount of mobile phase consisting of water/acetonitrile (75:25) for HPLC analysis under conditions described in Section 2.1.2.

2.1.5 Dialysis of brine samples

Brine samples were dialysed using a 22 mm x 35 feet dry diameter Snakeskin pleated dialysis tubing (Pierce) with a molecular weight cut off of 3 500 Da, against double distilled water in a beaker with continuous stirring using a magnetic stirrer. The water was changed at hourly intervals over a period of 5 hours. Brine samples were removed from the tubing and freeze-dried overnight. Freeze-dried samples were dissolved in small amount of double distilled water and HPLC analysis was carried out using conditions described in Section 2.1.2.

2.1.6 Carbohydrate assay

Carbohydrate analysis was conducted on the brine samples using two different tests, viz., the Molisch reaction and the Somogyi-Nelson's assay as prescribed in Clark & Switzer, Experimental Biochemistry, (1977). The Molisch reaction was carried out by adding a few drops of a 5% α-naphtol (Sigma) in ethanol solution to each brine sample followed by stratification with concentrated sulphuric acid (98% H_2SO_4) (BDH Laboratory Supplies). For Somogyi-Nelson's assay, a 0.05% glucose stock solution was prepared. From the stock solution, 0.1, 0.2, 0.3, 0.4 and 0.5ml was aliquoted into clean test tubes. Double distilled water was added to make up a volume of 6ml followed by addition of 1ml of Cu^{++} reagent. A blank sample containing 6ml distilled water and 1ml Cu^{++} reagent was prepared. To 2ml of each brine sample in separate tubes, 4ml of distilled water was added followed by addition of 1ml of Cu^{++} reagent. All tubes were heated on a water bath for 15 minutes at 70°C. 1ml of Arsenomolybdate reagent was added to each tube and the tubes were allowed to stand for 5 minutes. Absorbance was read at 510nm. All measurements were done in duplicates. Preparation of solutions is given in Appendix 1.

2.1.7 Total organic carbon and Total organic nitrogen determination

Since facilities for determining TOC and TON were not available in the department, brine samples were sent to LIRI Technologies, Rhodes University, for these analyses.

2.2 ANALYSES OF BRINE SAMPLES COLLECTED BEFORE RAINY SEASON

Seventeen brine samples from different stages of the soda ash process i.e. well field, solar evaporation ponds, storage ponds, crystallizer ponds and disposal area, collected before the rainy season, were obtained from Botswana Ash (Pty) Ltd and analysed.

2.2.1 Ultra-Violet spectrophotometry

The brine samples were centrifuged at 13 000 rpm for 5 minutes using a Heraeus biofuge and UV analysis was carried out on the supernatants using a Shimadzu UV-160A spectrophotometer. The absorption spectra of the samples were recorded in the range 200-800nm.

2.2.2 Carbohydrate assay

Brine samples were centrifuged as described in Section 2.2.1 and carbohydrate analysis before and after hydrolysis was performed on the supernatants according to the method of Somogyi-Nelson method. Analysis before hydrolysis was carried out as described in Section 2.1.6. For analysis after hydrolysis, 1ml of 1M HCl (BDH Laboratory Supplies) was added to each tube containing 2ml of each brine sample and was heated for 7 to 8 minutes in a boiling water bath. The tubes were then cooled in a beaker of cold distilled water and the contents were neutralized to pH 7 with 1ml of 1M NaOH (BDH Laboratory Supplies) and the assay was carried out as described in Section 2.1.6. All measurements were done in duplicate.

2.2.3 Protein assay

Brine samples were centrifuged as described in Section 2.2.1 and protein analysis was performed according to Bradford's method (Bradford 1976). Into 8 microfuge tubes, duplicate amounts of 5, 10, 15 and 20µl of 0.5mg/ml bovine serum albumin (BSA) (Boehringer Manheim) were aliquoted. The volume in each tube was adjusted to 100µl with 0.15M NaCl. 1ml of Coomassie Brilliant Blue solution (CBB) (Appendix 1) was added to the tubes and vortexed till thoroughly mixed. The solution was left to stand for 2 minutes at room temperature, the absorbance of the samples were measured at 595nm. To determine protein concentrations of the brine samples, duplicate amounts of 800µl of sample with 300µl of CBB was assayed using the same method. All measurements were done in duplicate.

2.2.4 Solvent extraction

From preliminary analysis, methyl ethyl ketone was found to be the best solvent, and hence further solvent extractions were carried out using this solvent as described in Section 2.1.1 followed by HPLC analysis.

2.2.5 HPLC analysis

HPLC analysis of the methyl ethyl ketone extracts was conducted at two wavelengths, 254nm and 300nm, using a Beckman System Gold HPLC with a 126 programmable solvent module, a 168 diode array detector module and a system gold software. From preliminary studies, a mobile phase of water/acetonitrile (75:25) was found to give the best separation, and hence it was used for analysis of the methyl ethyl ketone extracts. All other subsequent HPLC conditions used were as previously described in Section 2.1.2.

2.2.6 Analysis of Phenolics

Analysis of phenolics in the brine samples, before and after acidification, was performed according to the method of Garcia *et al.* (1997). For analysis after acidification, brine samples were acidified to pH 2 (Cyberscan 2000 pH meter, Wirsam Scientific) and 3 x 60ml of each brine sample was extracted with ethyl acetate. A lot of foaming was observed in the samples and they were allowed to settle before extraction. The extracts were pooled and dried with anhydrous sodium sulphate for approximately 40 minutes, filtered through Whatman No.1 filter paper and evaporated in a round-bottom flask (250ml) using a Buchi Rotavapor R-114. The precipitate was dissolved in 5ml of methanol/water (60:40).

2.2.6.1 Total phenol assay

Caffeic acid was used as a standard for total phenol analysis, with calibration concentrations ranging from 0 to 500mg/l. 1ml of each standard and sample (acidified or non-acidified) was pipetted into a series of 100ml volumetric flasks. 16ml of double distilled water was added to each flask and mixed well. 2.5ml Folin-Ciocalteau's reagent (Saarchem) was added and was mixed well for 30 seconds. 15ml of sodium carbonate solution (Appendix 1) was added, and the solution was mixed and brought to 100ml mark with dd H_20. A blank containing 17ml of distilled water, 2.5ml of Folin-Ciocalteau's reagent and 15ml of sodium carbonate solution brought to 100ml mark with double distilled water, was prepared. The flasks were allowed to stand for one hour at room temperature, and absorbance was measured at 765nm.

2.2.6.2 Diphenol assay

1mM catechol (Sigma) was used as a standard. A series of 0.5 fold dilutions were made from the stock solution. To 10ml of sample (acidifed or non-acidified), 2ml of 5% sodium molybdate in 50% ethanol solution (Appendix 1) was added. After 15 minutes standing at 20°C, the absorbance was measured at 370nm against a blank of 50% ethanol.

2.2.7 Treatment with oxidative enzymes

Methyl ethyl ketone extracts were treated with four enzymes, (a) Horseradish peroxidase (HRP) (24.8units/ml) from Sigma, (b) Polyphenol oxidase (PPO) (24units/ml) from *Agaricus bisporus*, (c) Lignin peroxidase (LiP) (33units/ml) from *Phanerochaete chrysosporium* and (d) Laccase (0.595 units/ml) from *Trametes versicolor*. In each case, one unit of activity is defined as the amount of enzyme required to produce 1µmol product per minute. Appropriate controls containing no enzyme were included in the experiments. 1ml of each extract was evaporated using nitrogen stream, and resuspended by addition of 1ml of respective buffers, 0.1M phosphate buffer (pH 6.1) (a and b), 0.25M sodium tartrate buffer (pH 2.5) (c) and 0.1M sodium acetate buffer (pH 5) (d) to obtain a final concentration of 2mg/ml. 100µl of each of the enzyme solutions was added separately to each sample. 0.9ml of buffer was added to PPO and laccase treated samples, whereas with HRP and LiP treated samples hydrogen peroxide was added to make up a final volume of 2ml. 30% hydrogen peroxide was added at 15 minute intervals with stirring using 100µl volumes (Roy-Arcand *et al.* 1991; Burton *et al.* 1993; Tien & Kirk 1993).

2.2.7.1 UV analysis of enzyme treated samples

UV analysis of the enzyme treated samples and their respective controls were carried out as described in Section 2.2.1. A blank with enzyme containing no sample was also analysed.

2.2.7.2 HPLC analysis of enzyme treated samples

HPLC analysis was conducted using the same system and conditions as described in Section 2.1.2 except that the detector wavelength was set at 300nm and a mobile phase of water/acetonitrile (75:25) was used.

2.2.7.3 Mass spectrometry

The enzyme treated samples that gave larger peak areas than their respective controls were selected for mass spectrometric analysis. All samples were purified using a silica (Silica Gel 60, Merck, South Africa) column followed by evaporation using a nitrogen stream. Precipitates were dissolved in small amount of the mobile phase, water/acetonitrile/acetic acid (75:25:0.1), to give a concentration of 0.05mg/ml. Two to three drops of acetic acid were added to each sample, to facilitate ionization. LC-MS analysis was carried out on the samples using a Finnigan Mat LCQ (U.S.A.) system, in negative ESI (Electron Spray Ionization) mode attached to a Finnigan MAT LCQ software system. The samples were injected into a Spectra system P2000 LC system attached to a Spectra system SN 4000 detector, using a 4.6 x 250mm Waters Spherisorb S5ODS1 Waters (U.S.A.) C_{18} Reverse phase column. Other conditions were the same as described in Section 2.1.2.

2.3 ANALYSES OF BRINE SAMPLES COLLECTED AFTER RAINY SEASON

20 brine samples which had been collected after the rainy season were received from Botswana Ash (Pty) Ltd and these samples were analyzed in exactly the same manner as the first set of samples i.e. the samples collected before the rainy season, using UV, carbohydrate and protein assays, extractions, HPLC analysis, total phenol and diphenol assays and oxidative biotransformations to facilitate comparison of the two sets of samples (Section 2.2.1 to 2.2.7.3).

2.4 ANALYSES OF BICARBONATE AND SODA ASH SAMPLES

Solid bicarbonate and soda ash samples were received from Botswana Ash (Pty) Ltd, and a number of analyses were carried out to determine the nature of the organic components present in the samples.

2.4.1 Solvent extraction

100g of bicarbonate or soda ash were extracted with 200ml of methyl ethyl ketone as described in Section 2.1.1. Components were isolated using column chromatography (Silica Gel 60), eluting with ethyl acetate and benzene (20:80). Thin Layer Chromatography (TLC) analysis was carried out on 20 x 20cm Silica gel 60 F_{254} (Merck) plates with the same solvent system mentioned above to confirm the presence of compounds in the fractions, and fractions containing the same compound were pooled and evaporated under reduced pressure. The compound isolated was very oily in nature and had a sweet smell, and was named compound A.

2.4.2 UV analysis

UV analysis of compound A was carried out as described in Section 2.2.1.

2.4.3 Nuclear Magnetic Resonance spectroscopy

For all NMR studies, compound A was dissolved in deuterated chloroform ($CDCl_3$) (Sigma). 400mHz ^{1}H-NMR, 100mHz ^{13}C-NMR and 2D-NMR analyses were carried out on a Bruker (Germany) AMX 400 instrument. Chemical shifts are quoted in the δ scale.

2.4.4. Infra-Red spectrophotometry

Compound A was spread on NaCl discs. IR spectra were recorded in MidIR (400-4000 cm^{-1}) region using a Perkin-Elmer FTIR spectrum 2000 spectrophotometer. Air was used as a background scan.

2.4.5 HPLC analysis

HPLC analysis of compound A was carried out as described in Section 2.1.2 except that the detector wavelength was set at 280nm and a mobile system of water/acetonitrile (50:50) was used.

2.4.6 HPLC-Mass spectrometry

Compound A (0.05mg/ml) was dissolved in a mobile phase of water/acetonitrile/acetic acid (50:50:0.1). LC-MS analysis was carried out as described in Section 2.2.7.3.

2.4.7 Gas chromatography-Mass spectrometry

Compound A was dissolved in chloroform to a final concentration of 0.1mg/ml. GC-MS analysis was conducted on a Finnigan MAT GC-mass spectrometer, using a J&W DB1 column (30m) and 1µl injections. The following operating conditions were used:

Initial temperature: 80°C for 5 minutes
Rate: 22°C/minute to 260°C with holding temperature for 7 minutes
Constant velocity: 40cm/second

2.4.8 Thin layer chromatography analysis

TLC analysis using the same solvent system mentioned in Section 2.4.1 was carried out on compound A and one sample each from each stage of the soda ash process to detect for the presence of Compound A in the brine samples.

2.4.9 Elemental analysis

Compound A was sent to the Chemistry department, UCT for elemental analysis to determine the composition of the isolated compound.

2.4.10 Confirmatory analysis

From all the analyses carried out, compound A was suggested to be benzyl butyl pthalate (BBP). Compound A, and one sample from each stage of the soda ash process, was spiked with benzyl butyl phthalate (BBP) (Merck) to confirm that the isolated compound was actually BBP and also to confirm the presence of BBP in the brine samples. A 1mM solution of BBP in methanol was prepared and a 1:10 dilution was made with the methyl ethyl ketone extracts and the isolated compound. HPLC analysis was carried out using a Beckman System Gold HPLC with a 126 solvent module, a 166 detector and a 32 Karat software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 PRELIMINARY INVESTIGATION OF THREE BRINE SAMPLES

This was carried out to extract the organic components from the samples and to develop appropriate analytical methodology for characterizing the organics.

3.1.1 Solvent extraction and HPLC analysis of extracts

The brine samples (W-Brine, N-Brine and T-Brine) were extracted with four different organic solvents: butanol, chloroform, ethyl acetate and methyl ethyl ketone to determine which solvent would would give the most efficient extraction of the organic components present in the brine samples. The HPLC results obtained show that the best extraction was obtained with the methyl ethyl ketone extracts (Figures 3.1), and separation was achieved using a mobile phase of water and acetonitrile (75:25); hence, this mobile phase was used for all further HPLC analysis.

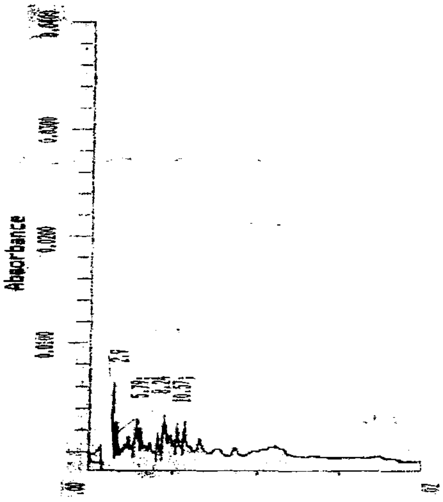
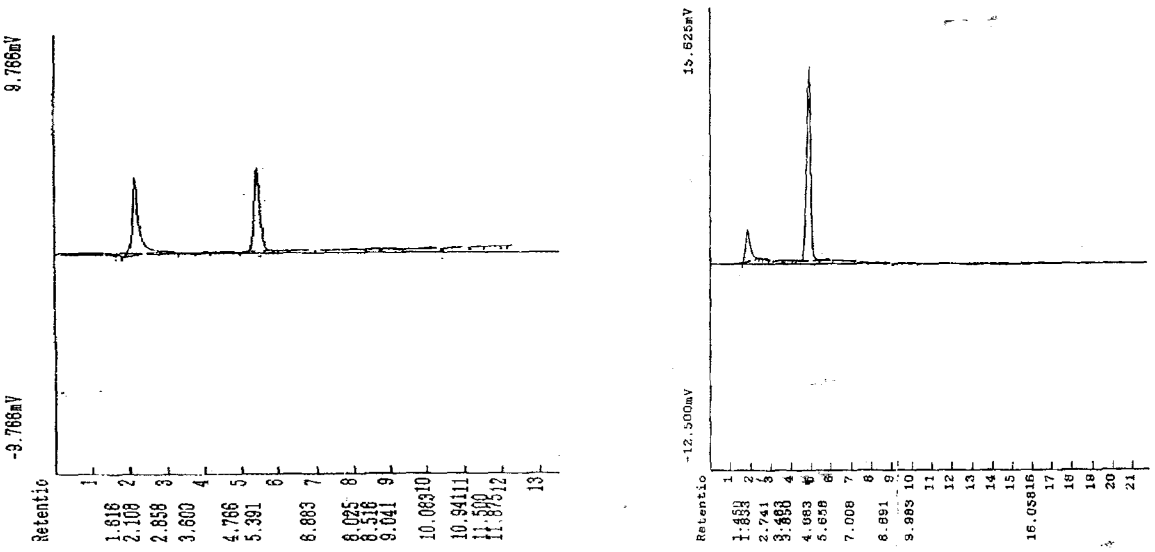


Figure 3.1. HPLC chromatogram of T-brine extracted with methyl ethyl ketone.

Samples extracted with butanol, chloroform and ethyl acetate did not give good separation profiles (Figure 3.2), hence, methyl ethyl ketone was used for all subsequent extractions since this solvent was found to give the most efficient extraction.



A B

Figure 3.2. HPLC chromatogram of T-brine extracted with chloroform (A) and ethyl acetate (B).

Similar separation profiles were observed amongst the three brine samples. This suggests that the same contaminating compounds are present in all the brine samples. Solvent extraction and HPLC analysis provided suitable methods for getting profiles of the brine samples which could then be used for comparative purposes.

