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Our Hands, Our Minds, Our Destiny

APPLICATION OF *EICHHORNIA CRASSIPES* ROOT EXTRACT AS AN ACID-BASE INDICATOR

BY

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R125797A

Submitted in partial fulfillment of the requirements for the degree of

Bachelor of Science Honours in Chemical Technology

Department of Chemical Technology in the

Faculty of Science and Technology at the

Midlands State University

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June 2016

DEDICATION

This research report has been dedicated to the Chemical Technology profession as a whole and everything that is affected by it. Most of all; for all individuals, groups, mentors, advisors, lecturers, students, supervisors, colleagues, family, friends and foes alike; and above all else God omnipotent. My mother, uncles, aunts and relatives fall in into the above mentioned categories somehow; however theirs is a special mention.

For Chemical Technology aspects, production aspects, mechanical aspects, instrumentation aspects, innovation, professionalism, knowledge acquired, relationships forged, love, life, peace, joy, happiness, pleasure, sorrow, prosperity, ingenuity, trust, loyalty, faith, hope, vision, aspirations and everything in between and not explicitly mentioned.

ACKNOWLEDGEMENTS

Firstly I acknowledge my creator, almighty God for his guidance, protection, provision and favour throughout the course of my first degree. I would also like to pay due homage and appreciation to the impartation and wisdom given to me by my supervisors, family, friends and colleagues alike. This research report and the experiences gained in the period specified would not have been possible without the energy, resilience and continued effort of the following individuals:

- Mr. Nharingo T. (Project main supervisor)
- Mrs. Zinyama N. (Project co-supervisor)
- Dr Guyo U. (lecturer)
- Philip Machinya
- Fellow students

ABSTRACT

Synthetic acid-base indicators used in titrimetric analysis are highly toxic and harmful to human beings and aquatic organisms. Due to the presence of anthocyanins, some plant extracts have been proved to exhibit solvatochromism properties that can be exploited for use as acid-base indicators in titrimetric analysis. In this research *Eichhornia crassipes (E. crassipes)* root extract was applied as an acid-base indicator in titrimetric analysis in comparison to standard synthetic acid-base indicators. Solvents of different polarities were used to optimise pigment extraction. *E. crassipes* root extracts were characterised with HPLC, FTIR and GC-MS and the best extracting solvent was determined. The root extract was tested for the presence of flavonoids and anthocyanins. The root extract was applied in titrimetric analysis for four neutralisation titrations- strong acid against strong base, strong acid against weak base, weak acid against strong base and weak acid against weak base. The best extracting solvent was found to be 0.1 % HCl (v/v) in ethanol by considering the peak areas of the components present in HPLC chromatograms. The root extract tested positive for the presence of flavonoids and anthocyanins. Functional groups that were found to be present were associated with carbohydrates, flavylium ion, phenols and anthocyanins. Cyanidin and perlagonidin derived anthocyanins were predicted to be present due to the presence of selected molecular ions of *m/z* 287 and 449, and 579 respectively. One way ANOVA showed that there was no significant difference between the mean titre volumes for titration of HCl- NaOH and CH_3COOH- NH_4OH. The end-points obtained from four neutralisation titrations using *E. crassipes* root extract were comparable to those obtained using methyl red, phenolphthalein and bromothymol blue. Based on the results in the research, *E. crassipes* root extract proved to be an effective substitute of phenolphthalein and bromothymol blue for strong acid-strong base and weak acid-weak base titrations respectively, hence it is recommended.

DECLARATION

I, **Nhapi Courage T.**, hereby declare that I am the sole author of this dissertation. I authorize Midlands State University to lend this dissertation to other institutions or individuals for the purpose of scholarly research.

Signature ……

Date …….

APPROVAL

This dissertation entitles "**Application of *Eicchornia crassipes* (water hyacinth) root extract as an acid-base indicator**" by **Nhapi Courage T.** meets the regulations governing the award of the degree of **Bachelor of Science in Chemical Technology Honours** of the Midlands State University, and is approved for its contribution to knowledge and literal presentation.

Supervisor ……

Date …….

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LIST OF ABBREVATIONS

HPLC	High performance liquid chromatograph
GC-MS	Gas chromatography- mass spectrometer
FTIR	Fourier transform infrared spectrometer
NTP	National Toxicology Programme
UV-Vis	Ultra violet- visible spectrometer
H^{+}NMR	Proton nuclear magnetic resonance
ZimAlloys	Zimbabwe Alloys
BAW	Butan-1-ol: acetic acid: water
NIST	National Institute of Standards and Technology
ANOVA	Analysis of variance
t-Test	Test statistics
ACE	Associated Chemical Enterprises
MSU	Midlands State University

CHAPTER ONE

INTRODUCTION

1.0 Background

Industrialisation and increase in use of synthetic products in chemical analysis has resulted in environmental pollution that poses health effects to both human beings and aquatic organisms for example the use of synthetic standard acid-base indicators in titrimetric analysis [1]. Synthetic acid-base indicators are highly toxic and harmful [3]. Effluents with synthetic acid-base indicators are non-biodegradable under normal conditions and sometimes degrade under anaerobic conditions producing more harmful compounds [4].

Many researches show that convectional synthetic acid-base indicators have carcinogenic, mutagenic and genotoxic effects to aquatic organisms and humans [6]. Methyl red causes gastrointestinal and respiratory tract irritation, phenolphthalein causes chronic ulcerative colitis, thinning of the intestinal wall and loss of normal mucosal pattern of terminal ileum and phenol red is harmful on the central nervous system and heart resulting in disrythmias, seizures and coma [5]. Hence the use of *E. crassipes* root extract as a substitute for the harmful synthetic acid-base indicators will reduce health effects associated with synthetic indicators.

Various waste treatment methods have failed to completely remove synthetic dyes from aqueous solutions [6]. Due to the problems associated with the use of synthetic acid-base indicator, there is need to look for alternative forms that can substitute synthetic indicators. Natural acid-base indicators from plants have the potential of becoming alternatives since there are less toxic, cheap, easily available and eco-friendly [3,7].

Eichhornia crassipes (water hyacinth) has been termed an invasive aquatic macrophyte due to its rapid growth and ability to adapt to different aquatic environments [8]. Its population can

double within 5-12 days leading to rapid generation of biomass within a short space of time [10]. The presence of *E. crassipes* in water bodies has social, economic and environmental impact as it causes reduced population of aquatic organisms and phytoplankton due to low concentration of dissolved oxygen. It also increases vector-borne diseases, boating and fishing problems, results in difficulty electricity generation, rapid water loss through evapotranspiration and siltation [10]. Hence finding a better use for the plant like the use of the root extract in titrimetric analysis will reduce or eliminate the effects associated with the plant.

Plant researches have shown that plants with beautiful colours have anthocyanins which are pH sensitive. These include *Bougainvillea spectabilis*, *Ipomea nil*, *Opuntia ficus indica* and *Ixora coccinea* [11] only to mention a few, that have been applied as acid-base indicators in titrations. *E. crassipes* has purple and violet flowers and its roots have been observed to show solvatochromism properties, hence can be investigated for use in titrimetric analysis [12].

The aim of the research was to find an alternative acid-base indicator which is environmentally friendly by using *E. crassipes* root extracts, thereby substituting harmful synthetic acid-base indicators and making use of the problematic plant [13].

1.1 Aims

- To optimise the extraction of pigments from *E. crassipes* roots.
- To apply the pigment as an indicator in acid-base titrations in comparison to standard synthetic indicators.

1.2 Objectives

- To extract *E. crassipes* root pigments using solvents of different polarities.
- To characterise the components of the crude extract using high performance liquid chromatography (HPLC), gas chromatography mass spectrometer (GC-MS) and Fourier transform infrared spectrometer (FTIR).
- To determine the end-point using extracted pigment in comparison to standard synthetic acid-base indicators in volumetric analysis.

1.3 Problem statement

Synthetic acid-base indicators that are currently used in titrimetric analysis are toxic to the environment, humans and aquatic organisms. They are carcinogenic and mutagenic to living organisms and there are also expensive, Shih *et al* [4], hence there is need for substituting them.

E. crassipes is a problematic, invasive plant that has social, economic and environmental problems to both humans and aquatic organisms [15]. Physical, chemical and biological methods have failed to eliminate the plant due to its high proliferation rate and drug resistance. There is massive generation of biomass that lowers oxygen concentration in water, prevent sunlight to phytoplankton, increase vector-borne diseases, cause problems in hydroelectric power generation systems, interfere with navigation of water flow, decrease water quality, cause decline in temperature, pH, nutrient level, increase in transportation costs, water loss through evapotranspiration and siltation [10]. Some of these problems have caused death of fish in Lake Chivero, Harare and Lake Victoria, Kenya [10]. Due to the problems associated with *E. crassipes* there is need to make use of the plant e.g. applying the root extract as acid-base indicator in titrimetric analysis.

1.4 Justification

The use of *E. crassipes* root extract as natural acid-base indicator in titrimetric analysis had provided an alternative to the use of toxic synthetic acid-base indicators [17]. Natural indicators are cheap, less toxic and environmentally friendly as compared to the synthetic indicators [18]. Once *E. crassipes* root extract have been established to show the indicator properties, they can find a broad spectrum of application including the textile industry.

Application of *E. crassipes* root extract as an acid-base indictor will make the problematic plant useful, thereby substituting toxic synthetic acid-base indicator. According to Zhang *et* al [19], *E. crassipes* have high proliferation rate, therefore there is always rapid generation of biomass that can be extracted and used in titrations.

Due to the effects associated with synthetic standard acid-base indicators, green chemistry can be applied to reduce or to eliminate production of toxic products, generation of large volumes of waste and use of too much energy [20]. The research seeks to eliminate or substitute use of synthetic acid-base indicators with *E. crassipes* root extract for sustainable development.

CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

The chapter focuses on the use of acid-base indicators in titrimetric analysis, synthetic acid-base indicators and their effects to humans and aquatic organisms. Review of studies that were carried out on the use of plant extracts as acid-base indicators and application of green chemistry for sustainable development are also discussed in this chapter. The sections also covers problems associated with *E. crassipes* and its possible application in the research and application of plant extracts in acid-base titrations.

2.1 Acid-base indicators and their use in volumetric analysis

A pH indicator is a halo-chromic chemical compound that is added in small amounts to the solution or analyst sample so that the acidity or alkalinity can be determined [18]. Bart and Pilz [21] defined pH indicators as chemical detectors of hydronium ions (H_3O^+) or hydrogen ions (H^+) in the Arrhenius model that changes colour depending on the concentration of these ions. There are usually weak acids or bases which have their acid or conjugate base forms with different colours due to differences in their absorption spectra [22]. Acid-base indicators changes colour over a range of pH e.g. methyl red pH range is from 4.2 to 6.2 and phenolphthalein from 8.3 to 10. The ability of the indicators to donate or accept electrons results in change of colour with change in pH (acidity or alkalinity).

Volumetric analysis is quantitative determination of the unknown substance by determining the volume of a solution of known concentration that reacts quantitatively with a measured volume of solution to be determined [5]. In volumetric analysis, pH indicators are used to determine the end-point of the reactions.

2.2 Synthetic acid-base indicators (dyes) and their effects to humans and aquatic organism

Organic dyes from industrial effluents are pollutants which makes it difficult in waste water treatment systems [6]. These dyes and their degradation products are toxic, mutagenic and carcinogenic to humans and aquatic organisms. This is due to the presence of carcinogens such as benzidine, naphthalene and other phenolic compounds which can remain in the environment for a long period of time [6]. Many synthetic dyes are highly soluble in water hence can be discharged with waste water in water bodies. Most of the dyes are resistant to normal treatment of waste water process i.e. bio-treatment of the effluents is ineffective due inability of the dye or acid-base indicator to degrade aerobically while anaerobic degradation yields carcinogenic aromatic amines as by-products [27].

Most acid-base indicators and dyes used in the pigmentation and titration processes in many industrial applications are derived from azo dyes as reported by Mahmoud *et al* [28]. Azo dyes are xenobiotic compounds which have one or more azo linkages and aromatic rings. They constitute the largest class of dyes with greatest colour varieties. There are electron-deficient xenobiotic compounds due to the presence of the azo linkage (N=N) and sulphonic (SO_3^-) groups which are electron withdrawing groups that generate electron deficiency within the molecule making the dye less susceptible to bio-degradation [31]. Azo dyes include methyl red, methyl orange, acid orange 6 and acid orange 7. In the human body synthetic azo compounds are metabolized by enzyme azoreductase in the intestines and liver to aromatic amines. Aromatic amine, benzidine induce urinary bladder cancer in humans and tumours in some experimental animals. Under anaerobic conditions, azo dyes can be reduced to aromatic amines which are toxic, mutagenic and carcinogenic [31]. Examples of azo dyes and other dyes that are applied as acid-base indicators in titrimetric analysis are shown in Table 1.1 together with health effects associated with them.

Due to chemical structures of dyes, they resist fading on exposure to UV light, water and many chemicals which makes them not easily degraded in the environment. Wastewater containing dyes causes intense colouration to water bodies which reduce sunlight transmission into water and also decreases solubility of gases in water hence affecting aquatic organisms and plants [32]. This occurs by preventing penetration of sunlight thereby preventing photosynthesis and other algal based biological treatment systems.

Coloured wastewater in the ecosystem causes aesthetic pollution, eutrophication and perturbations in aquatic life. Isiuk *et al* [25] and Luo *et al* [35] postulated that dyes are difficult to remove from waste water because they are stable to light, heat and oxidising agent, hence they are not easily degradable. These dyes require adequate treatment to abate them which increases the expenses of wastewater treatment processes.

Table 1.1: Synthetic indicators and their health effects

Synthetic indicators	Effects	Ref
Phenolphthalein	Causes chronic ulcerative colitis, thinning of the intestinal walls and loss of normal mucosal pattern of terminal ileum. It also causes tumours e.g. thymic lymphoma, connective tissue tumours, pheochromocytoma, renal-cell-adenoma, ovarian cancer NTP 1996.	[5,36]
Bromocresol green	Skin, eye, digestive and respiratory tract irritations are acute effects. Chronic effects include pneumoconiosis, breathlessness, lung shadows, hallucinations, coma, decreased	[44]

	appetite, nausea and vomiting, diarrhoea, memory loss, schizophreniform psychosis, profuse discharge from the nostrils (coryza).	
Bromothymol blue	Causes pneumoconiosis, breathlessness, hallucinosis, schizophreniform psychosis, induce sedation, irritability, delirim, memory loss, forgetfulness (aphasias), dysarthria, fatigue, coma, nausea and vomiting, bronchoderma, profuse discharge from the nostrils (coryza), foetal abnormalities and hyperreflexia.	[39]
Methyl red	Causes birth defects, skin irritation, cyanosis, gastrointestinal irritation with nausea, vomiting and diarrhoea, central nervous system depression, respiratory tract irritation, methemoglobinemia, narcotic effects in high concentration, dizziness or suffocation, dermatitis, reproductive defects, liver, kidney and heart damage, mutagenic effects and development of tumours.	[33,34]
Phenol blue	Causes harmful effects to the central nervous system and heart resulting in disrhythmias, seizures and comma.	[5]

Methyl orange	Causes eye and skin irritation, gastrointestinal irritation, vomiting and respiratory tract irritation, tumour formation, mutation that occur due to prolonged exposure may lead to development of bladder cancer, liver carcinomas, lung adenomas and hepatomas.	[48]

2.2.1 Toxic degradation products of methyl red acid-base indicator

Synthetic acid-base indicators are usually electron withdrawing compounds due to the presence of sulphonic (SO_3^-) groups. Sadeghi *et al* [29] and Seesuriyachan *et al* asserts that the presence of these groups on aromatic compounds result in electrons being withdrawn from the ring, generating electron deficiency within the molecule making the indicator less susceptible to bio-degradation. Methyl orange can be degraded to N,N-dimethyl-p-phenylene diamine and 4-aminosulphonic acid and methyl red can be degraded to 2-aminobezoic acid (anthranilic acid) and N, N-dimethyl-p-phenylene diamine (4-N, N-dimethylamino-aniline) which are toxic [50]. Figure 2.1 shows how methyl red degrade producing two toxic products which are 2-aminobenzoic acid and N, N-dimethyl-p-phenylene diamine.

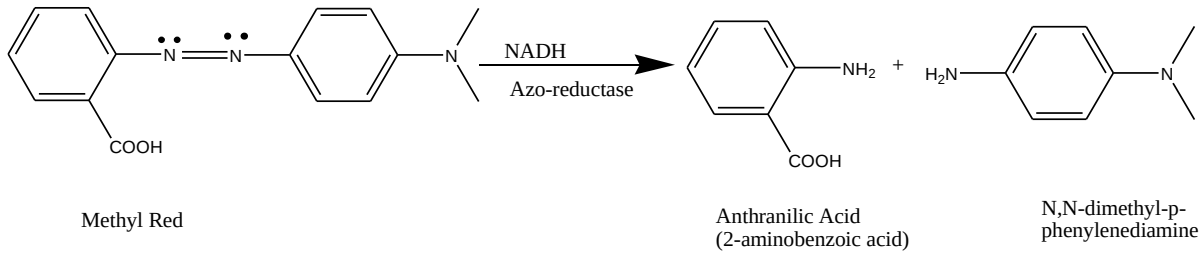


Fig. 2.1: Methyl red and its toxic metabolites

Anthranilic acid (2-aminobenzoic acid) is associated with gastro-enteritis with haemorrhages and necrosis of the liver [47]. N, N-dimethyl-p-phenylene diamine is associated with both acute

and chronic health effects. Acute health effects include fatality if swallowed due to methaeglobinemia as the substance can bind to haemoglobin inhibiting normal oxygen uptake. It can also produce toxic effects if inhaled. Chronic effects are irreversible i.e. mutations have been reported upon exposure long period of time, formation of sarcomas in the spleen, liver and kidney of humans [55].

2.3 Application of green chemistry for sustainable development

Green chemistry is a potential approach toward scientifically based environment protection. The application of green chemistry in chemical processes has significant impact on improving environmental monitoring. Application of green chemistry e.g. the use of natural dyes goes back to 1664 when sir Robert Boyle reported his collection assays on colours [50]. The research seeks to make use of *E. crassipes* root extracts by applying them as acid-base indicators, thereby improving environmental monitoring and enhancing sustainable development.

2.3.1 Review of studies that were carried out on natural acid-base indicators

2.3.1.0 Introduction

This section covers studies that have carried out, methodologies employed, extracting solvents used and data interpretation from the results generated.

2.3.1.1 Plants used to extract acid-base indicators

Many studies have been carried out on the use of plant extracts as substitute of synthetic acid-base indicators in titrations. Natural indicators have been extracted from *Aspilia Africana* and *Urena Labata* (Mgbo) flowers by Eze and Ogbuefi [20], *Gerbera jamasonii and Tagertes erecta* flowers by Shivaji *et al*, flowers of *Ipomea nil* and *ipomea biloba* by Abbas [7], flowers of *Bougainvillea Spectabilis* by Bagul *et al*, *Jacaranda acutifolia* and *Ixora Coccinea* flowers by Patrakar *et al* [14], flower sap of China rose by Gupta *et al* [50], petal sap of *Delonix regia*

by Jain [5], golden beet root, *mangifera indica* seed by Onwuachi *et al* [53] and blood leaf (*Iresine herbstii*) by Doctor [54].

2.3.1.2 Extraction methods employed to extract the acid-base indicators

The methods of extraction that were employed by many researchers on plant flowers are almost the same. They only differ in that some researchers used fresh flowers and some used dried flowers. The method used for fresh flowers include washing of the flowers with distilled water, maceration with the extracting solvent e.g. ethanol, water or methanol, then filtration and finally use of the crude extract as an indicator in titrimetric analysis [11,12].

In some cases where dried flowers were used, the method differed on that the flowers were grinded to fine powder using either a mechanical blender or a pestle and mortar. Extraction using an appropriate solvent, filtration and use of the crude extract as an indicator [5] are the basic methodological steps that were employed. Deshpande *et al* [11], Eze *et al* [18] and Patrakar *et al* [52] dried their samples away from the direct sunlight as they tried to prevent photo-degradation and oxidative loss of the dye [59], but Gupta *et al* dried the flower samples on direct sunlight [50] and they both found positive results.

Some researchers used other parts of plants to extract the indicators like the use of seeds, leaves and roots [60]. Methods employed for extraction of the dye were almost the same since drying of the sample was done for all these parts, extraction using a suitable solvents and use of the crude extracts as indicators in volumetric analysis. Suva [20] used fresh *Opuntia ficus indica* fruits to extract the pigment that was used as an indicator. In the research, *E. crassipes* roots were investigated for the acid-base indicator properties.

Of all studies that were carried out, very few isolated the active components that give the acid-base indicator properties [60]. The compounds that were being isolated were anthocyanins from the crude sample. Bondre *et al* [61] and Singh *et al* used n-butanol: acetic acid: water in a ratio

of 4:1:5 and separated the sample using thin layer chromatography (TLC). Separation of the anthocyanins from sample were also done in Food Science researches. The extracts were fully characterised with UV-Vis spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), high performance liquid chromatography (HPLC), gas chromatography- mass spectrometer (GC-MS) and proton nuclear magnetic resonance (H^+ NMR).