3.1.3 Standard humic compounds

Standard humic substances were used to enable determination of the nature of the components of brine samples and also to check whether any of these standards were present in the samples. The standards used gave different retention times with various mobile phases (Table 3.1). The retention times of certain of the model compounds corresponded to those observed in the brine samples, suggesting that some components in the brine samples were humic in nature. Common retention times were approximately 2, 3 and 5 minutes suggesting that the organic components could be phthalic acid or dimethyl ester derivatives

Table 3.1. Retention times of standard humic compounds using various mobile phase systems.

	water/acetonitrile (75:25)	**water/acetonitrile (10:90)**	**methanol/water (2:98)**	**acetonitrile/ phosphate buffer (pH2,10:90)**	**phosphate buffer (pH 6.4)**
1	5.616	1.933	1.966	5.083	6.275
2	2.025	2.083	1.975	5.066	2.5
3	1.891	3.283	1.983	7.6	3.05
4	1.766	1.925	1.991	2.758	3.683
5	2.25	2.466	4.516	13.741	3.941
6	2.133	2.283	2.219	3.741	3.783
7	2.008	2.406	2.475	4.2	2.475

1 = Malonic acid dimethyl ester
2 = Sebacic acid dimethyl ester
3 = Caprylic acid methyl ester
4 = Tridecanoic acid methyl ester
5 = Phthalic acid
6 = Tricarballyic acid
7 = 1,2,4,5-Benzenetetracarboxylic acid

3.1.4 Desalting of brine samples

Since the brine samples are heavily concentrated with salt, desalting using a Sephadex G-25 column was used as an alternative approach to solvent extraction, to remove excess salt in the brine samples that might affect the separation of the organic components present in the brine samples. HPLC analysis was used to observe the effect desalting would have on the separation of the organic components. From the HPLC analysis result obtained, desalting had little effect on the separation (Figure 3.3) and this approach was not pursued further.

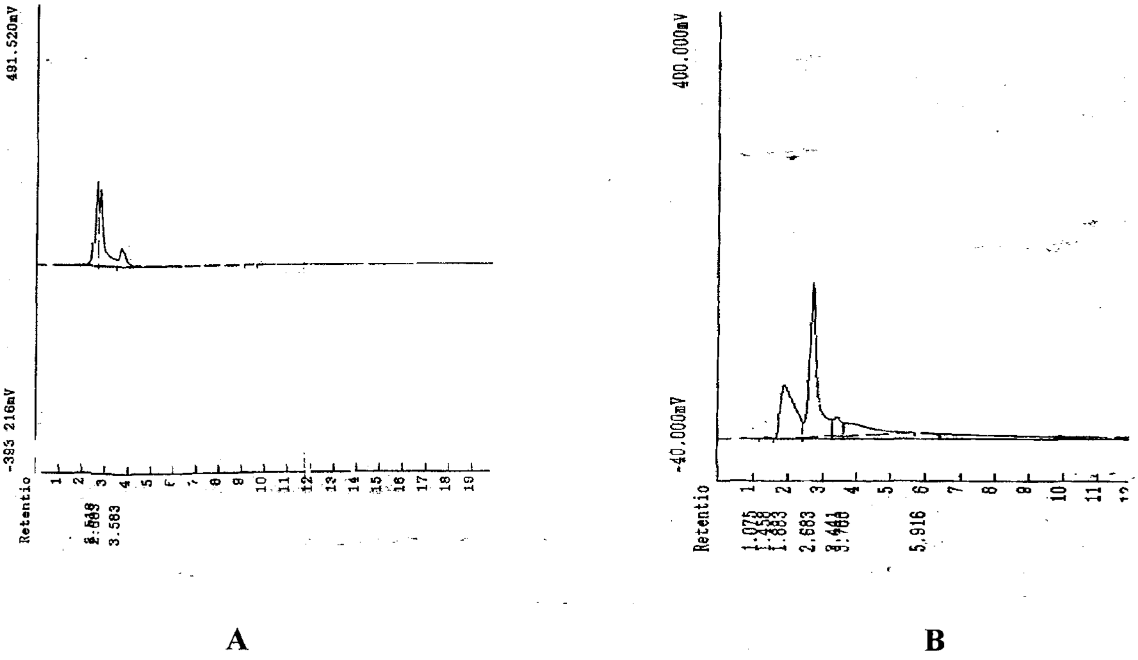
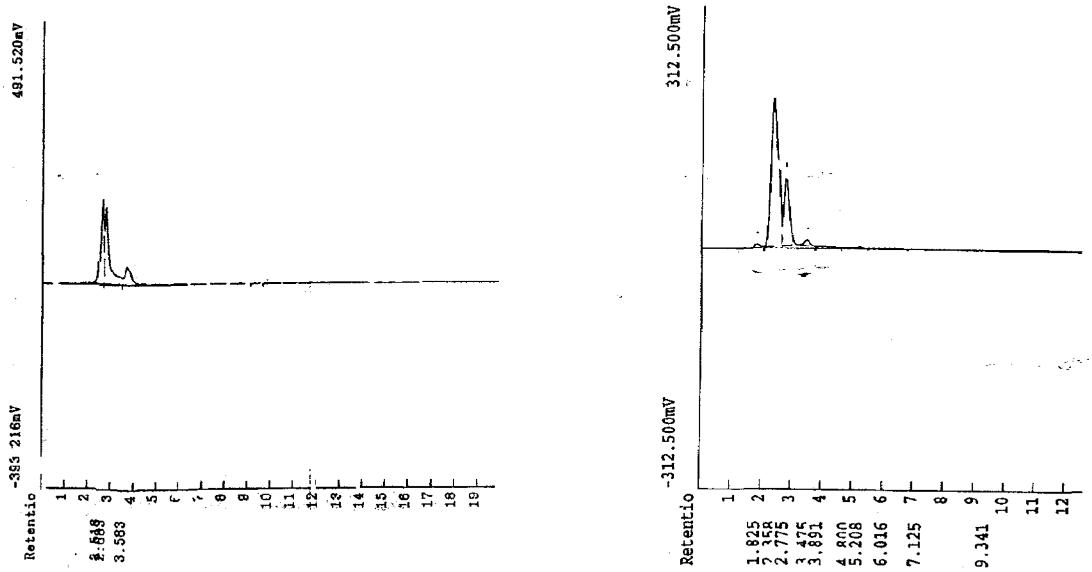


Figure 3.3. HPLC chromatogram of well-brine before (A) and after (B) desalting using a Sephadex G-25 column.

3.1.5 Dialysis of brine samples

Another method carried out to remove the excess salt present in the brine samples was dialysis using dialysis tubing. Dialysis had little effect on the separation of the brine components (Figure 3.4) and similar HPLC profiles were observed with the desalted and dialysed brine samples, suggesting that both these methods are equally ineffective.



A

B

Figure 3.4. HPLC chromatogram of well-brine before (A) and after (B) dialysis.

3.1.6 Carbohydrate analysis

Carbohydrate analysis was done to determine the nature of the components of the brine. Two methods were used for this, the Molisch test and Somogyi-Nelson's assay. In the Molisch test for the presence of carbohydrates, positive results were indicated by a red colour at the zone of contact, and this was observed in all the brine samples assayed using this reaction. Similarly, from the results obtained using the Somogyi-Nelson's assay, the presence of carbohydrates was observed in all the brine samples, with the highest concentration seen in T-brine (Table 3.2). The presence of high carbohydrate concentration in the T-brine could be a result of biological activity of *Dunaliella spp* in the solar evaporation ponds resulting in the production of exopolysaccharides

Table 3.2. Carbohydrate concentrations of brine samples.

Brine sample	**Concentration (mg/ml)**
W-brine	0.151
N-brine	0.111
T-brine	0.492

3.1.7 TOC and TON analysis

Total organic carbon and Total organic nitrogen analysis was also done to determine the nature of the brine components. Brine samples were sent to LIRI Technologies here at Rhodes University, since facilities for these experiments were not available in the department. All three brine samples gave positive results for the presence of organic carbon and organic nitrogen, measured as TOC and TON. The brine samples were found to contain higher organic carbon concentrations than organic nitrogen concentrations, with the highest concentrations of each seen in T-brine (Table 3.3). The high concentrations of TOC in the T-brine is expected since the T-brine is used for the production of the soda ash and the final soda ash product has been reported to contain very high amounts of TOC. The well-brine has been found to contain significant levels of ammonia hence the presence of nitrogen in the brine samples is also expected.

Table 3.3. Total organic carbon and Total organic nitrogen analysis of brine samples.

Brine sample	**TOC (mg/l)**	**TON (mg/l)**
W-brine	59.23	22
N-brine	77.77	27
T-brine	165.85	33

3.2 ANALYSES OF 17 BRINE SAMPLES COLLECTED BEFORE (FIRST SET) AND AFTER RAIN (SECOND SET)

3.2.1 UV analysis

UV analyses were carried out to verify the organic nature of the contaminating compounds present in the brine samples. In both sets of samples i.e the first set of samples collected before the rainy season and the second set of samples collected after the rainy season, the highest absorbances of each sample were detected in the UV range, at approximately 300nm, indicating the presence of aromatic compounds, and hence this was one of the wavelengths used for detection in HPLC analysis (Figure 3.5).

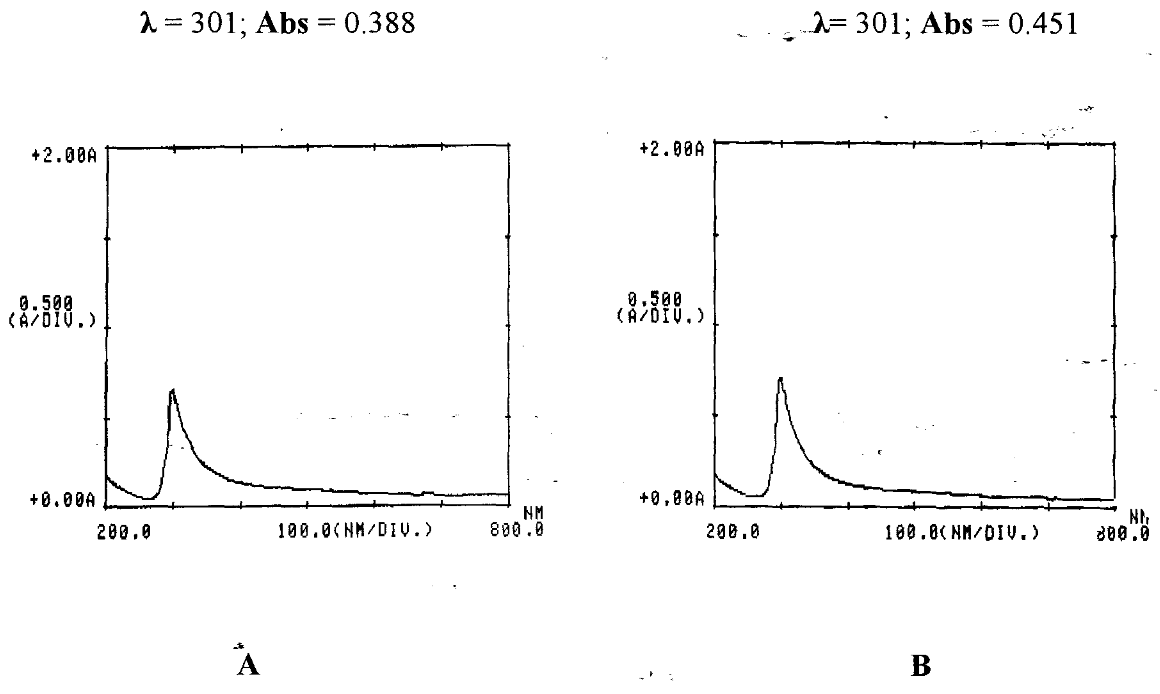


Figure 3.5. UV analysis of well brine collected before rainy season (first set) (A) and after rainy season (second set) (B).

In general, most of the samples from the second set had lower absorbances than samples from the first set. The decrease in the absorbances were almost 0.5. However, well-brine from the second set showed the highest absorbance amongst the second set of samples and the absorbance was higher than that of the well-brine from the first set indicating higher concentrations of organics in the raw brine collected after the rainy season rainfall (Figure3.6).

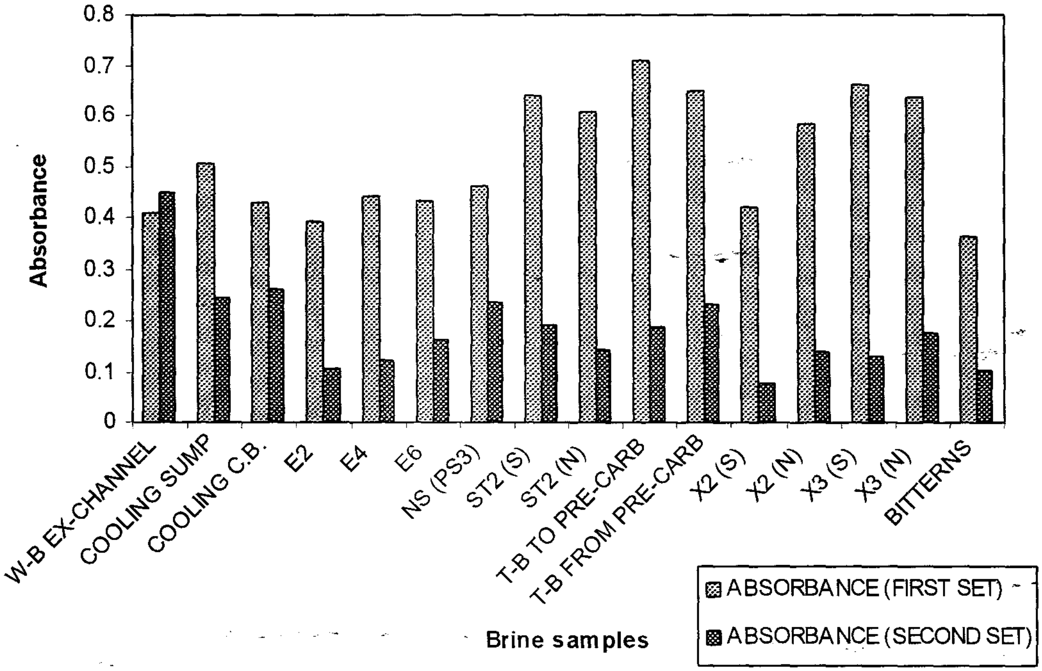


Figure 3.6. UV analysis of brine samples collected before (first set) and after rainy season (second set).

3.2.2 Carbohydrate analyses

Brine samples were assayed for carbohydrates using the Somogyi-Nelson's assay before and after hydrolysis with 1M HCl. Hydrolysis was carried out to convert polysaccharides present in the brine samples to simple carbohydrates. Before hydrolysis, the second set of samples from the well field, E2 out and E6 out had higher concentrations than the first set. In both sets of samples, there was an increase in carbohydrate concentrations after hydrolysis and there was a marked increase of 15 fold in the well-brine from the second set (Figure 3.7). This shows that there was an increase in the amount of polysaccharides present in the samples which could be converted to simple carbohydrates. This increase could be a result of the release of more exopolysaccharides by the *Dunaliella* spp in the solar ponds.

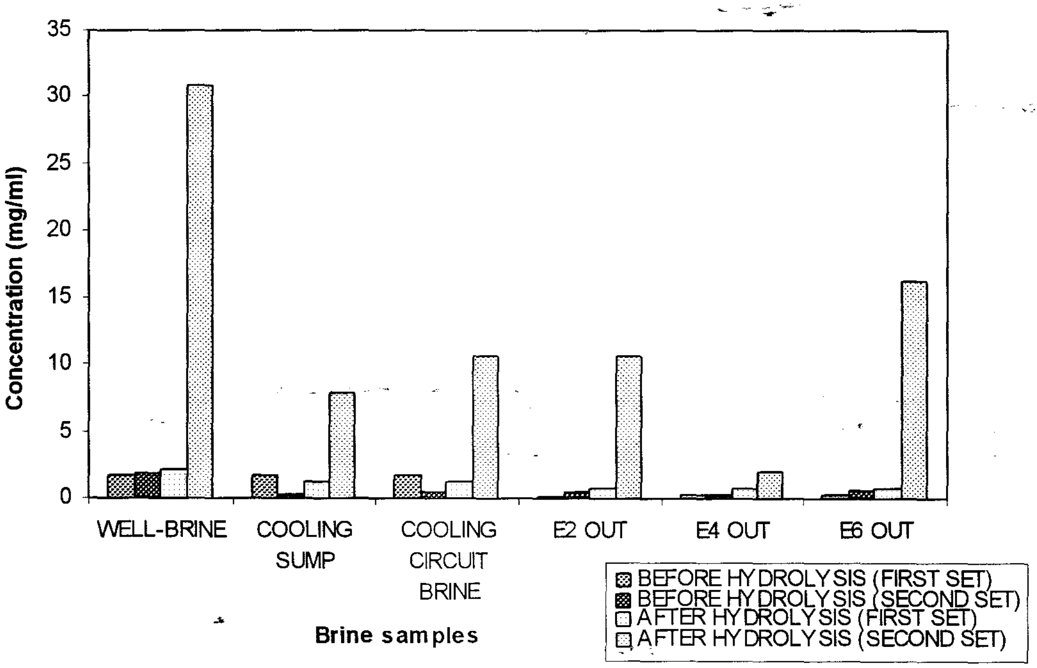


Figure 3.7. Carbohydrate concentrations before and after hydrolysis of brine samples collected before rain (first set) and after rain (second set) from well-field and solar evaporation ponds.

From the storage ponds, crystalliser ponds and disposal area, before hydrolysis, samples from the second set had higher carbohydrate concentrations than the first set, as was seen with samples collected from the well-field and solar evaporation ponds, suggesting higher contamination which is due to higher microbial activity after rainfall. After hydrolysis, both sets of samples showed an increase in concentrations with a marked increase in concentrations in the second set especially in samples collected from the storage ponds (Figure 3.8).

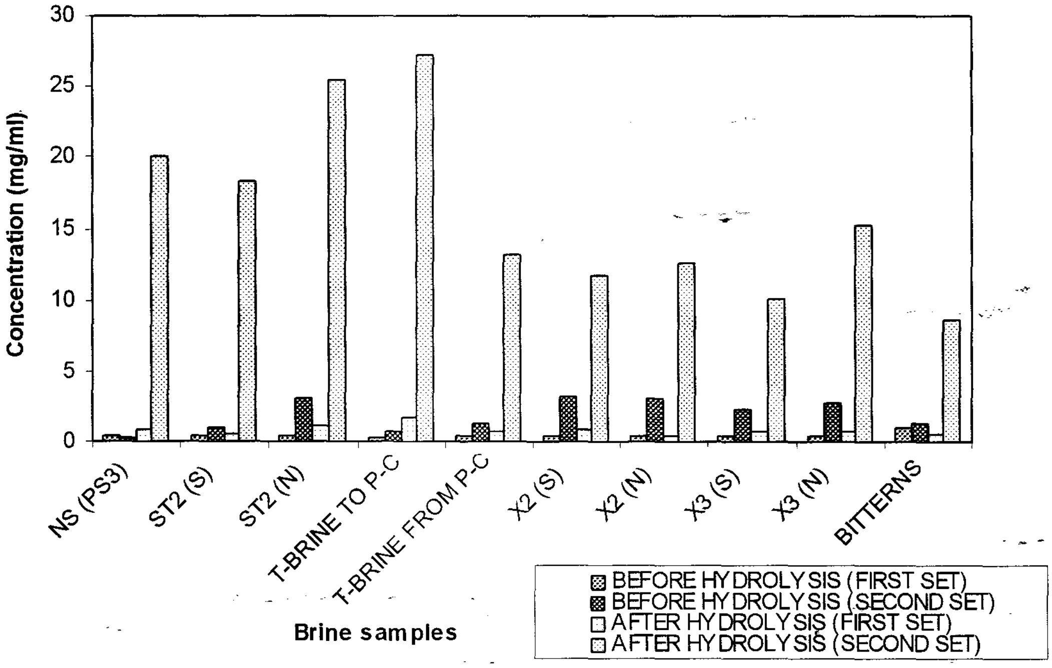


Figure 3.8. Carbohydrate concentrations before and after hydrolysis of brine samples collected before (first set) and after rain (second set) from storage ponds, crystalliser ponds and disposal area.