2.3.1.3 Solvents used to extract anthocyanins (pH sensitive compounds)

Anthocyanins are mostly extracted with acidified solvents like water, acetone, ethanol, methanol or mixtures of aqueous solvents. The acid will breakdown the cell membranes and release the anthocyanins, but harsh chemical treatment may break the innate anthocyanin structure like use of mineral acids such as 1% HCl. However, acidification with organic acids e.g. formic or acetic acid does not destroy the anthocyanin structure [64]. Many studies have shown that extraction of anthocyanins is more effective with acidified solvents than non-acidified solvents. Methanol is an environmental pollutant and more toxic as compared to other alcohols, hence ethanol is preferred by many researchers for the recovery of anthocyanins from plant material [64]. Sulphur water (aqueous SO_2) has also been used to extract anthocyanin from plant material such as red grape and black currents.

Solvents that were used in this research are 0.1 % HCl in ethanol, 0.1 % HCl in methanol, 4.5 % formic acid in water, butan-2-ol: glacial acetic acid: water (5:3:2) and ethanol.

2.3.1.4 Results generated from reviewed studies

Colour changes at different pH values of natural indicators has been attributed to the presence of anthocyanins and flavonoids which are pH sensitive [20]. Anthocyanins are organic compounds that are usually found in the aqueous sap of the vacuole of the epidermal plant cells. These compounds have a complex structure consisting of an aromatic three-ring molecular region, one or more attached sugar molecules and sometimes acyl groups attached

to the sugar molecules. Anthocyanins are water soluble and are usually more stable in acidic media than in alkaline solutions [3]. A general structure of an anthocyanin is shown in Figure 2.2.

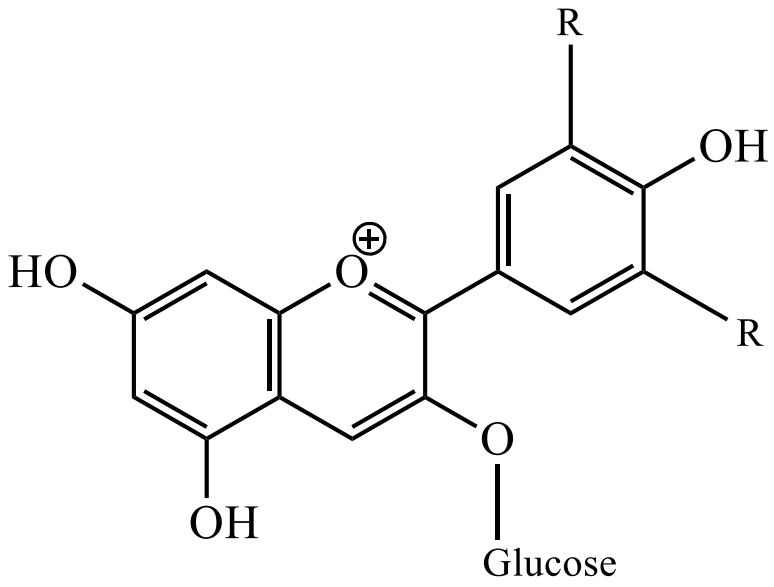


Fig. 2.2: General structure of anthocyanin.

Almost any plant that have blue, violet, purple or red flowered colours contains organic pigments, anthocyanins that changes colour with change in pH [50]. The colour stability of anthocyanins depend on structure of the anthocyanins, pH, temperature, oxygen, light and water activity [61]. They tend to be red in a more acidic solution and blue in basic solution.

Anthocyanins have several biological activities which include antioxidant, antihepatocarcinogenic, anti-inflammatory, anti-tumour, hypolidemic, cardioprotective and cancer chemopreventive, hence they are safe to use in acid-base titration [74].

2.3.1.4.1 Action of anthocyanins at different pH

The colour of anthocyanin pigments depend on pH, thus the acidity of the medium [61]. At acidic pH (1-3), anthocyanins exist predominantly in the form of the red or orange flavylium cation (2-phenylchromenylium cation). The colour intensity decrease as pH increases and also

the concentration of the flavylium cation decreases which undergoes hydration to produce the colourless pseudo base (hemiacetal or chromenol). This is due to kinetic and thermodynamic competition between hydration reaction of the flavylium cation and proton transfer reactions related to the acidic hydroxyl groups of the aglycone [68]. The conjugated 2-benzopyrilium system is disrupted due to a nucleophilic attack of water at position 2 of the anthocyanidin skeleton. Flavylium cation lose proton as the pH shifts higher. The equilibrium will now shift towards a purple quinoidal anyhydrobase at pH < 7 and a deep blue ionised anhydrobase at pH < 8. As the pH increases further, the carbinol form yields through opening of the central pyran ring and the light yellow chalcone will result. The anthocyanidin system undergoes a variety of molecular transformations as pH changes, thus in aqueous solutions, anthocyanidins exist as five molecular species in chemical equilibrium which are red flavylium cation, colourless carbinol pseudo base, purple quinoidal base, blue quinoidal base anion and yellowish chalcone [70,72]. These transformations are shown by the mechanism shown on Figure 2.3 and a three dimensional structural transformation shown on Figure 2.4.

Plant species containing anthocyanins can change colour in solution by undergoing these transformations due to change in the acidity or basicity of the solution [61]. Figure 2.5 shows the colour changes when *Rubus occidentalis* extract was added to twelve buffer solution of different pH values (1 to 12).

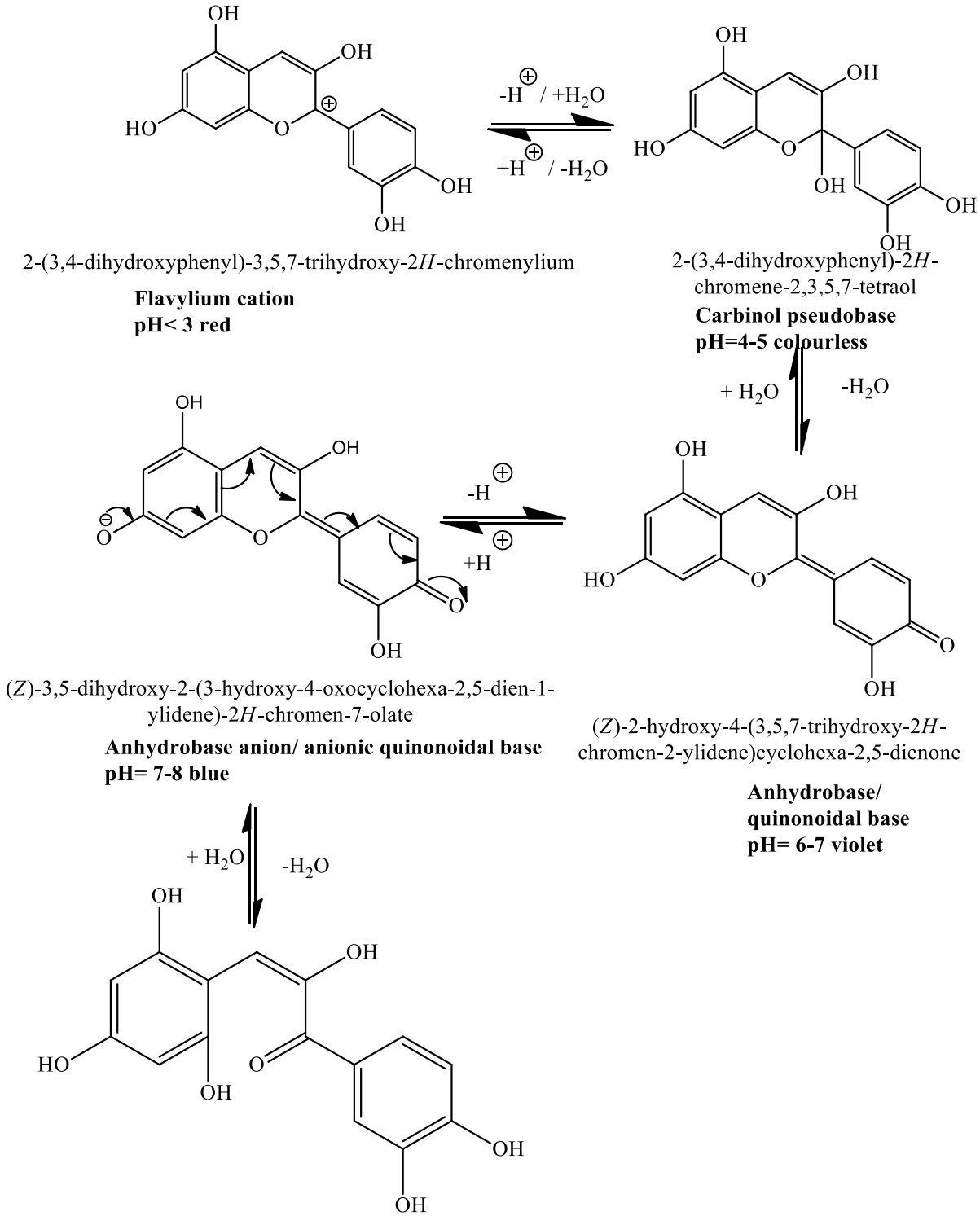


Fig. 2.3: Structure of cyanidins in aqueous solution under varying pH [61].

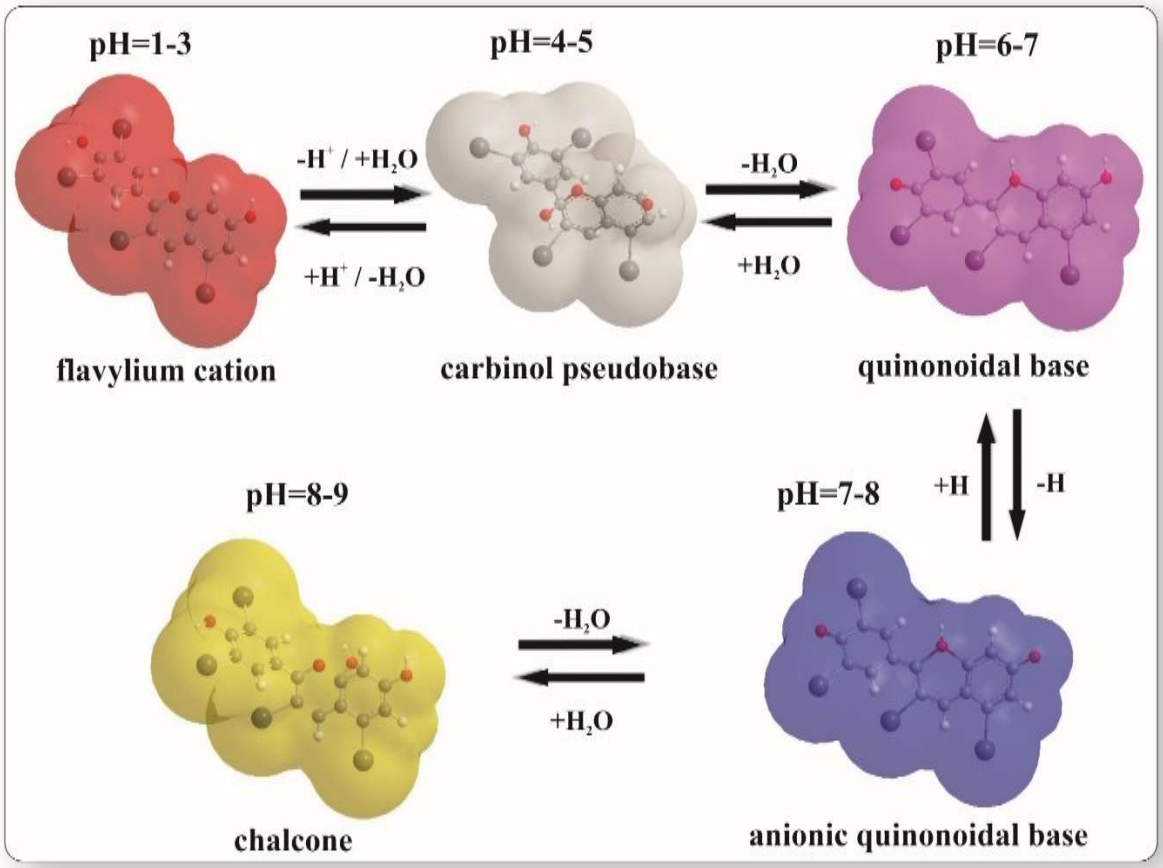


Fig. 2.4: Changes that occur to the anthocyanin structure as pH changes [70].

Fig. 2.5: Colour change results after addition of *Rubus occidentalis* extracts in buffer solutions of pH from 1-12. [54,61].

2.3.1.4.2 Titration results

The end point results of natural plant acid-base indicators obtained from titrations of strong acid vs strong base (HCl vs NaOH) ranges from 9.7 to 10.2, strong acid vs weak base (HCl vs

NH_4OH) ranges from 9.4 to 10.7, weak acid vs strong base (CH_3COOH vs NaOH) ranges from 9.46 to 9 9 and weak acid vs weak base (CH_3COOH vs NH_4OH) ranges from 9.7 to 11.4 when 10 ml of the titrate were used [7,12,14,62,68]. The results obtained from the natural plant acid-base indicators were compared with the results obtained from standard synthetic indicators. Almost all researches showed that the natural indicators can substitute the harmful standard synthetic indicators as the results were almost the same or had a slight deviation.

2.3.1.4.3 HPLC and GC-MS results

The results obtained by Qin *et al* [73] from HPLC analysis of mulberry extracts showed that four anthocyanins were present. Two peaks from the chromatogram were more pronounced compared to the other two as shown in Figure 2.6. A gradient mobile phase was used with acetonitrile and triflouroacetic acid as the eluting solvents. RP C18 column (250 × 4.6 mm, 5µm) and detection wavelength of 520 nm was employed during analysis.

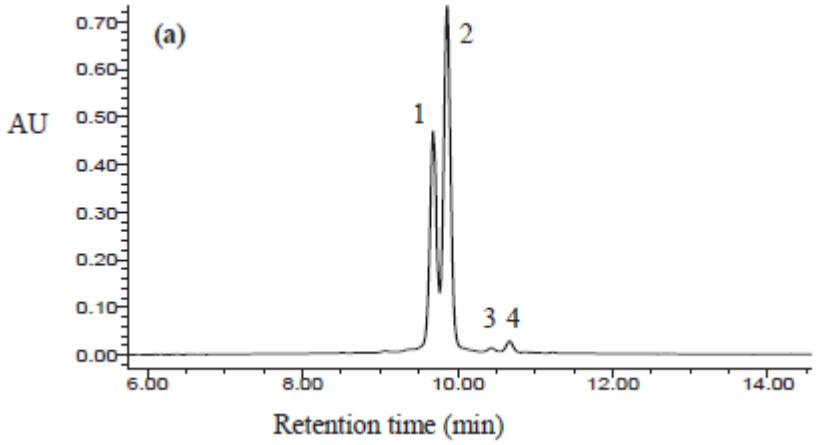


Fig. 2.6: HPLC chromatogram for mulberry fruit extract [73].

Stoj *et al* [74] proposed that the order of elution of anthocyanins is affected by the addition of a carbohydrate to the structure. This is due to increase in polarity of the compound, hence more glycosylated compounds have decreased retention times. Hydrophobic groups will tend

to increase retention times at which the compounds are eluted. The order of elution of aglycones: delphinidin < cyanidin < pelargodinin.

2.3.1.4.4 FTIR results

Infrared spectrometer was used in the analysis of vibrational frequencies of functional groups presence in plant extracts. The normal O-H stretching band occurs at 3634 cm^{-1} when not hydrogen bonded. The frequencies decrease when the hydroxyl group becomes involved in hydrogen bonding. The greater the strength of the hydrogen bond, the lower the absorption frequency. Vibrational frequencies between 3400-3300 can usually be related to sugar vibration and phenol OH groups. Bands from 2900 to 3450 cm^{-1} are usually assigned to CH and OH vibrational groups [75]. O-H stretching in glucose usually occur between 3876 to 3005 cm^{-1}, C-C and C=O usually produce several closely absorption bands between 1100 and 1000 cm^{-1} in the spectra of carbohydrates [75]. Absorption band at 840 and 898 cm^{-1} can be assigned CH and CH_2 complex vibrational modes of anomeric forms. CH_2, C-O-H and C-C-H groups are usually observed at 1432, 1334 and 1263 cm^{-1} respectively.

2.4 Eichhornia Crassipes (water hyacinth) features and its life cycle

Eichhornia crassipes also known as water hyacinth is a monocotyledonous freshwater aquatic plant, native of Brazil and some parts of South America [76]. The plant belongs to:

Kingdom: Plantae

Order: Commelnales

Family: Pontederiaceae

Genus: Eichhornia

Species: crassipes

Hence the scientific name of the plant is *Eichhornia crasssipes*. Figure 2.6 shows *E. crassipes* leaves, flowers and roots.

Fig. 2.7: Picture of *E. crassipes* flowers, leaves and roots.

E. crassipes was once used as an ornamental plant which lead to its spread across the globe due to the presence of attractive blue to violet coloured flowers. It has round to oblong curved leaves and waxy coated petioles. *E. crassipes* is a free floating plant due to presence of air filled sacs in its leaves and stem which aids to its buoyancy. It is the most abundant aquatic macrophyte which spread across water surfaces within a short period of time. Its population can double within 5-12 days [9]. The weed grows optimally in warm condition in temperature range of 28 to 30°C and environments with high nutrient levels where the water flow is slow to stagnant. It reproduces mostly by vegetative propagation [13]. Sexual reproduction also occurs where thousands of seeds are produced which can remain viable for over 20 years [9]. *E. crassipes* is termed an invasive plant whose capacity for growth and propagation causes major conservation problems with social and economic repercussions [10]. The aim of the research was to make use of the problematic plant by its application as an acid-base indicator in titrimetric analysis.

2.4.1 Effects of *E. crassipes* to the environment, humans and aquatic organisms

E. crassipes has high proliferation rate which results in massive generation of biomass that covers water surface interfering with water flow navigation and increased rate of water loss through evapotranspiration. The plant interfere with power generation, decreased temperature, pH, biological oxygen demand and chemical oxygen demand, affect water transport and fishing industry. *E. crassipes* forms marts on water surface restricting sunlight penetration that underwater native plants need for growth and photosynthesis and it is also a prime habitat for disease causing vectors such as mosquitoes, parasitic flatworms (schistosome) and even snakes [10].

2.4.2 Methods implemented to prevent the spread of *E. crassipes* in water bodies

Various researches have been carried out in Zimbabwe and Kenya on the effects of the noxious aquatic weed to the environment. In 1998 Zimbabwe held the First Global Working Group Meeting for the Biological and Integrated Control of *E. crassipes* in Harare in a way to combat the *E. crassipes* problems at Lake Chivero and Manyame [16]. Chemical, physical and biological control strategies have been employed, but the aquatic weed proved to be resilient to these strategies. Some chemicals that are used are of synthetic origin that increase environmental pollution. Despite all the efforts that are being undertaken to reduce infestation of *E. crassipes*, it still remains the world's most problematic waterweed [81]. Physical removal of *E. crassipes* from water bodies is being implemented at Lake Chivero in Zimbabwe. The plant seeds can be viable for more than 20 years, so the plant remains a problem. Making use of plant can reduce biomass generated in water bodies.

2.4.3 Application of green chemistry in environmental monitoring e.g. use of *E. crassipes* plant

Green chemistry can be applied to abate environmental pollution like in the removal of heavy metals, synthetic dyes and other contaminants. Mahamadi and Nharingo [95] used *Eirchhornia*

crassipes as a sorbent in the removal of heavy metals. Dyes were also removed using the plant e.g. methyl red and other cationic dyes [98]. Various researches where *E. crassipes* was applied in heavy metals have been carried out. The research focused on the application of *E. crassipes* root extract as acid-base indicator in titrimetric analysis.

CHAPTER THREE

METHODOLOGY

3.0 Introduction

This chapter focuses on the chronological steps that were carried out to generate data that answers the objectives of the study. The steps include sampling and preparation of *E. crassipes* roots and extraction of the pigment using solvents of different polarities. Characterisation, application of root extracts in acid-base titrations and data treatment were also discussed in this chapter. All experimental steps were carried out using apparatus, reagents and instruments listed in appendix A.

3.1 *E. crassipes* sample collection and preparation

E. Crassipes roots were collected from Mucheke River in Masvingo, Zimbabwe. The roots were removed by hand from the plant. The samples were washed and cleaned with tap water and then rinsed with distilled water. *E. crassipes* roots were placed in a room and left to dry for two months. The dried samples were pulverised at Zimbabwe Alloys (ZimAlloys) in Gweru, Zimbabwe. The powder was sieved through 75 µm test sieve and stored in a polyethylene bag before use [53].

3.2 Extraction of the pigments using solvents of different polarities

Powdered *E. crassipes* roots (1 g) were placed into a 250 ml volumetric flask. A volume of 50 ml of water was added and mixed with reciprocating vortex shaker for 2 h. The mixture was then filtered by vacuum suction filtration. The filtrate was collected and stored for analysis in high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). The procedure was repeated using ethanol, butan-2-ol: glacial acetic acid: water (BAW) (5:3:2) [20], 0.1 % HCl in methanol (v/v), 4.5 % formic acid in water (v/v) and 0.1 % HCl in ethanol (v/v).

3.3 HPLC and GC-MS analysis of the crude extracts

The section focuses on characterisation of *E. crassipes* root extracts using HPLC and GC-MS to obtain information which include determining the number of components present, the peak areas of the separated components and the presence of selected molecular ion in the root extracts.

3.3.1 Preparation of samples for HPLC analysis

A volume of 1 mL was taken from each of the extracts, 0.1 % HCl in ethanol extract, 0.1 % HCl in methanol extract, 4.5 % formic acid in water extract, ethanol extract and butan-2-ol: glacial acetic acid: water (5:3:2) extract and placed in six 50 mL volumetric flasks. The flasks were topped to the mark with HPLC grade acetonitrile and sonicated for 2 minutes. The samples were then analysed with GBC scientific HPLC (LC 200).

3.3.2 HPLC analysis of the crude extracts

GBC scientific HPLC with LC 200 UV-Vis detector, LC 1120 HPLC pump and Winchrome Lite software was used for analysing the following extracts: 0.1 % HCl in ethanol (v/v), 0.1 % HCl in methanol (v/v), 4.5 % formic acid in water (v/v), ethanol and butan-2-ol: glacial acetic acid: water (5:3:2) (v/v). RP C18 column (150×4.6 mm ID×5 µm), UV-Vis detector at 520 nm was used. The mobile phase of 90 % acetonitrile: 10 % water was used as the eluent solvents. The flow rate of 1 mL/min, injection volume of 20 µm and the ambient temperature (18-25 °C) were used [87]. Data were collected and processed with Winchrome Lite software in a personal computer.

3.3.3 GC-MS analysis of the crude extracts

Extracts of 0.1 % HCl in ethanol (v/v), 0.1 % HCl in methanol (v/v), 4.5 % formic acid in water (v/v), ethanol, butan-2-ol and glacial acetic acid: water (5:3:2) (v/v) were analysed with GC-MS.

Agilent ® 7890 Gas chromatograph was used with a capillary column length of 29.790 m, internal diameter of 320 µm, maximum temperature of 350 °C and film thickness of 0.25 µm with HPS phase. Helium carrier gas was used with a splitless front Intel mode. A constant pressure of 14 200000 psig was applied for the entire run with front Intel purge flow of 3 ml/minute. Temperature profile used was from initial temperature with a target temperature of 50 °C for 1 minute, initial rate of 20 °C/min with a target temperature of 170 °C and an initial rate of 8.75 °C/min with a target temperature of 240 °C. The transfer line temperature to mass spectrometer (MS) was set at 300 °C.

An acquisition time of 6 minutes was used for the MS detector to start detecting components separated by GC. A start mass of 50 and an end mass of 800 was set with an acquisition rate of 30 spectra per second. The detector voltage was set at 200 V with an electron energy of +70 volts. Temperature for the ion source was set at 250 °C.

3.4 Identification of the best solvent

The best solvent was determined basing on the total peak areas obtained from the chromatograms of HPLC analysis. Peak area under the chromatograms is proportional to concentration of the components present, hence solvent with the largest total peak area was found to be the best solvent. The best solvent was found to be acidified ethanol (0.1 % HCl in ethanol (v/v)).

3.5 Bulk extraction using acidified ethanol (0.1 % HCl in ethanol (v/v))

A mass of 100 g of powdered *E. crassipes* roots were placed in a 500 mL conical flask. A volume of 250 ml acidified ethanol (0.1 % HCl in ethanol (v/v)) was added to the sample and mixed with a reciprocating vortex shaker for 4 h. The mixture was then filtered by vacuum suction filtration. The filtrate was collected and stored for phytochemical test and titration processes.

3.6 Phytochemical test on crude extract

3.6.1 Test for flavonoids in acidified ethanol extract

i. NaOH test: A volume of 1 mL of the extract was treated with NaOH and HCl and the colour change was observed and recorded.

ii. Lead acetate test: A volume of 1 mL of the extract was treated with lead acetate and the colour change was observed and recorded.

iii. H_2SO_4 test: A volume of 1 mL of the extract was treated with concentrated H_2SO_4 and the colour change was observed and recorded [62,63].

3.6.2 Test for anthocyanins in acidified ethanol extract

i. NaOH test: A volume of 1 mL of the extract was treated with 2 M NaOH and the colour change was observed and recorded [62,63].

3.7 Application of the *E. crassipes* root extract and standard indicators in acid-base titration

The section focuses on determining colour changes of *E. crassipes* root extract when subjected to buffer solutions of different pH values and titration using the *E. crassipes* root extract and standard indicators.

3.7.1 Determination of colour change of *E. crassipes* root extract at different pH

Buffer solutions of pH 2.2 to 12.04 were prepared as shown in Table B1 and B2 of Appendix B. A volume of 2 ml of each buffer solution was placed in a test tube and a few drops of *E. crassipes* root extract were added [17,57]. The buffer solution and the root extract were mixed by swirling and the colours of the solutions were noted.

3.7.2 Titration using *E. crassipes* root extract, methyl red, phenolphthalein and bromothymol blue indicators.

Four titrations were performed i.e. strong acid vs strong base, strong acid vs weak base, weak acid vs strong base and weak acid vs weak base. The titrations were conducted in the order HCl and NaOH; HCl and NH_4OH; CH_3COOH and NaOH and CH_3COOH and NH_4OH. A volume of 10 ml of 1 M NaOH was placed in an Erlenmeyer flask and three drops of *E. crassipes* root extract indicator were added. HCl (1 M) was placed in a burette. The titrant (HCl) was added to titrate (NaOH) until a colour change was observed. Titrations were conducted in five replicate analyses [52,58]. The procedure was repeated for all titrations i.e. HCl-NH_4OH, CH_3COOH-NaOH and CH_3COOH-NH_4OH.

The procedure was repeated using methyl red, phenolphthalein and bromothymol blue respectively. The same aliquots were used for both titrations, hence the same set of glassware was used. The end point was noted and recorded in Appendix C.

3.8 Data treatment

All titrations were conducted in five replicate analyses, hence the mean and standard deviation were calculated. Standard test statistics (t-test) was used to analyse the mean titre volume of the natural indicator and that of each synthetic indicator relative to the equivalence point. One way ANOVA was used to test the mean and variance of the extracted natural bio-indicator and the synthetic indicators (phenolphthalein, methyl red and bromothymol blue). Differences at $p < 0.05$ were considered to be significant. Comparison of the data obtained from the use of extracted indicator and standard synthetic indicators during acid-base titrations was conducted.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 Introduction

The chapter focuses on presentation of results in different forms, evaluation and interpretation of experimental data, comparison of the results obtained using *E. crassipes* root extract and those obtained using synthetic acid-base indicators. It also focuses on significance, implications and conclusions of the findings.

4.1 Selection of the best extracting solvent results

The different extracting solvents used in an effort to determine the best extracting solvent indicated that the components of the extracts were pH sensitive. Acidified ethanol *E. crassipes* root extract was brick red, acidified methanol extract was faint yellow, 4.5 % formic acid in water extract was faint orange, butan-2-ol: glacial acetic acid: water (5:3:2) extract was brown and ethanol extract was yellow in colour. It showed that change of colour of the root extracts depended on the acidity or alkalinity of the extracting solvent. The pH sensitivity exhibited by the extracts can be attributed to the presence of anthocyanins. According to Khoddami *et al* [64] , acidified solvents were good extracting solvents for anthocyanins and flavonoids. It is also reported that optimum anthocyanin extraction is observed to occur when concentration of less than 1 % of strong mineral acids like HCl, HNO_3 and H_2SO_4 are used, hence 0.1 % HCl was used in this research [64].

The extracts obtained from different extracting solvents were observed to have different colours depending on the acidity of the solvent. During preliminary test, ethanol and acidified ethanol (0.1 % HCl in ethanol) produced distinct colour change after being subjected to solutions of different pH values. Ethanol extract showed brown colour in basic solutions and light yellow colour in acidic solutions. Acidified ethanol extract showed dark brown colour in

basic solutions and colourless in acidic solutions. This showed that ethanol and acidified ethanol solvents were able to extract the compounds that give acid-base characteristics more as compared to other solvents.

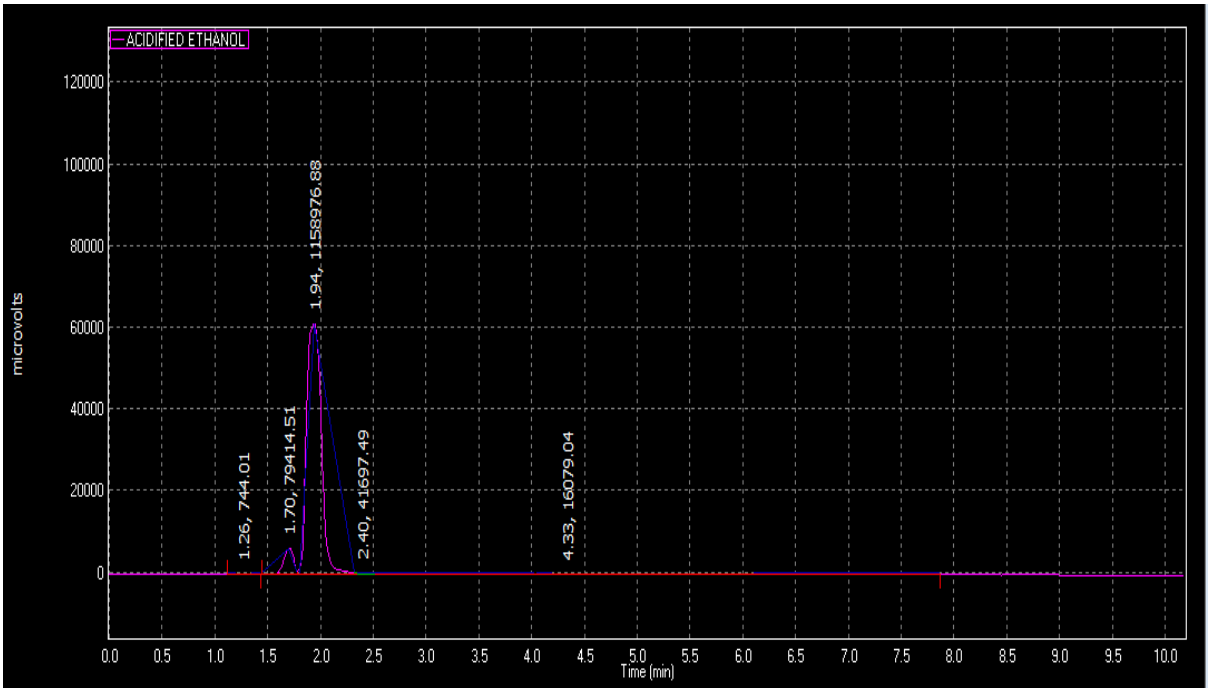
4.2 HPLC results of the crude extracts

Two components were observed to be present from all extracts as shown in HPLC chromatograms in Figure C2 to Figure C11 of Appendix C. The retention times for these two distinct peaks range from 1.55 minutes to 2.16 minutes for all extracts. Acidified ethanol extract produced five peaks with retention times of 1.26, 1.70, 1.94, 2.40 and 4.33 minutes. The two most pronounced peaks occurred at 1.70 and 1.94 minutes. Ethanol extract produced eight peaks with retention times of 1.63, 1.86, 2.22, 3.09, 3.47, 4.63, 5.73 and 8.00 minutes. The two most pronounced peaks occurred at 1.63 and 1.86 minutes. The extract of 4.5 % formic acid in water produced four peaks with retention times of 0.37, 2.16, 2.43 and 5.46 minutes. The two most pronounced peaks occurred at 2.16 and 2.43 minutes. Acidified methanol extract produced four peaks with retention times of 1.66, 1.86, 4.31 and 5.96 minutes. The two most pronounced peaks occurred at 1.66 and 1.86 minutes. BAW extract produced five peaks with retention times of 1.55, 1.75, 3.211, 4.41 and 9.96 minutes. The two most pronounced peaks occurred at 1.55 and 1.75 minutes.

The differences in retention times of the components in the extracts could be attributed to how the components interacted with the mobile and stationary phase. Due to differences in polarities of the extracting solvents used the retention times of the components present varied. This can be attributed to different components being eluted at different rate. The extracts that were extracted might be different that resulted in different retention times being observed.

Figure 4.1(a) and (b) show chromatograms of acidified ethanol extract obtained from an HPLC analysis of *E. crassipes* root crude extract.

a)



b)

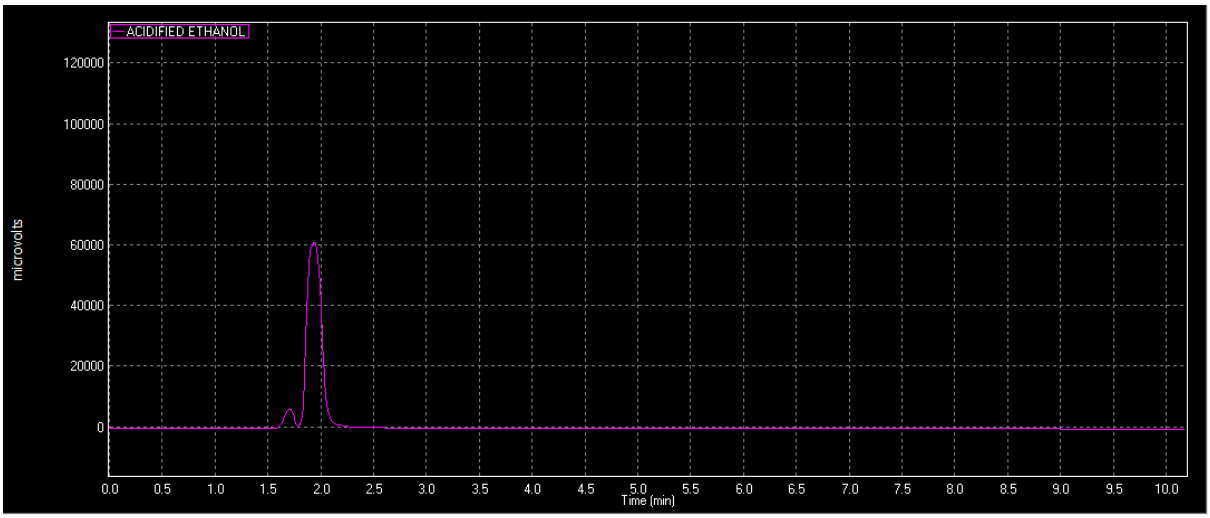


Fig 4.1: HPLC chromatograms (a) with calculated peak area and (b) without area of acidified ethanolic extract.

Basing on the area under the peaks of chromatograms in Figure 4.1, acidified ethanol was identified as the best extracting solvent. The area under the chromatogram is proportional to concentration of the components present in the *E. crassipes* root extracts. Hence acidified ethanol extracted large quantity of the components in the extracts.

The experimental results showed that the components that were present in the extracts might be anthocyanins. The HPLC chromatograms obtained in this research were almost similar to those obtained by Qin *et al* [73] from mulberry fruit extract which showed two most pronounced peaks and two which were not more pronounced. The retention times of the results obtained from this research differed from those obtained by Qin *et al*. This might be due to that different plant extracts were used in the two researches. According to Qin *et al* cyanidin and pelargonidin were found to be present in mulberry fruit extract. These might be the same compounds present in *E. crassipes* root extract which were observed in the chromatograms.

Differences in retention times might also be due to differences in the column parameters used, the eluting solvents used and the HPLC pump used. Qin *et al* used a longer column (250 × 4.6 mm, 5 µm), triflouroacetic acid and acetonitrile as the eluting solvents. In this research a shorter column (150 × 4.6 mm, 5 µm) and acetonitrile solvent was used. The mobile phases that were employed in this research was an isocratic mobile phase whilst in literature gradient mobile phase was used.

4.3 GC-MS results of the crude extracts

Selected molecular ions of *m/z* 287, 449 and 579 were found to be present. The presence of molecular ions of *m/z* 287 and 449 showed that cyanidin derived anthocyanins might be present in *E. crassipes* root extracts [88]. The presence of *m/z* 579 showed that perlagodinin derived anthocyanins might be present [73]. The chromatograms which showed the presence of the selected molecular ions were obtained from acidified ethanol and ethanol extracts. No peaks were observed from acidified methanol, 4.5 % formic acid in water and butan-2-ol: acetic acid: water extracts as shown in Figure C14 to C17 of Appendix C, hence acidified ethanol and ethanol were able to extract the proposed acid-base characteristic actives. This also helped in choosing the best extracting solvent by correlating the results obtained from HPLC.

Fragments associated with elimination of known moieties attached to aglycon parent molecule of *E. crassipes* anthocyanin which might be present were observed [89,90]. Peaks observed at a retention times of 14:06:4 min for acidified ethanol (unknown 48) and 14:06:1 min for ethanol extract (unknown 54) as shown in Figure 4.2 and 4.3 respectively showed that flavylium cation (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2H-chromenylium) might be present. From NIST library the compounds were identified as unknowns with chemical formula of $C_{15}H_{11}O_6$, for both acidified ethanol and ethanol extract, but based on literature the chemical formula $C_{15}H_{11}O_6^+$ showed the presence of cyanidin derived anthocyanins.

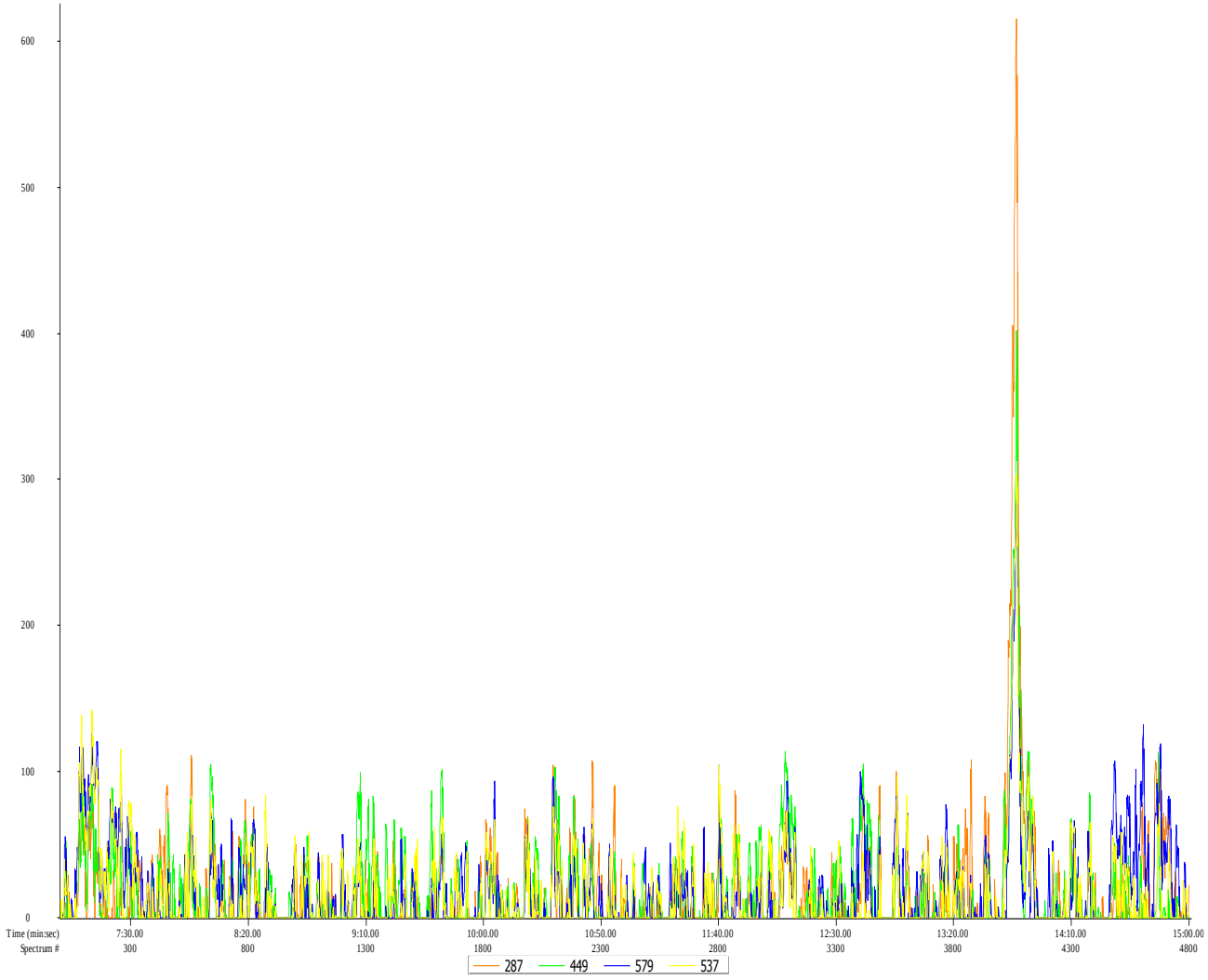


Fig. 4.2: Acidified ethanol (0.1 % HCl in ethanol (v/v)) extract GC-MS chromatogram.