3.2.3 Protein analyses

Protein analysis was done according to the method of Bradford (1976) to determine changes in the amount of proteins in the samples before and after the rainy season. Brine samples collected before and after rain gave positive results for proteins. There was a marked increase in protein concentrations after rain again suggesting higher contamination after rainfall. The higher concentrations after rainfall could be a result of higher microbial activity in the solar ponds resulting in the release of more extracellular proteins or it could be due to the presence of higher levels of ammonia in the well brine. A seven fold increase was observed in the well-brine (Figure 3.9).

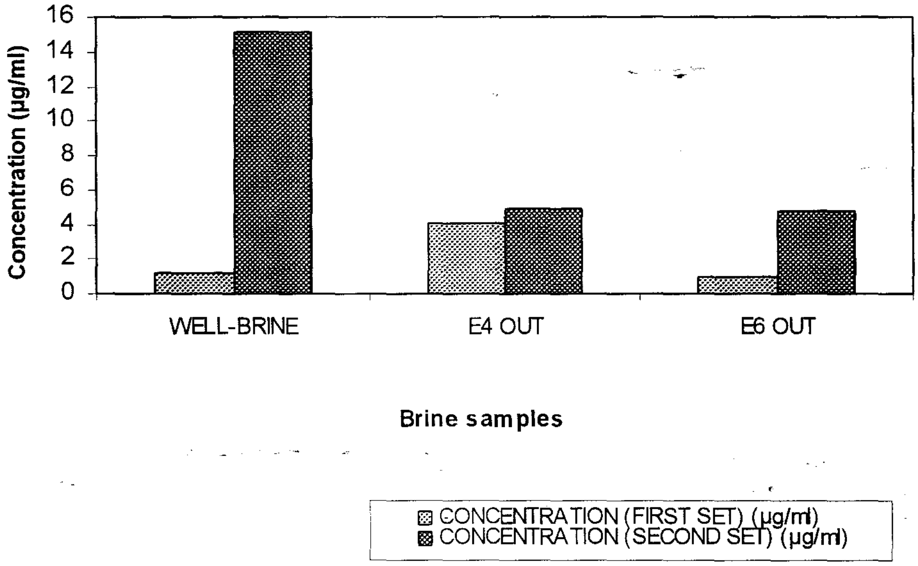


Figure 3.9. Protein concentrations of brine samples collected before (first set) and after rain (second set) from well field and solar evaporation ponds.

As was observed with samples collected from the well field and solar evaporation ponds, brine samples from the second set which were collected from the storage ponds and crystalliser ponds, had much higher protein concentrations than samples from the first set, this again suggests an increase in microbial activity after the rainy season (Figure 3.10).

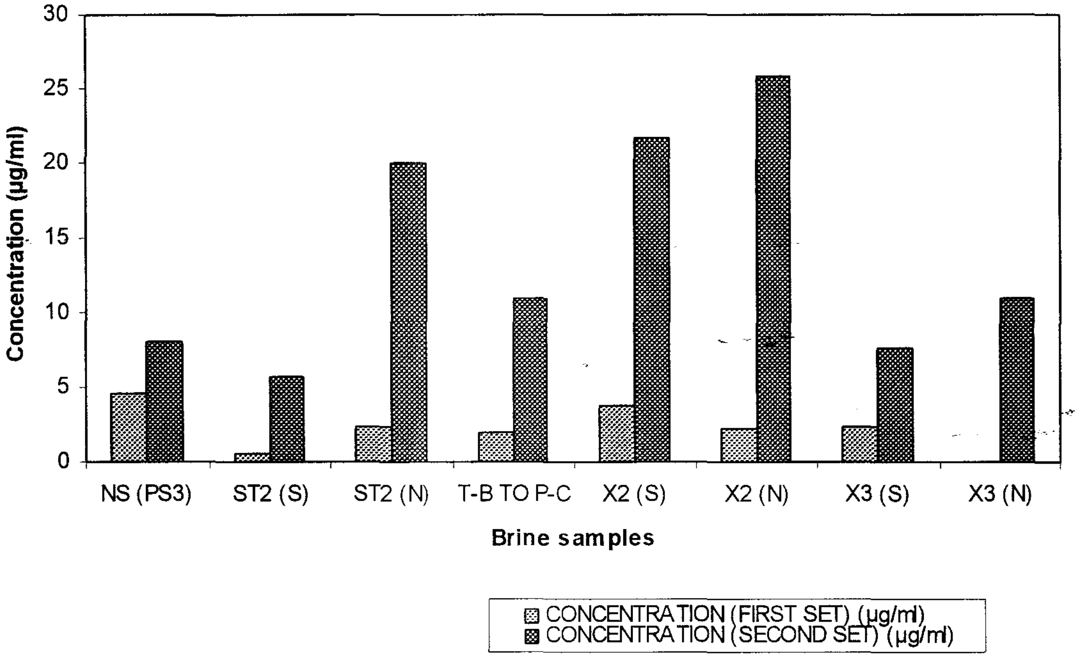


Figure 3.10. Protein concentrations of brine samples collected before rain (first set) and after rain (second set) from storage ponds and crystalliser ponds.

3.2.4 Solvent extraction and HPLC analysis

Solvent extraction was carried out to extract the organic components of the brine samples. Extractions were done with methyl ethyl ketone since from preliminary investigations this was found to be the solvent that gave the most efficient extraction. HPLC analyses were carried out to characterize the organic compounds in the brine samples. Profiles and the specific changes between the extracts from both sets were noted.

Results obtained from HPLC analyses at both wavelengths of 254nm and 300nm showed the presence of a number of components in both sets of samples, with a larger number of compounds detected at λ=254nm in both sets (Figure 3.12). This indicates the presence of non aromatic as well as aromatic compounds. An increase in the number of peaks as well as an increase in peak area was observed at both wavelengths in brine samples collected after the rainy season suggesting higher numbers of different components (Table 3.4). HPLC analysis of methyl ethyl ketone was also carried out to check the retention time of the solvent peak and any minor contaminants it might contain (Figure 3.11). The main peak in the extracts from the first set was at a retention time of approximately 2 minutes whereas with the extracts from the second set, main peaks were seen at retention times of approximately 4 and 7 minutes. The peak seen at a retention time of approximately 4 minutes could very likely be the solvent peak. Another set of samples (third set) was received in October this year just before the completion of this project. HPLC analysis of extracts from the third set showed similar profiles to those from the second set.

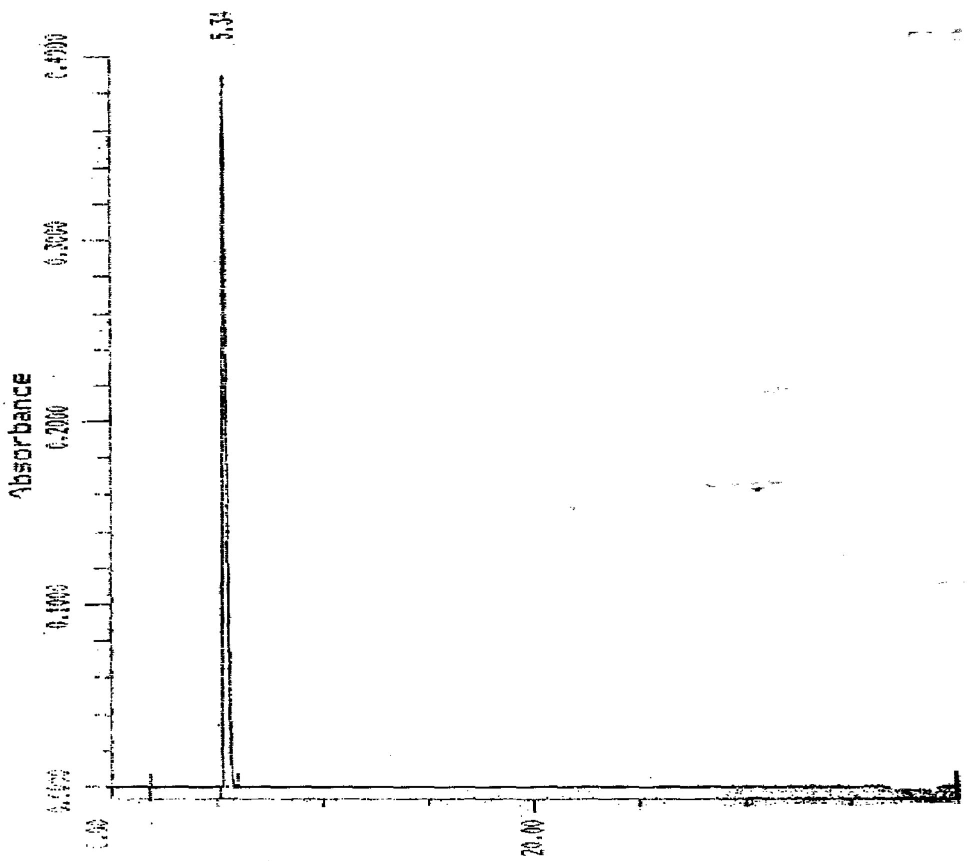


Figure 3.11. HPLC chromatogram showing methyl ethyl ketone peak at a retention time of 5.34.

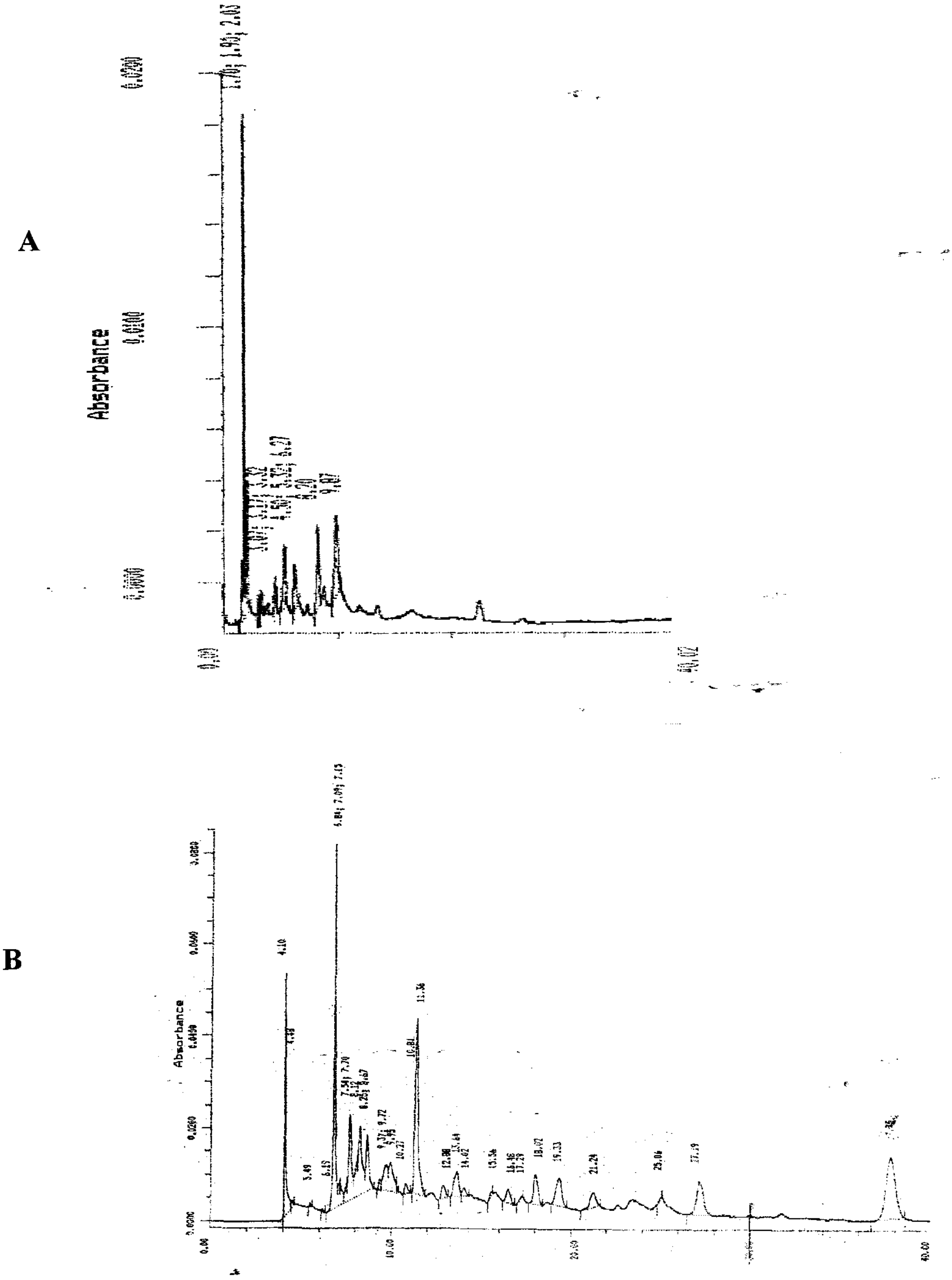


Figure 3.12. HPLC chromatograms (run over 40 minutes), of well-brine from first (A) and second set (B) extracted with methyl ethyl ketone and analysed at 300nm.

Table 3.4. HPLC peak areas at 300nm of brine samples collected before and after rain.

Sample	Retention time before rain	Peak area before rain	Retention time after rain	Peak area after rain
W-Brine	4.5	0.03	4.1	3.62
E6 (PS2)	4.74	0.14	4.04	3.62
NS (PS3)	4	0.11	3.96	3.17
X1 (S)	4.52	0.74	4.07	3.42
Bitterns	4.01	0.56	4.04	4.33

3.2.6 Analysis of Phenolics

3.2.6.1 Total phenol analysis

To determine whether the brine samples contained components which are phenolic in nature, the total phenol content was measured in solvent extracts obtained before and after acidification of the brine samples to pH 2. Brine samples were acidified to pH 2 to protonate the phenolics which should enhance the solubility in the organic phase. Total phenol concentrations before acidification, in both sets of samples, were found to be high, and the second set of samples showed higher concentrations than the first. However in samples extracted after acidification, there was a marked decrease in the concentrations. Only the well-brine from the second set and E2 out from the first set showed a nearly two fold higher total phenol concentration after acidification. This result shows that the brine samples contain phenolic compounds and there is an increase in the phenolic content after the rainy season (Figure 3.13).

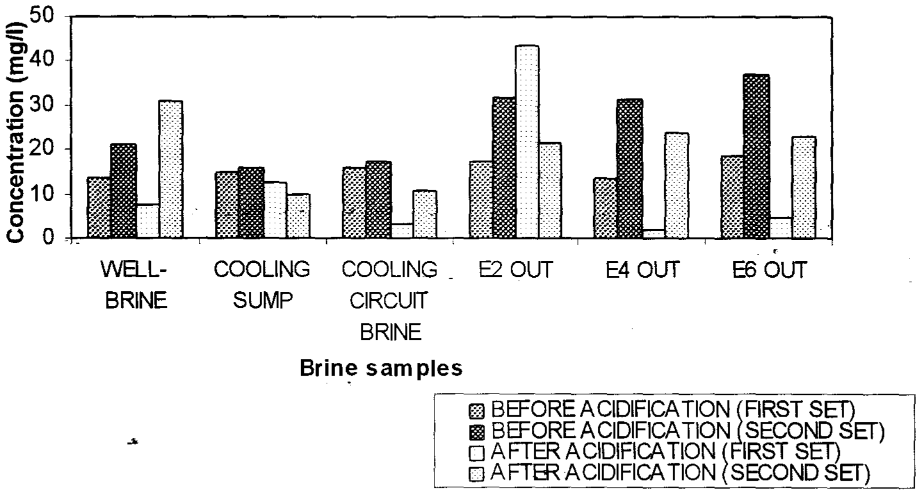


Figure 3.13. Total phenol concentrations of brine samples collected before rain (first set) and after rain (second set) from well field and solar evaporation ponds.

In most of the brine samples collected from the storage ponds, crystalliser ponds and disposal area after the rainy season there was higher total phenol concentration than before the rainy season, before acidification. After acidification there was a lower concentration in extracts in both sets of samples but the second set of samples gave higher concentrations in extracts after acidification than the first set of samples suggesting that concentration of phenolics was higher after the rainy season (Figure 3.14).