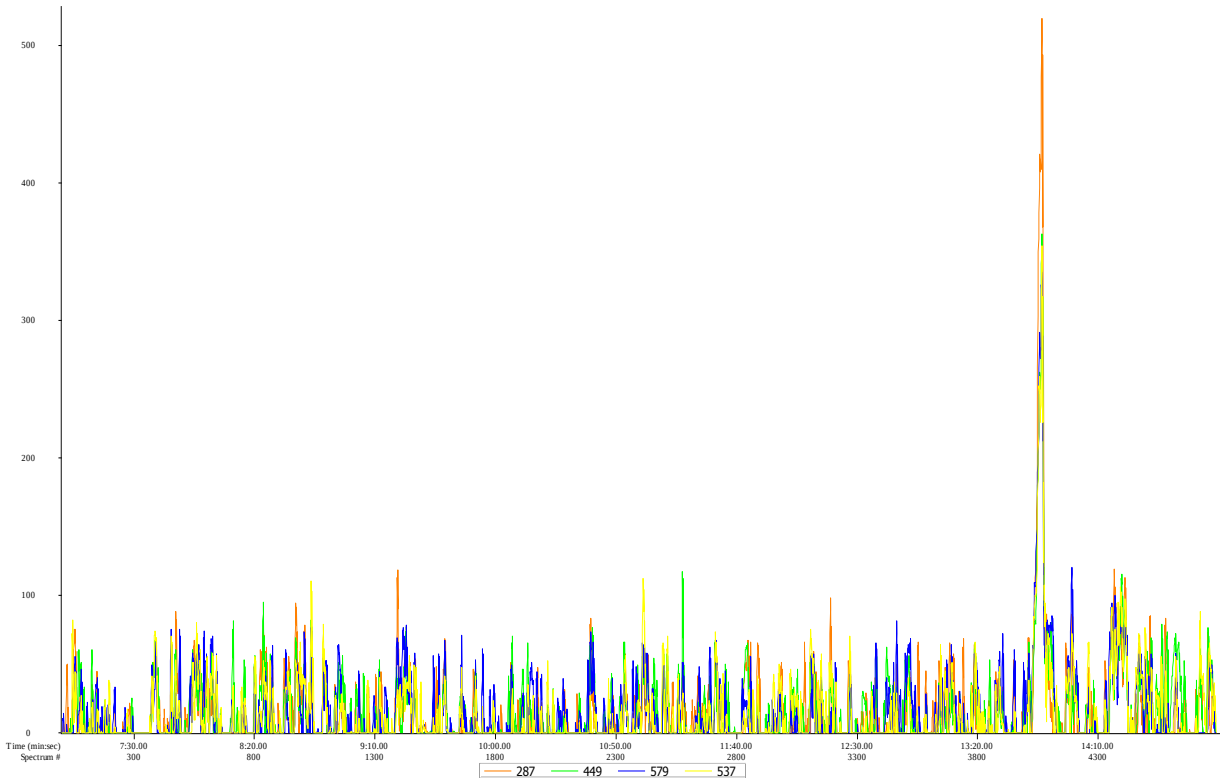


Fig. 4.3: Ethanol extract GC-MS chromatogram.

Figure 4.2 show the structure of cyanidin-3-O- glucoside and its fragments which are obtained in GC-MS analysis.

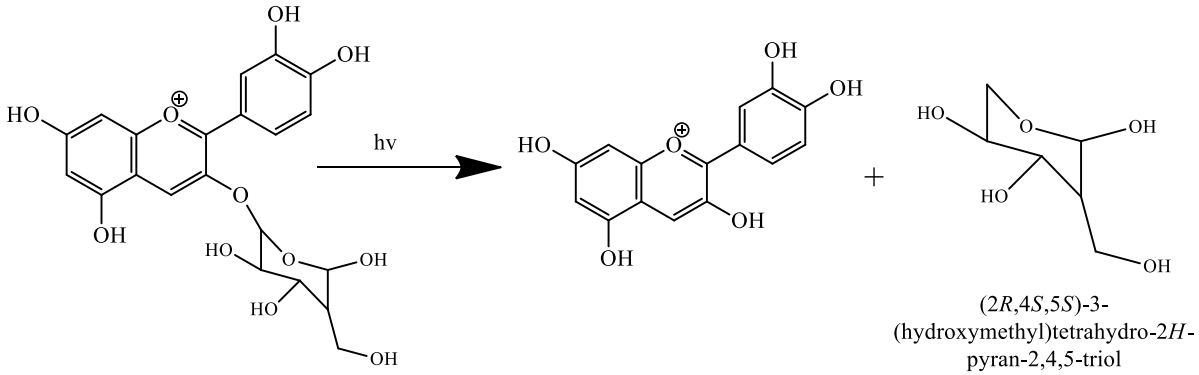


Fig. 4.4: Structure of cyanidin-3-O-glucoside, cyanidin and glucose moiety [68,70].

Co-elution might have occurred such that component at *m/z* 287 was eluted together with component at *m/z* 449 which had low intensity of approximately 400. The presence peak at *m/z*

287 might be due to the loss of a deoxy-glucosyl group at *m/z* 162 from *m/z* 449 as shown in Figure 4.4 [73,91]. Fragment ion of *m/z* 164 at a retention time of 8:27:7 minutes was also observed which showed that glucose molecules were also detected as fragment in the mass spectrometer. Glycones that could been fragmented can be glucose or galactose of *m/z* 162 [89].

Molecular ion of *m/z* 579 was also detected but due to the intensities shown on the chromatogram, it could be concluded that the compound was in low concentration. The presence of *m/z* 579 could be due to the presence of pelargodinin-3-O- rutinoside ($C_{27}H_{30}O_{14}^{+}$) [73,92] which usually elute before cyanidin derived anthocyanins as proposed by Stoj *et al* [74]. Fragment ions that may be associated with molecular ion $C_{27}H_{30}O_{14}^{+}$ are $C_{21}H_{21}O_{10}^{+}$ *m/z* 433 as a result of loss of deoxy-glucosyl and $C_{15}H_{11}O_5^{+}$ *m/z* 271 (rutinosyl) as a result of loss of another deoxy-glucosyl group as shown in Figure 4.5 [88].

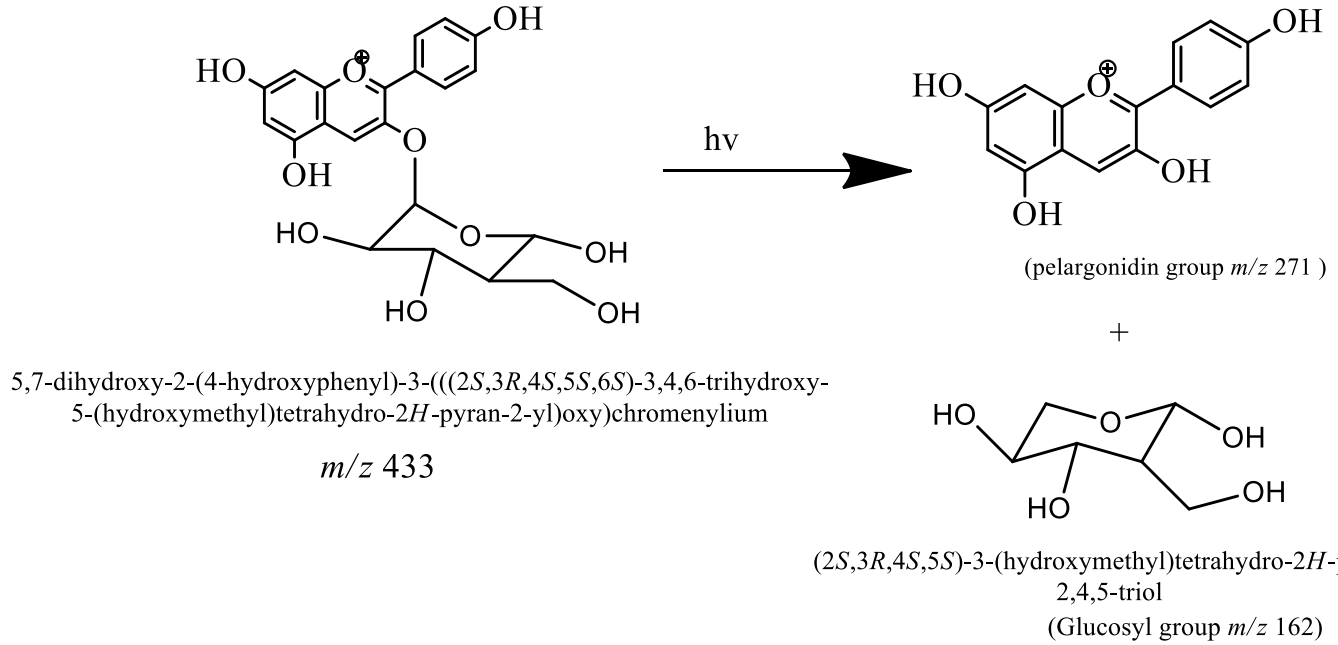


Fig. 4.5: Structure of pelargonodin-3-O-rutinoside and its fragments [70].

4.4 FTIR results of the acidified ethanol extract

This section focuses on FTIR characterisation of the acidified ethanol, *E. crassipes* root extract to determine the functional groups that are present.

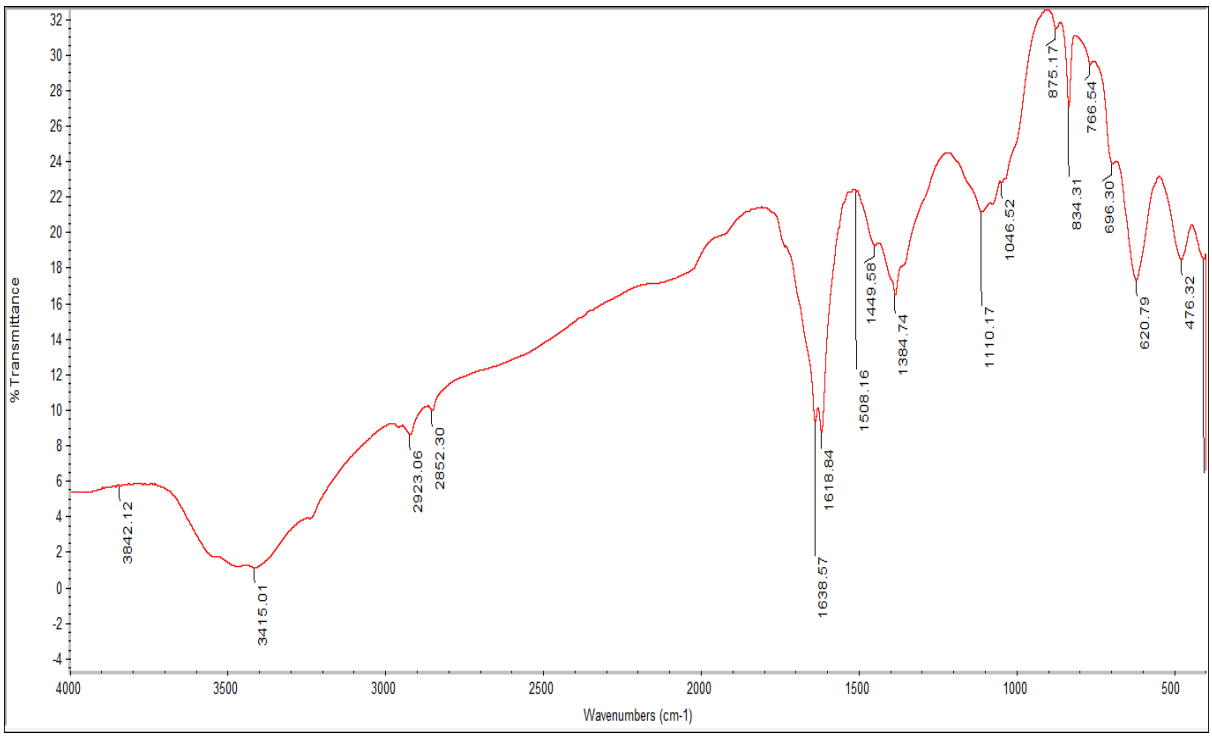


Fig. 4.6: FTIR results of *E. crassipes* root extract (0.1 % HCl in ethanol extract) vibrational frequencies.

FTIR spectrum in Figure 4.6 shows FTIR bands that were present in the *E. crassipes* root extract. Vibrational frequency at 3842.12 cm^{-1} can be assigned to O-H vibrational stretching. Vibrational band at 3415.01 can be due to the presence of C-H and O-H groups related to sugar vibration and phenol O-H groups. O-H group due to being bonded to other groups, the vibrational frequency decreased, hence the peak at 3415.01 might due to its presence [93]. At low wavenumber, the O-H stretching band overlaps with the C-H stretching peak. Peaks located at 2923.06 cm^{-1} and 2852.30 cm^{-1} can be assigned to symmetric and asymmetric stretching of $-CH_2$ and $-CH_3$ groups since it falls within the range 2935 – 2850 cm^{-1} of C-H of CH_2 vibrational band [94,95]. Vibrational band at 1638.57 cm^{-1} can be assigned to C=O group

since it falls within the range of C=O stretching, 1849 – 1634 cm^{-1}. Peaks at 1508.16 cm^{-1} can be due C=C in ring group, 1449.58 cm^{-1} can be assigned to α-CH_2 bending, 1384.84 cm^{-1} can be CH_2 or CH_3 deformation or O-H bending (in-plane) or CH_3 bending or can also be assigned to C-O-H bending [82].

Peaks at 1046. 52 and 1110.17 cm^{-1} can be C-C-C bending, 875.17 cm^{-1} was assigned to C-H bending and ring puckering whilst at 834.31 cm^{-1} can be C-H and =CH_2 out-of-plane bending [95]. Vibrational frequencies at 766.54 cm^{-1} can be assigned to O-H bending (out of plane), 696.30 and 620.79 cm^{-1} can be C-H deformation of the compounds present in the sample [94].

From the vibrational bands obtained from the extracts, it could be concluded that functional groups associated with carbohydrate molecules, phenols, flavylium cation and cyanidin derived anthocyanins might be present in the *E. crassipes* root extract.

4.5 Colours of *E. crassipes* root extract in buffered solutions

The colour changed from yellow to dark brown as shown in Table 4.1 when the extract was added to buffer solutions of pH 2.2 to 12.04 as shown in Figure C20 of Appendix C.

Table 4.1: Colour change of buffer solution after adding *E. crassipes* root extract.

pH	Colour
2.2	Yellow
3	Light brick red
4	Brick red
5	Redish purple
6	Purple
7	Colourless

8	Violet
9	Red violet
10	Light brown
11	Brown
12.04	Dark brown

The sensitivity of the extract to different pH can be attributed to the presence of anthocyanins. As described by Bondre *et al* [61], Iosub *et al* [69], Lalitha *et al* [63] and Fossen *et al* [68], different colour change presented by the *E. crassipes* root extract when subjected to different pH can be due to protonation or deprotonation of the indicator. The colour changed might be due to transformation shown in Figure 2.3 and 2.4 of section 2.3.1.4.1. The colours from literature changed from red to yellow whilst in the research they changed from yellow to dark brown. This might be attributed to different plant extracts being used in the researches and also the crude extract component matrix might be different between the two plant extracts.

4.5 Phytochemical results for the acidified ethanol crude extract

Table 4.2 show the results obtained from phytochemical test conducted to test for the presence of flavonoids and anthocyanins.

Table 4.2: Colour change from phytochemical test.

Test	Colour change
Flavonoids	
NaOH test	Yellow to orange
H_2SO_4 test	Orange
Lead acetate test	White precipitate

Anthocyanins	
NaOH test	Blue violet colour

The extract was found to contain anthocyanins and flavonoids as the colour change of solution gave positive test as shown in Table 4.2. The results obtained in this research on phytochemical test were the same as results obtained by Singh *et al* [62] and Lalitha and Jayanthi [63] when they conducted phytochemical test on *Delonix regia* and *Caesalpinia pulcherrima* [62], *Pistia stratiotes* and *Eichhornia crassipes* [63,97] extracts for antimicrobial activity, antioxidant activity and phytochemical test analysis. Due to confirmation of the presence of flavonoids and anthocyanins which are pH sensitive, *E. crassipes* root extract exhibited solvatochromic properties, hence can be applied in titrimetric analysis.

4.6 Titration results using *E. crassipes* root extract, methyl red, phenolphthalein and bromothymol blue as indicators

Table 4.3: End points mean titre volumes and colour change for the four titrations using *E. crassipes* root extract, methyl red, phenolphthalein and bromothymol blue.

Titration	Indicator	Mean ± SD	Colour
HCl vs NaOH	Methyl red	10.98 ± 0.13	Yellow to red
	Phenolphthalein	11.06 ± 0.09	Pink to colourless
	Bromothymol blue	11.16 ± 0.05	Blue to yellow
	E. crassipes root extract	11.6 ± 0.11	brown to colourless
HCl vs NH_4OH	Methyl red	7.8 ± 0.07	Yellow to red
	Phenolphthalein	7.12 ± 0.08	Pink to colourless
	Bromothymol blue	7.8 ± 0.07	Blue to yellow

	E. crassipes root extract	7.74 ± 0.09	brown to colourless
CH_3COOH vs NaOH	Methyl red	12.16 ± 0.05	Yellow to red
	Phenolphthalein	11.12 ± 0.08	Pink to colourless
	Bromothymol blue	10.9 ± 0.07	Blue to yellow
	E. crassipes root extract	11.58 ± 0.08	brown to colourless
CH_3COOH vs NH_4OH	Methyl red	9.54 ± 0.05	Yellow to red
	Phenolphthalein	7.6 ± 0.07	Pink to colourless
	Bromothymol blue	8.34 ± 0.05	Blue to yellow
	E. crassipes root extract	8.82 ± 0.08	brown to colourless

The end points for all titration conducted using *E. crassipes* root extract as an indicator were very close to the end points obtained using standard synthetic acid-base indicators i.e. methyl red, phenolphthalein and bromothymol blue as shown in in Figure 4.3 and in Appendix C. For strong acid against strong base titration (HCl and NaOH), the end point obtained using *E. crassipes* root extract indicator matched that of phenolphthalein with titre mean volumes of 11.06 ± 0.11 and 11.06 ± 0.09 respectively. Hence the root extract can be used as a substitute of phenolphthalein for strong acid against strong base titrations. The colour changed from dark brown in basic solution to colourless at the end point.

For strong acid against weak acid titration (HCl vs NH_4OH), the end point obtained using *E. crassipes* root extract was 7.74 ± 0.09 which is close to that obtained using methyl red (7.8 ± 0.07) and bromothymol blue (7.8 ± 0.07). The bio-indicator (*E. crassipes* root extract) can be a good substitute of methyl red and bromothymol blue for this type of titration. The colour changed from brown (basic) to colourless at the end point.

For weak acid against strong base titration (CH_3COOH vs NaOH), the end point obtained using the natural *E. crassipes* root extract was 11.58 ± 0.08 that is deviate significantly from the results other indicators. Hence the natural indicator cannot be used as a substitute of any of the indicators under study. The colour changed from dark brown to colourless at the end point.

For weak acid against weak base titration (CH_3COOH vs NH_4OH), the end point obtained using *E. crassipes* root extract as an indicator was 8.82 ± 0.08 that was close to the end point obtained using bromothymol blue 8.34 ± 0.05 as compared to the other standard indicators used. Hence the root extract can be used as a substitute for bromothymol blue in weak acid against weak base titrations. The colour changed from brown (basic) to colourless at the end point.

It was also observed that the extract act reversibly and gave distinct colour change in both directions when exposed to different solutions of varying pH [56].

4.5.1 Statistical analysis of generated data

The experimental data generated from titration was statistically analysed using one way ANOVA. The results are shown in Table C22 to C25 of Appendix C. From the results obtained from titrations of HCl-NaOH and CH_3COOH-NH_4OH, there was statistical significant evidence at 5 % confidence interval to show that there were no significant differences between the mean titre volumes of the four indicators (methyl red, phenolphthalein, bromothymol blue and *E. crassipes* root extract). For the titrations of strong acid against weak base and weak acid against strong acid, it was found that there were significant differences between the mean titre volumes of the four indicators [3].

Standard t-test was conducted for the titration combinations that showed that there were significant differences between the mean titre volumes form one way ANOVA, HCl-NH_4OH and CH_3COOH-NaOH. The results of t-test are shown in Table C17 to C21 of Appendix C.

Significant difference was observed in HCl-NH_4OH titration when phenolphthalein and the root extract mean titre volume were compared. In when methyl red and bromothymol blue were compared with the root extract, no significant difference were observed. This can be attributed to the pH range of phenolphthalein (8.4 – 10) as compared to that of bromothymol (6-7.6) and methyl red (4.4 - 6).

Titration of CH_3COOH-NaOH showed that there were significant differences between the mean titre volumes of all indicators. Hence the root extract cannot substitute methyl red, phenolphthalein and bromothymol blue in weak acid- strong base titrations. Figure 4.7 show bar graph of mean titre volumes obtained from the four titration, HCl-NaOH, HCl-NH_4OH, CH_3COOH-NaOH and CH_3COOH-NH_4OH.

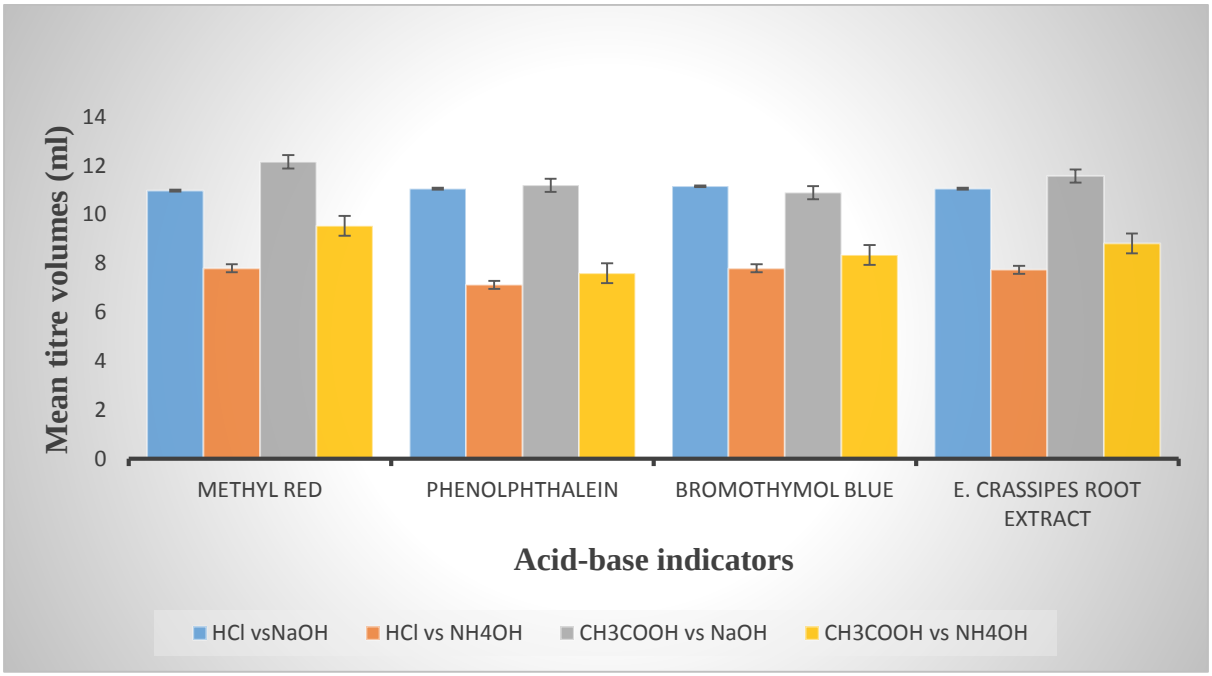


Fig. 4.7: Mean titre volumes obtained during four titration processes, HCl-NaOH, HCl-NH_4OH, CH_3COOH-NaOH and CH_3COOH-NH_4OH.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

Pigments from *E. crassipes* root extract were extracted and tested for their acid-base indicator properties. The best extracting solvent was found to be 0.1 % HCl in ethanol (v/v), hence bulk extraction was done use acidified ethanol. The root extract tested positive for the presence of flavonoids and anthocyanins. From GC-MS analysis it was predicted that cyanidin and pelargodinin derived anthocyanins were present. The extract proved to be a good substitute acid-base indicator to the synthetic indicators as the end point values were not significantly different. One way ANOVA showed that there was no significant difference between the mean titre volumes of the synthetic indicators and that of the extract for strong acid against strong base and weak acid against weak base titration. Standard t-test showed that there was significant difference between the means of *E. crassipes* root extract and phenolphthalein from weak acid against strong base titration. No significant difference was observed on all indicators under study from strong acid and weak base titration. Hence it could be concluded that *E. crassipes* root extract can be used as an effective substitute of phenolphthalein in strong acid-strong base titration and also bromothymol blue in weak acid- weak base titrations.

5.2 Recommendations

- Based on the findings of this research, use of *Eichhornia crassipes* root extract as acid-base indicator for strong acid against strong base titration as a substitute of phenolphthalein and for weak acid against weak base titration as a substitute of bromothymol is recommended.

5.2.1 Further research

- Isolation and identifying the active components of the extract.
- Exploring acid-base titrations using *E. crassipes* root extract in comparison to other synthetic acid-base indicators not used in the research.

REFERENCES

[1] Puvaneswari N, Muthukrishnan J, Gunasekaran P. Toxicity assessment and microbial degradation of azo dyes. Indian J Exp Biol 2006;44:618–26.

[2] Lavanya C, Dhankar R, Chhikara S, Sheoran S. Degradation of toxic dyes. Int J Curr Microbiol Appl Sci 2014;3:189–99.

[3] Mohd P, Khan A, Farooqui M. Analytical applications of plant extract as natural pH indicator. J Adv Sci Res 2011;2:20–7.

[4] Shih Y, Tso C, Tung L. Rapid degradation of methyl orange with nanoscale zerovalent iron particles. J Environ Eng Manag 2010;20:137–43.

[5] Jain PK, Jain P, Jain P. Green chemistry: petal sap of *Delonix regia* as a substitute for hazardous internal indicators in volumetric analysis. Biosci Biotechnol Res Commun 2013;6:186–9.

[6] Sarangerel D, Altangerel A, Munkhzaya T, Sukhbaatar B, Amarzaya B. Equilibrium and kinetic studies for reval of reactive blue 50 from aqueous media using zero valent Iron. Int J Civ Environ Eng 2014;14:38–45.

[7] Abbas SK. Study of acid-base indicator property of flowers of *Ipomoea biloba*. Int Curr Pharm J 2012;1:420–2. doi:10.3329/icpj.v1i12.12452.

[8] Kateregga E, Sterner T. Indicators for an invasive species : Water hyacinths in Lake Victoria. Ecol Indic 2007;7:362–70. doi:10.1016/j.ecolind.2006.02.008.

[9] Moyo P, Chapungu L, Mudzengi B. Integrated management approach to the control of water hyacinth, The case of Shagashe river in Masvingo, Zimbabwe. Greener J Phys Sci 2013;3:229–40.

[10] Téllez TR, Martín E, López DR, Granado GL, Pérez EA, López RM, et al. The Water

Hyacinth , *Eichhornia crassipes*, an invasive plant in the Guadiana River Basin (Spain). Reabic Aquat Invasions 2008;3:42–53. doi:10.3391/ai.2008.3.1.8.

[11] Deshpande A, Jadge D, Dhawale S, Patrakar R, Gadgul A. Flower extract of *Ixora coccinea* as a natural indicator in acid base titration. J Pharm Res 2010;3:2512–3.

[12] Mwangi T. Water hyacinth – Can its aggressive invasion be controlled. UNEP Glob Environ Alert Serv 2013:1–17.

[13] Rezania S, Ponraj M, Talaiekhozani A, Mohamad SE, Md Din MF, Taib SM, et al. Perspectives of phytoremediation using water hyacinth for removal of heavy metals, organic and inorganic pollutants in wastewater. J Environ Manage 2015;163:125–33. doi:10.1016/j.jenvman.2015.08.018.

[14] Masoud MS, Hagagg SS, Ali AE, Nasr NM. Solvatochromic behavior of the electronic absorption spectra of gallic acid and some of its azo derivatives. Spectrochim Acta Part A 2012;94:256–64. doi:10.1016/j.saa.2012.03.037.

[15] Aloo P, Ojwang W, Omondi R, Njiru JM, Oyugi D. A review of the impacts of invasive aquatic weeds on the bio-diversity of some tropical water bodies with special reference to Lake Victoria (Kenya). Biodivers J 2013;4:471–82.

[16] Chikwenhere GP. Current strategies for the management of water hyacinth on the Manyame river system in Zimbabwe. Plant Prot Res Inst 2001;102:105–8.

[17] Abbas SK. A study on pH indicator property of flowers of *Ipomea nil*. J Innov Pharm Biol Sci 2014;1:72–6.

[18] Eze SO, Ogbuefi RA. Analytical potontials of dye extarcts from *Aspilia*. Asian J Nat Appl Sci 2014;3:54–60.

[19] Zhang Y-Y, Zhang D-Y, Barrett SC. Genetic uniformity characterizes the invasive spread of water hyacinth (*Eichhornia crassipes*), a clonal aquatic plant. Mol Ecol

2010;19:1774–86. doi:10.1111/j.1365-294X.2010.04609.x.

[20] Suva MA. *Opuntia ficus indica* (*L* .) fruit extract as Natural Indicator in acid-base titration. J PharmaSciTech 2014;3:3–5.

[21] Bart HJ, Pilz S. Industrial scale natural products extraction. Ind Scale Nat Prod Extr 2011:1–26. doi:10.1002/9783527635122.

[22] Bhise SH, Shinde NG, Surve BS, Pimpodkar N V, Shikalgar SS. *Acalypha wilkesiana* as natural pH indicator. Int J Nat Prod Res 2014;4:33–5.

[23] Pimpodkar N V, Surve BS, Bhise SH. Use of *Argyreia cuneata* flower extract as a natural indicator in acid base titration. J Curr Pharma Res 2014;4:1124–7.

[24] Carmen Z, Daniela S. Textile organic dyes-characteristics, polluting effects and separation/ elimination procedures from industrial effluents. Org Pollut Ten Years After Stock Conv – Environ Anal Updat 2012:55–86.

[25] Isiuku B, Horsfall M, Spiff A. Removal of methyl red from aqueous solution by NaOH-activated Cassava peels carbon in a fixed-bed column. Res J Appl Sci 2014;9:238–43.

[26] Surve BS, Humbe P V, Kale RG. Use of *Cordyline fruticosa* leaves extract as a natural jndicator in acid-base titration. Int J Curr Pharm Sci 2015;1:194–7.

[27] Piroi D, Magureanu M, Mandache N, Parvulescu V. Decomposition of organic dyes in water using non-thermal plasma. Bucharest: 2009.

[28] Mahmoud MA, Poncheri A, Badr Y, Wahed MGA El. Photocatalytic degradation of methyl red dye. S Afr J Sci 2009;105:299–303.

[29] Sadeghi A, Aboutaleb M, Bazardehi K, Raffe S, Zarif B. Biotransformation of carmoisine and reactive black 5 dyes using *saccharomyces cerevisiae*. J Sci Res

2014;6:859–64.

[30] Sahasrabudhe M, Pathade G. Decolourization and degradation of C. I. reactive red 195 by Georgenia sp. CC-NMPT-T3. Indian J Exp Biol 2012;50:290–9.

[31] Vijaya, P P, Padmavathy P, Sandhya S. Decolourization and Biodegradation of Reactive Azo Dyes by Mixed Culture. Indian J Biotechnol 2003;2:259–63.

[32] Kumar M, Prakash SO, Kumar SM. Photochemical treatment of methyl red waste water by Photo-Fenton reagent. Int J Res Chem Environ 2012;2:87–92.

[33] Dim PE. Adsorption of methyl red and methyl orange using different tree bark powder. Acad Res Int 2013;4:330–8.

[34] Giwa A, Giwa FJ, Ifu BJ. Microbial decolourization of an anthraquinone dye C. I. reactive blue 19 using *Bacillus cereus*. Am Chem Sci J 2012;2:60–8.

[35] Luo X, Zhan Y, Huang Y, Yang L, Tu X, Luo S. Removal of water-soluble acid dyes from water environment using a novel magnetic molecularly imprinted polymer. J Hazard Mater 2011;187:274–82. doi:10.1016/j.jhazmat.2011.01.009.

[36] NTP. Report on carcinogens, Thirteenth edition. 2010.

[37] Breckland Scientific Supplies. Safety data sheet-bromocresol green. BSB Sci Solut 2015.

[38] International V. Bromocresol indicator ACS , Reag . Ph Eur. MSDS. Chemwatch Mater Saf Data Sheet 2012:1–7.

[39] Biotechnology SC. Material safety data sheet bromothymol blue. St Cruz Biotechnol 2011:1–11.

[40] Chen H, Hopper SL, Cerniglia CE. Biochemical and molecular characterization of an azoreductase from *Staphylococcus aureus*, a tetrameric NADPH-dependent

flavoprotein. J Microbiol 2005;151:1433–41. doi:10.1099/mic.0.27805-0.

[41] LabChem. Material safety data sheet of methyl red-methylene blue. LabChem 2010:1–8.

[42] MSDS P. Material safety data sheet methyl orange 2006:1–3.

[43] Santa Cruz Biotechnology. Material safety data sheet Methyl orange 2011:1–11.

[44] Mas RH, Haris M, Sathasivam K. The removal of methyl red from aqueous solutions using banana pseudostem fibers. Am J Appl Sci 2009;6:1690–700.

[45] Ramya M, Iyappan S, Manju A, S JJ. Biodegradation and decolorization of acid red by Acinetobacter radioresistens. J Bioremediation Biodegrad 2010;1:1–6. doi:10.4172/2155-6199.1000105.

[46] So K, Wong P, Chan K. Decolourization and Biodegradaation of Methyl Red by Acetobacter Liquefaciens. Toxic Assess An Int J 1990;5:221–35.

[47] Sanner T. Scientific committee on consumer products (SCCP) Opinion on 4-Aminobenzoic acid (PABA). 2006.

[48] Biotechnology SC. Material data sheet N,N-Dimethyl-p-phenylenediamine 2012:1–9.

[49] America T. Material safety data sheet of N,N-dimethyl-1,4-phenylenediamine 2005:1–6.

[50] Gupta P, Jain P, Jain PK. Flower sap : A natural resource as indicator in acidimetry and alkalimetry. Int J CheTech Res 2012;4:1619–22.

[51] Eze S, Obguefi R. Analytical potentials of dye extracts from *Urena lobata (Mgbo)* flowers. Commun Appl Sci 2014;2:25–35.

[52] Patrakar R, Gond N, Jadge D. Flower extract of *Jacaranda acutifolia* used as a natural indicator in acid base titration. Int J PharmTech Res 2010;2:1954–7.

[53] Onwuachu UI, Aboh UJUA, Iwuoha G. Extraction, characterisation and workability of some local plant dyes as acid-base indicators. Internatin J Res Applied, Nat Soc Sci 2014;2:1–6.

[54] Doctor T, Cababat R. Blood leaf (*Iresine herstii*) extract used as an indicator of soil pH. Internatin J Sci Res Publ 2014;4:10–4.

[55] Bagul SB, Dabhade MP, Dabhade P, Bhamare TD. "A herbal pH indicator from bracts extract of *Bougainvillea spectabilis*." A Genes J Pharmagene 2012;2:11–3.

[56] Shivaji PT, Rajaram PS, Singh S, Shyale SS. Acid-Base indicator property of Dye obtained from *Gerbera jamesonii* and *Tagertes erecta* 2014;3:1–4.

[57] Saptarini NM, Suryasaputra D, Nurmalia H. Application of butterfly bea (*Clitoria ternatea Linn*) extract as an indicator of acid-base titration. J Chem Pharm Res 2015;7:275–80.

[58] Kadam S, Yadav A, Raje V, Waghmare K. Comparative study of natural and synthetic indicators. Der Pharma Chem 2013;5:296–9.

[59] Paul C, Chakraborty DD, Ghosh A, Chakraborty P. Natural indicator as a substitute to synthetic indicator-A developmental approach. J Appl Pharm Sci 2014;4:120–2. doi:10.7324/JAPS.2014.40921.

[60] Thote P, Mandir GV. Green chemistry, study of acid-base indicator property. Int J Res Granthaalayah 2015;3:1–6.

[61] Bondre S, Patil P, Kulkarni A, Pillai MM. Study on isolation and purification of anthocyanins and its applications as pH indicator. Int J Adv Biotechnol Res 2012;3:698–702.

[62] Singh S, Bothara SB, Singh S, Patel R, Ughreja R. Preliminary pharmaceutical characterization of some flowers as natural indicator, acid-base titration. Pharmacogn J

2011;3:39–43. doi:10.5530/pj.2011.22.8.

[63] Lalitha P, Jayanthi P. Preliminary studies on phytochemicals and antimicrobial activity of solvent extracts of *Eichhornia crassipes (Mart.) Solms*. Asian J Plant Sci Res 2012;2:115–22.

[64] Khoddami A, Wilkes MA, Roberts TH. Techniques for analysis of plant phenolic compounds. Molecules 2013;18:2328–75. doi:10.3390/molecules18022328.

[65] Aishah B, Nursabrina M, Noriham A, Norizzah AR, Shahrimi MH. Anthocyanins from Hibiscus sabdariffa, Melastoma malabathricum and *Ipomoea batatas* and its colour properties. Int Food Res J 2013;20:827–34.

[66] He F, Liang N, Mu L, Pan Q, Wang J, Reeves MJ, et al. Anthocyanins and their variation in red wines I. monomeric anthocyanins and their color expression. Molecules 2012;17:1571–601. doi:10.3390/molecules17021571.

[67] Raki VP, Ota AM, Skrt MA, Miljkovi MN, Kosti DA, Sokolovi DT, et al. Investigation of fluorescence properties of cyanidin and cyanidin 3-O-β-glucopyranoside. Hem Ind 2015;69:155–63. doi:10.2298/HEMIND140203030R.

[68] Fossen T, Cabrita L, Andersen M. Colour and stability of pure anthocyanins infuenced by pH including the alkaline region. Food Chem 1998;63:435–40.

[69] Iosub SD, Meghea A, Geana I. Solvatochromic parameters of some anthocyanin derivatives concentrated from selected natural extracts. UPB Sci Bull, Ser B 2014;76:25–34.

[70] Ananga A, Georgiev V, Ochieng J, Phills B, Tsolova V. Production of anthocyanins in grape cell cultures, potential source of raw material for Pharmaceutical, Food and Cosmetic industries. Mediterr. Genet. Code-Grapvine Olive, Florida: 2013, p. 247–87.

[71] Agrawal S, Chouhan K, Raj CN, Jain S, Balasubramaniam A. Isolation of herbal acid-

base indicator from the seeds of *Punica granatum*. J Chem Pharm Res 2011;3:168–71.

[72] Elumalai A, Eswaraiah MC, Kasarla R, Ravi P. An alternative to synthetic acid base indicator- *Tagetes erecta linn.* Asian J Res Chem 2012;5:218–20.

[73] Qin C, Li Y, Niu W, Ding Y, Zhang R. Analysis and characterisation of anthocyanins in *Mulberry fruit*. Czech J Food Sci 2010;28:117–26.

[74] Stój A, Malik A, Targoński Z. Comparative analysis of anthocyanin composition of juices obtained from selected spicies of berry fruits. Polish J Food Nutr Sci 2006;15:401–7.

[75] Ibrahim M, Alaam M, El-haes H, Jalbout AF, Leon A De. Analysis of the structure and vibrational spectra of glucose and fructose. Eclet Quim 2006;31:15–22.

[76] Kunatsa T, Mufundirwa A. Biogas production from water hyacinth case of Lake Chivero- Zimbabwe. Int J Recent Technol Eng 2013;2:138–42.

[77] Dellagreca M, Previtera L, Zarrelli A. Structures of new phenylphenalene-related compounds from *Eichhornia crassipes* (water hyacinth). Tetrahedron 2009;65:8206–8. doi:10.1016/j.tet.2009.07.069.

[78] Adebayo AA, Briski E, Kalaci O, Hernandez M, Ghabooli S, Beric B, et al. Water hyacinth (*Eichhornia crassipes*) and water lettuce (Pistia stratiotes) in the Great Lake. Aquat Invasions 2011;6:91–6. doi:10.3391/ai.2011.6.1.11.

[79] Mironga JM. Lessons for effective management of water hyacinth (*Eichhornia crassipes (Mart.)* solms) in Kenya. Int J Humanit Soc Sci 2014;4:118–26.

[80] Mironga JM. The effect of water hyacinth, *Eichhornia crassipes*, infestation on phytoplankton productivity in Lake Naivasha and the status of control. 2003.

[81] Mironga JM, Mathooko JM, Onywere SM. Effects of spreading patterns of water

hyacinth (*Eichhornia crassipes*) on zooplankton population in lake Naivasha, Kenya. Int J Dev Sustain 2014;3:1971–87.

[82] Mahamadi C, Nharingo T. Modelling the kinetics and equilibrium properties of cadmium biosorption by river green alga and water hyacinth weed. Toxicol Environ Chem 2007;89:297–305. doi:10.1080/02772240601010063.