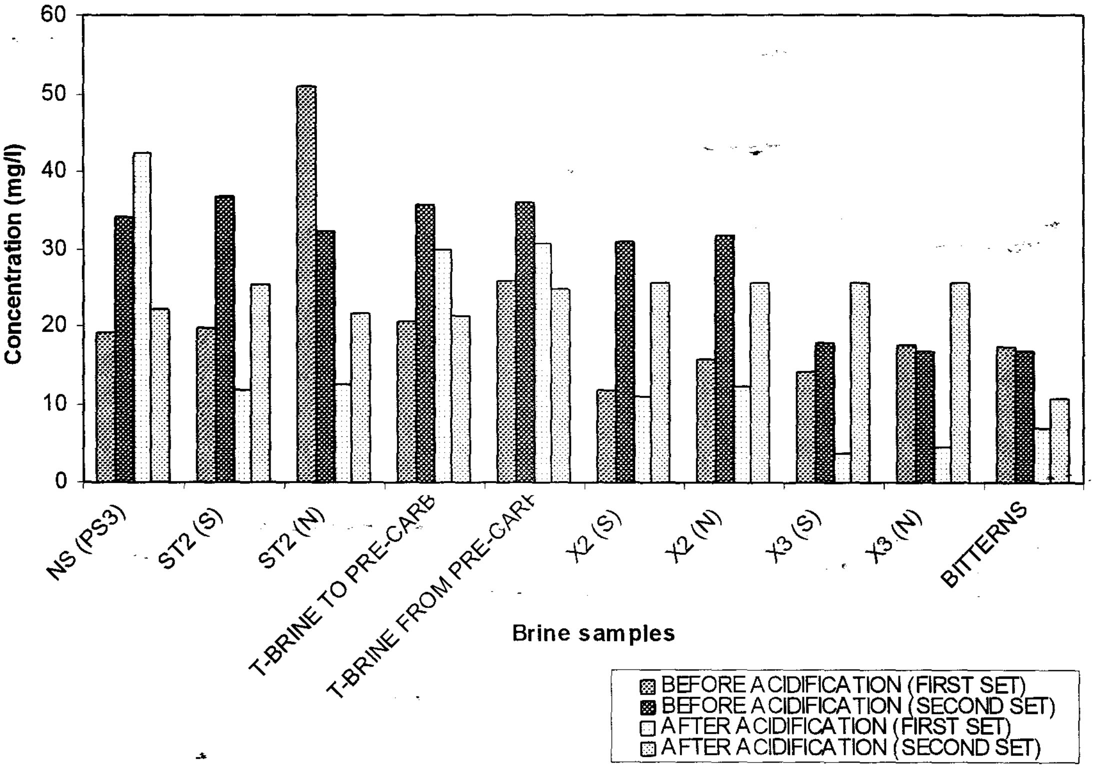


Figure 3.14. Total phenol concentrations of brine samples collected before rain (first set) and after rain (second set) from storage ponds, crystalliser ponds and disposal area.

3.2.6.2 Diphenol analyses

Diphenol analysis was carried out using catechol as a standard to check for the presence of more specific components such as catechols. Acidification to pH 2 was done to protonate the phenolic compounds which should then enhance the solubility in the organic phase. Before acidification, the brine samples collected after the rainy season (second set) had higher diphenol concentrations than the samples collected before the rainy season. However, after acidification there was decrease in diphenols extracted, in most of the samples from both sets. Samples from the first set gave almost negligible extractable diphenols after acidification. Only well-brine and cooling sump brine extracts showed higher concentration after acidification. Phenolic components are present in the raw brine in detectable amounts, but during the process, there is a marked decline in the concentrations (Figure 3.15).

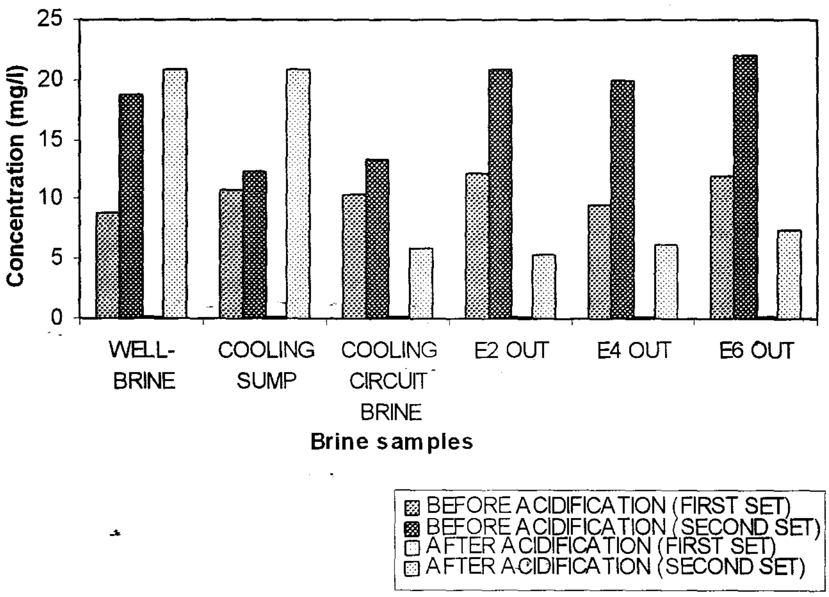


Figure 3.15. Diphenol concentrations of brine samples collected before rain (first set) and after rain (second set) from well field and solar evaporation ponds.

The same pattern as observed with samples from the well field and evaporation ponds was seen with samples from the other three stages from the soda ash process. Higher concentrations in extracts of the second set of samples before acidification were seen, and a marked decrease in concentrations in extracts from both sets after acidification with a higher diphenol concentration in the second set. This again suggests that after the rainy season, the concentration of phenolics was higher (Figure 3.16).

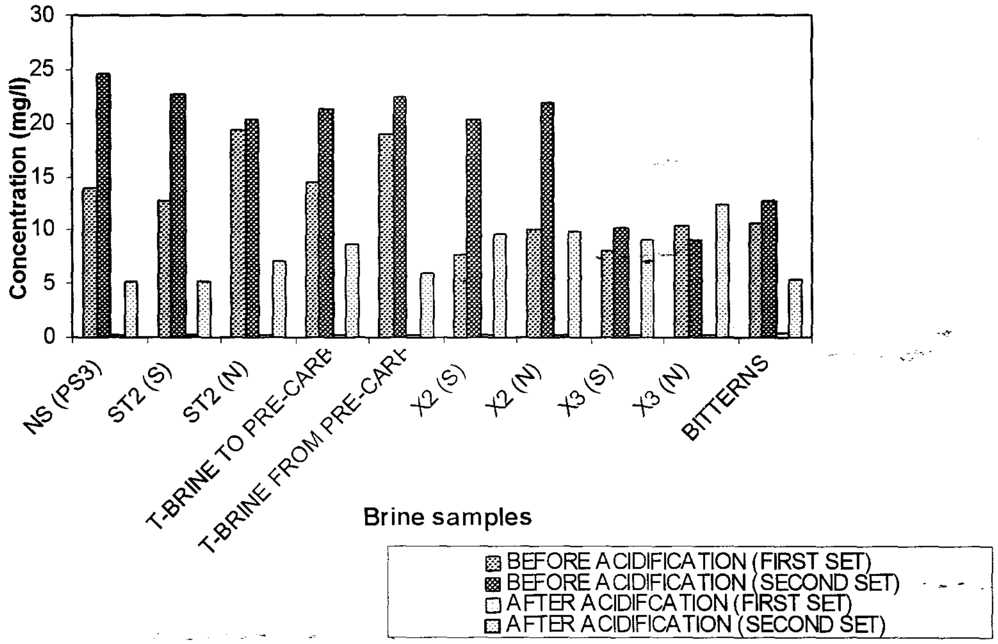


Figure 3.16. Diphenol concentrations of brine samples collected before rain (first set) and after rain (second set) from storage ponds, crystalliser ponds and disposal area.

3.2.7 Oxidative biotransformations

Enzyme oxidations, with appropriate controls containing no enzyme, were carried out on methyl ethyl ketone extracts of all the brine samples. The concept behind the enzyme oxidations studies was to determine whether there were any components present in the brine which could act as suitable substrates for the enzymes used. For example, if the brine components were phenolic in nature then PPO would act upon some of these phenolic compounds. Phenolic substrates were expected to be present since the assays that were done for phenolic compounds gave positive results. PPO and laccase were expected to react with these substrates to produce products such as quinones.

3.2.7.1 UV analyses of enzyme treated samples

UV analyses were carried out to determine changes in the wavelengths as well as absorbances of brine components as a result of treatment with the oxidative enzymes. In both sets, samples treated with laccase and PPO showed higher absorbance peaks than their respective controls, which is an indication that suitable substrates for these enzymes were present, and enzymatic reactions were taking place, yielding more highly conjugated products (Figure 3.17). Laccase oxidizes various aromatic compounds resulting in the synthesis of humic substances and PPO is involved in polymeriztion of naturally occurring phenols resulting in the formation of humic acids. Since the raw brine is humic in nature, it was expected that suitable substrates for these enzymes would be present. With the HRP and LiP treated samples, very few samples showed higher absorbances than their controls. This indicates that few suitable substrates were present for these enzymes.

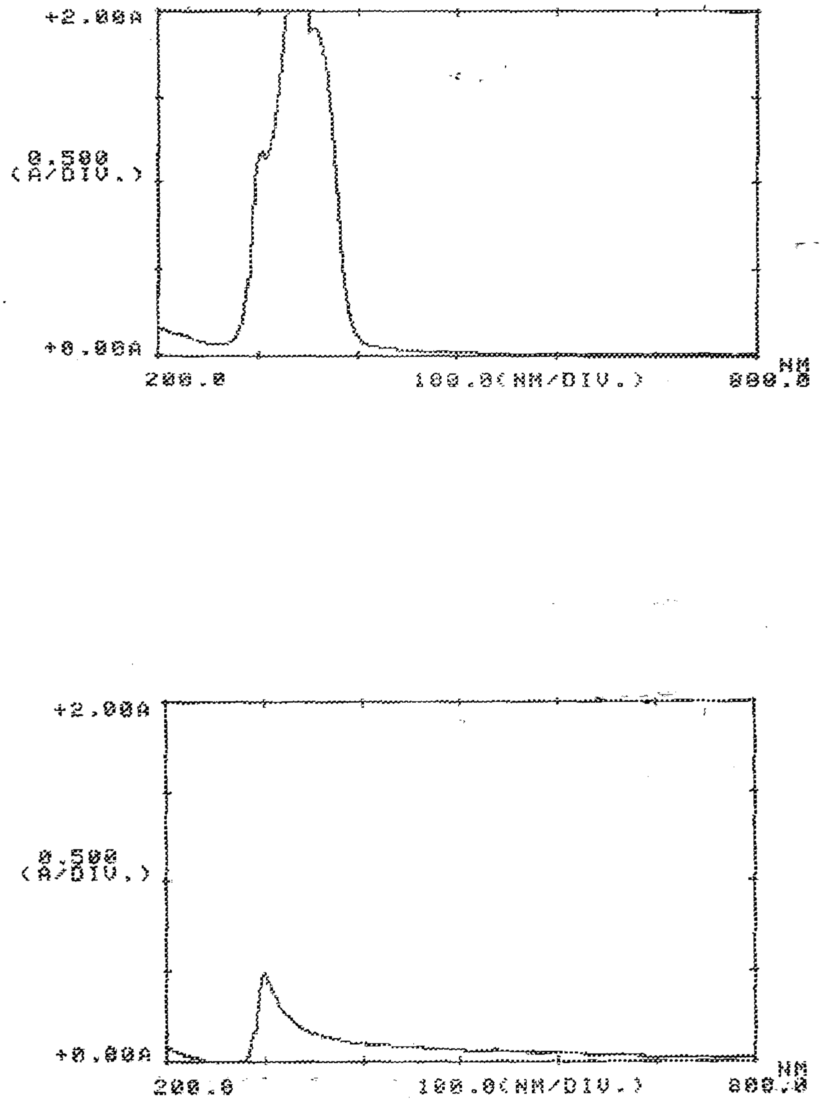


Figure 3.17. UV analysis of well-brine treated with laccase (A) and the respective control containing no enzyme (B).

3.2.7.2 HPLC analyses of enzyme treated samples

HPLC analysis of the enzyme treated samples and their respective controls from both sets were carried out to detect the presence of any products that could have been formed as a result of the enzyme oxidations. In the first set of samples, in all the samples treated with the four different enzymes, a peak at approximately 3 minutes was seen and no product peak was observed. The peak at approximately 3 minutes could very likely be the buffer peak (Figure 3.18). With the second set of samples, the same was observed with the PPO, HRP and LiP treated samples (Figure 3.19).

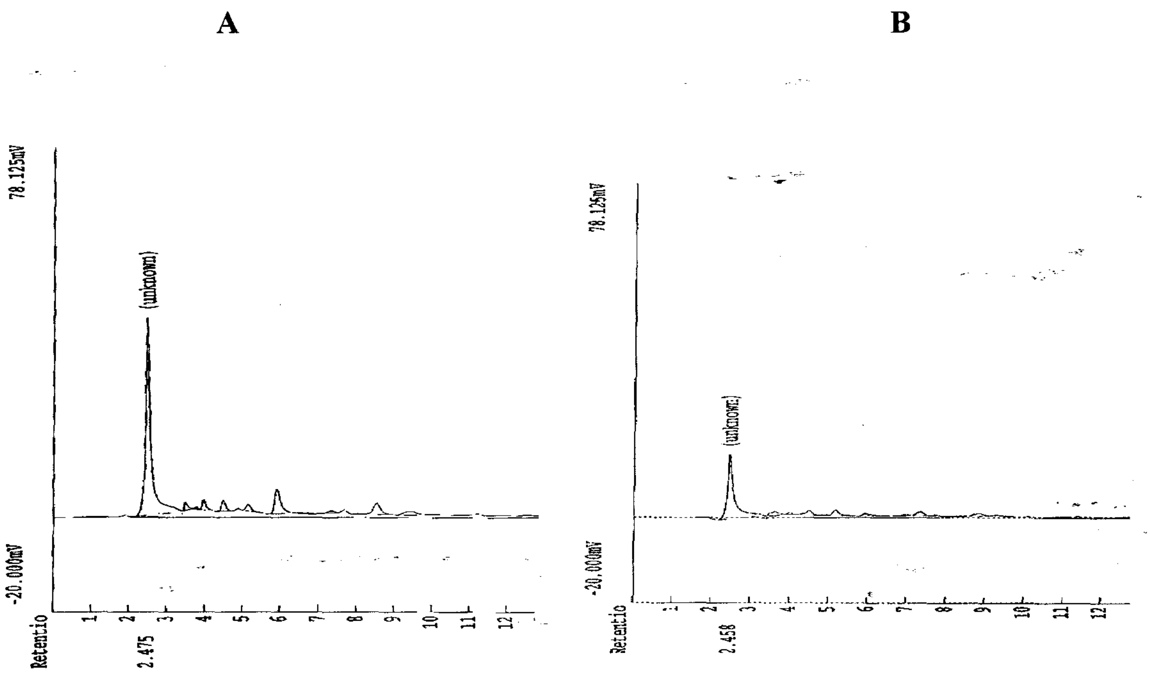


Figure 3.18. HPLC analysis of well-brine treated with PPO (A) and the control containing no enzyme.

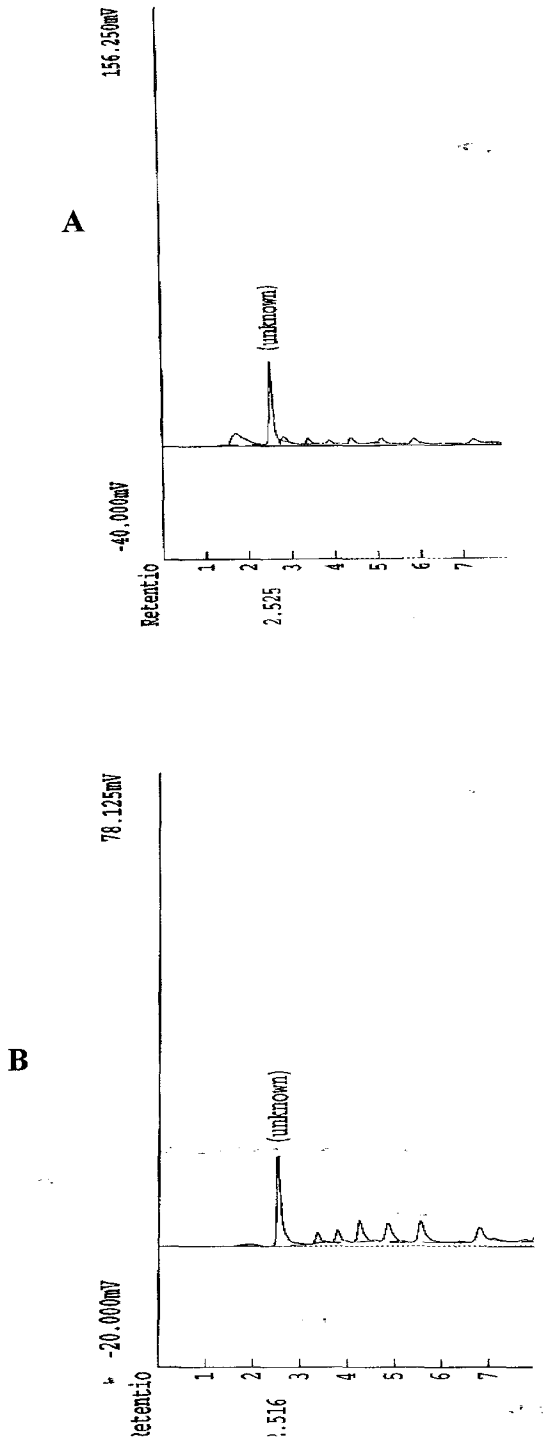


Figure 3.19. HPLC analysis of well-brine treated with laccase (A) and the control containing no enzyme (B).

However, the laccase-treated samples from the second set showed a difference; a peak at approximately 27 minutes was observed. This could be a humic substance formed as a result of oxidation of the organics in the brine samples by laccase. However, this peak could also be BBP because later investigations demonstrated the presence of this compound in all samples from the second set, as well as in solid bicarbonate and soda ash. The absence of this peak in the HRP, LiP and PPO treated samples of this set of HPLC data may be due to dilution rather than the absence of any BBP.