[83] Mahamadi C, Nharingo T. Competitive adsorption of Pb2+, Cd2+ and Zn2+ ions onto *Eichhornia crassipes* in binary and ternary systems. Bioresour Technol 2010;3:859–64. doi:10.1016/j.biortech.2009.08.097.

[84] Mahamadi C, Nharingo T. Utilization of water hyacinth weed (*Eichhornia crassipes*) for the removal of Pb (II), Cd (II) and Zn (II) from aquatic environments : An adsorption isotherm study. Environ Technol 2010;31:1221–8. doi:10.1080/09593331003646604.

[85] Abdullah N. Removal of methyl red from aqueous solution using dried water hyacinth. Univ Malaysia J 2010:1–25.

[86] Saltabas O, Teker M, Konuk Z. Biosorption of cationic dyes from aqueous solution by water hyacinth roots. Glob NEST J 2012;14:24–31.

[87] Baublis A, Spomer A, Berber-Jimenez MD. Anthocyanin pigments : Comparison of extract stability. Urbana: 2000.

[88] Junqueira-gonçalves MP, Yáñez L, Morales C, Navarro M, Contreras RA, Zúñiga GE. Isolation and characterization of phenolic compounds and anthocyanins from *Murta* (*Ugni molinae Turcz.*) fruits. assessment of antioxidant and antibacterial activity. Molecules 2015;20:5698–713. doi:10.3390/molecules20045698.

[89] Guimarães R, Barros L, Carvalho AM, Queiroz MJRP, Santos-buelga C, Ferreira ICFR. Characterization of phenolic compounds in wild fruits from North eastern

Portugal. Minho, Portugal, 2014.

[90] Aza-gonzález C, Ochoa-Ajelo N. Characterization of anthocyanins from fruits of two Mexican chili peppers (*Capsicum annuum L* .). J Mex Chem Soc 2012;56:149–51.

[91] Dias ALS, Rozet E, Chataigné G, Oliveira AC, Rabelo CAS, Hubert P, et al. A rapid validated UHPLC–PDA method for anthocyanins quantification from *Euterpe oleracea* fruits. J Chromatogr B 2012;907:108–16. doi:10.1016/j.jchromb.2012.09.015.

[92] Shah NM, Morris M, Grijalva A, Chapman JM. Characterization of anthocyanins and flavonol glycosides from *Delonix regia* and *Ixora casei* hance by LC-ESI-MS-MS. 2006.

[93] Venkatesan S, Pugazhendy K, Sangeetha D, Vasantharaja C, Prabakaran S, Meenambal M. Fourier transform infrared (FT-IR) spectoroscopic analysis of *Spirulina*. Int J Pharm Biol Arch 2012;3:969–72.

[94] Kiefer J, Frank K, Zehentbauer FM, Schuchmann HP. Infrared spectroscopy of *Bilberry* extract water-in-oil emulsions sensing the water-oil interface. Biosensors 2016:1–13.

[95] Stelian R, Chirugiu L. Physico-chemical research on *Sambucus emulus L.* species. University of Medicine and Pharmacy of Craiova, 2014.

[96] Nikonenko N, Buslov D, Sushko N, Zhbankov R. Analysis of the structure of carbohydrates with useof the regularized deconvolution method of vibrational spectra. BAU Fen Bil Enst Derg 2002;4:13–6.

[97] Tulika T, Mala A. Pharmaceutical potential of aquatic plant *Pistia stratiotes (L)* and *Eichhornia crassipes*. J Plant Sci 2015;3:10–8.

APPENDIX A

a) Apparatus

Burette (50 ml), volumetric flasks (50 ml, 100 ml, 250 ml, 500 ml and 1000 ml), beakers (100 ml, 250 ml and 500 ml), Erlenmeyer flasks (250 ml and 500 ml), pipette, pipette filler, Pasteur pipette, filter papers, spatula, petri dishes, weighing crucibles, measuring cylinders (25 ml, 100 ml and 250 ml), polythene bags, wash bottles, 75 µm sieve, test tubes, test tube holder, Buchner funnel, vials,

b) Reagents

Table A1: Reagents used

Chemical name	Chemical formula	Manufacturer	Mass/Conc.
Sodium hydroxide	$NaOH$	ACE	40.0013g (1M)
Hydrochloric acid (32 %)	HCl	Glassworld	98 ml/L (1M)
Glacial acetic acid	CH_3COOH	Glossworld	57.5 ml/ L (1M)
Ammonium hydroxide	NH_4OH	Scientific Masters	74.6 ml/ L (1M)
Ethanol	C_2H_5OH	Chisumbanje	99 %
Methanol	CH_3OH	Fisher Chemicals	55 %
Butan-2-ol	C_4H_9OH	ACE	25 ml/ 50 ml
Methyl red	$C_{15}H_{15}N_3O_2$	ACE	0.0203g
Phenolphthalein	$C_{20}H_{14}O_4$	Skylabs	0.1018g
Bromothymol blue	$C_{27}H_{28}Br_2O_5S$	ACE	5 ml
Formic acid	$H. COOH$	Glassworld	4.5 %
Acetonitrile	CH_3CN	PROLABO	55 %
Sodium hydrogen phosphate	Na_2HPO_4	ACE	28.3937g (0.2M)
Citric acid	$C_6H_8O_7$	ACE	21.0098g (0.1M)
Glycine	CH_2NH_2COOH	ACE	7.5071g

Sodium chloride	NaCl	ACE	5.8441g
Sulphuric acid	HSO_4	Cosmo Chemicals	98 %
Lead acetate	$(CH_3COO)_2$ Pb. $3H_2O$	ACE	18.9725g (1 M)
Distilled water	H_2O	MSU Lab	-

c) Instrumentation

Table A2: Instrumentation used

Name	Model	Manufacturer	Use
Analytical balance	JJ224BC	G & G	weighing
Electric water bath	CE-600	CE	heating
HPLC	LC 200	GBC	separation
GC-MS (GC)	Agilant	7890 series	separation
(MS)	LECO	Pegasus HT	identification of molecular ions
FTIR Spectrometer	Nicolet 6700	Thermo Scientific	identification of functional groups
pH meter	Az-8601	Thermo Scientific	measuring pH of solutions
Hot plate	MSH 10	Labcon	heating
Tema mill	T 750 k	Tema machines	pulverizing
Sonicator	KQ-250B	China Corp.	Ultra-mixing
Vortex shaker	RS-12R	Bajendra	mixing

APPENDIX B

a) Preparation of extracting solvents

Preparation of 1M hydrochloric acid

A volume of 98 ml, 32 % HCl was added to a 1 L volumetric flask with approximately 500 ml of distilled water. The flask was filled up to the mark with distilled water and inverted several times.

Preparation of acidified methanol

A volume of 5 ml (0.1 %) 1 M hydrochloric acid was added to 45 ml of methanol in a 50 ml volumetric flask. The flask was inverted several times to mix the contents.

Preparation of acidified ethanol

For preliminary extraction

A volume of 5 ml (0.1 %) 1 M hydrochloric acid was added to 45 ml of ethanol in a 50 ml volumetric flask. The flask was inverted several times to mix the contents.

For bulk extraction

A volume of 25 ml (0.1 %) 1 M hydrochloric acid was added to 225 ml of ethanol in a 250 ml volumetric flask. The flask was inverted several times to mix the contents.

Preparation of butan-2-ol: glacial acetic acid: water (BAW)

Butan-2-ol: glacial acetic acid: water was mixed in a ratio of 5: 3: 2 to make up 50 ml i.e. 25 ml butanol, 15 ml glacial acetic acid and 10 ml distilled water. The flask was inverted several times to mix the contents.

Preparation of ethanol

A volume of 50 ml ethanol was taken from 98 % ethanol solution.

Preparation of 4.5 % formic acid in water

A volume of 11.1 ml of formic acid was transferred into a 50 ml volumetric flask containing approximately 35 ml of distilled water. Distilled water was added up to the mark and the flask was inverted to mix the contents.

b) Preparation of solvent to be used as the mobile phase in HPLC analysis

Preparation of 90 % acetonitrile

A volume of 225 ml acetonitrile was placed in a 250 ml volumetric flask. Distilled water was added up to the mark. The contents were mixed by inverting the flask.

c) Preparation of buffer solutions

Preparation of Glycine-NaOH buffer (pH 8.53 – 12.9)

A mass of 7.5075 g (0.1 mol) of glycine (aminoethanoic acid) and 5.8443 g (0.1 mol) of sodium chloride were mixed and placed in 1 L volumetric flask. Distilled water was added up to the mark and the flask was inverted several times to mix the contents.

A mass of 4.1020 g (0.1 M) of NaOH was placed in a 1 L volumetric flask and distilled water was added up to the mark. The flask was inverted several times to mix the contents until they dissolve.

To make up 100 ml of buffer solution, mixture of glycine and sodium chloride and 0.1 M NaOH were mixed in the following portions shown in Table B1 to make the buffer solutions of pH 9.31 to 12.04.

Table B1: Volumes used to make Glycine-NaOH buffer solutions from pH 9.31 to 12.04.

pH	Volume of Glycine (ml)	Volume of NaOH (ml)
9.31	80	20
10.09	60	40

11.01	51	49
12.04	45	55

Preparation of Na_2HPO_4 – citric acid buffer (pH 2.2 – 8.0)

A mass of 28.3937 g (0.2 M) of Na_2HPO_4 was placed in a 1 L volumetric flask and filled up to the mark with distilled water. The flask was inverted several time to mix and dissolve di-sodium hydrogen orthophosphate anhydrous.

A mass of 21.0098 g (0.1 M) of citric acid was placed in a 1 L volumetric flask and filled up the mark with distilled water. The contents were mixed by inverting several times.

To make 100 ml of the buffer 0.2 M Na_2HPO_4 was added to 0.1 M citric acid in the proportions shown in Table B2 below.

Table B2: Volumes used to make Na_2HPO_4- citric acid buffer of pH 2.2 to 8.0.

pH	Volume of Na_2HPO_4 (ml)	Volume of citric acid (ml)
2.2	2	98
3.0	20.55	79.45
4.0	38.55	61.45
5.0	51.50	48.50
6.0	63.15	36.85
7.0	82.35	17.65
8.0	97.25	2.75

d) Preparation of phytochemical test solutions

Preparation of 1 M and 2 M sodium hydroxide (NaOH)

A mass of 40. 0019 g (0.1 mol) of NaOH was placed in a 1 L volumetric flask and distilled water was added up to the mark. The flask was inverted several times to mix and dissolve NaOH.

A mass of 8.0037 g (2 mol) of NaOH was placed in a 100 ml volumetric flask and distilled water was added up to the mark. The flask was inverted several times to mix and dissolve NaOH.

Preparation of 1 M hydrochloric acid (HCl)

A volume of 98 ml of hydrochloric acid was added to a 1 L volumetric flask with approximately 500 ml of distilled water. Distilled water was then added up to the mark. The flask was inverted several times to mix HCl and distilled water.

Preparation of 1 M lead acetate [$(CH_3COO)_2 Pb. 3H_2O$]

A mass of 18.9725 g (1 mol) of lead acetate was placed in a 50 ml volumetric flask and filled up to the mark with distilled water up to the mark. The flask was inverted several times to mix the contents.

e) Preparation of titration solutions

Preparation of 1 M hydrochloric acid (HCl)

A volume of 98 ml of hydrochloric acid was added to a 1 L volumetric flask with approximately 500 ml distilled water. Distilled water was added up to the mark. The flask was inverted several times.

Preparation of 1 M sodium hydroxide (NaOH)

A mass of 40.0008 g (1 M) of NaOH was placed in a 1 L volumetric flask and distilled water was added up to the mark. The flask was inverted several times.

Preparation of 1 M acetic acid (CH_3COOH)

A volume of 57.5 ml of glacial acetic acid was added in a 1 L volumetric flask with approximately 500 ml distilled water. Distilled water was added up to the mark. The flask was inverted several times.

Preparation of 1 M ammonium hydroxide (NH_4OH)

A volume of 74.6 ml of ammonia was added to a 1 L volumetric flask with approximately 500 ml distilled water. Distilled water was added up to the mark. The flask was inverted several times.

APPENDIX C

RESULTS

Extracts of different solvents.

Fig. C1: Extracts obtained using different extracting solvents.

HPLC results

The following figures C2 to C10 shows HPLC chromatograms together with calculated area of five different extracting solvents (4.5 % formic acid in water, acidified ethanol, acidified methanol, butan-2-ol: glacial acetic acid: water (5:3:2) and ethanol) .

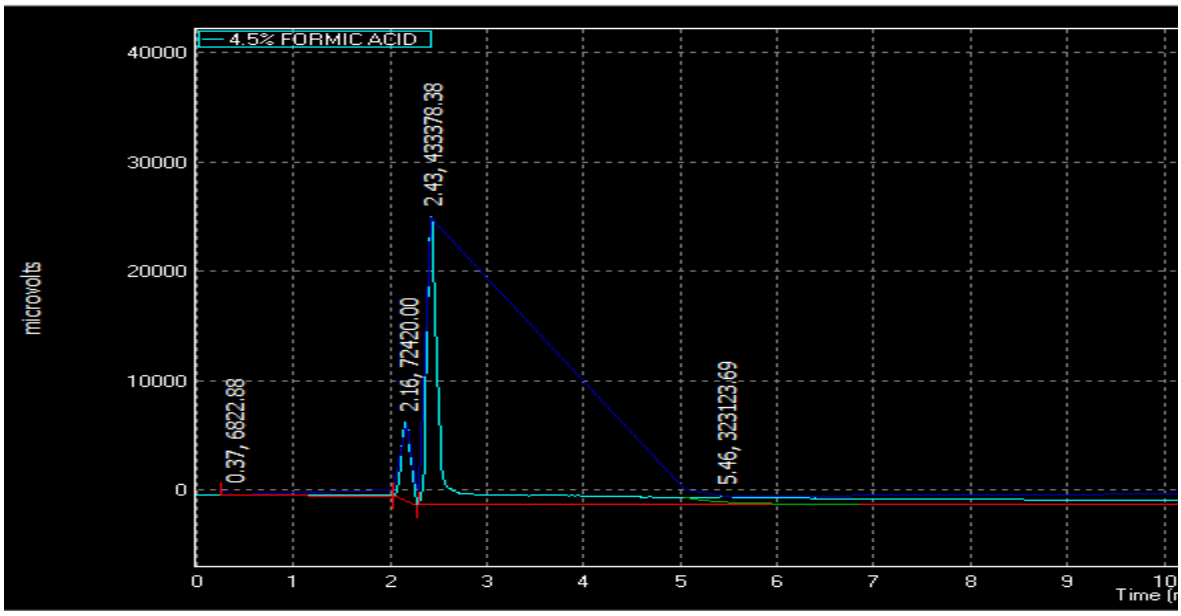


Fig. C2: HPLC chromatogram with calculated area for 4.5 % formic acid in water.

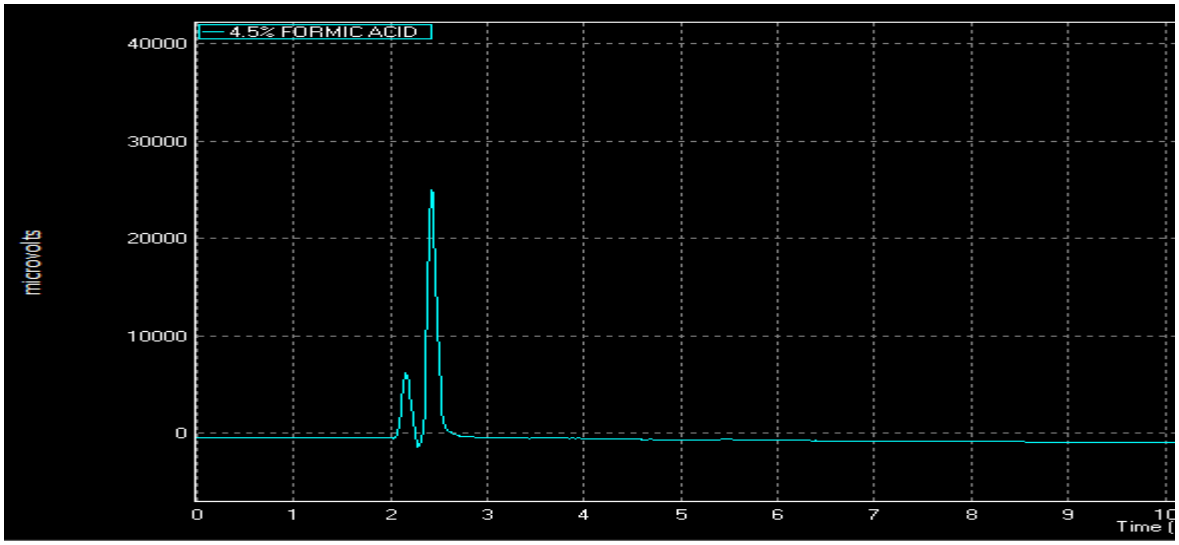


Fig. C3: HPLC chromatogram for 4.5 % formic acid in water.

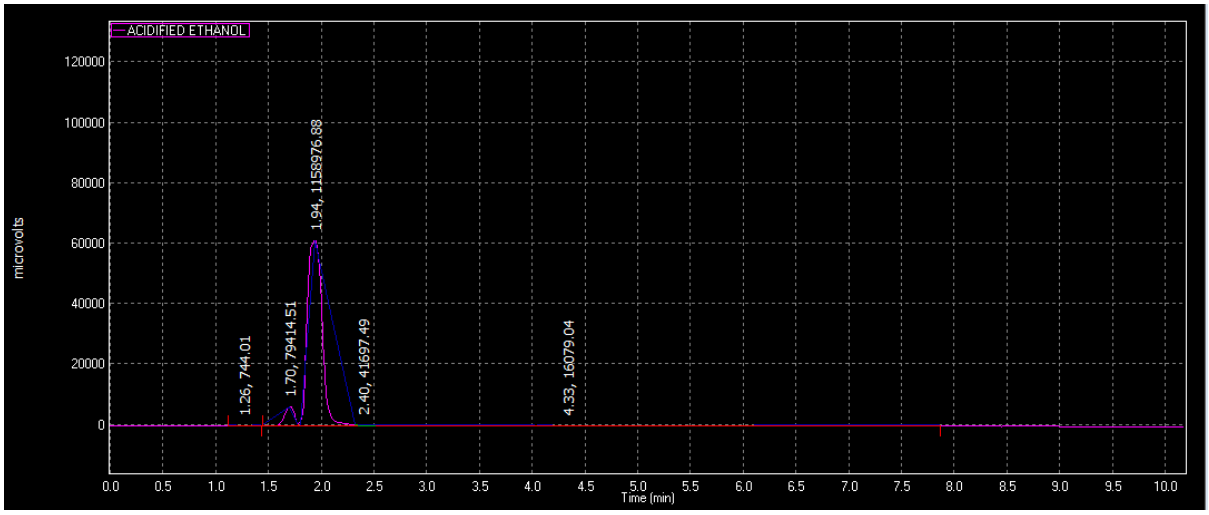


Fig. C4: HPLC chromatogram with calculated area for acidified ethanol.

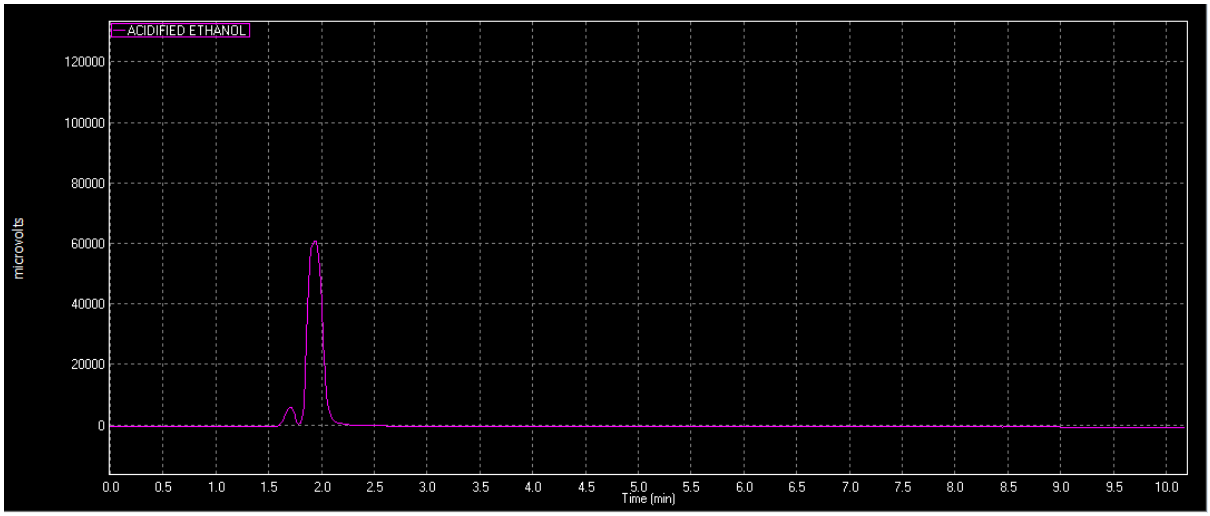


Fig. C5: HPLC chromatogram for acidified ethanol.