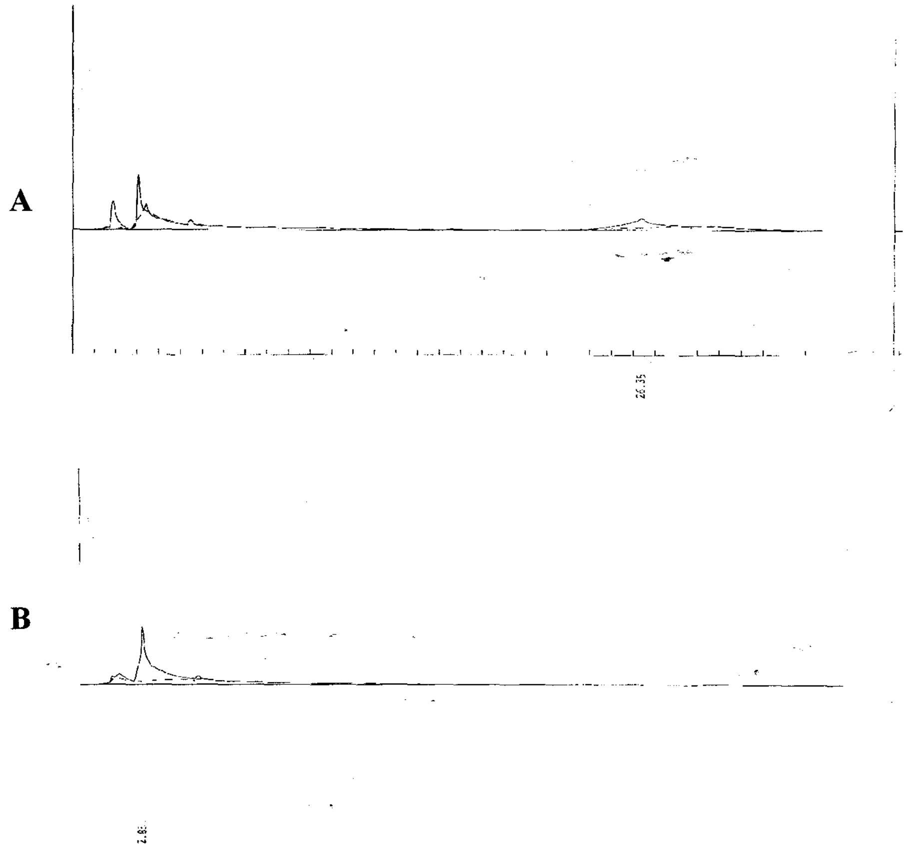


Figure 3.20. HPLC chromatograms of well-brine from second set treated with laccase (A) and the control containing no enzyme (B).

3.2.7.3 HPLC-MS analyses of enzyme treated samples

Mass spectral analysis was carried out on all the methyl ethyl ketone extracts and all the enzyme treated samples from the first set to determine the range of the molecular weights of the brine components. Analysis was also done on the laccase treated samples from the second set since a marked difference in retention times between components in the first and second set of laccase treated samples was observed. The molecular weights of humic substances typically range from a few hundreds to thousands of Daltons. Analysis of the methyl ethyl ketone extracts show the presence of compounds with molecular weights ranging from 200 to about 2000 Da which is characteristic of humic substances (Figure 3.21).

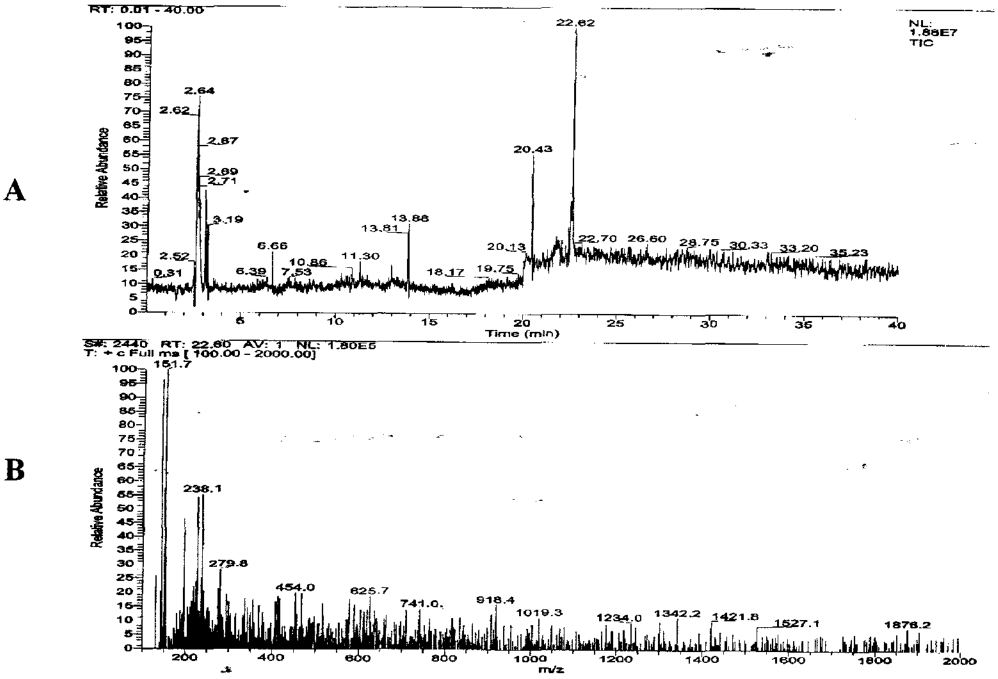


Figure 3.21. HPLC-MS chromatogram (A) and corresponding mass spectrum (B) of NS (PS3) extract after methyl ethyl ketone extraction and purification using a silica column.

In all the enzyme treated samples, i.e. HRP, LiP, PPO from the first set and Laccase from both sets a main peak at a retention time of approximately 3 minutes was seen. In the mass spectra of the methyl ethyl ketone extracts, a number of other major peaks at retention times of approximately 3, 15, 20, 23 minutes were seen (Figure 3.21). Retention times of minor components were also observed in the spectra of the enzyme treated samples (Figure 3.22). High molecular weights were observed in the methyl ethyl ketone extracts which is typical of humic substances (Figure 3.21).

HRP and LiP treated samples showed a slight increase in molecular weights compared to the untreated methyl ethyl ketone extracts (Figure 3.22). The increase in molecular weight could be a result of polymerization reactions since peroxidases have been reported to oxidize phenolic compounds in their catalytic cycle in the presence of hydrogen peroxide, generating phenoxy radicals which then react to form insoluble polymers independent of the enzyme. However, PPO treated samples from the first set and laccase treated samples from both sets showed a decrease in molecular weights (Figure 3.22). This decrease could be a result of enzymic degradation of the organic components in the brine samples.

No products peaks were observed in the first set of enzyme treated samples during HPLC analysis, but with the laccase-treated samples from the second set, HPLC analysis showed a peak at approximately 27 minutes which could be a humic product formed as a result of oxidations with laccase, since this enzyme is known to oxidize aromatic compounds leading to the formation of humic substances or this peak could also be BBP which was observed in methyl ethyl ketone extracts from the second set collected after the rainy season and the third set which was received just before the completion of this project and was also isolated from solid bicarbonate and soda ash samples.

This section of the work shows that some of the brine components are likely to be humic in nature since they are of high molecular weight which is typical of humic substances. The observation that certain of the brine components which can be converted by laccase and PPO enzymes, and some were apparently polymerized by HRP and LiP, indicates their aromatic, and probably phenolic nature.

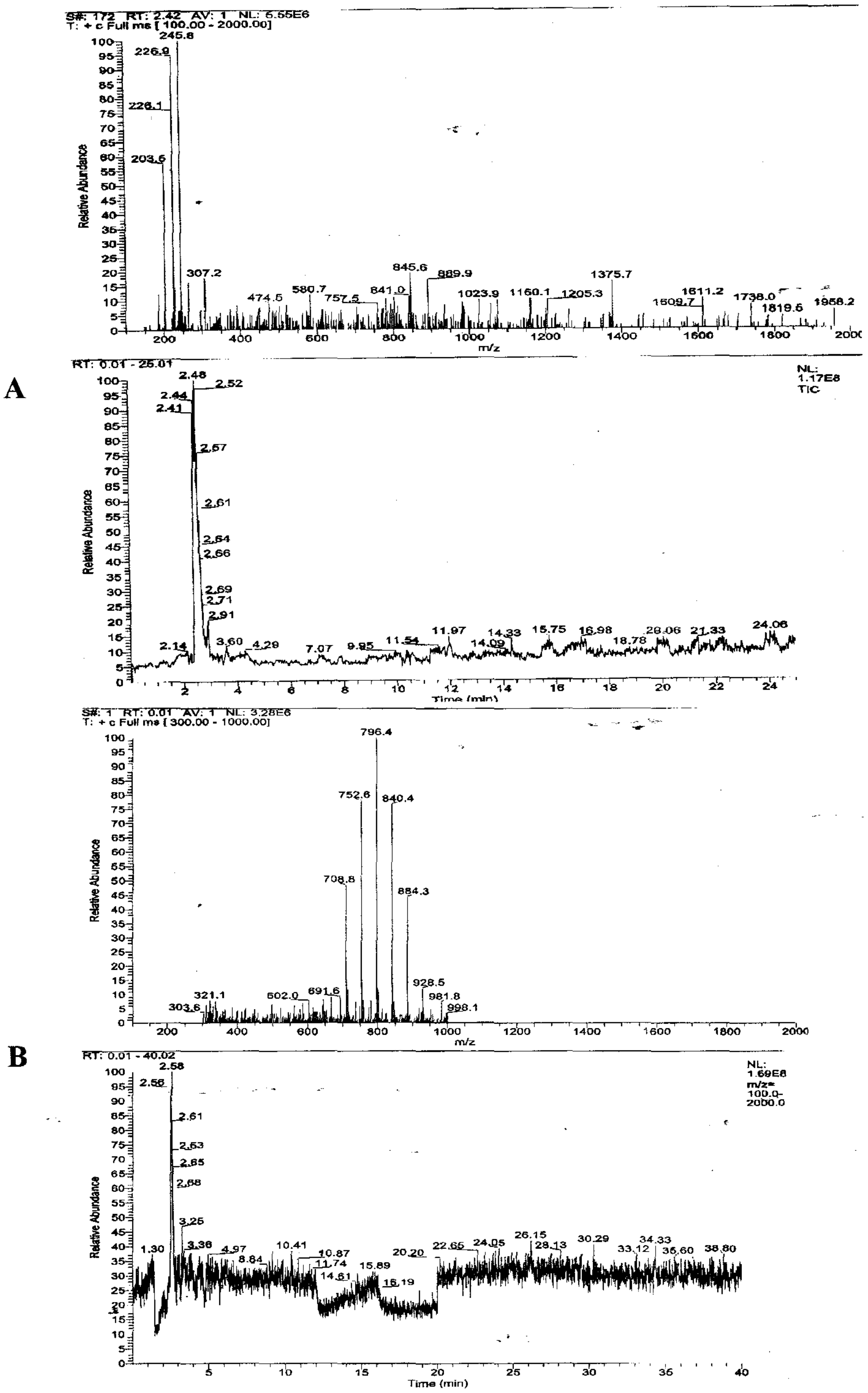


Figure 3.22. Mass spectra and corresponding HPLC-MS chromatograms of NS (PS3) extract treated with HRP (A) and PPO (B).

3.3 ANALYSES OF BICARBONATE AND SODA ASH SAMPLES

3.3.1 Solvent extraction

Solvent extraction was carried out on the bicarbonate and soda ash samples using methyl ethyl ketone to extract the organic contaminants present in the samples. The bicarbonate extract was yellowish in colour and the soda ash extract was clear.

3.3.2 UV analysis

UV analysis of the extracts from bicarbonate and soda ash samples were carried out to investigate the organic nature of the contaminating compound. The extracts gave their highest absorbance at a wavelength of 280nm, which indicates that the contaminating compound is aromatic in nature and also is further conjugated as indicated by the absorbance at higher wavelength (Figure 3.23).

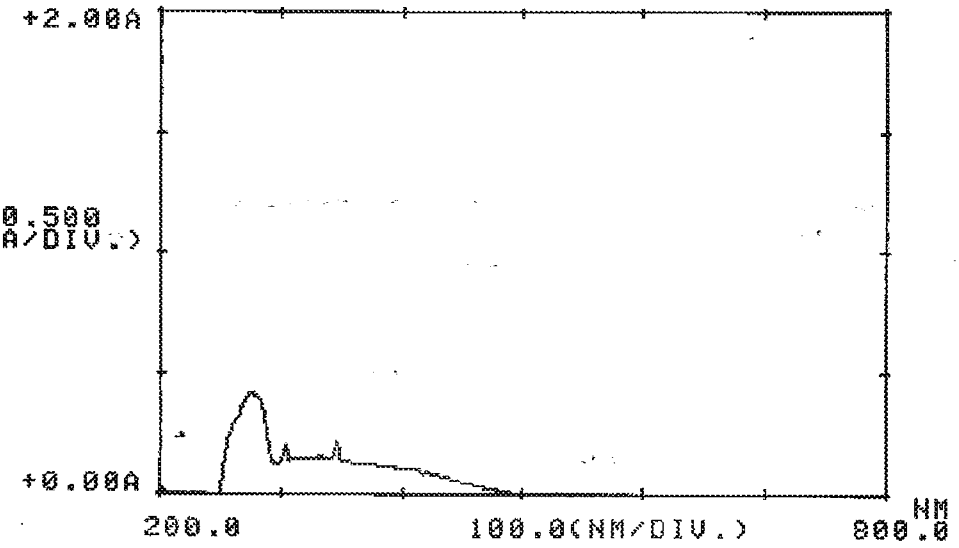


Figure 3.23. UV scan of extract from solid bicarbonate after purification.

3.3.3 NMR analysis

3.3.3.1 ^{1}H-NMR

^{1}H-NMR analysis of the isolated compound (compound A) from the bicarbonate and soda ash samples was carried out to determine the number of hydrogen atoms present in the isolated compound (Figures 3.24 and 3.25). The same profiles were observed in the extracts from both samples. NMR analysis has been used elsewhere for the determination of the structures of humic substances (Stevenson 1994; Saiz-Jimenez 1996). For example determination of the number of hydrogen atoms would enable working out the structure of compound A.

Impurities were observed in the ^{1}H-NMR spectra of the bicarbonate and soda ash extracts in the region of 0.5 to 2.5 ppm (Figures 3.24 and 3.25), and hence purification of the extracts, using a silica column, was carried out. The ^{1}H-NMR analysis was repeated to enable accurate determination of the number of hydrogen atoms. Again, the same profiles were seen for the bicarbonate and soda ash samples (Figures 3.25 and 3.26). The results obtained showed that compound A contains 20 hydrogen atoms. The number of hydrogen atoms and the chemical shifts in ppm are shown in Table 3.5.

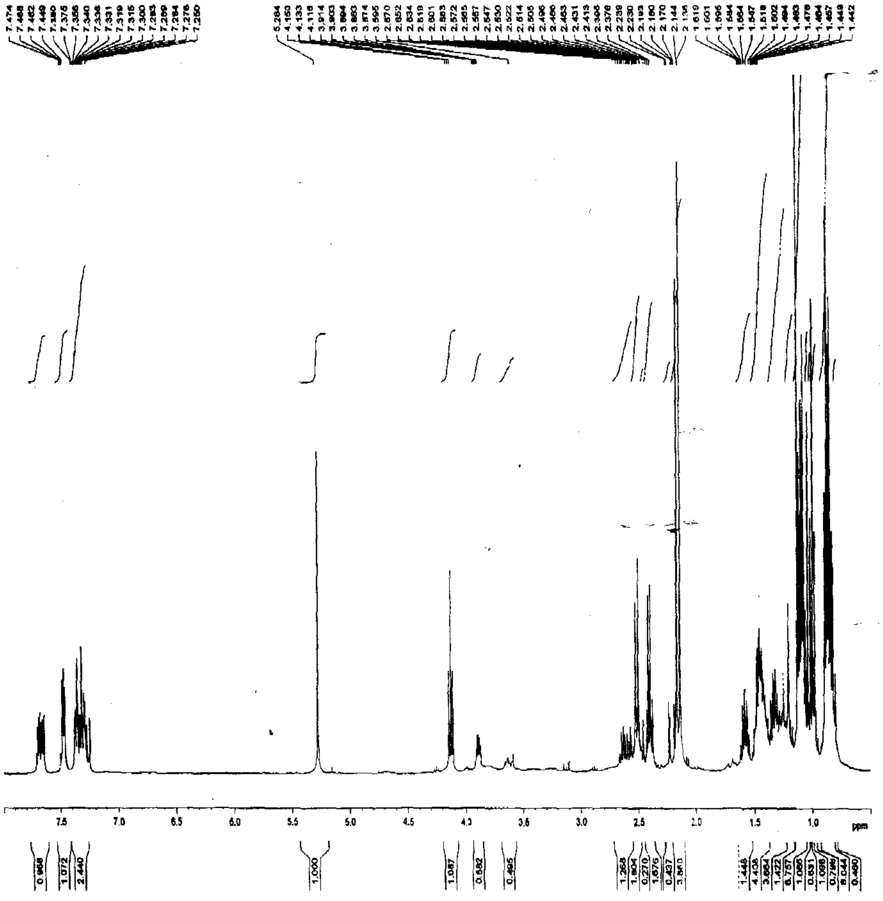


Figure 3.24. ¹H-NMR spectrum of extract of bicarbonate, unpurified.

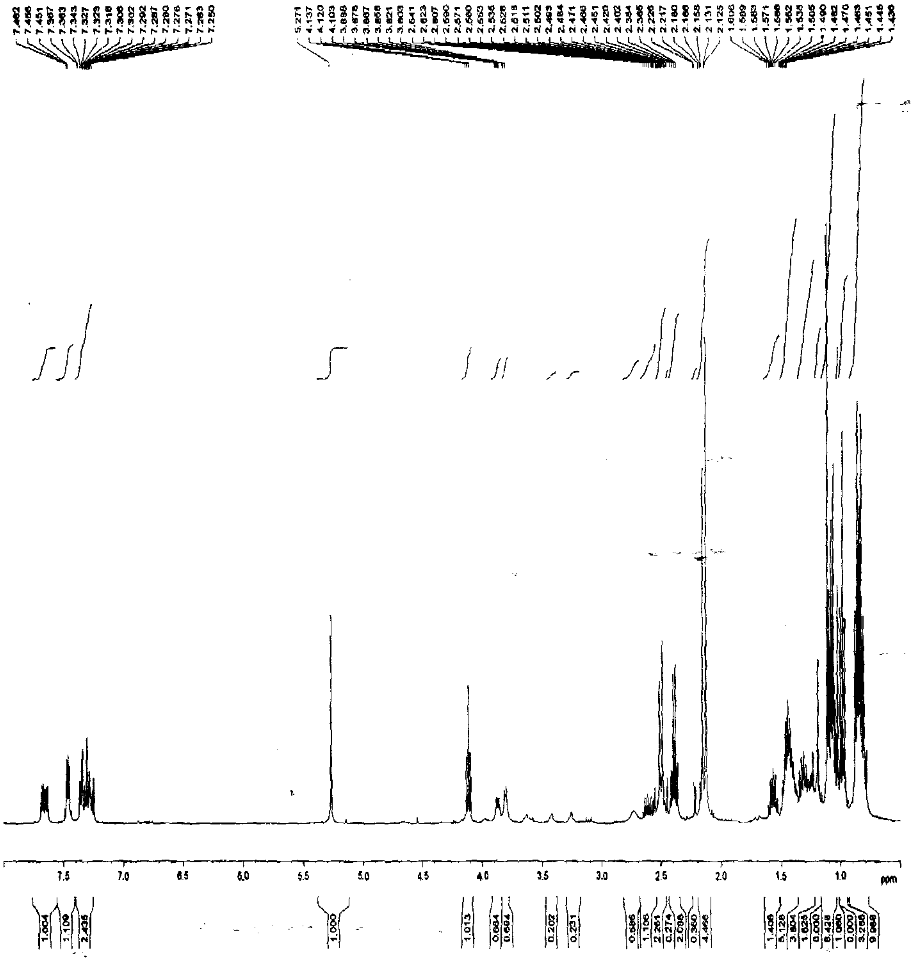


Figure 3.25. ^{1}H-NMR spectrum of extract of soda ash, unpurified.

Table 3.5. H^1-NMR analysis of compound A isolated from bicarbonate and soda ash samples.

Proton	**Chemical shift (ppm)***
(a)	t (0.9) 3H
(b)	m (1.35) 2H
(c)	m (1.6) 2H
(d)	t (4.2) 2H
(e)	s (5.3) 2H
(f)	m (7.3-7.8) 9H

*: s = singlet; m = multiplet; t = triplet

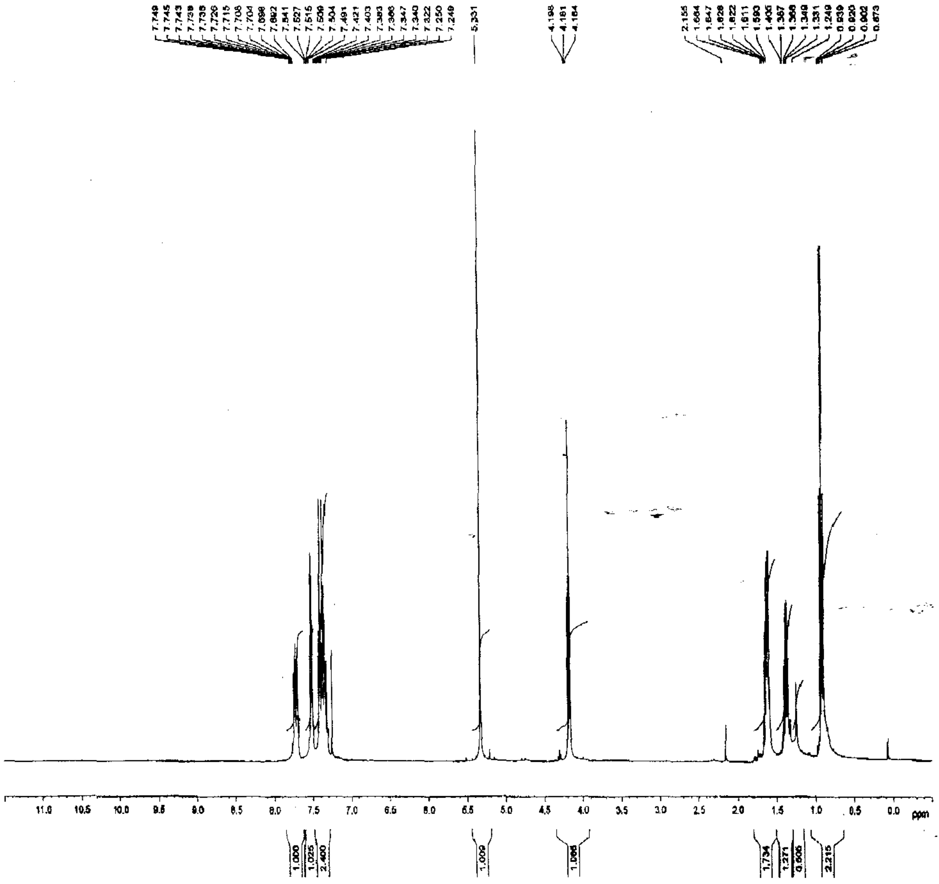


Figure 3.26. ^{1}H-NMR spectrum of extract of bicarbonate after purification.

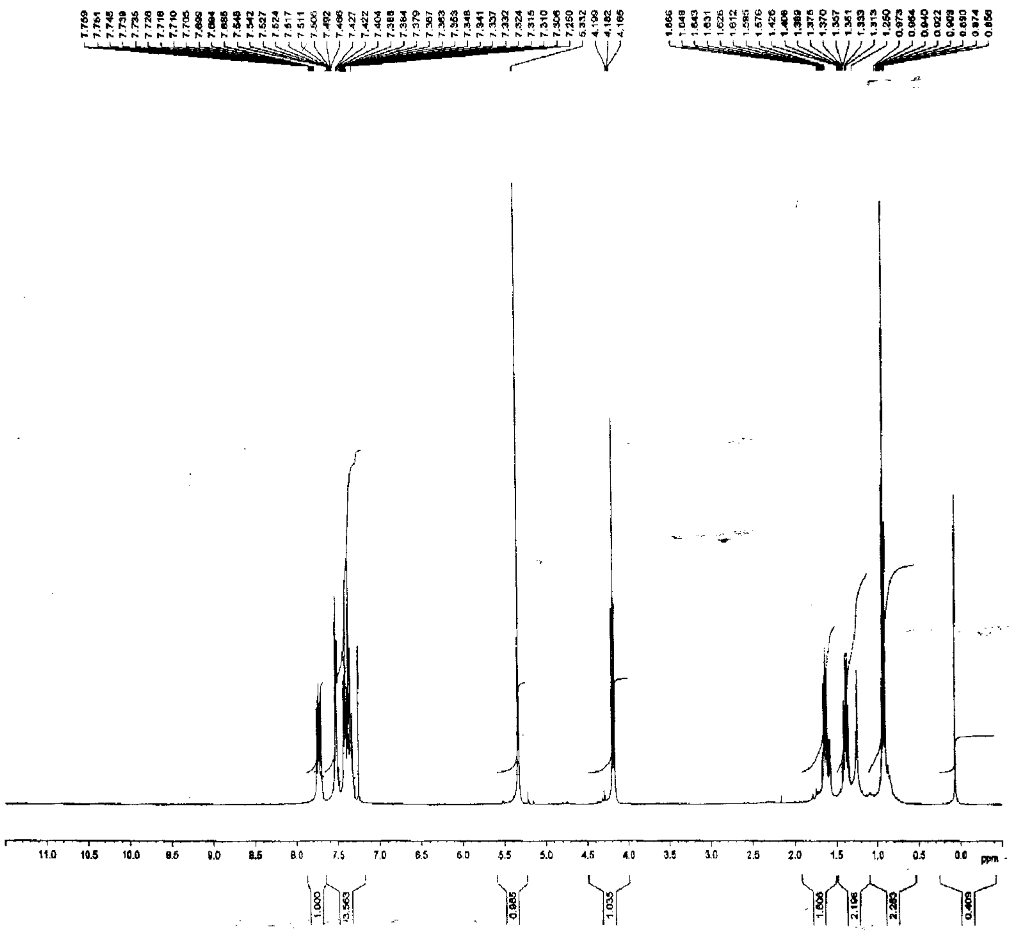


Figure 3.27. ^{1}H-NMR analysis of extract of soda ash after purification.

^{13}C-NMR analysis

^{13}C-NMR analysis was conducted to determine the number of carbon atoms present in the bicarbonate and soda ash extracts. This would help get a clearer understanding of the structure of the isolated compound. The same profiles were seen with both the extracts. The presence of 8 carbon atoms, in both the aliphatic region and the aromatic region, were observed (Figures 3.28 and 3.29). This suggests that compound A is aromatic with an aliphatic linkage in it.

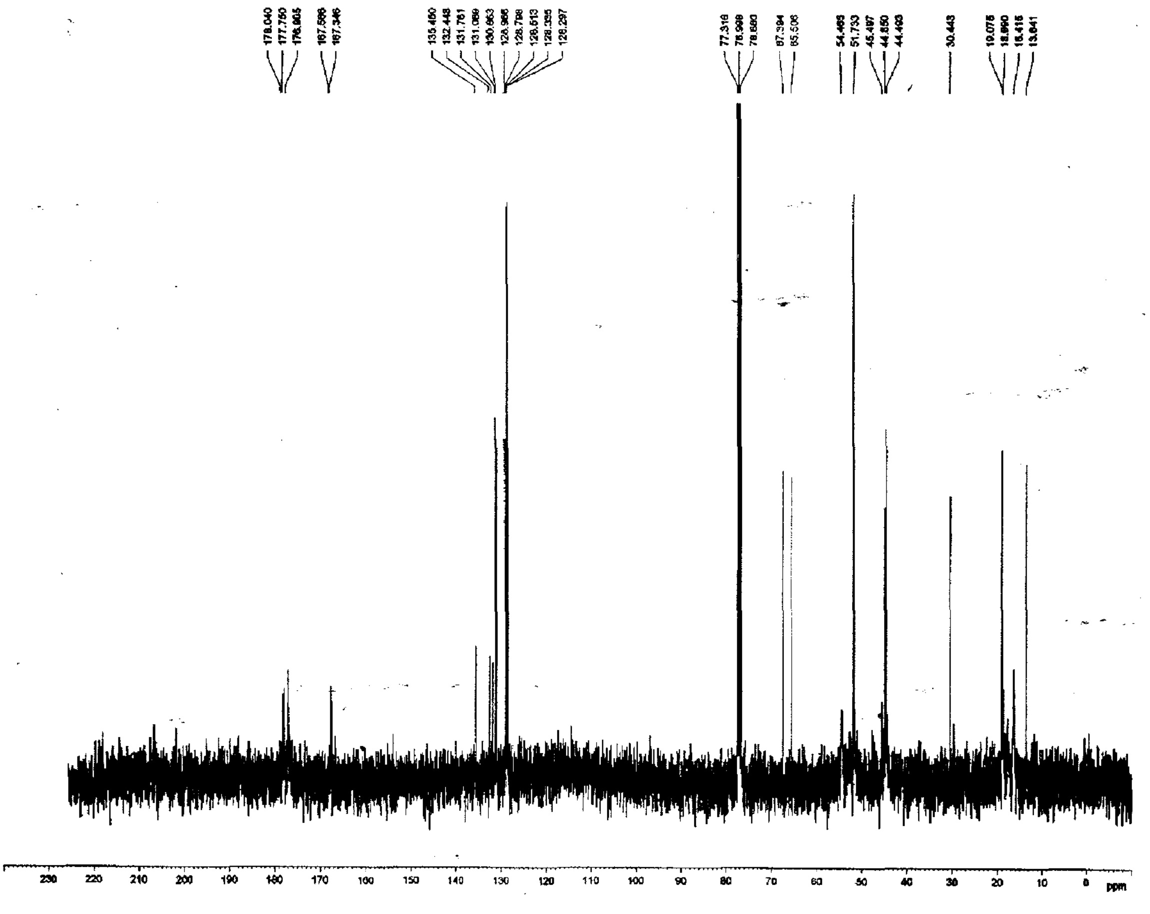


Figure 3.28. ^{13}C-NMR spectrum of extract of bicarbonate after purification.

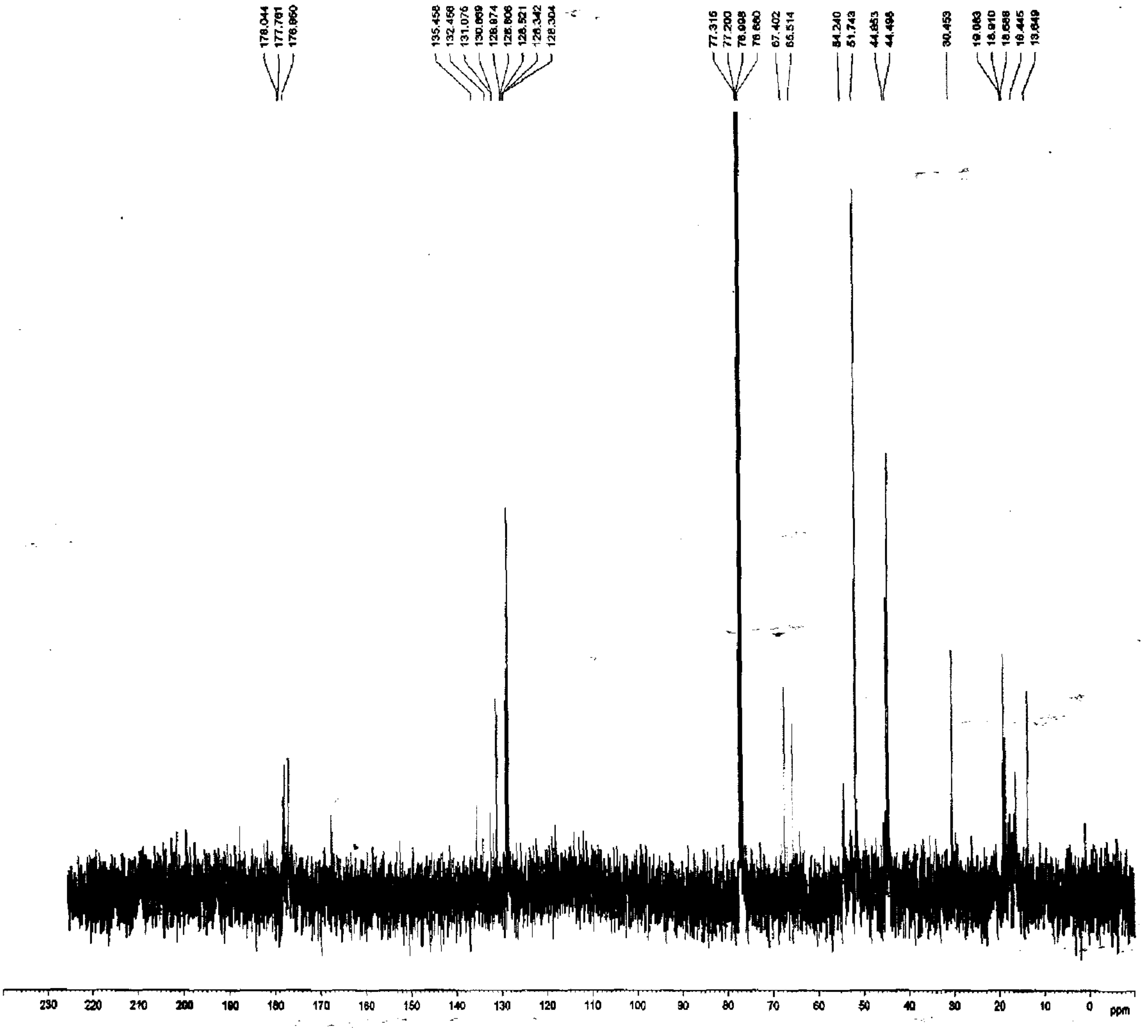


Figure 3.29. ^{13}C-NMR spectrum of extract of soda ash after purification.

3.3.4 IR analysis

IR analysis was carried out to determine functional groups present in compound A. Before purification of compound A, the IR spectrum showed the presence of a number of functional groups, however it was difficult to deduce a structure from the information obtained. Purification of compound A was carried out to eliminate the impurities present, in order to facilitate determination of the structure (Figure 3.30). IR analysis after purification gave a clearer spectrum indicating the functional groups present which suggested that the contaminating compound is an aromatic *ortho* disubstituted compound with the definite presence of an ester group (Table 3.6).

Table 3.6. IR analysis of compound A isolated from bicarbonate and soda ash samples.

Functional group	**IR stretching**
 $C=O$	1725 cm^{-1}
-C-O-	1200 cm^{-1}
-C-O-C	1040 cm^{-1}
Aromatic C-H	2900 cm^{-1}
1,2 distribution	700-800 cm^{-1}

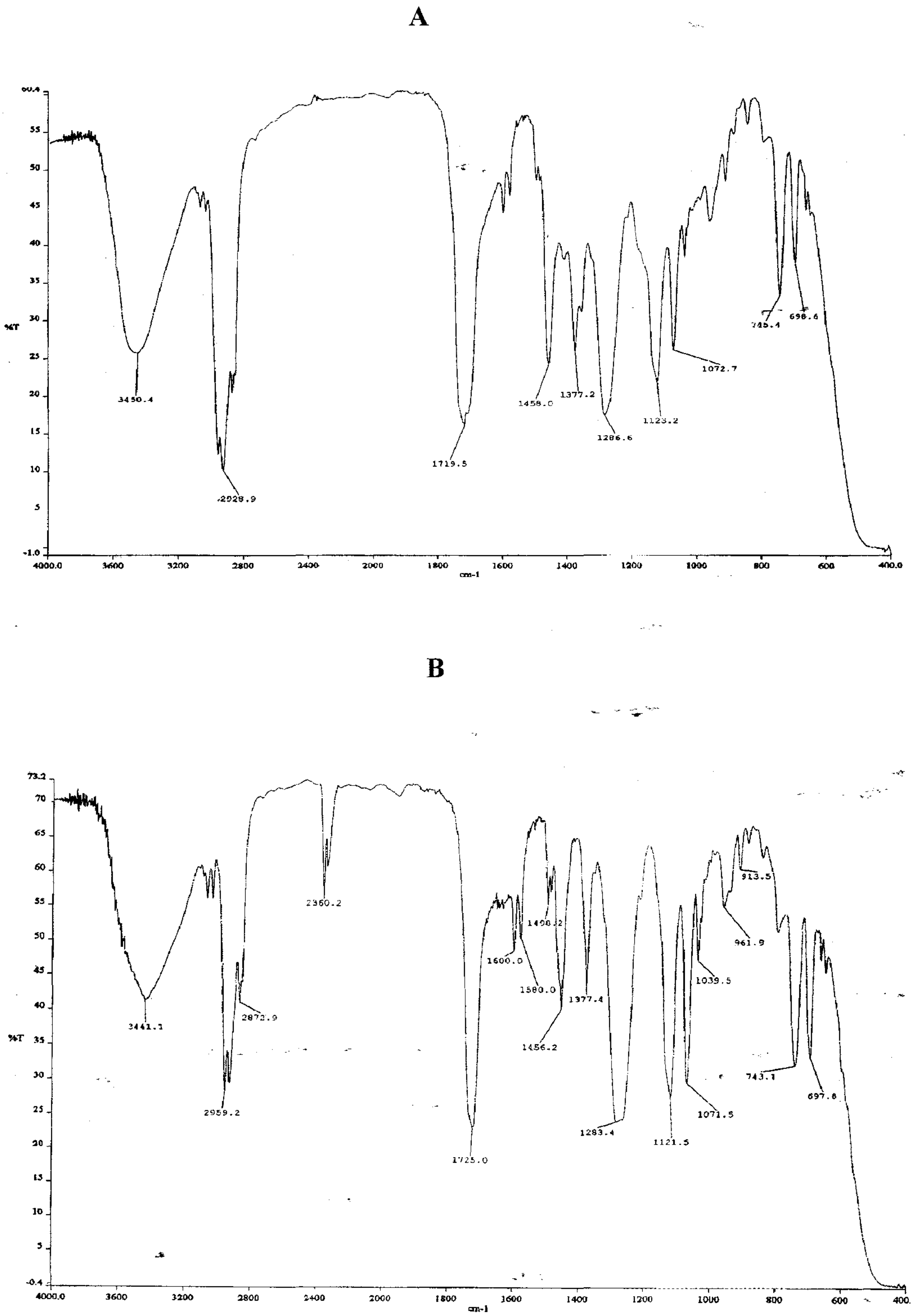


Figure 3.30. IR spectra of extract of bicarbonate before (A) and after purification (B).