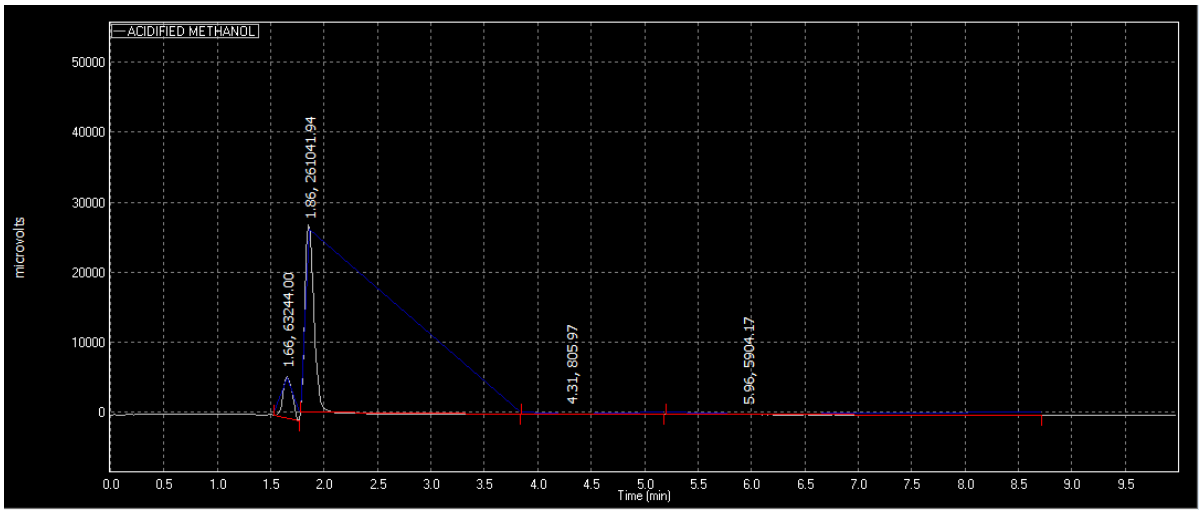


Fig. C6: HPLC chromatogram with calculated area for acidified methanol.

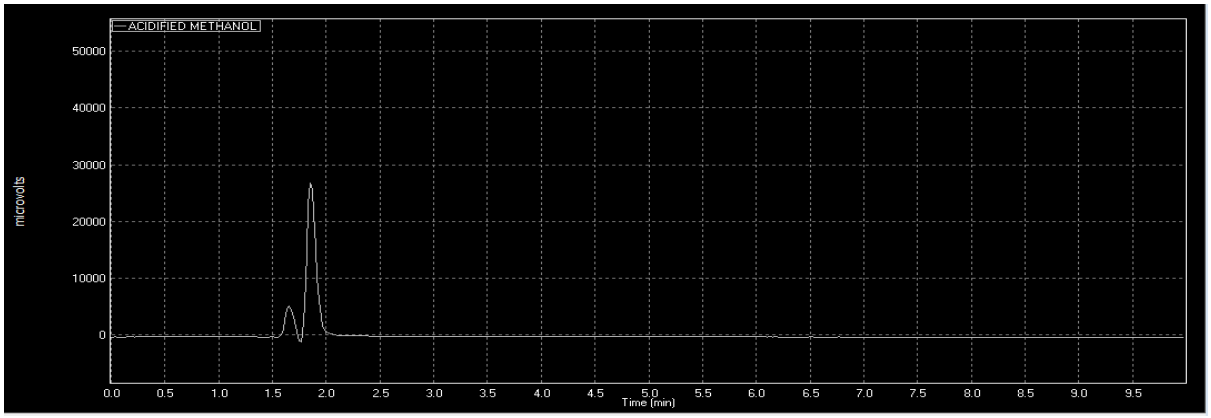


Fig. C7: HPLC chromatogram for acidified methanol.

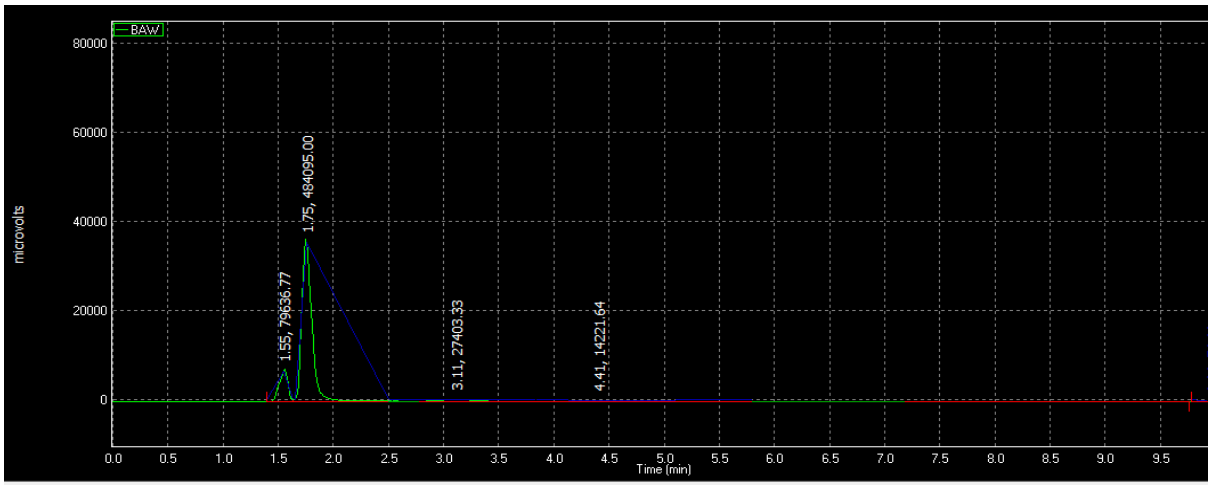


Fig. C8: HPLC chromatogram with calculated area for BAW.

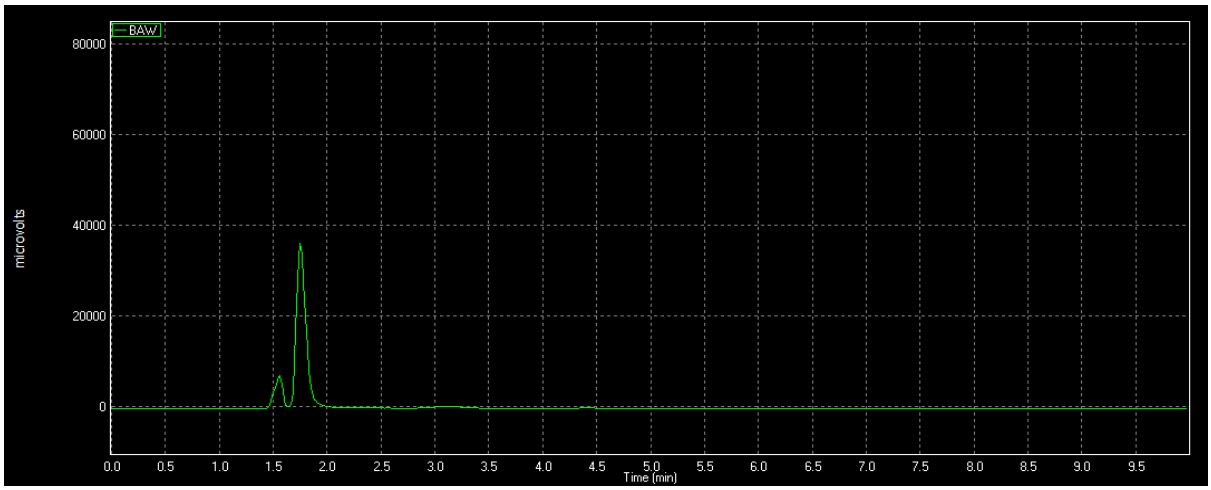


Fig. C9: HPLC chromatogram for BAW.

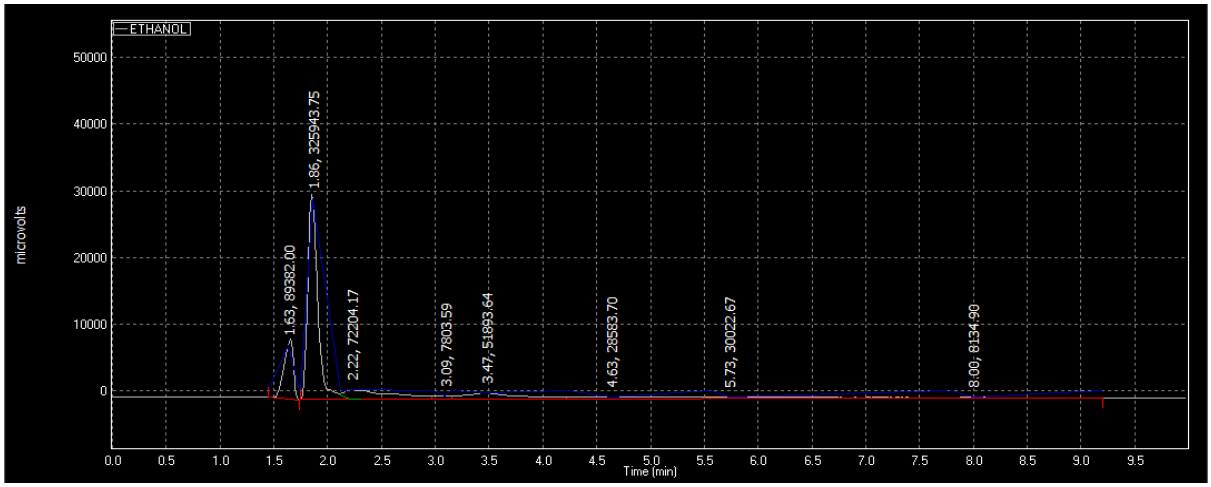


Fig. C10: HPLC chromatogram with calculated area for ethanol.

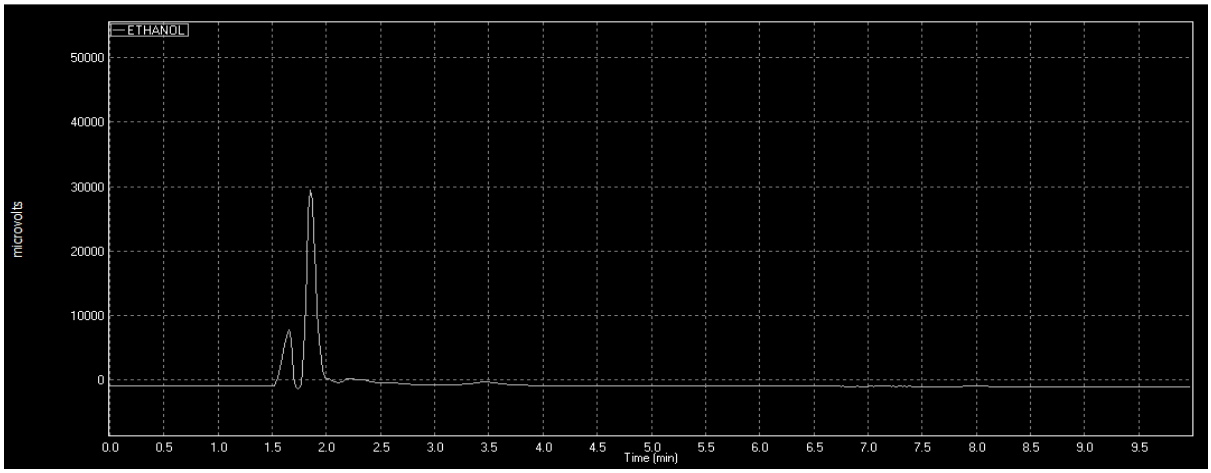


Fig. C11: HPLC chromatogram for ethanol.

GC-MS results

The following figures C13 to C17 shows GC-MS chromatograms for selected masses of molecular ions together with their corresponding retention times.

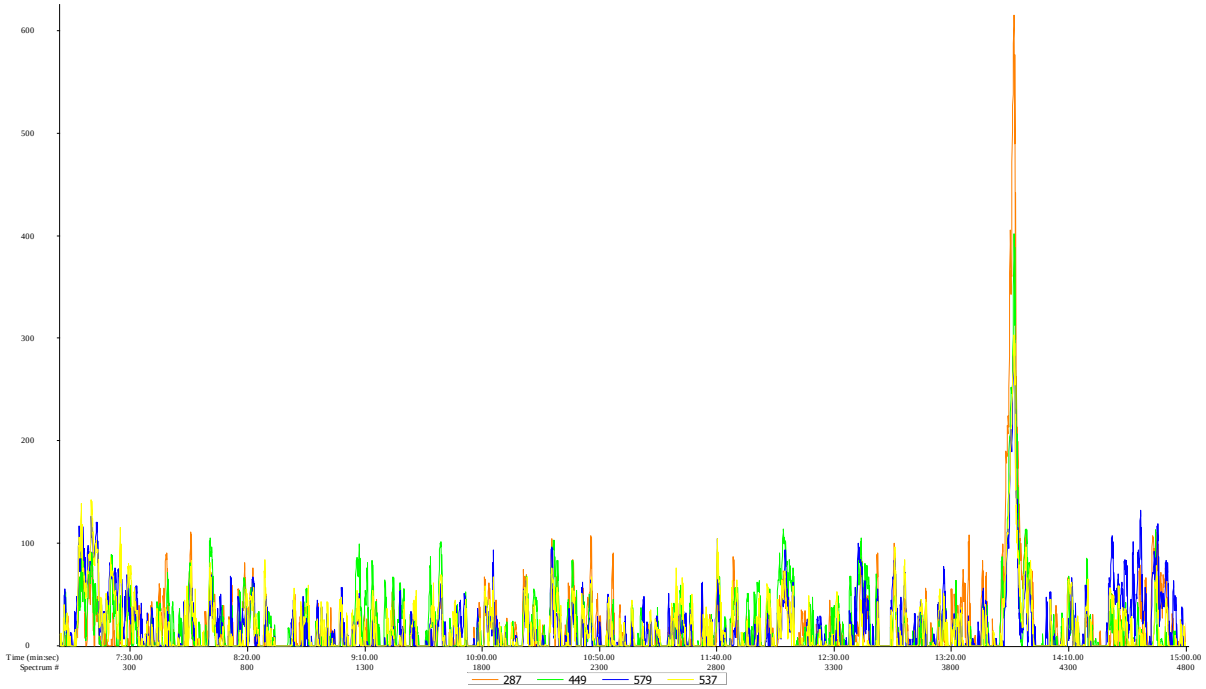


Fig. C12: GC-MS chromatogram for acidified ethanol.

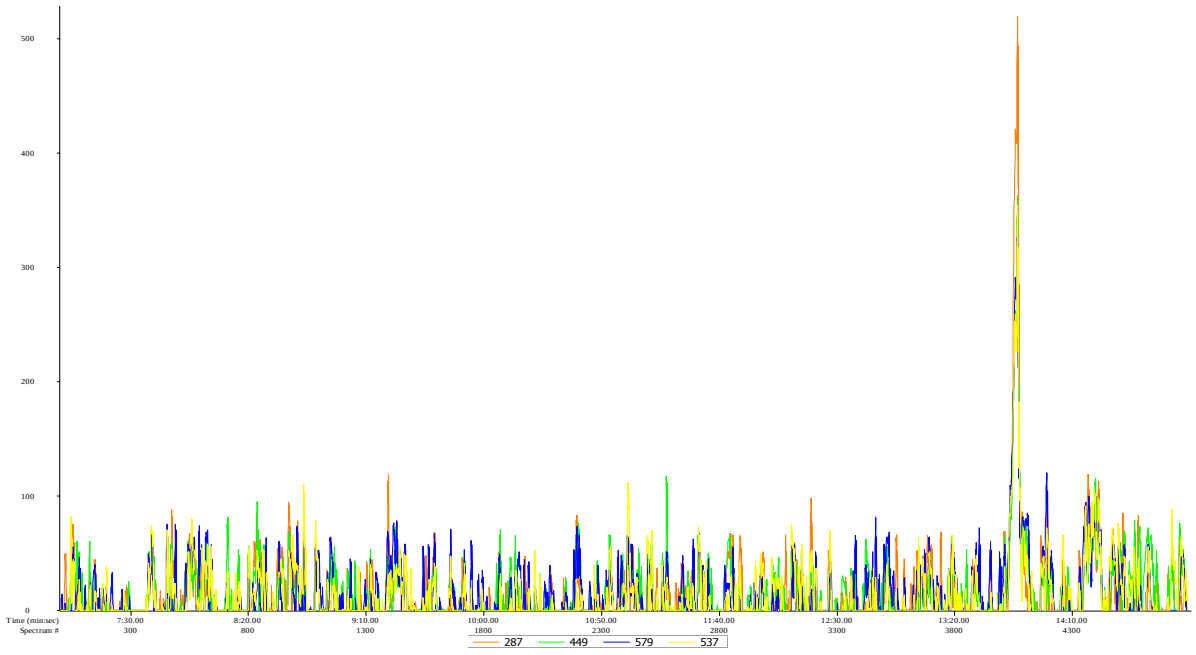


Fig. C13: GC-MS Chromatogram for ethanol

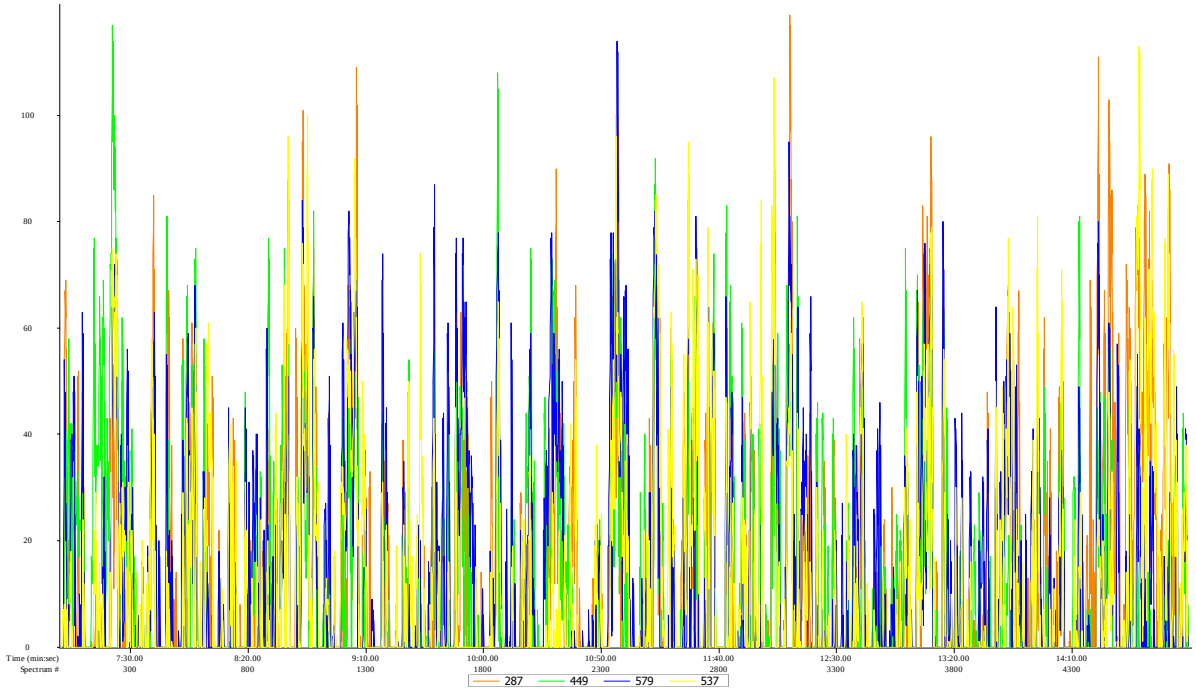


Fig. C14: GC-MS chromatogram for acidified methanol.

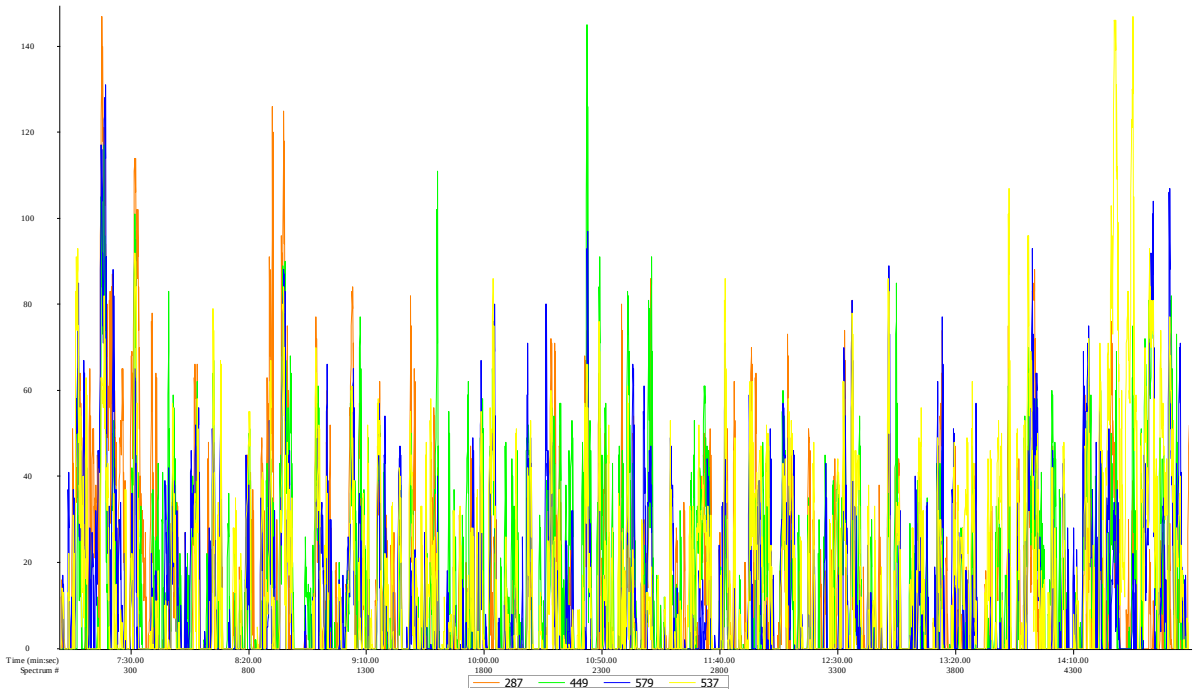


Fig. C15: GC-MS chromatogram for 4.5 % formic acid in water.

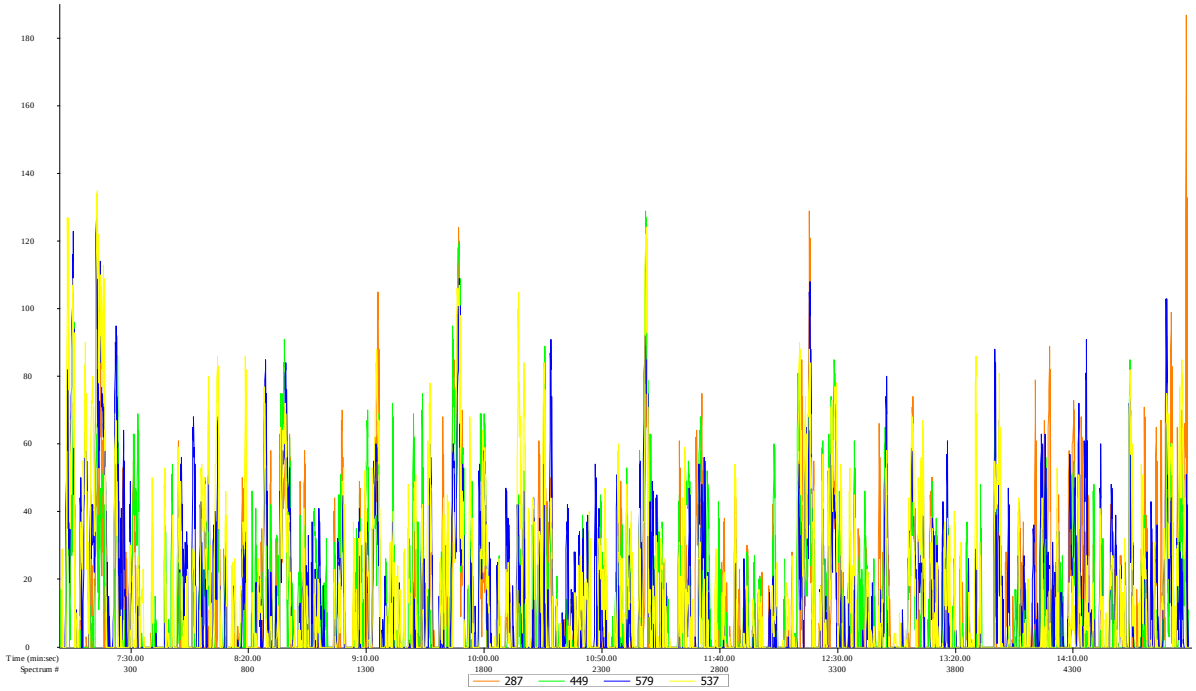


Fig. C16: GC-MS chromatogram for BAW.

Fourier transform infrared spectrometry (FTIR) results

Figure C17 shows vibrational frequencies obtained from *E. crassipes* roots extract.

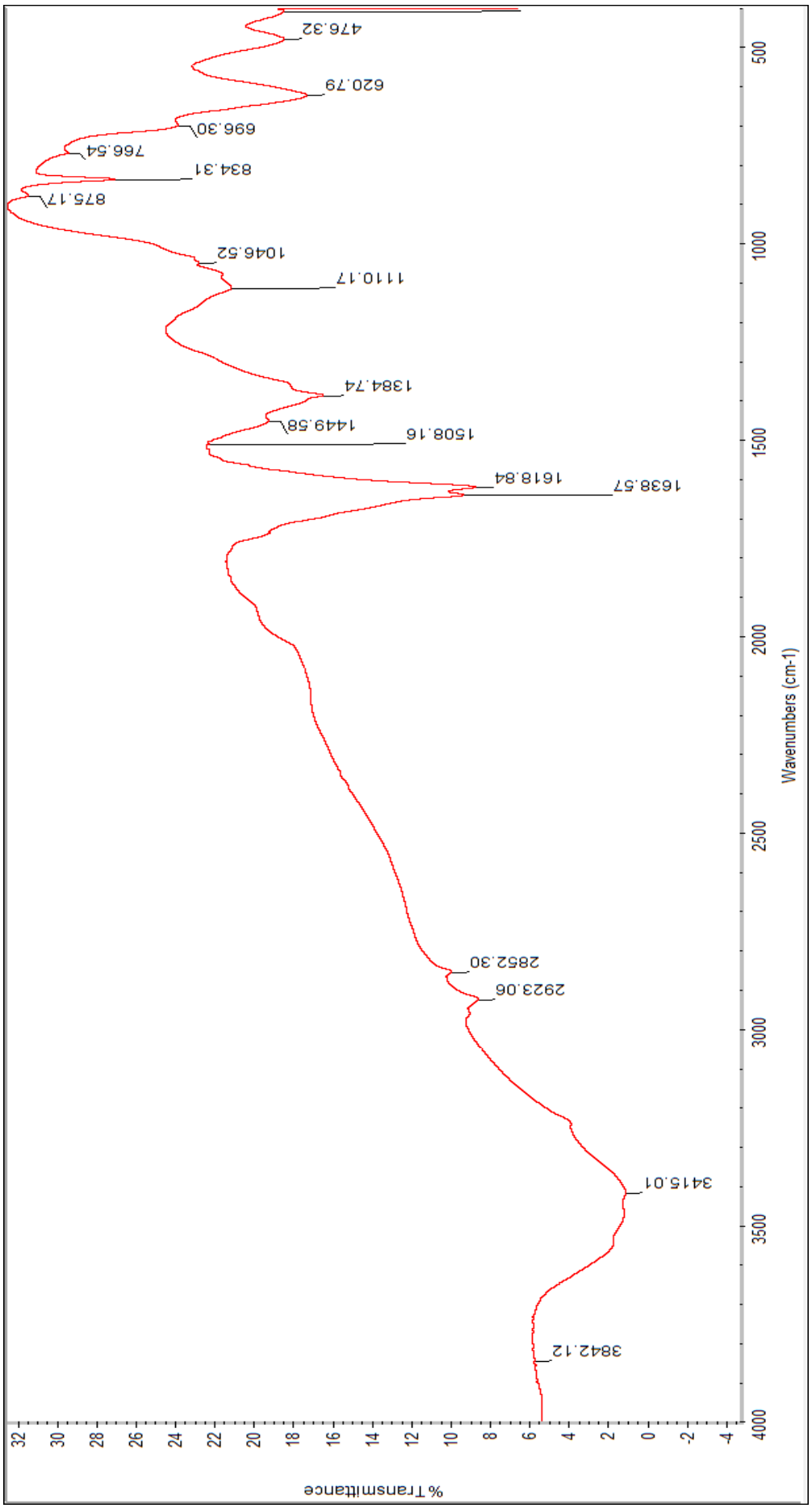
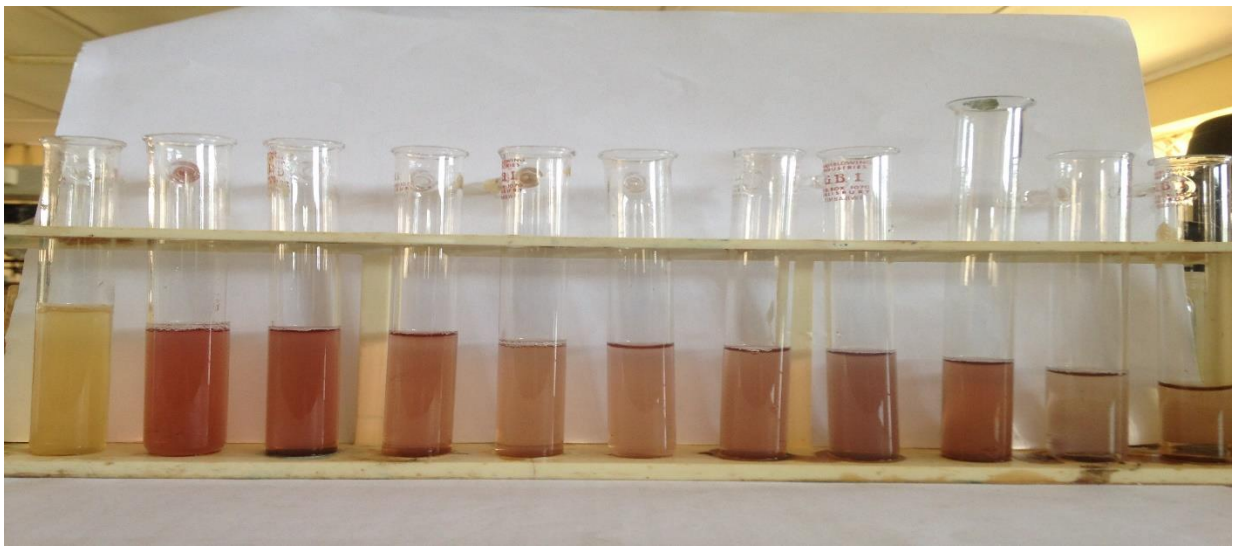


Fig. C17: FTIR vibrational frequencies of *E. crassipes* root extract.

Colour changes of the extract at different pH values.

The following figure shows colour changes when the *E. crassipes* root extract indicator was added to buffer solutions of pH 2.2 to 12.04.



2.2 3.0 4.0 5.0 6.0 7.0 8.0 9.31 10.09 11.01 12.04

Fig. C18: Colour changes when the *E. crassipes* root extract was added to buffer solutions of pH 2.2 to 12.04

Titration results

a) Strong acid vs strong base (1 M HCl vs 1 M NaOH)

Table C1: Titration using methyl red as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	0.8	12.1	23.1	34.3	6.0		
Final volume	12.0	23.0	34.0	45.3	16.9		
Titre volume	11.2	10.9	10.9	11.0	10.9	10.98	0.13

Table C2: Titration using phenolphthalein as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	16.8	28.5	5.6	16.6	27.6		
Final volume	28.0	39.6	16.6	27.6	38.6		
Titre volume	11.2	11.1	11.0	11.0	11.0	11.06	0.09

Table C3: Titration using bromothymol blue as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	14.5	25.7	7.4	18.5	29.7		
Final volume	25.7	36.9	18.5	29.6	40.9		
Titre volume	11.2	11.2	11.1	11.1	11.2	11.16	0.05

Table C4: Titration using *E. crassipes* root extract as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	35.4	6.1	17.2	28.3	3.5		
Final volume	46.6	17.2	28.3	39.3	14.4		
Titre volume	11.2	11.1	11.1	11.0	10.9	11.06	0.11

b) Strong acid vs weak base (1 M HCl vs 1 M NH_4OH)

Table C5: Titration using methyl red as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	2.2	10.3	18.1	25.8	33.4		
Final volume	10.1	18.1	25.8	33.6	41.4		

Titre volume	7.9	7.8	7.7	7.8	7.8	7.8	0.07

Table C6: Titration using phenolphthalein as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	33.8	3.5	10.6	17.9	25.0		
Final volume	41.0	10.6	17.6	25.0	32.2		
Titre volume	7.2	7.1	7.0	7.1	7.2	7.12	0.08

Table C7: Titration using bromothymol blue as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	25.2	33.0	4.2	12.1	20.0		
Initial volume	33.0	40.9	11.9	19.9	27.8		
Titre volume	7.8	7.9	7.7	7.8	7.8	7.8	0.07

Table C8: Titration using *E. crassipes* root extract as an indicator

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	7.7	15.3	23.0	30.8	38.6		
Final volume	15.3	23.0	30.8	38.6	46.4		
Titre volume	7.6	7.7	7.8	7.8	7.8	7.74	0.09

c) Weak acid vs strong base (1 M CH_3COOH vs 1 M NaOH)

Table C9: Titration using methyl red as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	3.6	15.8	28.0	3.0	15.2		

Final volume	15.8	27.9	40.2	15.1	27.4		
Titre volume	12.2	12.1	12.2	12.1	12.2	12.16	0.05

Table C10: Titration using phenolphthalein as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	10.3	27.5	5.1	16.3	27.6		
Final volume	21.3	38.6	16.3	27.4	38.7		
Titre volume	11.0	11.1	11.2	11.2	11.1	11.12	0.08

Table C11: Titration using bromothymol blue as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	4.3	15.4	26.5	37.5	4.7		
Final volume	15.2	26.3	37.5	48.3	15.6		
Titre volume	10.9	10.9	11.0	10.8	10.9	10.9	0.07

Table C12: Titration using *E. crassipes* root extracts as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	21.5	16.2	28.0	4.9	16.8		
Final volume	33.2	27.8	39.5	16.5	28.3		
Titre volume	11.7	11.6	11.5	11.6	11.5	11.58	0.08

d) Weak acid vs weak base (1 M CH_3COOH vs 1 M NH_4OH)

Table C13: Titration using methyl red as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	0.5	10.1	19.6	29.4	39.2		
Final volume	10.1	19.6	29.2	38.9	48.6		
Titre volume	9.6	9.5	9.6	9.5	9.5	9.54	0.05

Table C14: Titration using phenolphthalein as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	2.4	10.1	17.9	25.4	33.0		
Final volume	10.1	17.7	25.4	33.0	40.6		
Titre volume	7.7	7.6	7.5	7.6	7.6	7.6	0.07

Table C15: Titration using bromothymol blue as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	11.7	20.2	28.5	36.8	6.0		
Final volume	2.01	28.5	36.8	45.2	14.3		
Titre volume	8.4	8.3	8.3	8.4	8.3	8.34	0.05

Table C16: Titration using *E. crassipes* root extract as an indicator.

Run	1	2	3	4	5	$\bar{\chi}$	S
Initial volume	6.6	15.5	24.6	34.6	3.8		
Final volume	15.5	24.4	33.4	43.3	12.6		

Titre volume	8.9	8.9	8.8	8.7	8.8	8.82	0.08

One way ANOVA and t-test results

Table C17: T-test results of HCl-NH_4OH using *E. crassipes* root extract and methyl red and also root extract and phenolphthalein.

HCL-NH_4OH (*E. crassipes* vs methyl red)	*Variable 1*	*Variable 2*	HCl-NH_4OH (*E. crassipes* vs phenolphthalein)	*Variable 1*	*Variable 2*
Mean	7.8	7.74	Mean	7.12	7.74
Variance	0.005	0.008	Variance	0.007	0.008
Observations	5	5	Observations	5	5
Pooled Variance	0.0065		Pooled Variance	0.0075	
Hypothesized Mean Difference	0.05		Hypothesized Mean Difference	0.05	
Df	8		df	8	
t Stat	0.196116		t Stat	-12.2325	
P(T<=t) one-tail	0.424705		P(T<=t) one-tail	9.26E-07	
t Critical one-tail	1.859548		t Critical one-tail	1.859548	
P(T<=t) two-tail	0.849409		P(T<=t) two-tail	1.85E-06	

t Critical two-tail	2.306004	t Critical two-tail	2.306004

Table C18: T-test results of HCl-NH_4OH titration using *E. crassipes* root extract and phenolphthalein

t-Test: Two-Sample Assuming Equal Variances

HCl-NH_4OH (*E. crassipes* root extract vs bromothymol blue)	*Variable 1*	*Variable 2*	
Mean	7.8		7.74
Variance	0.005		0.008
Observations	5		5
Pooled Variance	0.0065		
Hypothesized Mean Difference	0.05		
df	8		
t Stat	0.196116		
P(T<=t) one-tail	0.424705		

t Critical one-tail	1.859548
P(T<=t) two-tail	0.849409
t Critical two-tail	2.306004

Table C19: T-test results of CH_3COOH-NH_4OH titration using *E. crassipes* root extract and methyl red

t-Test: Two-Sample Assuming Equal Variances

CH_3COOH-NH_4OH (*E.crassipes* root extract vs methyl red)	*Variable 1*	*Variable 2*
Mean	12.16	11.58
Variance	0.003	0.007
Observations	5	5
Pooled Variance	0.005	
Hypothesized Mean Difference	0.05	
Df	8	
t Stat	11.85116	
P(T<=t) one-tail	1.18E-06	
t Critical one-tail	1.859548	
P(T<=t) two-tail	2.36E-06	
t Critical two-tail	2.306004	

Table C20: T-test of CH_3OH-NH_4OH titration using *E. crassipes* root extract and phenolphthalein

t-Test: Two-Sample Assuming Equal Variances

CH_3COOH-NaOH (*E. crassipes* root extract vs phenolphthalein)	*Variable 1*	*Variable 2*
Mean	11.12	11.58
Variance	0.007	0.007
Observations	5	5
Pooled Variance	0.007	
Hypothesized Mean Difference	0.05	
Df	8	
t Stat	-9.63809	
P(T<=t) one-tail	5.58E-06	
t Critical one-tail	1.859548	
P(T<=t) two-tail	1.12E-05	
t Critical two-tail	2.306004	

Table C21: T-test of CH_3COOH-NH_4OH titration using *E. crassipes* root extract and bromothymol blue

t-Test: Two-Sample Assuming Equal Variances

CH_3COOH-NH_4OH (*E. crassipes* root extract vs bromothymol blue)	*Variable 1*	*Variable 2*
Mean	10.9	11.58

Variance	0.005	0.007
Observations	5	5
Pooled Variance	0.006	
Hypothesized Mean Difference	0.05	
Df	8	
t Stat	-14.9011	
P(T<=t) one-tail	2.03E-07	
t Critical one-tail	1.859548	
P(T<=t) two-tail	4.06E-07	
t Critical two-tail	2.306004	

Table C22: One way ANOVA results of HCl-NaOH titration

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0815	3	0.027167	2.650407	0.084125	3.238872
Within Groups	0.164	16	0.01025			
Total	0.2455	19				

Table C23: One way ANOVA results of HCl-NH_4OH titration

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.6455	3	0.5485	87.76	3.77E-10	3.238872
Within Groups	0.1	16	0.00625			
Total	1.7455	19				

Table C24: One way ANOVA results of CH_3COOH-NaOH titration

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.66	3	1.553333	282.4242	4.61E-14	3.238872
Within Groups	0.088	16	0.0055			
Total	4.748	19				

Table C25: One way ANOVA results of CH_3COOH-NH_4OH titration

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1271.538	3	423.846	1.160937	0.355304	3.238872

Within			
Groups	5841.432	16	365.0895
Total	7112.97	19	

APPENDIX D

Equations

D1 - Mean

$\bar{\chi} = \frac{\sqrt{\chi}}{n}$ Where $\bar{\chi}$ is the mean value, χ are the experimental values and n is the number of measurements.

D2- Standard deviation

$S = \frac{\sqrt{(\chi - \bar{\chi})}}{n-1}$ Where S is the standard deviation, χ are experimental values, $\bar{\chi}$ is the mean and n is the number of measurements.

D3- t-Test

$$T_0 = \frac{\overline{X} - \mu_0}{S/\sqrt{n}}$$ where $\bar{X}$ is the population mean, μ is the sample mean, S is the standard deviation and n is the number of values.

D4-One way ANOVA

$SSB = \Sigma n(\bar{x} - \overline{X})^2$ where $\bar{X}$ is the group mean and $\bar{X}$ is the grand mean

$SSW = \Sigma(x - \bar{x})^2$ where x is group values and $\bar{x}$ is the group mean

$F = \frac{SSB}{SSW}$ where SSB is the sum of squares between groups and SSW is the sum of squares within groups