3.5 HPLC analysis

HPLC analysis was carried out to confirm the presence of compound A in the bicarbonate and soda ash extracts. From the results obtained, compound A shows a retention time of approximately twenty seven minutes (Figure 3.31). This suggests that Compound A is polar in nature since polar compounds generally tend to elute later during an HPLC analysis run. This peak, with this retention time, was seen in all the brine samples collected after the rainy season but not in any of the brine samples collected before the rainy season.

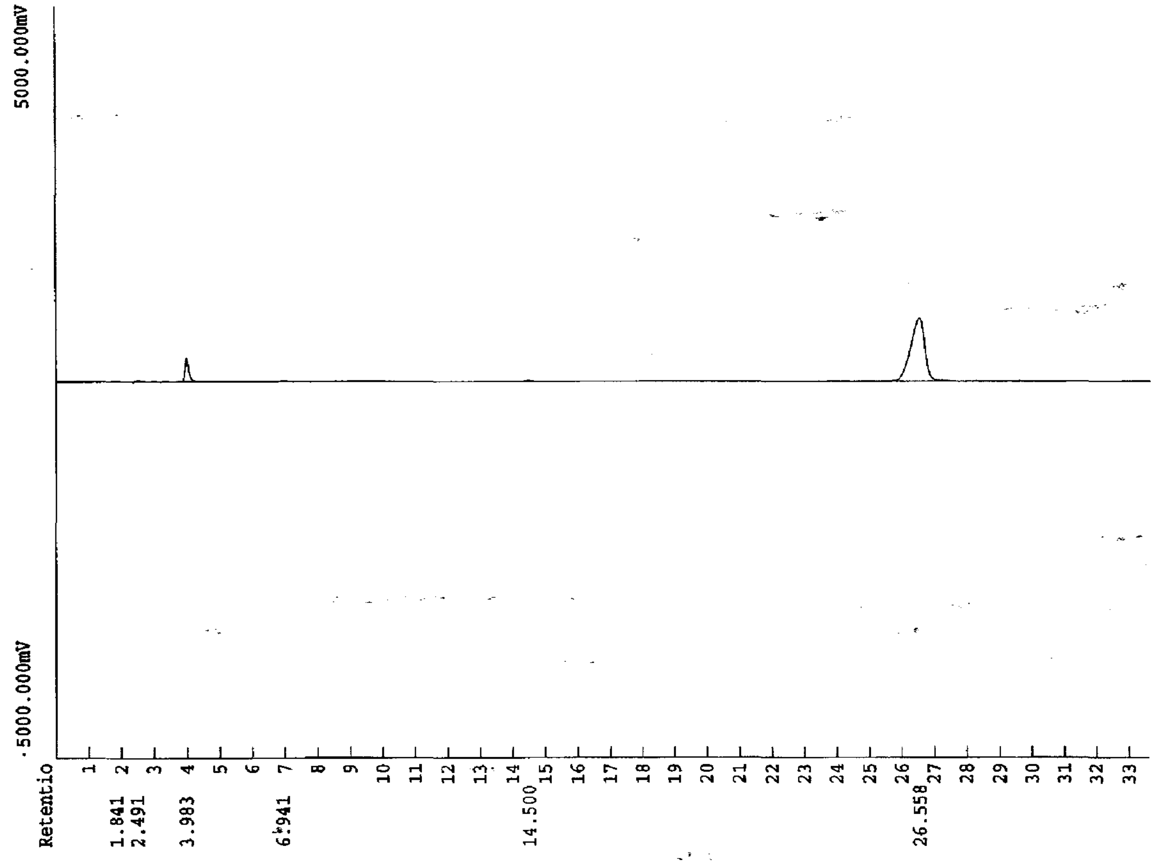


Figure 3.31. HPLC chromatogram of extract of bicarbonate after purification.

3.3.6 HPLC-MS analysis

HPLC-MS was carried out to determine the molecular weight of compound A. The LC-MS result showed a peak at a retention time of 2.48 and the molecular weight was 333 (Figure 3.32). The odd molecular weight number suggested that compound A might contain nitrogen, hence this isolated compound was sent to UCT for elemental analysis. However, the elemental analysis and GC-MS analysis confirmed that compound A is not nitrogen containing.

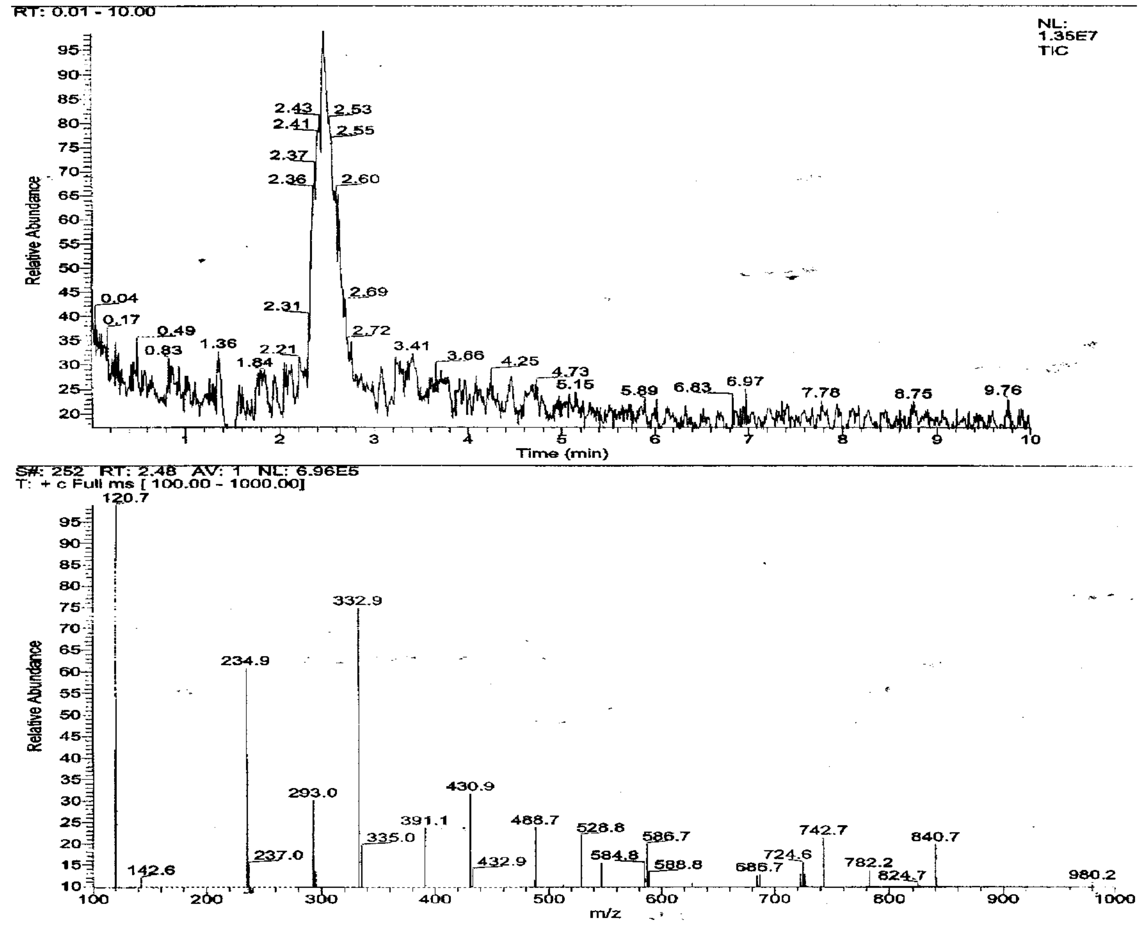


Figure 3.32. Mass spectrum of extract of bicarbonate after purification.

3.3.7 GC-MS analysis

For mass spectral analysis of compound A, GC-MS was used instead of LC-MS because of its higher sensitivity and speed. The GC-MS result (Figure 3.33) showed that the molecular weight of the compound was 312. The above result together with a detailed library search indicated that the contaminating compound was benzyl butyl phthalate (BBP) which has a molecular formula of $C_{19}H_{20}O_4$. This corresponds with the theoretical molecular weight of benzyl butyl phthalate. The structure of the compound is shown below in Figure 3.34 and proposed cleavage reactions of this compound are shown in Figure 3.35.

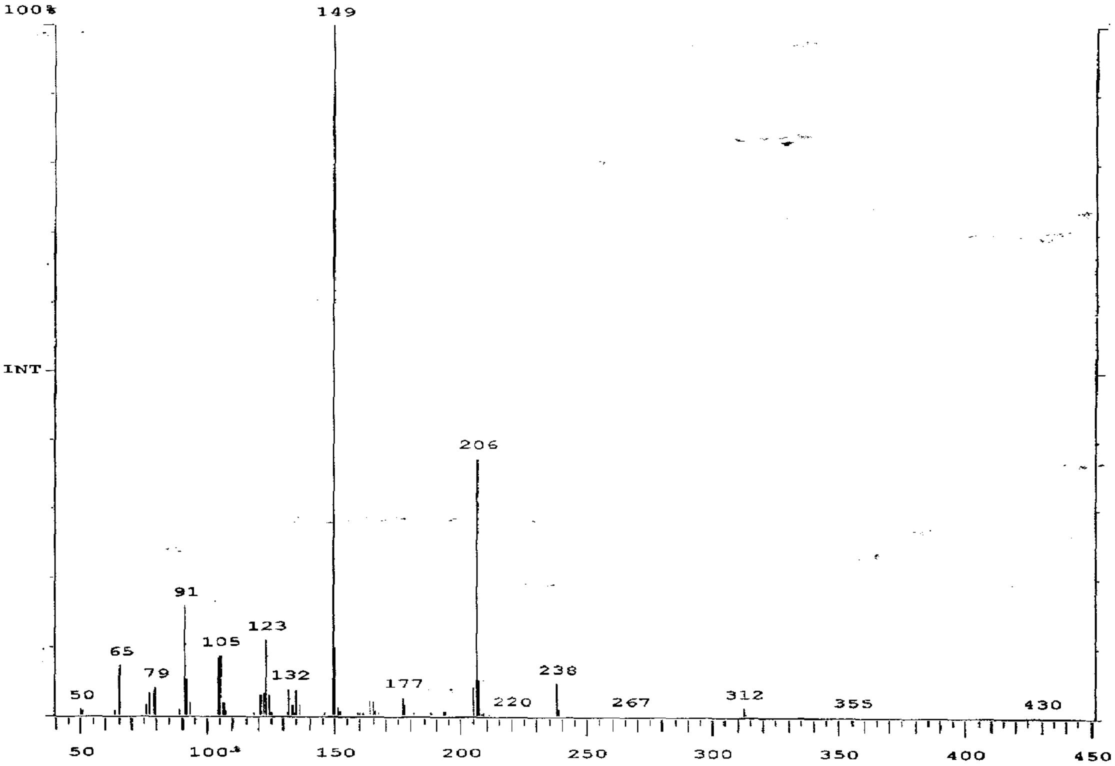
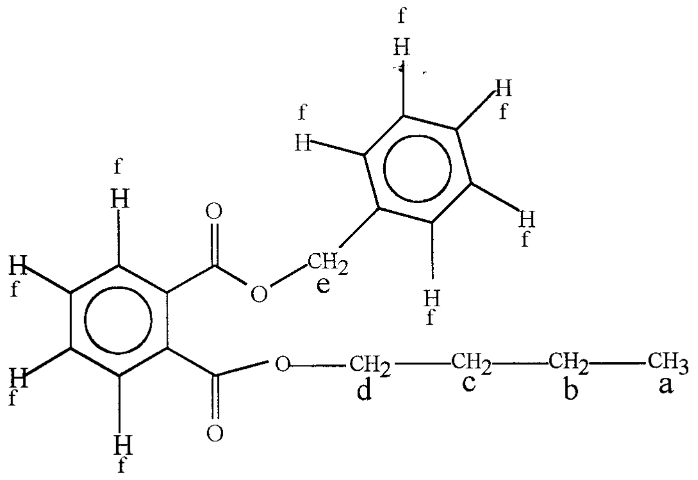
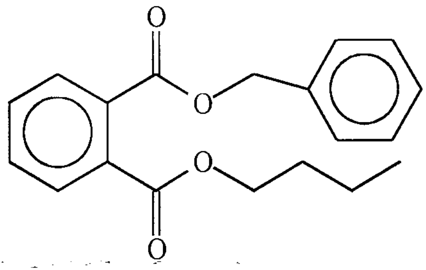


Figure 3.33. GC-MS of extract of bicarbonate after purification.



Compound A (determined from NMR studies)



Benzyl butyl phthalate

Figure 3.34. Proposed structure of compound A.

The molecular weights of the products from the suggested cleavage reaction correspond with the molecular weights seen in the GC-MS spectrum, confirming that compound A is benzyl butyl phthalate.

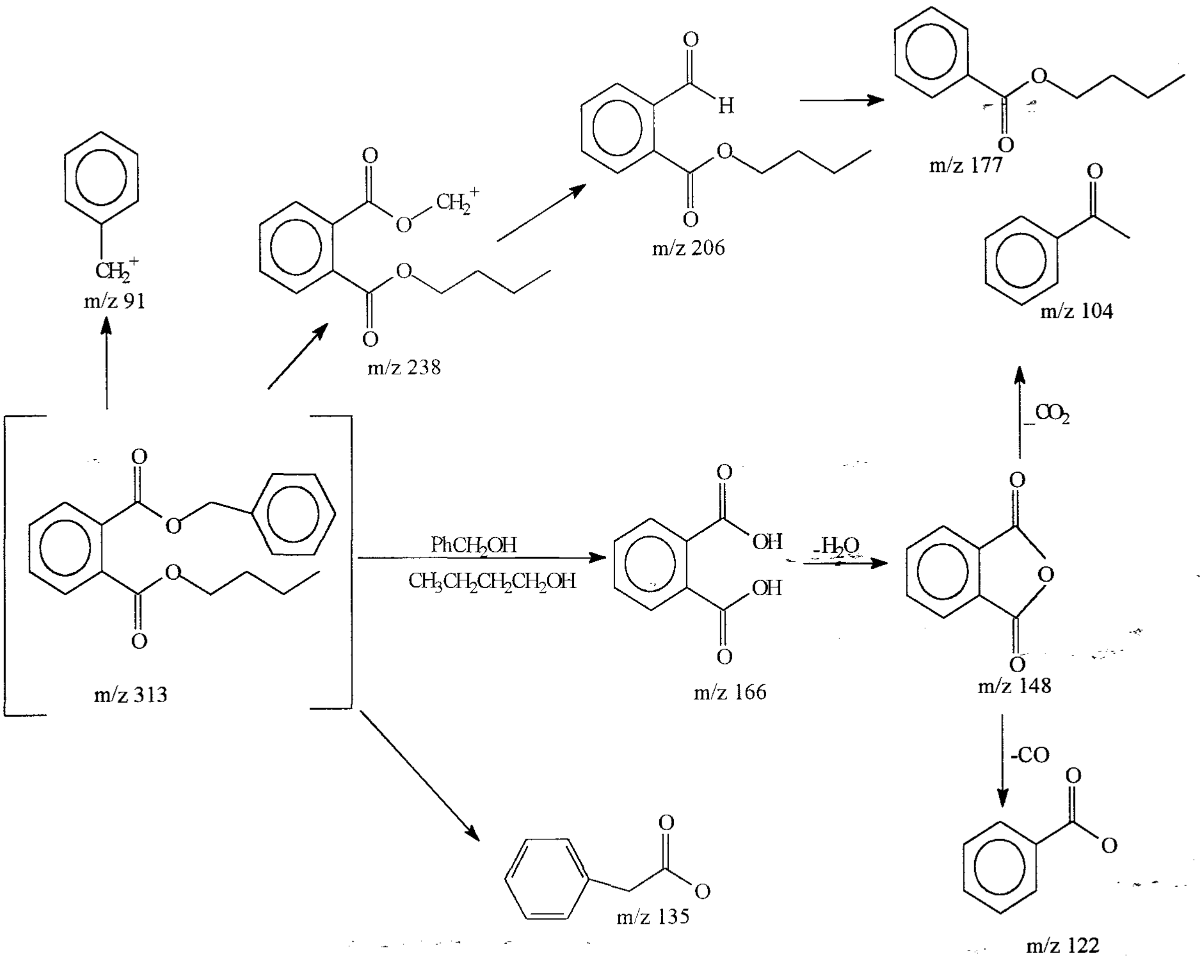
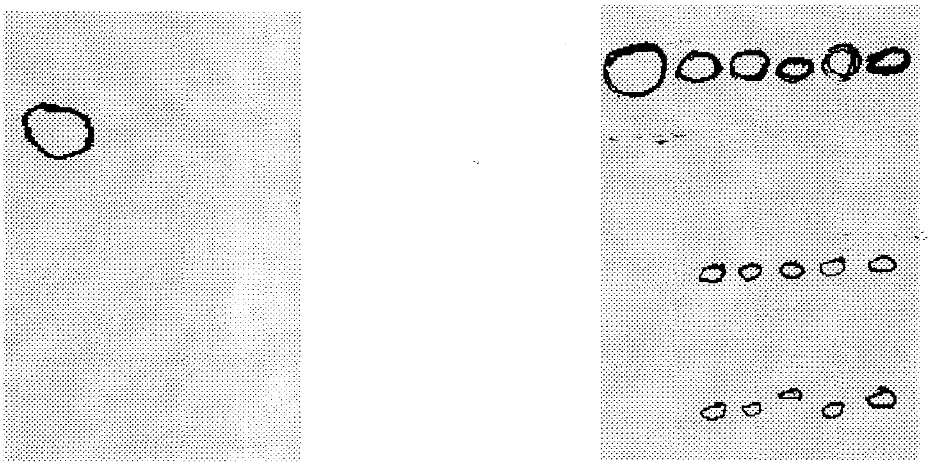


Figure 3.35. Suggested cleavage reactions of benzyl butyl phthalate.

3.3.8 TLC analysis

Thin layer chromatography (TLC) is a method used for identifying substances and testing the purity of compounds. TLC is a useful technique because it is relatively quick and requires small quantities of materials. TLC analysis of methyl ethyl ketone extracts, compared with compound A as a standard, was carried out to detect the presence of compound A in the brine samples, and to establish if this particular compound was present in the raw brine or if it was formed during the soda ash process. From the results obtained, it is clear that compound A was present in the brine samples, confirming that it is not formed during the processing (Figure 3.36).



Purified compound A

Figure 3.36. TLC plates showing compound A and compound A with brine samples well-brine (1), E6 (PS2) (2), NS (PS3) (3), X1 (S) (4) and bitterns (5).

3.3.9 Elemental analysis

From the micro analysis results obtained, only the elements C and H are clearly present (Table 3.7). The presence of N and S was not seen. This further confirms that compound A is BBP since BBP does not contain any nitrogen or sulphur.

Table 3.7. Elemental analysis of compound A.

Element	**Ratio**
C	73.31
H	6.89
N	0
S	0

3.3.10 Confirmatory analysis

HPLC analysis of commercially available benzyl butyl phthlate (BBP) was carried out to determine the retention time at which the compound would elute and also to see if compound A, and the component in the brine samples would elute at the same retention time as authentic BBP. The HPLC chromatogram in Figure 3.37 shows that the retention time of BBP is 29.35 minutes, under the run conditions used.

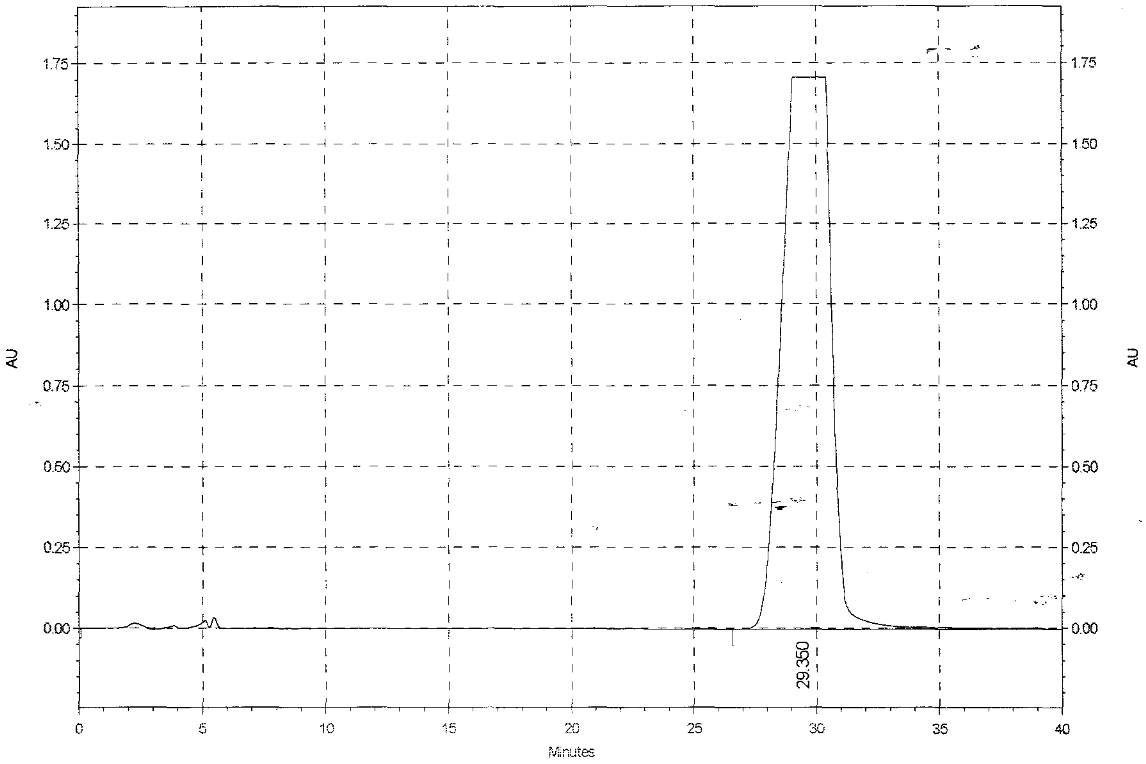


Figure 3.37. HPLC chromatogram showing retention time of 1mM benzyl butyl phthalate.

Under the same conditions, brine samples showed peaks with retention times of approximately 29 minutes, and after spiking with 1mM BBP, a peak with a much higher peak area at the same retention time was seen in all the brine samples, confirming the presence of BBP in the brine samples (Table 3.7). (The slight change in retention time from 26.558 to 29 minutes is because a different HPLC system was used. The Merck Lachrom system that was used for most of the HPLC analysis in this study was temporarily out of order and hence a Beckman System Gold machine had to be used).

Table 3.7. Peak areas of brine samples before spiking and after spiking with BBP.

Sample	Retention time before spiking	Peak area before spiking	Retention time after spiking	Peak area after spiking
W-Brine	29.1	1964043	29.05	3749305
E6 (PS2)	29.167	154288	29.383	8246322
NS (PS3)	29.167	328824	29.167	14089886
X1 (S)	29.617	77293	29.483	9707056
Bitterns	29.367	184695	29.333	687458

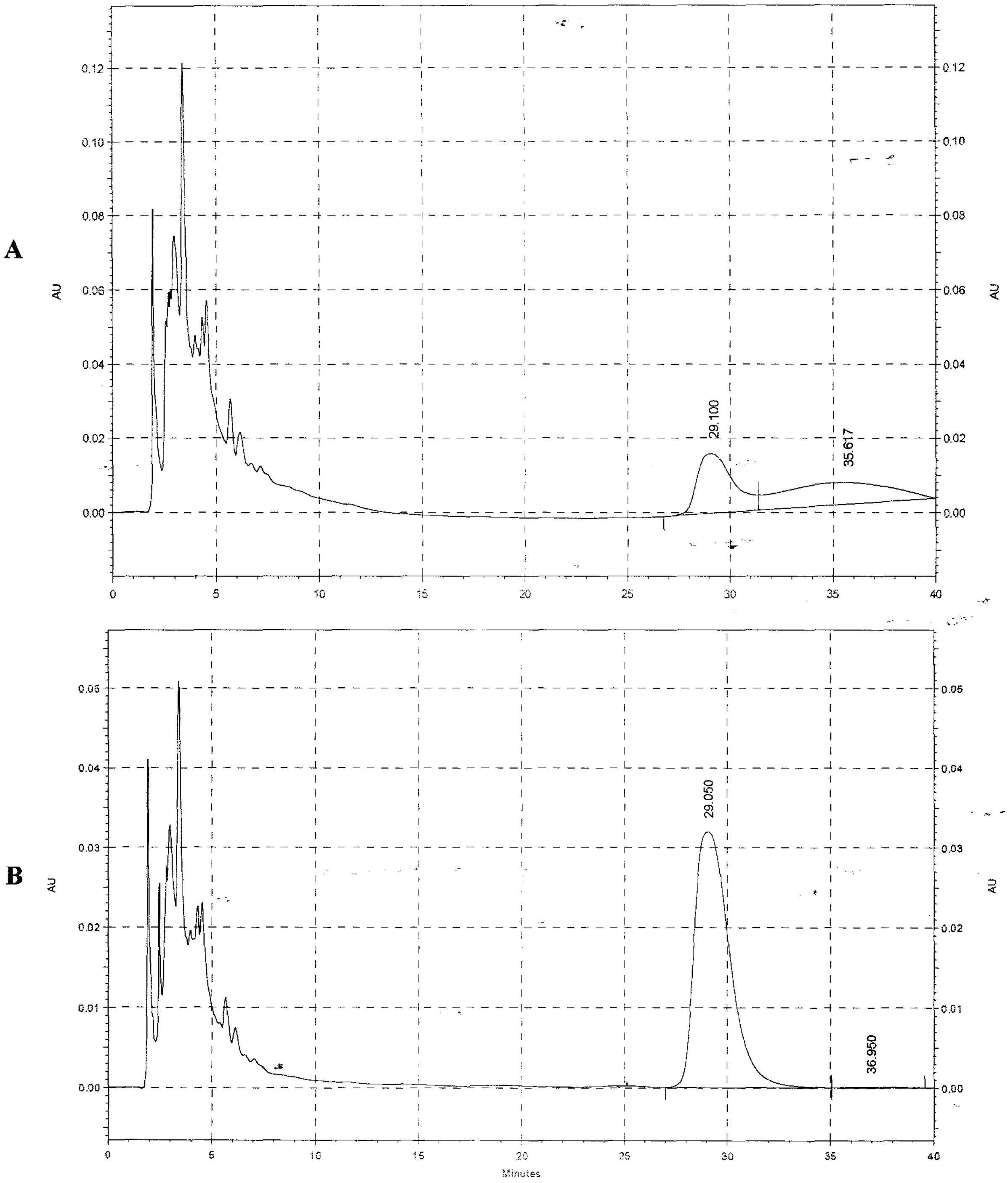


Figure 3.38. HPLC chromatograms of well-brine before (A) and after spiking (B) with BBP.

CHAPTER 4

CONCLUSION

This project has provided a suitable method for developing profiles and characterizing the organics in the brine, bicarbonate and soda ash samples and has allowed the identification of certain humic components.

Solvent extractions, dialysis and desalting were investigated as methods for isolating the organics from the brine samples, solid bicarbonate and soda ash samples, as these methods have also be used elsewhere for the isolation of humic substances (Aiken 1985). Solvent extraction was found to be the best method of extracting the organics in the samples. Desalting and dialysis were found not to aid the removal of excess salt from the brine in that these treatments did not result in improved separation of the organic components of the brine. The analytical methodology used for characterizing the brine samples, and the bicarbonate and soda ash samples, was High performance liquid chromatography (HPLC), which proved to be a suitable technique in that differences between the various brine samples were readily identifiable. Clear differences were also identified between the first set collected before the rainy season and the second set collected after the rainy season. The standard humic substances and the brine components showed similar retention times hence it can be suggested that the components of the brine were humic in nature.

UV analysis of the brine samples, bicarbonate, and soda ash samples clearly indicated that the components were aromatic in nature, since highest absorbances were detected in the range of 280 to 300nm. Carbohydrates and proteins were also found to be present in the brine samples, with much higher concentrations of both seen in the set of samples collected after the rainy season. According to Norton (1992), once rains comes, microorganisms in saltworks reestablish themselves. The higher carbohydrate and protein concentrations observed after rainfall could be a result of higher biological activity by the microorganisms in the solar ponds resulting in the production of high levels of exopolysaccharides and extracellular proteins. Phenolic substances are present in significant amounts in the brine and previous investigations have demonstrated that the discolouration of the final soda ash product i.e. the browning, could be a result of the presence of phenolic compounds (Rose 1993; Rose 1998). The total phenol and diphenol measurements showed high concentrations of both in the two sets of samples and after acidification a marked decrease in the extracted phenol and diphenol concentrations were observed in the two sets, but the second set of samples gave higher concentrations than the first set. The higher total phenol and diphenol concentrations observed in the second set after acidification could be a result of the presence of higher concentrations of phenolics in the brine after the rainy season.

Oxidative biotransformations were carried out with four oxidoreductase enzymes, on their basis of their known selectivities. Several enzymes including HRP, LiP, PPO and laccase have been used elsewhere for the oxidation of aromatic compounds (Aitken *et al.*, 1994; Perez *et al.*, 1996), and these enzymes are also involved in the polymerization of naturally occurring phenolics, resulting in the formation of humic substances (Randtke & Larson 1984). In this study, the enzymes, HRP, LiP, PPO and laccase were used as a novel means of demonstrating the presence of specific compounds known, on the basis of expert knowledge available in our laboratory, to be substrates for the enzymes. The results of the oxidation experiments with the methyl ethyl ketone extracts show that no products were formed in the first set using all four enzymes. In the second set of enzyme treated samples, PPO, HRP and LiP treated samples did not form any products. However, with the laccase-treated samples from the second set, a peak at a retention time of approximately 27 minutes was observed and this was not observed in the laccase treated extracts from the first set. This peak was later found to be present in all the methyl ethyl ketone extracts from the second set and in the bicarbonate and soda ash extracts. This compound, extracted from the bicarbonate and soda ash extracts, was identified as benzyl butyl phthalate. The peak present in the second set of laccase treated samples might be a humic product formed as a result of laccase oxidations since laccase has been reported to oxidize aromatic compounds resulting in the formation of humic substances or this peak could be BBP. The absence of this particular peak in the PPO, HRP and LiP treated samples from the second set might be due to dilution rather than the absence of BBP.

The solid bicarbonate and soda ash samples were extracted with methyl ethyl ketone to isolate the contaminating organic components present. The bicarbonate and soda ash extracts were oily in nature and had a sweet smell suggesting the presence of an ester. The bicarbonate extract was yellowish in colour suggesting the presence of N. The extracts were submitted for NMR analysis to obtain an indication of the nature of the compounds. Several different chemical and spectroscopic methods including NMR, IR and GC-MS have been used for the characterization of humic substances (Stevenson 1994; Saiz-Jimenez 1996). The NMR profiles of the bicarbonate and soda ash extracts showed exactly the same profile which suggests that the same contaminating compound is present in both of them. Purification using a silica column was carried out to get rid of unwanted impurities and with the aid of TLC analysis, the same compound was demonstrated to have been isolated from both the bicarbonate and soda ash. TLC analysis was used since it is a commonly used, rapid method for identifying substances and testing the purity of compounds. A thorough study of the isolated compound (compound A) from the bicarbonate and soda ash was carried out. Characterization of the compound was carried out by use of various spectroscopic methods such as NMR, IR, HPLC, LC-MS and GC-MS.

Compound A was identified as benzyl butyl phthlate (BBP) and spiking experiments with commercially available BBP confirmed that the isolated compound was actually BBP. This compound was not only present in the bicarbonate and soda ash but was also shown to be present in all the brine samples collected after the rainy season. This compound was not seen in any of the brine samples collected before the rainy season. This suggests that BBP occurs in the raw well brine and is not formed during the soda ash production process. Compound A was sent for elemental analysis to further analyse for the presence of nitrogen since from previous studies on the Sua Pan project, it was concluded that the contaminating compound might contain N. The elemental analysis results showed no N in the pure compound, again confirming that the isolated compound is BBP.

On this basis of the findings of this study, the contamination problem of the soda ash product apparently originates in the raw material which is the well brine. Thus, continuous monitoring of the well brine by analytical methods such as HPLC would be essential for the monitoring of the contamination problem.

Future research would involve establishing the origin of the BBP in the raw well brine by analysing for the presence of BBP in virgin or unused well brine over defined time intervals in order to pin point the exact stage at which the presence of BBP in the well brine is depicted.

APPENDIX 1

THE SOMOGYI-NELSON METHOD FOR CARBOHYDRATE DETERMINATION (Clark & Switzer 1977)

Materials

Reagent A

Na_2CO_3	25g
Potassium sodium tartrate	25g
$NaHCO_3$	20g
Na_2SO_4	200g
Distilled water	1000ml

Reagent B

$CuSO_4.5H_2O$	30g
Distilled water	200ml
Concentrated H_2SO_4	0.4ml

Reagent C

1 part B + 25 parts A - made fresh before use.

Arsenomolybdate reagent

Solution A

Ammonium molybdate	25g
Distilled water	450ml
Concentrated H_2SO_4	21ml

Solution B

$Na_2HAsO_4.7H_2O$	3g
Distilled water	25ml

Mix solutions A and B, make up to 500ml with distilled water and incubate at 55°C for 30 minutes and store in a dark bottle.

BRADFORD'S PROTEIN ASSAY (Bradford 1976)

Bradford's Protein Assay Reagent

Coomassie brilliant blue G-250	100mg
95% ethanol	50ml
Phosphoric acid	100ml
Distilled water	made up to 1000ml

The Coomassie blue is dissolved in ethanol before addition of phosphoric acid.

PHENOL ASSAY (Garcia Garcia *et al.* 1997)

Total phenol assay reagents

Folin-Ciocalteau's reagent (commercially available)

Sodium carbonate solution	100g
Distilled water	made up to 1000ml

Diphenol assay reagents

50% ethanol solution

100% Ethanol	50ml
Distilled water	made up to 100ml

5% Sodium molybdate solution in 50% ethanol

Sodium molybdate	5g
50% ethanol	made up to 100ml

APPENDIX II

BUFFERS FOR ENZYME OXIDATIONS

0.1M Phosphate buffer (pH 6.1)

KH_2PO_4	12.52g
K_2HPO_4	1.39g
Distilled water	made up to 1000ml

pH was adjusted to 6.1 with 0.25M NaOH

0.1M Sodium acetate buffer (pH 5.0)

Sodium acetate	13.6g
Distilled water	made up to 1000ml

pH was adjusted to 5.0 with glacial acetic acid

0.25M Sodium tartrate buffer (pH 2.5)

Sodium tartrate	57.52g
Distilled water	made up to 1000ml

pH was adjusted to 2.5 with 0.25M tartaric acid

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